

Jamian E^{1,3}, Sanip Z², Ramli M³, Mohd Daud K³,
Mohamad S³, Hassan R^{3,*}

¹Department of Pathology,
Hospital Sultanah Aminah,
Johor Bahru, Johor,
Malaysia

²Central Research
Laboratory, School of
Medical Sciences, Universiti
Sains Malaysia, Kubang
Kerian, Kelantan, Malaysia

³Department of
Haematology, School of
Medical Sciences, Universiti
Sains Malaysia, Kubang
Kerian, Kelantan, Malaysia

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Corresponding author:
Rosline Hassan
Email: roslin@usm.my

Reticulocyte Haemoglobin as a Biomarker for the Detection of Iron Deficiency Anaemia in Haemodialysis Patients on Recombinant Human Erythropoietin

Abstract— Iron deficiency anaemia (IDA) frequently occurs in haemodialysis (HD) patients undergoing recombinant human erythropoietin (rHuEPO) therapy and is commonly associated with rHuEPO hypo-responsiveness. However, the conventional iron indices are inadequate to exhibit the status or utilisation of iron during erythropoiesis. The aim of this study was to elucidate the accuracy and usefulness of the reticulocyte haemoglobin (RET-He) test for diagnosing IDA in HD patients undergoing rHuEPO therapy. Methods: In this cross-sectional study, fifty-five blood samples of HD patients on rHuEPO therapy were collected and analysed for haematological and biochemical parameters. A receiver operating characteristics curve was also plotted for sensitivity and specificity analysis. IDA detection rates by RET-He, soluble transferrin receptor (sTfR) and serum ferritin were 63.64%, 3.64% and 0%, respectively. RET-He level was significantly correlated with sTfR level, mean cell volume, mean cell haemoglobin level and the transferrin receptor-ferritin index. The sensitivity and specificity of RET-He in detecting IDA were 78.3% and 92.0%, respectively, with an area under the curve of 0.864. IDA was more frequently detected by RET-He than by ferritin or sTfR in HD patients undergoing rHuEPO therapy. The RET-He level also showed higher sensitivity and specificity for the iron status in these patients. Therefore, RET-He is a useful biomarker for the detection of IDA in HD patients undergoing rHuEPO therapy.

Keywords—chronic kidney disease, haemodialysis, iron deficiency anaemia, recombinant human erythropoietin, reticulocyte haemoglobin.

1 INTRODUCTION

Chronic kidney disease (CKD) is typically associated with normochromic, normocytic anaemia that manifests as a reduced reticulocyte response and bone marrow erythroid hyperplasia. The anaemia associated with CKD has many consequences for patients; including reduced quality of life, increased cardiovascular disease and increased mortality [1]. Recombinant human erythropoietin (rHuEPO) therapy is an effective therapy and can treat anaemia due to renal failure [2]. Other types of anaemia occurring in CKD are iron deficiency anaemia and megaloblastic anaemia. The underlying causes of iron deficiency anaemia are chronic blood loss occurring during phlebotomy, blood trapping in the dialysis apparatus, chronic bleeding from uraemia-associated platelet dysfunction and impaired dietary iron absorption.

In addition to the absolute iron deficiency, functional iron deficiency (FID) also develops in

patients with CKD. The underlying mechanism of FID is due to an excess of a protein called hepcidin which impairs dietary iron absorption and causes reticuloendothelial cell iron blockade. It is characterized by impaired iron release from body stores, so the body is unable to meet the demand for erythropoiesis. The undetected iron deficiency may limit the efficacy of rHuEPO and eventually lead to hypo-responsiveness to this therapy [3]. In these circumstances, detection of iron deficiency becomes important to ensure that the optimal dosage of rHuEPO is given and that iron therapy is concurrently incorporated to improve anaemia in these patients [4].

Generally, iron staining of bone marrow biopsy is cited as the gold standard to diagnose iron deficiency; but there is a huge limitation due to the invasive nature of this procedure. Therefore, the diagnosis of iron deficiency is traditionally based on a combination of iron metabolism parameters and hematologic indices

[5]. The development of iron deficiency anaemia (IDA) is evident by changes in haematological and biochemical parameters, such as red blood cell mean cell volume (MCV), mean cell haemoglobin (MCH), serum ferritin, serum transferrin saturation and serum iron levels; which will normally fall, and conversely, the transferrin level will rise [6]. The Kidney Disease Outcomes Quality Initiative (K/DOQI) anaemia working group had decided that the serum ferritin and the transferrin saturation levels should be the primary tools for assessing iron management in patients with anaemia and chronic kidney disease (CKD), including end stage renal disease [1]. The serum ferritin reflects on storage iron; and an absolute iron deficiency, according to the K/DOQI guidelines, correlates with a serum ferritin level of less than 100 ng/ml. Absolute iron deficiency is distinguished from FID, which is defined as a response to intravenous iron with an increase in haemoglobin (Hb) or a decrease in the erythropoiesis-stimulating agent requirement. Absolute iron deficiency can occur in patients with serum ferritin levels greater than 100 ng/ml. However, serum ferritin is highly associated with pathological conditions, such as inflammation which commonly occurs in CKD, thus leading to misinterpretation of its normal or high levels even though iron deficiency has developed [4].

In chronic kidney disease, the biochemical markers of iron deficiency [serum iron, ferritin, transferrin saturation and soluble transferrin receptor (sTfR)] have limited value in the diagnosis of functional iron deficiency, since the markers are inconclusive depending on the course of disease and rHuEPO therapy. Since these conventional biochemical parameters are ineffective in detecting iron deficiency, our aim was to evaluate reticulocyte haemoglobin (RET-He) as a marker for IDA in CKD patients undergoing haemodialysis and rHuEPO therapy.

2 METHODOLOGY

2.1 Subjects

A total of 55 participants were included in this cross-sectional study. They were recruited from patients who attended Haemodialysis Unit in Hospital Raja Perempuan Zainab II, Kota Bharu, Kelantan and were diagnosed with end stage renal failure on haemodialysis (HD). The diagnosis was confirmed by the medical history, physical examination and laboratory

investigations of each participant. Inclusion criteria included treatment with rHuEPO for at least 3 months and being in the maintenance phase of the rHuEPO doses for a minimum of 4 weeks. Exclusion criteria included thalassemia trait, malignancy, acute or chronic bleeding, and blood transfusion in the previous 3 months, vitamin B12 or folate deficiency and symptomatic coronary heart disease with haemoglobin (Hb) less than 7 g/dl. The protocol for this study was approved by the Research Ethics Committee (Human) of Universiti Sains Malaysia, and the study was conducted according to the Declaration of Helsinki. All subjects provided signed informed consent before any study procedures were performed.

2.2 Study Protocol

The study was divided into two visits. The first visit served as a screening period to identify candidates who fulfilled the inclusion criteria and who had been maintained on the rHuEPO therapy dose for the past 4 weeks. The personal and medical backgrounds as well as the haemoglobin and folate levels of the candidates were investigated. The screening period continued for 2 weeks after the first screening visit, during which time neither rHuEPO treatment nor iron supplementation were modified. At the end of the screening period, the eligible participants subsequently underwent a 4-week iron washout period; during which intravenous iron treatment was not given. At the end of the iron-free period, the participants returned for a second follow-up, where they underwent blood investigations, including haematological investigations [haemoglobin (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH) and (RET-He)] and iron parameters (serum ferritin and sTfR). Participants whose Hb levels increased by more than 1 g/dl during the iron-free period were omitted from the study. The increase in Hb level indicated that they did not have IDA. The rHuEPO dose remained unchanged throughout the study period.

2.3 Blood Sample Analysis

Peripheral blood was withdrawn and collected in EDTA tubes for a full blood picture consisting of blood counts (Hb concentration, MCV, MCH and red cell distribution width, RET-He) and peripheral blood film examination, and in plain tubes for iron parameters (serum ferritin and sTfR). The peripheral blood counts were measured using an automated haematology analyser (Sysmex XE-2100, Sysmex Corporation,

Kobe, Japan). Serum ferritin analysis was performed on an AxSYM immunochemical automated analyser (Abbot Laboratories, USA) using a commercially available two-site immunoenzymatic (sandwich) assay. The reference values were 29-278 µg/l for males and 5-96 µg/l for females. The sTfR concentration (reference value, 1.0-2.9 µg/ml) was measured using a commercial human sTfR ELISA kit (BioVendor, Czech Republic). The transferrin receptor-ferritin (TfR-F) index was defined as the ratio of sTfR to log ferritin.

Participants were classified as having iron deficiency anaemia (IDA) when they had Hb < 12 g/dl for males and < 11 g/dl for females, MCV < 80 fl, MCH < 27 pg, serum ferritin < 29 µg/l for males and < 5 µg/l for females, ferritin index (sTfR/log ferritin) > 1.5, sTfR > 2.9 mg/l and RET-He < 33 pg [7,8].

2.4 Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Science (SPSS) Software version 12.0 (SPSS, Chicago, IL, USA). Data were presented as mean +/- (SD) and statistical significance was sited at *P* < 0.05. The independent *t*-test was used to compare the mean differences between the two groups, whereas the correlation between RET-He and other haematological and iron parameters were determined by Pearson's Correlation analysis. Kappa coefficient analysis was performed to determine the agreement between RET-He and iron parameters, and a receiver operating characteristics (ROC) curve was plotted to identify the sensitivity and specificity of RET-He in diagnosing IDA.

Table I: Comparison of haematological and iron parameters in IDA defined by RET-He

Parameters	IDA (n=35)	Non-IDA (n=20)	<i>P</i> -value
Hb (g/dl)	9.9 (1.53)	11.1 (1.99)	> 0.05
RET-He (pg)	29.9 (2.29)	34.6 (1.39)	< 0.01*
MCV (fl)	83.9 (5.57)	90.5 (4.98)	< 0.01*
Ferritin (µg/l)	657.4 (597.38)	683.7 (492.31)	< 0.01*
sTfR (mg/l)	1.7 (0.83)	1.5 (0.49)	< 0.01*
TfR-F index	0.66 (0.31)	0.65 (0.31)	< 0.01*

Values presented as mean (SD)

Hb: haemoglobin, RET-He: reticulocyte haemoglobin, MCV: mean cell volume, sTfR: soluble transferrin receptor, TfR-F index: transferrin receptor-ferritin index (sTfR/log ferritin)

*Independent *t*-test (significant level at *P* < 0.05)

3 RESULTS

Fifty-five participants completed this study; 26 were male and 29 were female. Of these participants, 90.0% had anaemia, with 76.3%, 12.7% and 1.8% having hypochromic microcytic, normochromic normocytic and megaloblastic anaemia, respectively.

The RET-He, sTfR and serum ferritin levels revealed 35 (63.6%), 2 (3.6%) and no participants with IDA, respectively. In those participants with IDA (n=35) based on RET-He < 33 pg, the Hb levels were low but not significantly different than the levels in the non-IDA group (n=20). The RET-He, MCV and serum ferritin values were significantly lower, whereas the sTfR and the TfR-F index were significantly higher, in the IDA group than in the non-IDA group (Table I).

Table II and Fig.I show that, at a cut-off value of 30.4 pg, the RET-He parameter had the highest sensitivity, specificity and AUC for detecting IDA, when compared to the other parameters. In addition, after an intravenous iron challenge assessed by RET-He, 38 patients (69.1%) showed an increase in haemoglobin equal to or higher than 1 g/dl from the baseline, with increases to 10.69 (1.9) g/dl; these patients were considered iron deficient (responders). The remaining 17 patients (30.9%) showed no haemoglobin changes or a change of less than 1 g/dl from baseline, with increases to 9.62 (1.52) g/dl; these patients were considered iron replete (non-responders).

Significant positive correlations were also observed between RET-He and MCV (*r* = 0.544, *P* < 0.01) and MCH (*r* = 0.483, *P* < 0.01), whereas sTfR (*r* = -0.575, *P* < 0.01) and the TfR-F index (*r* = -0.497, *P* < 0.01) were significantly inversely correlated with RET-He. However, no significant correlation was found between RET-He and Hb or serum ferritin. Significant agreements were also evident between RET-He and sTfR (*κ* = 0.042) and the TfR-F index (*κ* = 0.036) for the detection of IDA, but no significant agreement was noted between RET-He and serum ferritin.

Table II: Sensitivity, specificity and AUC of the haematological and iron parameters

Parameters	Sensitivity (%)	Specificity (%)	Cut off Value	Positive Predictive Value, PPV (%)	Negative Predictive Value, NPV (%)	Area under ROC curve (AUC)
RET-He (pg)	78.3	92.0	30.4	74.47	50.0	0.864
MCV (fl)	60.9	58.3	83.6	85.7	54.05	0.558
MCH (pg)	73.9	58.3	26.6	53.3	57.12	0.616
sTfR (mg/l)	56.5	58.3	1.4	50.0	59.96	0.518
TfR-F index	56.5	50.0	0.53	66.6	60.0	0.489
Ferritin (µg/l)	IDA not detected					

ROC: receiver operating characteristic, RET-He: reticulocyte haemoglobin, MCV: mean cell volume, MCH: mean cell haemoglobin, sTfR: soluble transferrin receptor, TfR-F index: transferrin receptor-ferritin index (sTfR/log ferritin)

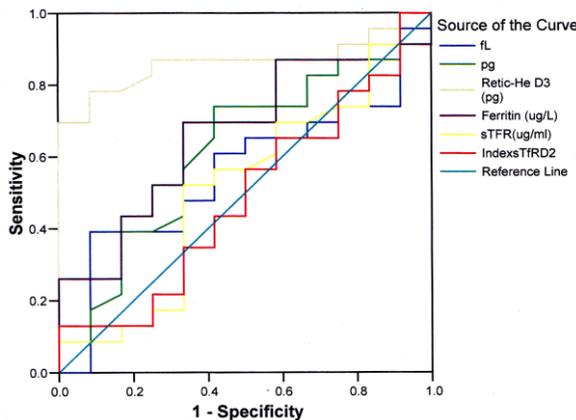


Figure 1: ROC curve for sensitivity and specificity of iron parameters in diagnosing IDA

4 DISCUSSION

In the present study, 90% of the participants were anaemic and 76.3% had hypochromic microcytic anaemia. The RET-He value showed the highest detection of iron deficiency, whereas the normal MCV and MCH values did not allow exclusion of iron deficiency in patients with CKD.

Of the 55 participants with CKD, 63.6% were diagnosed with IDA by RET-He, with a mean of 29.9 (SD = 2.29) pg. The RET-He measures haemoglobin in reticulocytes that are released from bone marrow about 18 to 36 hours before they form into mature erythrocytes. The RET-He parameter therefore offers a direct index of the iron availability. In HD patients undergoing rHuEPO therapy, a direct measurement of iron status provides many advantages compared to indirect measurements, such as serum ferritin and sTfR. For example, measuring the haemoglobin content of the reticulocytes is a direct assessment of the iron used for the synthesis of haemoglobin; therefore, it can

indicate whether sufficient iron is available for erythropoiesis. It can also play the role as an early marker of iron-deficiency erythropoiesis [9]. Thus, RET-He has an appreciable capability to provide high accuracy reading regarding the iron status of HD patients.

Conversely, only 3.6% of the participants were diagnosed with IDA by sTfR, with a mean of 1.67 (SD = 0.83) mg/l, and no participants were diagnosed with IDA by serum ferritin. A study by Skikne in 2011 showed that sTfR levels were significantly higher in patients with IDA or anaemia of chronic disease (ACD) with IDA than in patients with ACD only [10]. Further analysis also showed a higher detection of IDA with the use of sTfR than with ferritin alone, and this was particularly helpful in situations where routine markers provided inconclusive results. However, the value of sTfR as a parameter for assessing erythropoietin therapy response and the adequacy of an iron treatment is considered limited. The sTfR level has been used to assess the adequacy of iron supply to erythrocytes, but it is of less value for assessing the efficacy of rHuEPO therapy. In the present group of patients, the increased erythropoiesis due to the therapy raised the sTfR levels [11], so sTfR failed to identify iron deficiency in these HD patients. The increase in erythropoietic activity resulting from rHuEPO therapy could apparently mask a deficient iron status during rHuEPO treatment.

Measurement of serum ferritin was also not helpful in detecting IDA, as the serum ferritin values were 'inappropriately high' in the participants. Serum ferritin is an acute-phase reactant that is affected by pathological conditions such as chronic renal failure, so its level may be normal or even high even though iron deficiency has developed [4]. The serum

ferritin levels were higher in HD patients with CKD than in non-HD patients and healthy controls. Hence, under conditions where chronic inflammation was induced by renal failure, the measurement of serum ferritin was not a reliable marker for body iron supply [12]. Consequently, although serum ferritin has been used widely, many patients remained undiagnosed and inadequately treated.

In the present study, ROC curve analysis showed that RET-He had the largest area under the curve, followed by MCH, MCV, sTfR and the TfR-F index (Refer to Table II). With 78.3% sensitivity, 92.0% specificity and 0.864 AUC, the RET-He test proved to be more sensitive and specific for detecting IDA when compared to the other tests. This finding agrees with other studies, in which RET-He showed excellent diagnostic performance and was generally superior for diagnosis of IDA among HD patients when compared to traditional parameters, including the percentage of hypochromic red cells with various cut-off level between 29.8-33.0 pg [13]. The sTfR level had a sensitivity of 56.5% and specificity of 58.3%, but despite this potential discriminative value, sTfR tests are not widely available and are more costly than red cell indices, such as MCV and MCH, which are parts of a full blood count and are routinely available. The serum ferritin test had a low sensitivity for detecting IDA among our participants; in fact, the specificity could not be determined because none of the subjects could be diagnosed with IDA based on serum ferritin.

A significant positive correlation was observed between RET-He and both MCV and MCH. This suggests that low MCV and MCH were consistent with an IDA diagnosis by RET-He, although the mean of both indices was higher than the value used for IDA screening in the general population. A significant inverse correlation was also found between RET-He and sTfR and the TfR-F index. A previous study reported that both the sTfR and the TfR-F indices increased whereas that of RET-He fell during iron deficiency [14]. Our finding of a poor agreement between RET-He and sTfR was consistent with the results of another previous study [8] and suggested that the classic iron markers are relatively insensitive for diagnosing IDA.

There were several limitations in this study. This study was considered as a pilot and relatively small study; therefore, the reported findings may not represent the whole population. Small sample population also might not reflect the true prevalence of IDA and the actual differences

in haematological parameters tested between IDA and non-IDA groups. In the post-analytical data interpretation, the ranges of normal values in CKD patients were taken based on the reference range in the product insert and also from other references but not from the local population as it is not yet available.

5 CONCLUSION

In conclusion, iron deficiency anaemia is a common disorder among HD patients with CKD undergoing rHuEPO therapy. However, RET-He can be used as a biomarker for the detection of IDA in these patients. This test is easily available as part of the differential peripheral blood counting using an automated haematology analyser.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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