THE IMPORTANCE OF RET-He IN THE DIAGNOSIS OF IRON DEFICIENCY AND IRON DEFICIENCY ANEMIA AND THE EVALUATION OF RESPONSE TO ORAL IRON THERAPY

ZNAČAJ RET-He U DIJAGNOSTIKOVANJU NEDOSTATKA GVOŽĐA ANEMIJE IZAZVANE NEDOSTATKOM GVOŽĐA, KAO I U PROCENI REAKCIJE NA PERORALNU TERAPIJU GVOŽĐEM

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Summary

Background: The purpose of this study is to investigate whether or not reticulocyte hemoglobin equivalent (RET-He) is a superior indicator of blood count and other iron parameters in terms of diagnosing iron deficiency (ID) and iron deficiency anemia (IDA), and thus evaluating a patient's response to oral iron treatment.

Methods: The research population consisted of 217 participants in total: 54 control, 53 ID, 58 non-ID anemia, and 52 IDA patients. A hemoglobin (Hb) value of < 130.0 g/L was defined as indicating anemia for men, while an Hb value of < 120.0 g/L was defined as indicating anemia for women. All patients were administered 270 mg oral elemental iron sulphate daily.

Results: The RET-He was significantly lower in the IDA group, compared to other groups (IDA: 21.0 ± 4.1, ID: 26.0 ± 4.9, non-ID anemia: 32.1 ± 6.8, control: 36.6 ± 7.0; < 0.001). The ID group had a lower RET-He compared to the non-ID anemia group and the control group. On the 5th day of treatment, the ID and IDA group showed no significant differences in terms of Hb while the RET-He level demonstrated a significant increase. The increase in the RET-He level observed in the IDA group on the 5th day was significantly higher compared to the increase observed in the ID group. A RET-He value of 25.4 pg and below predicted ID diagnosis with 90.4% sensitivity and 49.1% specificity in IDA patients, compared to the ID group.

Kratak sadržaj

Uvod: Svrha ove studije je utvrđivanje da li je retikulocitni ekvivalent hemoglobina (RET-He) superiorni indikator krvne slike i drugih parametara gvožđa u smislu dijagnostikovanja deficita gvožđa (ID) i anemije usled nedostatka gvožđa (IDA), kako bi se na taj način procenila reakcija pacijenata na peroralni tretman gvožđem.

Metode: Populacija na kojoj je vršeno ispitivanje se sastojala od ukupno 217 učesnika: 54 kontrolna učesnika, 53 sa ID, 58 anemičnih (ali ne u vezi sa ID) i 52 IDA pacijenta. Vrednost hemoglobina Hb < 130,0 g/L je definisana kao indikacija anemije kod muškaraca, dok je vrednost Hb < 120,0 g/L definisana kao indikacija anemije kod žena. Svim pacijentima je dnevno oralno davano 270 mg elementarnog gvožđa sulfata.

Rezultati: U poređenju sa drugim grupama RET-He je bio značajno niži u IDA grupi (IDA: 21,0 ± 4,1; ID: 26,0 ± 4,9; ne-ID anemična grupa: 32,1 ± 6,8; kontrola: 36,6 ± 7,0; < 0,001). ID grupa je imala niži RET-He u poređenju sa ne-ID grupom anemije i kontrolnom grupom. Petog dana tretmana ID i IDA grupa nisu pokazale značajne razlike u smislu Hb dok je RET-He nivo pokazao značajno povećanje. Porast RET-He nivoa koji je uočen u IDA grupi petog dana bio je značajno veći u poređenju sa porastom koji je primećen u ID grupi. Vrednost RET-He od 25,4 pg i manje je predvidela ID dijagnozu sa 90,4% osetljivosti i 49,1% specifičnosti kod IDA pacijenata, u poređenju sa ID grupom.

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Conclusions: The results of our study, therefore, suggest that RET-He may be a clinically useful marker in the diagnosis of ID and IDA.

Keywords: reticulocyte hemoglobin equivalent (RET-He), iron deficiency anemia (IDA), iron

Zaključak: Rezultati našeg istraživanja sugerišu da RET-He može biti klinički koristan marker u dijagnostici ID i IDA.

Ključne reči: retikulocitni ekvivalent hemoglobina (RET-He), anemija usled nedostatka gvožđa (IDA), gvožđe

Introduction

Iron deficiency (ID) is globally the most common nutritional deficiency and is also the most common cause of anemia. Hemoglobin (Hb) levels may remain normal for a while after iron deposits are diminished, i.e., iron deficiency may be observed without anemia, and only the plasma ferritin level and the plasma transferrin saturation are reduced in this period. Once iron deposits are depleted, the hemoglobin level begins to drop. This means that this condition of diminished iron deposits in the body is referred to as iron deficiency (ID), and a continuation of this condition and the consequent development of anemia is referred to as iron deficiency anemia (IDA) (1).

The gold standard in ID diagnosis is the staining of bone marrow macrophages and erythroid precursors with Prussian blue in bone marrow aspiration. However, this procedure is quite expensive and invasive. Serum ferritin concentration, serum iron level (Fe), total iron binding capacity (TIBC), and transferrin saturation (TSAT), are the most common alternative biochemical tests (2), but these tests may be influenced by certain conditions. Serum iron falls in IDA, as well as in chronic disease anemia, and also fluctuates during the day depending on the iron intake. Since TSAT is calculated based on Fe and TIBC, changes in these values are also reflected on TSAT. The serum ferritin level shows the iron deposited in the body, and very low values indicate iron deficiency. However, serum ferritin is also an acute phase protein, and may, therefore, appear to be normal or high in cases such as infectious, inflammatory conditions and malignancy (3).

Reticulocytes are immature erythrocytes which lack a nucleus and contain ribosomal RNA residues. Reticulocytes are generated in bone marrow and released into the peripheral circulation after three days of maturation, becoming fully-matured erythrocytes the following day. Reticulocytes account for approximately 1% (0.5–2.5%) of erythrocytes in circulation. Measuring the reticulocyte count shows the erythropoiesis level in bone marrow and the response of bone marrow to anemia, i.e., erythropoietic activity in bone marrow. In moderate anemia cases, erythrocyte generation is expected to increase 2–3 fold within 10 days in healthy bone marrow, thanks to the erythropoietin hormone (4).

Automatic blood count devices, such as Sysmex, include a reticulocyte count channel. A fluorescent dye is used for blood count, and the RNA content of reticulocyte is measured simultaneously. In addition to the classification of reticulocytes via a scatter plot, depending on their nucleic acid concentration, this channel is used to measure the erythrocyte size and Hb amount in each cell (5). In cases of iron deficiency, the hemoglobin synthesis is firstly reduced in reticulocytes. The RET-He level is a useful parameter which allows for the diagnosing of anemia before it develops in a patient. It is also a parameter which can be identified long before the increase in Hb and classical reticulocyte count, which is useful for treatment and treatment follow-up. RET-He is the first parameter to evaluate functional ID in particular and show that the patient is benefiting from the treatment (6, 7).

This study aims to investigate the usability and reliability of RET-He as a parameter in the diagnosis of iron deficiency and iron deficiency anemia, as well as in the evaluation of short-term treatment response.

Materials and Methods

This study was performed in the Department of Hematology, xxx Training and Research Hospital between May 2017 and May 2018. Our study was approved by Research Ethics Committee, Ankara Numune Training and Research Hospital (E-18-2041-2018). Informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

217 patients, who had been admitted to the hematology clinic and had been diagnosed with anemia, were included in the study regardless of their sex. Patients under the age of 18, as well as those who had a chronic disease, were pregnant, or had received iron treatment within the previous three months, were excluded.

An Hb level below 120.0 g/L was defined as indicating anemia in women, and an Hb level below 130.0 g/L was defined as indicating anemia in men. A serum ferritin level of < 26.96 pmol/L was accepted as indicating ID within anemia patients. Only 105 patients with ID and IDA were administered 270 mg oral iron sulphate daily. Changes in Hb and RET-He levels were recorded after five days. The hemoglobin of reticulocytes (RET-He: reticulocyte Hb

equivalent) was also measured via this channel of the blood count device. The average value is 30.8 pg, while the lower limit is 28 pg. RET-He is the equivalent of the CHr (reticulocyte Hb content) measured with Siemens brand devices and shows the condition of iron deposits in bone marrow (8).

Blood samples were taken in tubes containing ethylenediaminetetraacetic acid dipotassium salt (EDTA-2K), and an automatic device (XN-1000, Sysmex) was used for blood count and RET-He measurement. Blood samples were taken in regular tubes for serum Fe, TIBC, serum ferritin and other biochemical parameters, and were analyzed with an automatic chemical analysis device (Cobas® 6000 analysis series Cobas® modular platform). TSAT was calculated as »serum Fe/TIBC x 100«.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS) for Windows 20 (IBM SPSS Inc., Chicago, USA) was used for statistical analysis, and the normal distribution of data was evaluated using the Kolmogorov-Smirnov test. Numeric variables with normal distribution were presented as mean ± standard deviation, and numeric variables without normal distribution were presented as median (min-max). Categorical variables were presented as numbers and percentages. To identify variables which show differences in attack numbers, ANOVA (post hoc: student t-test with Bonferroni correction) was used for numeric variables with normal distribution, and the Kruskal-Wallis test (post hoc: Mann-Whitney U test with Bonferroni correction) was used for numeric variables without normal distribution. The Chi-square test and the Fisher's exact Chi-square test were used in the comparison of categorical data. The relation between numerical variables was examined with Pearson and Spearman's correlation analysis. The changes in laboratory findings of ID and IDA groups on the 5th day were assessed with repeated measures of ANOVA. ROC Curve analysis was used for the assessment of the diagnostic performance. Predictive values were calculated with the Youden index, and p < 0.05 was considered to be statistically significant in statistical analysis.

Results

The research population consisted of 217 participants in total; 54 control, 58 non-ID anemia, 53 ID, and 52 IDA patients. No difference was observed between the groups in terms of age and sex distribution. The median ferritin level, average iron level, average saturation percentage, average mean corpuscular volume (MCV), and average mean corpuscular hemoglobin concentration (MCHC) were lower (p < 0.05), and the average TIBC and average platelet count (PLT) were higher in the IDA group and

the ID group, compared to the control group and the non-ID anemia group. The average Hb level was similar between the non-ID anemia group and the IDA group, and lower in these groups compared to the control group and the ID group. RET-He was significantly lower in the IDA group compared to other groups. The ID group had a lower RET-He compared to the non-ID anemia group and the control group (*Table I*).

No significant correlation was found between the baseline RET-He value and baseline laboratory findings in the control group and the non-ID anemia group. In the ID group, the baseline RET-He level was positively correlated with the baseline ferritin level (r = 0.357; p = 0.043) and the baseline Hb level (r = 0.345; p = 0.045). In the IDA group, the baseline RET-He level was positively correlated with the baseline ferritin level (r = 0.506; p < 0.001), the baseline iron level (r = 0.452; p = 0.001), the baseline saturation level (r = 0.351; p = 0.011), the baseline Hb level (r = 0.682; p < 0.001), the baseline MCV level (r = 0.560; p < 0.001), and the baseline MCHC level (r = 0.576; p < 0.001), and negatively correlated with the baseline TIBC (r = -0.548; p < 0.001) and the baseline PLT levels (r = -0.340; p = 0.044). *Figure 1* shows the correlation of the baseline RET-He with the baseline ferritin, the baseline TIBC, the baseline saturation, and the baseline iron levels.

There was no significant difference between the baseline and the 5th-day Hb, MCV, MCH and PLT levels in the ID group and the IDA group, while the RET-He level demonstrated significant increases. The increase in RET-He level on the 5th day was more significant in the IDA group compared to the ID group (*Figure 2*).

Figure 3 shows the RET-He performance assessment. According to this assessment, a RET-He value of 25.7 pg and below predicted ID diagnosis with 71.4% sensitivity and 100% specificity in all ID patients (IDA & ID), compared to the control group (AUC ± SE: 0.931 ± 0.02; +PV: 100%, -PV: 64.3%; p < 0.001). A RET-He value of 35.5 pg and below predicted ID diagnosis with 100% sensitivity and 55.6% specificity in only ID patients (non-ID anemia) compared to the control group (AUC ± SE: 0.881 ± 0.03; +PV: 68.8%, -PV: 100%; p < 0.001). A RET-He value of 25.4 pg and below predicted ID diagnosis with 90.4% sensitivity and 100% specificity in IDA patients, compared to the control group (AUC ± SE: 0.981 ± 0.01; +PV: 100%, -PV: 91.5%; p < 0.001). A RET-He value of 25.4 pg and below predicted ID diagnosis with 90.4% sensitivity and 49.1% specificity in IDA patients, compared to the ID group (AUC ± SE: 0.779 ± 0.04; +PV: 63.5%, -PV: 83.9%; p < 0.001). *Figure 4* shows the diagnostic performance diagram of RET-He.

Table I Demographic and clinical findings of the patients.

Variables	Control n = 54	Non-ID anemia n = 58	ID n = 53	IDA n = 52	p
Age (years)	42 ± 14.2	40.7 ± 8.2	43.2 ± 12.4	41.8 ± 12.8	0.748
Gender, n (%)					
Female	40 (74.1)	47 (81.0)	42 (79.2)	42 (80.8)	0.807
Male	14 (25.9)	11 (19.0)	11 (20.8)	10 (19.2)	
Ferritin (pmol/L)	51.01 (33.7–122.91)	43.37 (28.31–86.96)	20.22 (11.68–26.74)	13.93 (5.17–26.51)	<0.001*
Iron (µmol/L)	15.59 ± 3.54	15.07 ± 1.88	3.97 ± 1.59	2.72 ± 1.06	<0.001*
TIBC (µmol/L)	56.26 ± 11.83	57.57 ± 16.09	73.14 ± 8.59	76.72 ± 12.82	<0.001*
Transferrin saturation (%)	27.3 (16.8–39.2)	25.2 (2.4–35.7)	4.6 (1.9–11.8)	3.5 (1.4–8.2)	<0.001*
Hemoglobin (g/L)	152 ± 4.0	97 ± 11	152 ± 6.0	96 ± 13	<0.001*
MCV (fL)	90.5 ± 3.9	89.7 ± 5.5	74.3 ± 6.4	72.0 ± 7.8	<0.001*
MCHC (g/dL)	40.1 ± 1.6	39.4 ± 3.2	33.8 ± 2.4	28.4 ± 1.9	<0.001*
Platelet (103 µ/L)	337.1 (146.5–472)	322 (145.6–496.4)	416.3 (161.7–652.6)	396.5 (159-720)	<0.001*
RET-He (pg)	36.6 ± 7.0	32.1 ± 6.8	26.0 ± 4.9	21.0 ± 4.1	<0.001

*Normally distributed numerical variables were shown as mean ± standard deviation.
Numerical variables that do not show normal distribution were shown with median (min-max).
Categorical variables were shown as number (%).
* p < 0.005 shows statistical significance.
Abbreviations: ID, Iron Defiency; IDA, Iron Defiency Anemia; TIBC, Total Iron Binding Capacity; MCV, Mean Corpuscular Volume; MCHC, Mean Corpuscular Hemoglobin Concentration; RET-He, Reticulocyte Hemoglobin Equivalent.
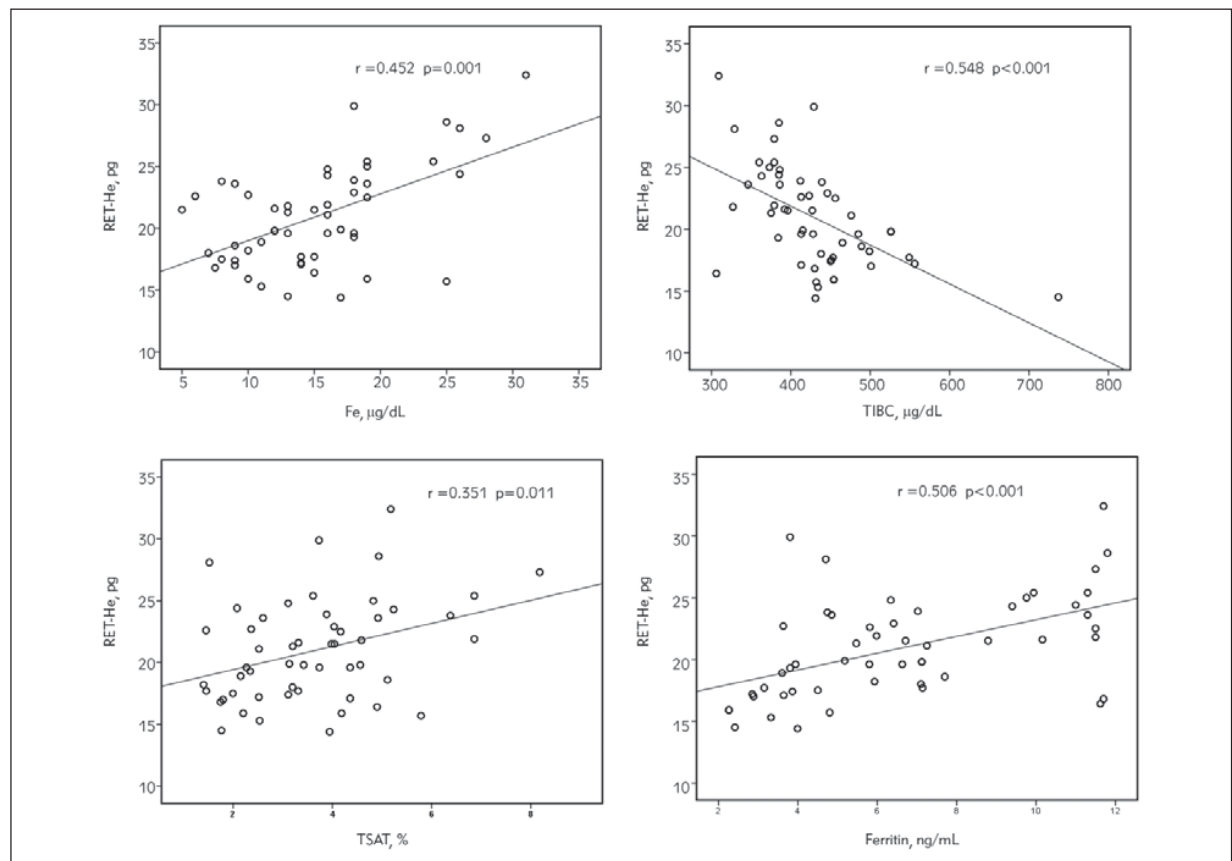

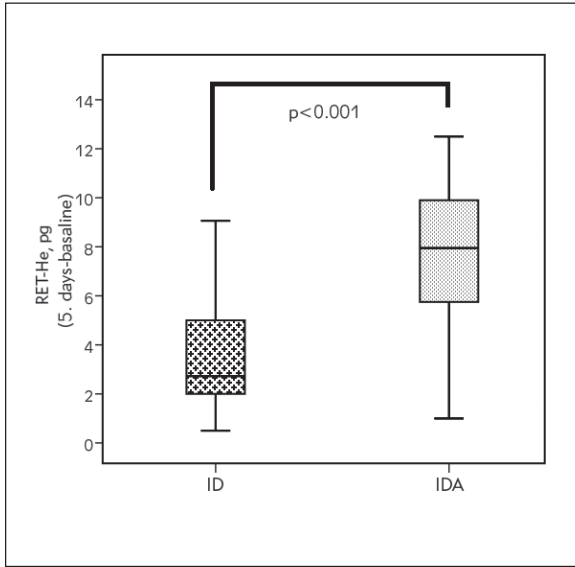**Figure 1** The relationship between RET-He and FE, TIBC, Transferrin saturation and Ferritin.

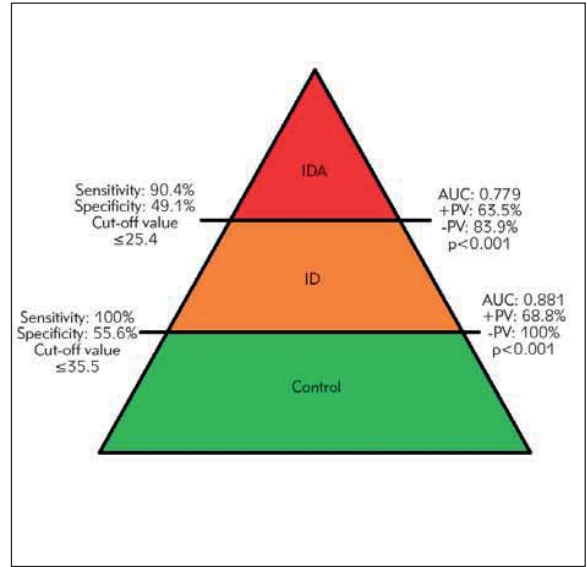**Figure 2** Differences in the RET-He level in IDA and ID patients.

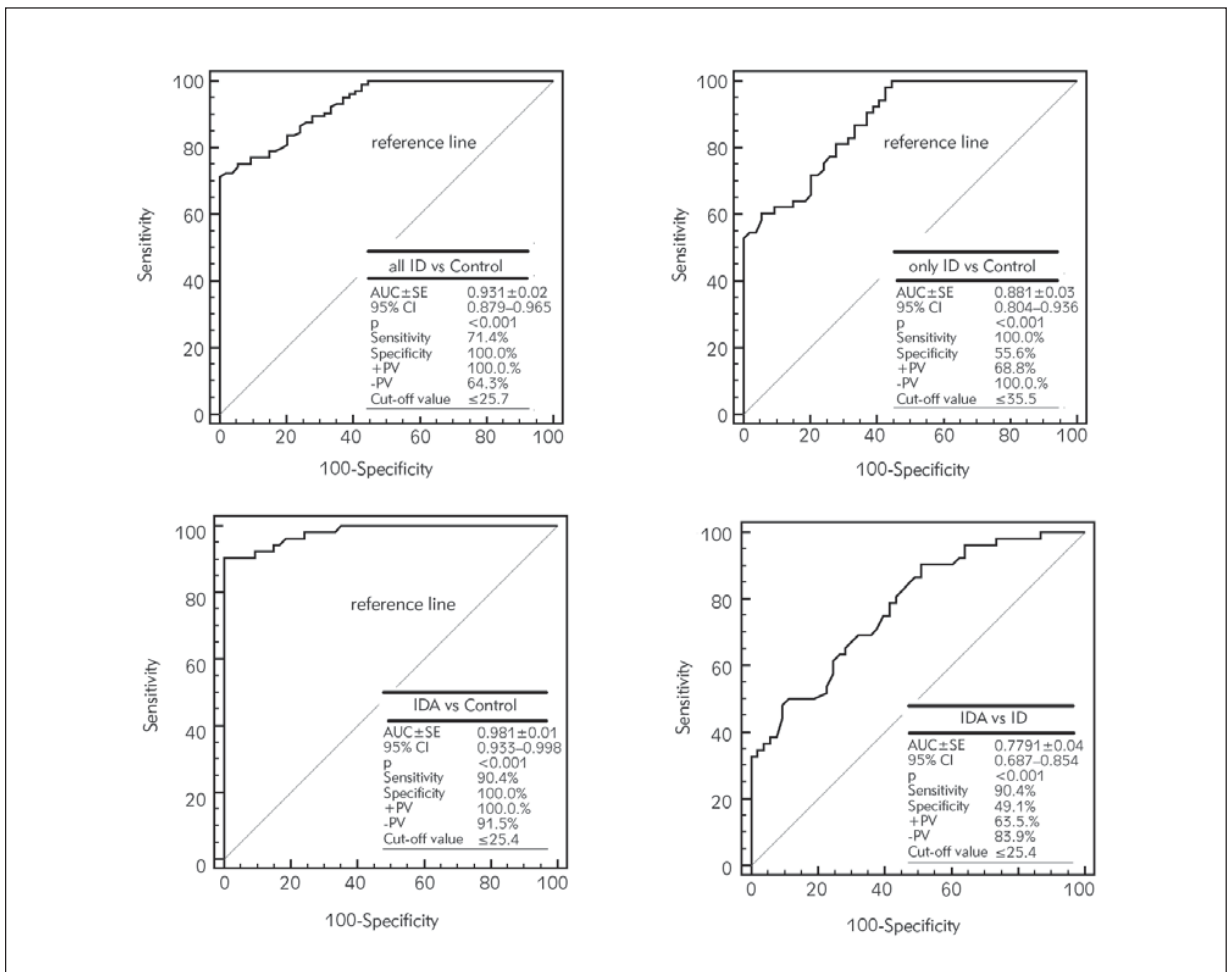**Figure 4** RET-He diagnostic performance diagram.

Figure 3 Prediction of Ret-He in the diagnosis of ID.

Discussion

The iron parameters that were assessed together with complete blood count to diagnose IDA include serum Fe level, serum ferritin level, TIBC, and TSAT. In addition to these parameters, reticulocyte count and percentage were used to assess treatment response in IDA cases. However, these parameters do not yield meaningful diagnostic findings in the later stages of iron deficiency. In the treatment of iron deficiency anemia, reticulocyte count and percentage are expected to increase significantly. This study demonstrates that the RET-He value is superior to other parameters regarding both diagnosis and the assessment of treatment response (3, 9).

RET-He is also used because it provides results in a very short period, does not incur additional costs, can be measured simultaneously in automatic blood count devices, and is not affected by other chronic diseases. RET-He has been shown to be useful in the diagnosis and treatment of IDA in infants, patients with chronic renal failure, and pregnant women (10–12).

This study evaluated 217 patients assigned into ID, IDA, non-ID anemia, and control groups. RET-He was found to be significantly lower in the ID group and the IDA group, compared to the other two groups, at the time of application. It was also significantly lower in the IDA group compared to the ID group. There was a positive correlation between low RET-He values and low ferritin levels, and it was shown that RET-He supported ID and IDA. Based on the previously mentioned information, RET-He can be said to be a very effective parameter in the diagnosis of ID and IDA, as well as in other differential diagnoses.

After the 5-day treatment administered to the DE group and the IDA group, no improvement was observed in Hb and other complete blood count parameters, while a significant increase in RET-He was observed. The increase in the RET-He value of the IDA group after the treatment was more significant compared to the increase in the ID group. This shows that RET-He may be a useful marker in the assessing of hematopoietic response to iron treatment at a very early stage in ID and IDA patients (4, 9, 12).

Considering RET-He performance assessment, a RET-He value of 25.7 pg and below predicted ID (ID and IDA) diagnosis with 71.4% sensitivity and 100% specificity. In ID (non-ID anemia) patients only, a RET-He value of 35.5 pg and below predicted ID diagnosis with 100% sensitivity and 55.6% specificity. A RET-He value of 25.4 pg and below predicted ID diagnosis with 90.4% sensitivity and 100% specificity in IDA patients, compared to the control group. This result is supported by another study in which a RET-He value of 28.5 was found to predict ID with over 90% sensitivity (9).

Twari et al. (13) demonstrated that RET-He might be a suitable marker for indicating latent iron deficiency in blood donors. Toki et al. (9) found RET-He to be a useful marker in the diagnosis of overt iron deficiency anemia. Eckhardt et al. (14) performed a study with chronic renal failure patients and found RET-He to be a good marker to diagnose iron deficiency.

The limitations of our study include the exclusion of pregnant women, patients with chronic inflammatory disease, and patients below the age of 18. There is also the limitation of the study being conducted at a single centre in a region with the same geographical conditions (15).

Our results clearly show that RET-He is quite a useful marker in the diagnosis of ID and IDA, and in the assessment of treatment response, as well as in the differential diagnosis of ID and IDA since it gives results very quickly and is not influenced by other infectious and chronic conditions. Randomized, prospective, and multi-centred studies, including different age groups, different geographical regions, and chronic inflammatory diseases, are required for RET-He to be safely used in clinical practice.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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