RETICULOCYTE HAEMOGLOBIN EQUIVALENT (RET-HE) : ESTABLISHING REFERENCE INTERVAL AND COMPARISON OF RET-HE CUT-OFF VALUE IN IRON DEFICIENCY ANAEMIA AND ANAEMIA IN CHRONIC KIDNEY DISEASE

RESEARCH PROJECT REPORT IN PARTIAL FULFILLMENT OF THE DEGREE OF MASTER OF PATHOLOGY (HAEMATOPATHOLOGY)

UNIVERSITY MALAYA

Candidate: Dr Ermi Neiza binti Mohd Sahid (MGA 110002)

Supervisor: Dr Hemalatha Shanmugam (University Malaya)

Dr Mimi Azura Aziz (Hospital Kuala Lumpur)

Department of Pathology

Faculty of Medicine

University Malaya

2016

DISCLAIMER

I declare that this research report was carried out in accordance with the regulation of University Malaya. It is original and as the result of my own research work unless otherwise indicated or acknowledge as reference work.

Name of candidate : Ermi Neiza binti Mohd Sahid

Candidate's ID number : MGA110002

Programme : Master of Pathology (Haematology)

Faculty : Faculty of Medicine

Research Report Title :

Reticulocyte Haemoglobin Equivalent (RET-He): Establishing Reference Interval and Comparison of RET-He Cut-Off Value in Iron Deficiency Anaemia and Anaemia in Chronic Kidney Disease.

Signature of Candidate :

Date : 1st April 2016

CONTENTS **Page Number**

LIST OF TABLES AND FIGURES :

RETICULOCYTE HAEMOGLOBIN EQUIVALENT (RET-HE) : ESTABLISHING REFERENCE INTERVAL AND COMPARISON OF RET-HE VALUE IN IRON DEFICIENCY ANAEMIA AND ANAEMIA IN CHRONIC KIDNEY DISEASE

MS ERMI NEIZA *MBBS*, [1]S HEMALATHA *MPath, FRCPA* and [2]A MIMI AZURA *MPath.*

[1]*Haematology Unit, Department of Pathology, Faculty of Medicine University Malaya, Kuala Lumpur.*
[2]*Haematology Unit, Department of Pathology, Hospital Kuala Lumpur.*

ABSTRACT

Iron deficiency anaemia is the most common cause of anaemia in the general population. The red cell parameters such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and conventional laboratory test such as serum ferritin, transferrin and transferrin saturation are widely used in diagnosis of iron deficiency anaemia. However, in chronic kidney disease patients, complication of anaemia particularly in dialysis patient is not easily defined by those conventional tests. During iron replacement and human recombinant erythropoietin therapy, the target haemoglobin level with appropriate ferritin level need to be standardised as to achieve the optimum result while avoiding the risk of iron over load. The problem with many conventional tests is inaccuracy in measuring iron deficiency anaemia due to interference of value by acute phase protein. Recently, the usage of new reticulocyte parameter, Reticulocyte Haemoglobin Content (CHr) was proven to be able to reflect iron availability especially in chronic kidney disease and recognised as a reliable marker in defining functional iron deficiency. CHr analysis was performed using ADVIA 120 and 2120 (Siemens Healthcare Diagnostics, Tarrytown, New York, U.S.A) haematology analyser based on flowcytometry principle and recently recommended in several guidelines including National Kidney Foundation (NKF) - Kidney Disease Outcomes Quality Initiative (KDOQI) and NICE Guidelines for Anaemia Management in Chronic Kidney Disease (CKD). In British Guidelines for Laboratory Diagnosis of Functional Iron Deficiency, CHr is one of the recommended

tests with a proposed cut-off value of CHr <29 pg for the diagnosis of functional iron deficiency (FID) in patients receiving erythropoiesis-stimulating agents (ESA) therapy.[9] Subsequently, a more novel reticulocyte parameter, Reticulocyte Haemoglobin Equivalent (RET-He) was recently introduced which also uses the principle of flowcytometry in its measurement and has been studied to have parallel function comparable to CHr. RET-He is available in the Sysmex XE and XN series hematology analysers (SYSMEX Corporation, Kobe, Japan). RET-He value <25 pg is suggestive of classical iron deficiency and also predicts FID in those receiving ESA therapy.[1] The aims of our study were to establish reference interval for RET-He and to assess its diagnostic performance in the detection of iron deficiency in anaemia study population as well as detection of functional iron deficiency in chronic kidney disease patients. Validation of reference interval for RET-He was done in 89 normal subjects and the results show values of 27.0 – 35.9 (pg). The ROC analysis of iron deficiency anaemia study revealed that by using a RET-He cut-off level of 26.6 (pg), iron deficiency could be diagnosed with a sensitivity of 90.53% and a specificity of 92.45%. For functional iron deficiency study in chronic kidney patients, our study show a less favourable result with the cut-off level of RET-He obtained was 29.6 (pg) with sensitivity and specificity of 50% and 68.3% respectively. In conclusion, RET-he proved to be a reliable marker of cellular haemoglobin content and can be used to accurately identify iron deficiency.

Keywords: *Reticulocyte Haemoglobin Equivalent (RET-He), reference interval, iron deficiency anaemia, functional iron deficiency.*

INTRODUCTION

Anaemia is the most common global haematological condition that gives rise to major consequences for human health as well as social and economic development. To date, nutritional anaemia is still considered to be among the most important contributing cause of anaemia in the world especially in under developed countries.[2]

The definition of anaemia by World Health Organisation is haemoglobin level of less than 130g/L in men, haemoglobin less than 120g/L in non-pregnant women and haemoglobin of less than 110g/L in pregnant women.[2] Iron deficiency is defined as a condition in which there is no mobilisable iron stores and in which signs of a compromised supply of iron to tissues, including the erythron, are noted. The events of reduced iron intake, lack of intestinal absorption, reduced iron stores and lack of mobilisation to peripheral tissues occurs in continuum. In time, the result of long-term negative iron balance will cause iron deficiency. At this point, iron stores in the form of haemosiderin and ferritin are progressively diminished and no longer meet the needs of normal iron turnover. Subsequently, the supply of iron to the transport protein apotransferrin is compromised and will further result in a decrease in transferrin saturation and an increase in transferrin receptors in the circulation and on the surface of cells, including the erythron.[3]

Once depletion of iron stores is established, the lack of mobilisable iron stores will eventually cause a detectable change in classical laboratory tests, including measurement of haemoglobin (Hb), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), total iron-binding capacity, transferrin saturation, and zinc-erythrocyte protoporphyrin.[3,4] There are many different ways on classifying the types of anaemia by laboratory tests but most of the classical haematological parameters are only able to reflect the iron status over periods of weeks or months as iron deficiency develops slowly in sequential changes of negative iron balance.

Another type of anaemia with high prevalence other than iron deficiency is anaemia secondary to functional iron deficiency. This is a condition whereby incorporation of

iron into erythroid precursors is insufficient despite adequate body iron stores, as defined by the presence of stainable iron in the bone marrow together with a serum ferritin value within normal limits[3,5]. Functional iron deficiency occurs when there is an imbalance between the iron needs for erythropoiesis and the iron supply, with the latter not maintained at sufficient rate for adequate haemoglobinisation of reticulocytes and mature erythrocytes.[3] This concept encompasses the definition of anaemia of chronic disease (ACD), the most frequent anaemia among hospitalized patients. ACD is a hypoproliferative anaemia, defined by low plasma iron concentrations in the presence of high reticuloendothelial iron stores and is seen in chronic inflammatory disorders such as chronic infections, malignancy and autoimmune disorders. Cytokines are implicated in the ACD increasing iron sequestration in the reticuloendothelial system[4], results in hyposideraemia. One of the most important iron regulatory peptide recognised in ACD is hepcidin, a 25-amino acid peptide synthesized in the liver[6]. Hepcidin is upregulated in the setting of chronic inflammation and cancer; resulting in its increased synthesis in the liver stimulated by cytokines of which interleukin-6 (IL-6) is the most important. By degrading ferroportin, hepcidin decreases iron absorption from the gastrointestinal tract and decreases the accessibility of stored iron from macrophages. This results in limited availability of iron for erythroid progenitor cells and iron restricted erythropoiesis. A particular case of ACD is represented by anaemia of chronic kidney disease (CKD)[5,7].

In iron deficiency anaemia (IDA) iron supply depends on the quantity of iron storage in the body, while in functional iron deficiency (iron restricted erythropoiesis) supply depends on the rate of mobilization of iron from the stores. The diagnosis of iron deficiency or functional iron deficiency is particularly challenging in patients with acute or chronic inflammatory conditions because most of the biochemical markers for iron metabolism such as serum ferritin and transferrin saturation are affected by the presence acute phase protein. These conventional tests are not reliable to be used for evaluation of iron availability in this situation and the published sensitivity and specificity of these markers are highly variable[8]. Transferrin, on the other hand is a negative acute phase reactant which fluctuates due to the diurnal variation of

serum iron and is affected by nutritional status, leading to a lack of sensitivity and specificity in assessing iron's availability.[9]

The established gold standard in determining the accurate iron status is assessment of stainable marrow iron[9]. This is not convenient to be done on a frequent basis for evaluation of iron status. More recently, many automated analysers have introduced new red cell parameters for early detection of iron deficiency. One of the parameter is reticulocyte indices that analyse the maturity characteristics of reticulocytes by measuring the fluorescence intensity of the cell. Other parameters evaluate the haemoglobin content of reticulocytes such as reticulocyte haemoglobin content (CHr) and reticulocyte haemoglobin equivalent (RET-He), which reflects the amount of iron available for haemoglobin production in bone marrow[10].

CHr is a parameter available on the ADVIA (Siemens Healthcare Diagnostics, Tarrytown, New York, U.S.A) automated haematology analyser. It was approved for clinical use on Advia analysers in the United States by United States Food and Drug Administration (FDA) in 1997. CHr was the first reticulocyte parameter that evidently had better predictive value for iron depletion among patients undergoing marrow examination compared to MCV, serum ferritin, or transferrin saturation[11]. CHr is determined from measurements of light scatter at two different angles following isovolumetric sphering of oxazine 750-stained reticulocytes. The volume and hemoglobin concentration of individual reticulocytes are independently measured from the amount of light scattered at two different angles.[12]

The newer parameter reticulocytes haemoglobin equivalent (RET-He) which is generated by all Sysmex (SYSMEX Corporation, Kobe, Japan) haematology analysers also has been recognized to have parallel function comparable to CHr. Ret-He reflects a direct assessment of the incorporation of iron into erythrocyte haemoglobin and can estimate recent functional availability of iron into the erythron[10]. RET-He is measured on the basis of automated fluorescent flow cytometry using a polymethine dye in the reticulocyte channel. It also measures the mean value of the forward light scatter intensity of mature red blood cells and reticulocytes[13]. At this moment, only Ret-He is useful as alternative to CHr for

evaluating iron availability and the need for iron supplementation during ESA treatment of CKD patients undergoing dialysis. A comparison study was done by Brugnara et al (2006) and Maconi et al (2009) between CHr and Ret-He methods in assessment of iron deficient states. Both studies shows a very good level of agreement between the cellular haemoglobin measurements performed with the Sysmex XE 2100 and those performed with the Siemens ADVIA instrument. Normal range values for the reticulocyte and red cell haemoglobin content parameters are superimposable between the two different methods and found to be concordant to each other[10,14].

Apart from assessment of functional iron deficiency and response to iron supplementation during erythropoietin stimulation agent (ESA) therapy, measurement of Ret-He is also useful in providing information in the diagnosis of iron deficiency and iron-restricted erythropoiesis.

Currently, CHr as reticulocyte parameter was already included in few guidelines to distinguish functional iron deficiency such as European Best Practice Guideline.[15] Ret-He, although is still being evaluated for standardised use in certain laboratory was found to show almost similar result compared to CHr. Therefore, the role of Ret-He in diagnosing disturbances of iron metabolism and assessing response to iron supplementation therapy particularly in chronic kidney disease is very promising and useful.

MATERIAL AND METHODS

Samples

This is a retrospective study analysing complete blood count (CBC) samples performed on the XN series Sysmex haematology analysers in the Haematology Unit Department of Pathology University Malaya Medical Centre. 86 subjects for normal control (male 40 and female 46) were selected from sequential request forms within a period of April 2015 to December 2015. The criteria for normal adult subjects are red cells parameters within the normal range for haemoglobin level (Hb), MCV, MCH and normal iron profile defined by normal serum ferritin level (Hb ≥ 120 g/L in women, Hb ≥ 130 g/L in men, MCV ≥ 80 fL, MCH ≥ 27 pg and serum ferritin ≥ 30 - 200 ug/L). This study has been approved by the Medical Ethics Committee of UMMC (Ref. no. 20161-2008).

222 subjects were selected for anaemia group according to WHO criteria of Hb ≤ 120 g/L in women and Hb ≤ 130g/L in men. Out of these 222 anaemic patients, 163 subjects were categorised as iron deficient by the cut-off level of serum ferritin less than 30 ug/L based on RCPA Guidelines for Iron Studies Standardised Reporting Protocol 2013. The remaining of 59 anaemic patients were selected through the pre-existing diagnosis of Chronic Kidney Disease with anaemia, based on prior medical records (haemoglobin ≤ 120 g/L in women, haemoglobin ≤ 130 g/L in men).

We considered borderline ferritin concentration (30 - 100 ug/L for adults) in anaemia chronic kidney disease as indication of iron deficiency (RCPA Guidelines for Iron Studies Standardised Reporting Protocol 2013).[16] For definition of ferritin level in functional iron deficiency, we used serum ferritin level of >100 ug/L and < 800ug/L as recommended by the guideline for the laboratory diagnosis of functional iron deficiency.[1]

The elevation in serum ferritin is this subgroup population is due to inflammation or acute phase protein response. The confirmation of inflammatory phase in these cases ideally should be done by assessment of CRP level. However due to study

limitation, we only use clinical presentation and established diagnosis of chronic kidney disease to select the subgroup population of functional iron deficiency. All of these request forms were received from various wards and outpatient clinics in University Malaya Medical Centre.

Measurement of parameters

All of the sample analysis in this study was conducted at Division of Laboratory Medicine, University Malaya Medical Centre. Samples for full blood count parameters and RET-He were analysed by Sysmex XN series analysers (SYSMEX Corporation, Kobe, Japan). RET-He is measured on the basis of automated fluorescent flow cytometry using a polymethine dye in the reticulocyte channel. It also measures the mean value of the forward light scatter intensity of mature red blood cells and reticulocytes. The serum ferritin was analysed by chemiluminescence technique using Advia Centaur XP analyser (Siemens Healthcare Diagnostics, Tarrytown, New York, U.S.A) while serum iron and serum transferrin were assayed spectrophotometrically by Dimension Vista 1500 analyser (Siemens Healthcare Diagnostics, Tarrytown, New York, U.S.A).

Statistical Analyses

Statistical software package for Social Sciences version 20.0 and MedCalc software version 16.2 were applied for statistical analysis of the results. Reference interval value calculation for RET-He was performed on normal subjects and Kolmogorov-Smirnoff test was applied to verify the normality of these data. Meanwhile, descriptive analysis to obtained values of mean and standard deviation were calculated for Haemoglobin, MCV, MCH, Serum iron, Serum Ferritin, Serum transferrin, Transferrin saturation and RET-He for all groups.

Receiver operating characteristic (ROC) curve analysis was utilised to illustrate the diagnostic performance of RET-He with the ROC curves.

Independent samples *t*-test was performed to detect statistical deviation between the groups of patients; P values < 0.05 were considered to be statistically significant.

RESULTS

Descriptive analysis of red cell parameters (Hb, MCV, MCH, RET-He, Serum Ferritin, Serum Iron, Serum Transferrin and Transferrin Saturation) for each group of normal subjects, iron deficiency anaemia and anaemia in chronic kidney disease are shown in Table 1, Table 2 and Table 3 respectively. Mean and standard deviation values for each parameter are calculated.

In normal subjects, RET-He level was 31.4 ± 2.2 (pg), while chronic kidney disease group shows RET-He level of 30.3 ± 3.8 (pg). Iron deficiency anaemia group has much lower RET-He value of 19.9 ± 5.0 (pg).

Table 1. Descriptive haematological data in normal subjects

	Hb (g/L)	MCV(fL)	MCH (pg)	RET-He (pg)	Se Ferritin (ug/L)	Se Iron (umol/L)	Transferrin (g/L)	TSat %
N	86	86	86	86	86	86	86	86
Mean	13.921	85.680	28.584	31.422	90.760	18.817	2.733	23.462
95% CI	13.574 to 14.268	84.729 to 86.631	28.211 to 28.957	30.944 to 31.900	61.774 to 119.747	16.732 to 20.903	2.614 to 2.851	21.398 to 25.525
SD	1.6172	4.4364	1.7401	2.2278	135.2000	9.7279	0.5519	9.6257
Minimum	11.400	73.200	23.800	24.100	5.100	6.700	2.000	8.000
Maximum	18.000	95.100	33.100	36.900	898.800	82.200	5.000	84.000

Table 2. Descriptive haematological data in iron deficiency anaemia group

	Hb (g/L)	MCV(fL)	MCH (pg)	RET-He (pg)	Se Ferritin (ug/L)	Se Iron (umol/L)	Transferrin (g/L)	TSat %
N	163	163	163	163	163	163	163	163
Mean	8.921	68.021	21.252	19.929	11.605	6.064	3.669	6.687
95% CI	8.636 to 9.207	66.395 to 69.648	20.319 to 22.184	19.157 to 20.701	7.402 to 15.808	4.287 to 7.842	3.509 to 3.828	4.884 to 8.491

SD	1.8461	10.5167	6.0310	4.9919	27.1749	11.4900	1.0331	11.6595
Minimum	2.800	7.300	12.900	4.200	0.500	0.300	1.800	1.000
Maximum	12.900	91.800	81.800	36.400	275.000	77.900	11.300	89.000

Table 3. Descriptive haematological data anaemia in chronic kidney disease group

	Hb (g/L)	MCV(fL)	MCH (pg)	RET-He (pg)	Se Ferritin (ug /L)	Se Iron (umol/L)	Transferrin (g/L)	TSat %
N	59	59	59	59	59	59	59	59
Mean	9.725	85.839	29.988	30.344	1145.181	14.000	1.944	30.237
95% CI	9.102 to 10.349	82.875 to 88.803	27.633 to 32.344	29.353 to 31.335	700.922 to 1589.440	11.134 to 16.866	1.762 to 2.126	24.333 to 36.142
SD	2.3928	11.3734	9.0388	3.8019	1704.7448	10.9966	0.6996	22.6566
Minimum	4.500	36.300	20.300	15.100	7.400	1.600	0.200	3.000
Maximum	16.600	106.500	92.400	36.200	8699.000	57.000	4.600	87.000

Reference range

A total of 86 healthy subjects (40 males, 46 females) were evaluated for calculation of the normal ranges for RET-He. They were all adult patients with mean age of 40 years old. The reference values obtained for RET-He in the healthy subjects ranged from 27.0 pg – 35.9 pg. We validated our reference interval against the published and established reference from a previous study. The reference established reference value ranges from 28.61 – 36.33 pg.[4] We used the method for validation of reference interval due to the small sample size of normal subjects in this study. Our reference interval values were determined at 95 percentile following the guidelines by Clinical and Laboratory Standards Institute (C28-A2) with 90% confidence interval. The data showed normal distribution and calculated using robust method. An independent test was applied to check for any significant difference between male and female subgroups.

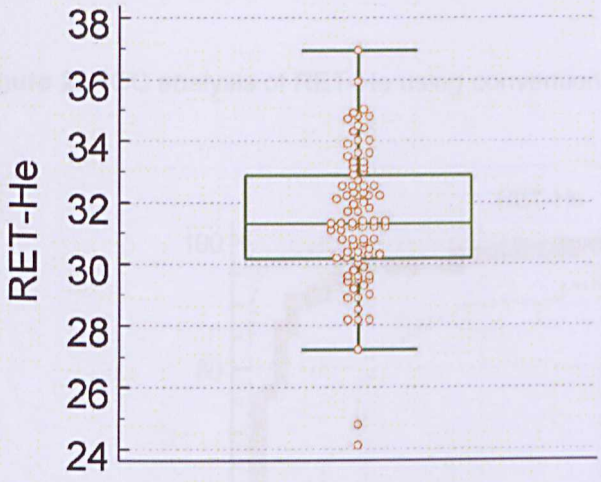


Figure 1. Box-and-Whisker plot of reference interval in 86 normal subjects.

Figure 1 shows Box-and-Whisker plot of reference interval in 86 normal subjects with mean value of RET-He of 31.4 pg and range values of 27.0 pg – 35.9 pg.

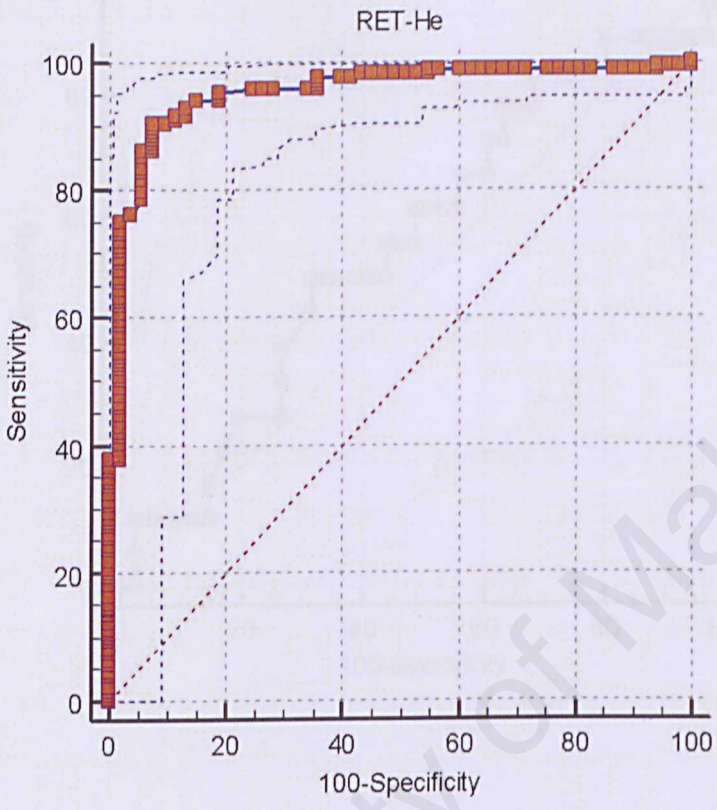
Cut-off level for diagnosis of Iron Deficiency

In this study, the objective is to determine the diagnostic performance of the RET-He parameter against the conventional diagnostic tests for Iron Deficiency.

We include the following conventional criteria for presence of iron deficiency in ROC curve generation: Hb ≤ 130g/L in men, Hb ≤ 120g/L in women, TSat ≤ 20% and Serum Ferritin ˂30ug/L. As for Iron Deficiency Anaemia in established chronic kidney disease, the criteria used for ROC generation are: Hb ≤ 110 g/L, TSat ≤ 20% and Serum Ferritin ˂100ug/L.

169 of the 222 subjects were categorised as having iron deficiency. The data from these cases were analysed to determine the cut off level of RET He. ROC curve analysis revealed that by using a RET-He cut-off level of 26.6 (pg), iron deficiency could be diagnosed with a sensitivity of 90.53% and a specificity of 92.45% (Figure 2). The area under the curve was 0.952 (P<0.0001).

Figure 2. ROC analysis of RET-He using conventional criteria of Iron Deficiency

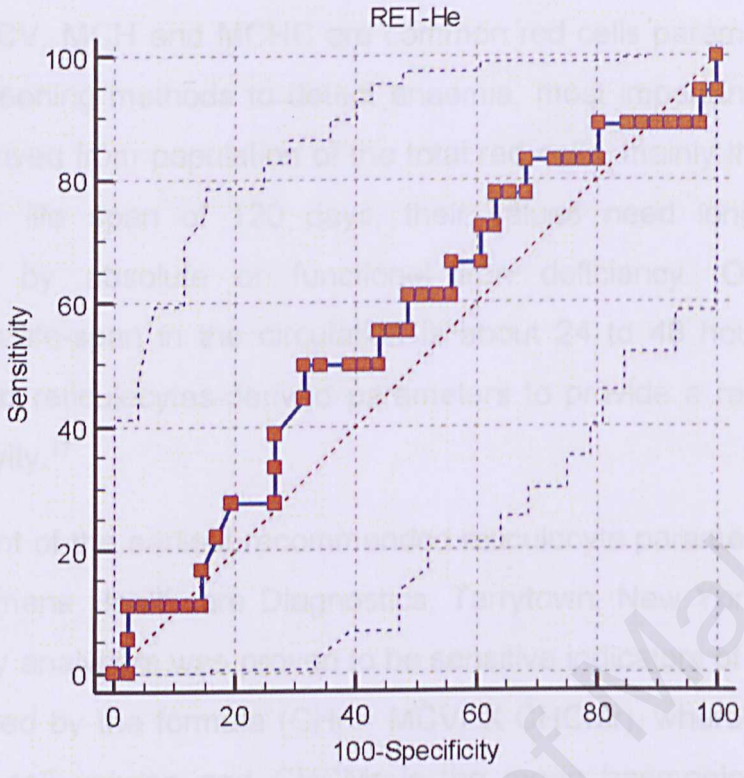


Functional Iron Deficiency

The results from 59 patients with diagnosis of chronic kidney disease with anaemia were analysed and 18 patients found to fulfil the definition of Functional Iron deficiency. The criteria used are: Hb ≤ 11 g/dL, TSat ≤ 20% with Serum Ferritin of 100 – 800 ug/L.

ROC analysis was carried out to evaluate the capability to of RET-He to diagnose functional iron deficiency in Chronic Kidney Disease patients (Figure 3). A similar cut-off level of 26.6 (pg) as per cut-off level in iron deficiency was used and the diagnostic performance of RET-He show sensitivity of 11.1% and a specificity of 87.8%. When the cut-off level of RET-He was set at 29.6 (pg), sensitivity and specificity were 50% and 68.3% respectively. The area under the curve was 0.562 ($P<0.0001$).

Figure 3. ROC analysis of RET-He for Functional Iron Deficiency



DISCUSSION

Hb level, MCV, MCH and MCHC are common red cells parameters that are still be used as screening methods to detect anaemia, most importantly IDA. However, as they are derived from population of the total red cells, mainly the mature population which have life span of 120 days, their values need longer time to change significantly by absolute or functional iron deficiency. On the other hand, reticulocytes life-span in the circulation is about 24 to 48 hour; therefore give an advantage to reticulocytes-derived parameters to provide a real-time view of bone marrow activity.[17]

Measurement of the earliest recommended reticulocyte parameter, CHr, obtained on ADVIA (Siemens Healthcare Diagnostics, Tarrytown, New York, U.S.A) automated haematology analysers was proven to be sensitive indicators of early iron deficiency. CHr is defined by the formula (CHr = MCVr X CHCMr), wherein MCVr is the mean reticulocyte cell volume and CHCMr is the mean haemoglobin concentration of reticulocytes, which is obtained by an optical cell-by-cell haemoglobin measurement.[6]

The aim of this study is to establish the reference interval for newer reticulocyte parameter, namely RET-He which available on Sysmex XE and XN-series analysers (SYSMEX Corporation, Kobe, Japan) for our own laboratory use at the Division of Laboratory Medicine University Malaya Medical Centre (UMMC). Ideally, a minimum of 120 samples from each group (male and female) is advised to establish the reference range as per the Clinical and Laboratory Standards Institute (CSLI) guideline (C28-A2). This has the advantage of allowing 90% confidence limits to be computed non-parametrically for each reference limit. Moreover, if separate intervals were needed for different subclasses (by sex or age-class, for example), each such interval should be based on the recommended number (at least 120) of reference observations. However, CLSI recommends that whatever number of values are obtained, the data should still be analysed by the non-parametric method and reported by percentiles appropriate to the number of values acquired.

For fewer samples of 86 normal subjects (40 male and 46 female), we use CLSI guideline (C28-A3) to validate a previously established reference interval by performing robust method. In this study, it was difficult to obtain appropriate group of targeted subjects in sufficient numbers. The distribution of data for reference interval in RET-He shows nearly similar values for male and female with P value of 0.0552 indicating no statistically significant difference between males and females healthy population in this study (Figure 4). The reference interval obtained in this study (27.0 – 35.9 pg) show mild variation from the previous equated published study by Brugnara et al. 2006 (28.61 – 36.33 pg).[10] This may be due to pre-analytical variables such as age group or more importantly the different populations selected by different institutes. These variables create different reference intervals; although, the differences are quite small. A bigger sample size for healthy population will also provide a more satisfying result due to more power of the sample size studied.

In this study, the optimal cut-off obtained for RET-He in 222 subjects for diagnosis of iron deficiency anaemia in general population is 26.6 (pg) with very good sensitivity of 90.53% and specificity of 92.46%. This value was slightly higher than the proposed cut-off value for iron deficiency in a study done by Canals et al. (2005) with cut-off point of 25 pg, specificity of 0.81 and a sensitivity of 0.76.[18] The general results deducted from IDA group are parallel to other conventional tests by reduction in values as reflection of the state of iron depletion (low ferritin), low iron availability (low MCH) and low iron availability for erythropoiesis (low RET-He).

The overall situation is different in renal anaemia where in some patients, functional iron deficiency takes place. Functional iron deficiency happens when there is failure to release iron rapidly enough to keep pace with the demands of the bone marrow for erythropoiesis which can happen during the use of erythropoietin-stimulating agents (ESAs) that rapidly deplete circulating iron. Any concurrent inflammation can also cause FID by the release of hepcidin, which decrease the availability of transferrin-bound iron in the blood. Hence, conventional methods of estimating iron stores such as serum ferritin and transferrin saturation are inadequate to evaluate functional iron deficiency.

In our study, we tried to show the clinical utility of the RET-He parameter as an index for iron status in the patient with renal anaemia. However, with study sample of 59 chronic kidney disease patients, we found that the diagnostic performance of RET-He, when compared with conventional parameters was not as impressive. We obtained a much smaller ROC area under the curve of 0.562 ($P<0.0001$) with cut-off RET-He level of 29.6 (pg). More or less similar results are obtained in a study by Brugnara et al. (2006) with a larger sample size of 1500 patients.

Overall factors that may influence our results are mainly the patient factors. Due to clinical limitation, we did not stratify chronic kidney disease patients based on whether they are undergoing routine haemodialysis or exclusion of patient on Recombinant Human Erythropoietin (rHuEPO). Small sample size also influences the outcome of our study. Furthermore, in renal patient, the presence of inflammation and uraemia makes this diagnosis particularly challenging. Evaluation of serum CRP levels might be useful in excluding patients with confirmed inflammatory disease or CKD patients with underlying inflammatory conditions.

In conclusion, RET-He is considered as a useful indicator in detecting iron deficiency in general population and particularly not useful to be used as single independent value for renal patient. Besides providing easy access of using peripheral blood for measurement, this parameter can also be added to existing reticulocyte channel in automated Sysmex blood analyser with requirement of additional reagent. There is a huge potential in using RET-he as a tool in monitoring responses to intravenous iron treatment in dialysis patient as already established by the other reticulocytes parameter, CHr. The study of RET-He in diagnosis of functional iron deficiency particularly in renal patients need to be improvised and specific in term of sample study and patients criteria to achieve better outcome in the future.

ACKNOWLEDGEMENT

I would like to thank all the people who contributed in some way to the work described in this thesis. First and foremost, I thank my academic advisors, Dr Hemalatha Shanmugam from Pathology Department University of Malaya and Dr Mimi Azura Aziz from Hematology Unit Hospital Kuala Lumpur. Throughout the period of my study, their constant advise, insight and monitoring had made this research work possible.

I would also like to express my gratitude to Associate Prof. Dr. Nazarina Binti Abdul Rahman, as the Head Department of Pathology, University Malaya, for allowing this study to be carried out.

Special thanks go to my family who always support and encourage me in the process to complete this study.

REFERENCES

1. D. Wayne Thomas, Rod F. Hinchliffe, Carol Briggs, Iain C. Macdougall, Tim Littlewood, Ivor Cavill. Guideline for the laboratory diagnosis of functional iron deficiency. British Journal of Hematology. 2013; 161: 639-648.

2. World Health Organisation. Iron deficiency anaemia: assessment, prevention and control: a guide for programme managers. Geneva: World Health Organisation; c2001. 114p.

3. Cavill, I., Macdougall, I.C. Functional iron deficiency. Blood. 1993; 82, 1377.

4. G Weiss, LT. Goodnough. Anaemia of Chronic Disease. N Engl J Med. 2005; 352: 1011-23.

5. Buttarello, M., Pajola, R., Novello, E., et al. Diagnosis of iron deficiency in patients undergoing hemodialysis. American Journal of Clinical Pathology. 2010; 133: 949–954.

6. Goodnough, L. T., Nemeth, E., Ganz, T. Detection, evaluation, and management of iron-restricted erythropoiesis. Blood Journal. 2010; 116: 4754-4761.

7. E. Urrechaga, L. Borque, and J. F. Escanero. Assessing iron status in CKD patients: new Laboratory parameters in Chronic Kidney Disease. 1st ed. InTech. Zagreb, Croatia; 2012. pp. 225–250,

8. Alan E. Mast, Morey A. Blinder, Dennis J. Dietzen. Reticulocyte hemoglobin content. Am. J. Hematol. 2008; 83: 307–310.

9. Fishbane, S., Kowalski, EA., Imbriano, LJ., et al. The evaluation of iron status in hemodialysis patients. J Am Soc Nephrol. 1996; 7: 2654-2657.

10. Brugnara, C., Schiller, B. & Moran, J. Reticulocyte haemoglobin content (Ret He) and assessment of iron-deficient states. Clinical and Laboratory Haematology. 2006; 28: 303–308.

11. Mast, A.E., Blinder, M.A., Lu, Q., Flax, S. & Dietzen, D.J. Clinical utility of the reticulocyte hemoglobin content in the diagnosis of iron deficiency. Blood. 2012; 99: 1489–1491.

12. Mohandas, N., Kim, YR., Tycko, DH., et al. Accurate and independent measurement
of volume and hemoglobin concentration of individual red cells by laser
light scattering. Blood. 1986; 68:506–513.

13. Thomas, L., Franck, S., Messinger, M., et al. Reticulocyte hemoglobin measurement – comparison of two methods in the diagnosis of iron-restricted erythropoiesis. Clinical Chemistry Laboratory Medicine. 2005; 43: 1193–1202.

14. M. Maconi, L. Cavalca, P. Danise, et al. Erythrocyte and reticulocyte indices in iron deficiency in chronic kidney disease: comparison of two methods. (2009)The Scandinavian Journal of Clinical & Laboratory Investigation. 2009; 69: 365–370.

15. L. Francesco, A. Pedro, B. Peter, et al. European Best Practice Guidelines for the Management of Anaemia in Patients with Chronic Renal Failure. Nephrol Dial Transplant. 2004; 19 [Suppl 2]: ii2–ii5.

16. The Royal College of Pathologists of Australasia (RCPA). Iron studies standardised reporting protocol. 1st ed. RCPA; c2013

17. Urrechaga, E. The new mature red cell parameter, low haemoglobin density, of the Beckman Coulter LH750: clinical utility in the diagnosis of iron deficiency. International Journal of Laboratory Haematology. 2010; 32: 144–150.

18. Canals, C., Remacha, A.F., Sarda, M.P., et al. Clinical utility of the new Sysmex XE2100 parameter–reticulocyte hemoglobin equivalent in the diagnosis of anemia. Hematologica. 2005; 90: 1133–1134.

19. Brugnara, C. Iron deficiency and erythropoiesis: new diagnostic approaches. Clinical Chemistry. 2003; 49: 1573–1578.

20. Buttarello, M., Pajola, R., Novello, E., et al. Diagnosis of iron deficiency in patients undergoing hemodialysis. American Journal of Clinical Pathology. 2010; 133: 949–954.

21. Urrechaga, E. Clinical utility of the new Beckman-Coulter parameter red blood cell size factor in the study of erythropoiesis. International Journal of Laboratory Haematology. 2009; 31: 623–629.

22. Urrechaga, E., Borque, L., Escanero, J.F. Erythrocyte and reticulocyte indices in the assessment of erythropoiesis activity and iron availibility. International Journal of Laboratory Medicine. 2013; 47: 1411–1416.

APPENDIXES

Reference interval for RET-He from healthy subjects (male and female)

Sample size	86
Lowest value	24.1000
Highest value	36.9000
Arithmetic mean	31.4221
Median	31.3000
Standard deviation	2.2278
Coefficient of Skewness	-0.3934 (P=0.1265)
Coefficient of Kurtosis	1.1236 (P=0.0678)
Kolmogorov-Smirnov test[a] for Normal distribution	D=0.0505 accept Normality (P>0.10)

[a] Lilliefors significance correction

Suspected outliers[a]

None

[a] Reed, 1971.

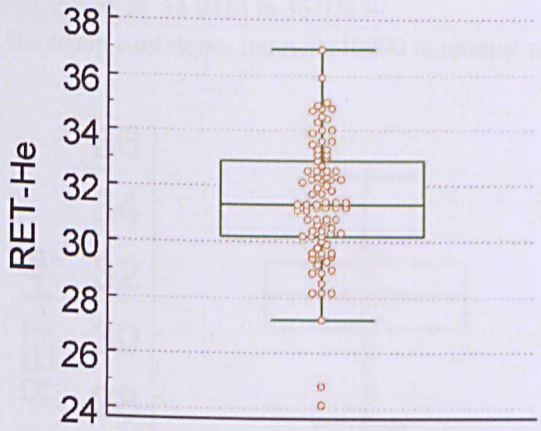
95% Reference interval, Double-sided

A. Method based on Normal distribution	
Lower limit	27.0558
90% CI	26.3687 to 27.7428
Upper limit	35.7884
90% CI	35.1013 to 36.4755

B. Non-parametric percentile method (CLSI C28-A3)	
Lower limit	25.2200
90% CI	
Upper limit	35.7425
90% CI	

C. Robust method (CLSI C28-A3)	
Lower limit	27.0418
90% CI[a]	26.3248 to 27.7927
Upper limit	35.9631
90% CI[a]	35.1141 to 36.7425

[a] Bootstrap confidence interval (10000 iterations; random number seed: 978).



Reference interval for RET-He in female

Sample size	46
Lowest value	24.8000
Highest value	35.9000
Arithmetic mean	30.9935
Median	31.2000
Standard deviation	1.9631
Coefficient of Skewness	-0.3847 (P=0.2576)
Coefficient of Kurtosis	1.7278 (P=0.0487)
Kolmogorov-Smirnov test[a] for Normal distribution	D=0.1039 accept Normality (P>0.10)

[a] Lilliefors significance correction

Suspected outliers[a]

None

[a] Reed, 1971.

95% Reference interval, Double-sided

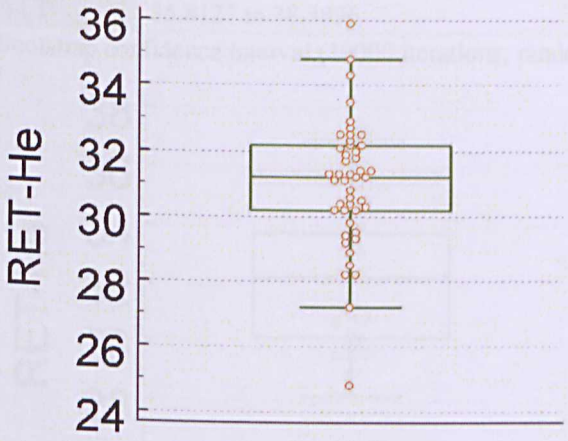
A. Method based on Normal distribution	
Lower limit	27.1458
90% CI	26.3151 to 27.9765
Upper limit	34.8412
90% CI	34.0105 to 35.6719

B. Non-parametric percentile method (CLSI C28-A3)	
Lower limit	25.2200
90% CI	
Upper limit	35.7075
90% CI	

C. Robust method (CLSI C28-A3)	
Lower limit	27.0579
90% CI[a]	26.1298 to 28.1355
Upper limit	35.0788

90% CI[a]	34.0414 to 36.0282

[a] Bootstrap confidence interval (10000 iterations; random number seed: 978).



Reference interval of RET-He in male

Sample size	40
Lowest value	24.1000
Highest value	36.9000
Arithmetic mean	31.9150
Median	32.2000
Standard deviation	2.4298
Coefficient of Skewness	-0.6670 (P=0.0748)
Coefficient of Kurtosis	1.3210 (P=0.1072)
Kolmogorov-Smirnov test[a] for Normal distribution	D=0.0974 accept Normality (P>0.10)

[a] Lilliefors significance correction

Suspected outliers[a]

None

[a] Reed, 1971.

95% Reference interval, Double-sided

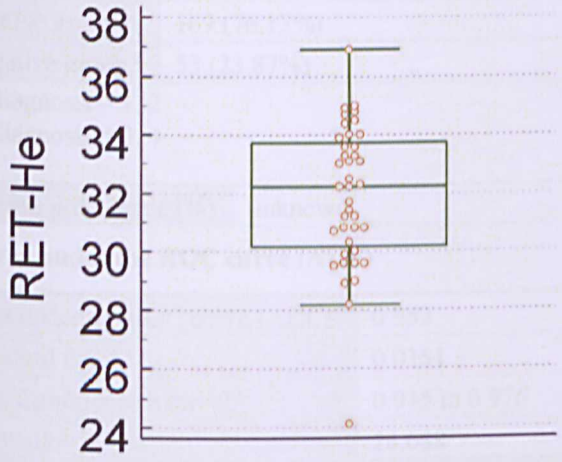
A. Method based on Normal distribution	
Lower limit	27.1527
90% CI	26.0489 to 28.2565
Upper limit	36.6773
90% CI	35.5735 to 37.7811

B. Non-parametric percentile method (CLSI C28-A3)	
Lower limit	24.2025
90% CI	
Upper limit	36.8525
90% CI	

C. Robust method (CLSI C28-A3)

Lower limit	27.0661
90% CI[a]	25.7712 to 28.4042
Upper limit	37.0900
90% CI[a]	35.8127 to 38.3896

[a] Bootstrap confidence interval (10000 iterations; random number seed: 978).



Independent T-test between female reference interval and male reference interval

Sample 1	
Variable	RET-He (male)
Sample 2	
Variable	RET-He (female)

	Sample 1	Sample 2
Sample size	40	46
Arithmetic mean	31.9150	30.9935
95% CI for the mean	31.1379 to 32.6921	30.4105 to 31.5765
Variance	5.9039	3.8540
Standard deviation	2.4298	1.9631
Standard error of the mean	0.3842	0.2895

F-test for equal variances	P = 0.168

T-test (assuming equal variances)

Difference	-0.9215
Standard Error	0.4739
95% CI of difference	-1.8640 to 0.02095
Test statistic t	-1.944
Degrees of Freedom (DF)	84
Two-tailed probability	P = 0.0552

ROC analysis in determining sensitivity and specificity of RET-He in diagnosis of Iron Deficiency anaemia

Sample size	222
Positive group [a]	169 (76.13%)
Negative group [b]	53 (23.87%)

[a] diagnosis = 1, 2
[b] diagnosis = 3, 4

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	0.952
Standard Error [a]	0.0161
95% Confidence interval [b]	0.915 to 0.976
z statistic	28.088
Significance level P (Area=0.5)	<0.0001

[a] DeLong et al., 1988
[b] Binomial exact

Youden index

Youden index J	0.8299
95% Confidence interval [a]	0.7212 to 0.8901
Associated criterion	≤26.6
95% Confidence interval [a]	≤25.6 to ≤28.676817384
Sensitivity	90.53
Specificity	92.45

[a] BC_a bootstrap confidence interval (1000 iterations; random number seed: 978).

Criterion values and coordinates of the ROC curve [Hide]

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
<4.2	0.00	0.0 - 2.2	100.00	93.3 - 100.0		1.00
≤4.2	0.59	0.01 - 3.3	100.00	93.3 - 100.0		0.99
≤10.7	1.18	0.1 - 4.2	100.00	93.3 - 100.0		0.99
≤11.5	1.78	0.4 - 5.1	100.00	93.3 - 100.0		0.98
≤11.7	2.37	0.6 - 5.9	100.00	93.3 - 100.0		0.98
≤11.8	4.14	1.7 - 8.3	100.00	93.3 - 100.0		0.96
≤12	4.73	2.1 - 9.1	100.00	93.3 - 100.0		0.95

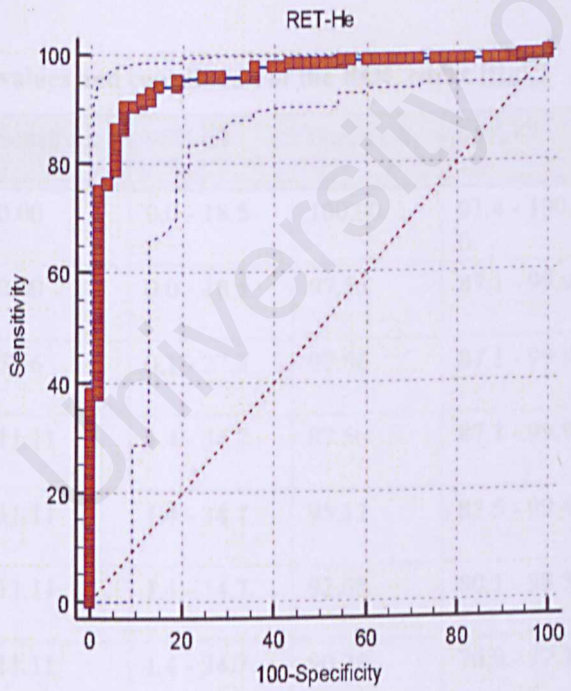
≤12.1	5.92	2.9 - 10.6	100.00	93.3 - 100.0		0.94
≤12.3	6.51	3.3 - 11.3	100.00	93.3 - 100.0		0.93
≤12.6	7.69	4.2 - 12.8	100.00	93.3 - 100.0		0.92
≤12.8	8.28	4.6 - 13.5	100.00	93.3 - 100.0		0.92
≤12.9	9.47	5.5 - 14.9	100.00	93.3 - 100.0		0.91
≤13.1	10.06	6.0 - 15.6	100.00	93.3 - 100.0		0.90
≤13.2	10.65	6.4 - 16.3	100.00	93.3 - 100.0		0.89
≤13.5	11.24	6.9 - 17.0	100.00	93.3 - 100.0		0.89
≤13.6	12.43	7.9 - 18.4	100.00	93.3 - 100.0		0.88
≤13.8	13.61	8.8 - 19.7	100.00	93.3 - 100.0		0.86
≤13.9	14.20	9.3 - 20.4	100.00	93.3 - 100.0		0.86
≤14	14.79	9.8 - 21.1	100.00	93.3 - 100.0		0.85
≤14.1	15.38	10.3 - 21.7	100.00	93.3 - 100.0		0.85
≤14.3	16.57	11.3 - 23.0	100.00	93.3 - 100.0		0.83
≤14.4	18.34	12.8 - 25.0	100.00	93.3 - 100.0		0.82
≤14.5	19.53	13.8 - 26.3	100.00	93.3 - 100.0		0.80
≤15.1	20.12	14.4 - 27.0	100.00	93.3 - 100.0		0.80
≤15.3	20.71	14.9 - 27.6	100.00	93.3 - 100.0		0.79
≤15.4	21.89	15.9 - 28.9	100.00	93.3 - 100.0		0.78
≤15.7	23.67	17.5 - 30.8	100.00	93.3 - 100.0		0.76
≤15.9	24.26	18.0 - 31.4	100.00	93.3 - 100.0		0.76
≤16.3	25.44	19.1 - 32.7	100.00	93.3 - 100.0		0.75
≤16.4	26.04	19.6 - 33.3	100.00	93.3 - 100.0		0.74
≤16.6	27.22	20.7 - 34.6	100.00	93.3 - 100.0		0.73
≤16.8	27.81	21.2 - 35.2	100.00	93.3 - 100.0		0.72
≤16.9	28.40	21.7 - 35.8	100.00	93.3 - 100.		0.72

				0		
≤17.1	29.59	22.8 - 37.1	100.00	93.3 - 100.0		0.70
≤17.2	30.77	23.9 - 38.3	100.00	93.3 - 100.0		0.69
≤17.3	31.36	24.5 - 38.9	100.00	93.3 - 100.0		0.69
≤18	32.54	25.5 - 40.2	100.00	93.3 - 100.0		0.67
≤18.2	33.73	26.6 - 41.4	100.00	93.3 - 100.0		0.66
≤18.3	34.32	27.2 - 42.0	100.00	93.3 - 100.0		0.66
≤18.4	34.91	27.8 - 42.6	100.00	93.3 - 100.0		0.65
≤18.5	36.09	28.9 - 43.8	100.00	93.3 - 100.0		0.64
≤18.6	36.69	29.4 - 44.4	100.00	93.3 - 100.0		0.63
≤18.7	37.87	30.5 - 45.6	100.00	93.3 - 100.0		0.62
≤18.9	37.87	30.5 - 45.6	98.11	89.9 - 100.0	20.07	0.63
≤19	38.46	31.1 - 46.2	98.11	89.9 - 100.0	20.38	0.63
≤19.1	40.24	32.8 - 48.0	98.11	89.9 - 100.0	21.33	0.61
≤19.2	41.42	33.9 - 49.2	98.11	89.9 - 100.0	21.95	0.60
≤19.4	42.01	34.5 - 49.8	98.11	89.9 - 100.0	22.27	0.59
≤19.5	43.20	35.6 - 51.0	98.11	89.9 - 100.0	22.89	0.58
≤19.6	44.97	37.3 - 52.8	98.11	89.9 - 100.0	23.83	0.56
≤19.7	46.15	38.5 - 54.0	98.11	89.9 - 100.0	24.46	0.55
≤19.8	46.75	39.0 - 54.6	98.11	89.9 - 100.0	24.78	0.54
≤19.9	47.93	40.2 - 55.7	98.11	89.9 - 100.0	25.40	0.53
≤20	49.11	41.4 - 56.9	98.11	89.9 - 100.0	26.03	0.52
≤20.1	50.30	42.5 - 58.1	98.11	89.9 - 100.0	26.66	0.51
≤20.2	50.89	43.1 - 58.6	98.11	89.9 - 100.0	26.97	0.50
≤20.3	52.07	44.3 - 59.8	98.11	89.9 - 100.0	27.60	0.49
≤20.4	52.66	44.9 - 60.4	98.11	89.9 - 100.0	27.91	0.48

≤20.5	53.25	45.4 - 61.0	98.11	89.9 - 100.0	28.22	0.48
≤20.6	53.85	46.0 - 61.5	98.11	89.9 - 100.0	28.54	0.47
≤20.9	54.44	46.6 - 62.1	98.11	89.9 - 100.0	28.85	0.46
≤21	55.62	47.8 - 63.2	98.11	89.9 - 100.0	29.48	0.45
≤21.1	56.80	49.0 - 64.4	98.11	89.9 - 100.0	30.11	0.44
≤21.2	57.99	50.2 - 65.5	98.11	89.9 - 100.0	30.73	0.43
≤21.5	59.17	51.4 - 66.7	98.11	89.9 - 100.0	31.36	0.42
≤21.7	59.76	52.0 - 67.2	98.11	89.9 - 100.0	31.67	0.41
≤21.8	60.95	53.2 - 68.3	98.11	89.9 - 100.0	32.30	0.40
≤21.9	62.13	54.4 - 69.5	98.11	89.9 - 100.0	32.93	0.39
≤22	62.72	55.0 - 70.0	98.11	89.9 - 100.0	33.24	0.38
≤22.1	63.31	55.6 - 70.6	98.11	89.9 - 100.0	33.56	0.37
≤22.2	64.50	56.8 - 71.7	98.11	89.9 - 100.0	34.18	0.36
≤22.5	65.68	58.0 - 72.8	98.11	89.9 - 100.0	34.81	0.35
≤22.6	66.27	58.6 - 73.4	98.11	89.9 - 100.0	35.12	0.34
≤22.7	67.46	59.8 - 74.5	98.11	89.9 - 100.0	35.75	0.33
≤22.9	68.64	61.1 - 75.5	98.11	89.9 - 100.0	36.38	0.32
≤23	69.23	61.7 - 76.1	98.11	89.9 - 100.0	36.69	0.31
≤23.2	70.41	62.9 - 77.2	98.11	89.9 - 100.0	37.32	0.30
≤23.3	71.01	63.5 - 77.7	98.11	89.9 - 100.0	37.63	0.30
≤23.5	72.19	64.8 - 78.8	98.11	89.9 - 100.0	38.26	0.28
≤23.6	73.37	66.0 - 79.9	98.11	89.9 - 100.0	38.89	0.27
≤23.8	73.96	66.7 - 80.4	98.11	89.9 - 100.0	39.20	0.27
≤23.9	75.15	67.9 - 81.5	98.11	89.9 - 100.0	39.83	0.25
≤24	76.33	69.2 - 82.5	96.23	87.0 - 99.5	20.23	0.25
≤24.2	78.70	71.7 - 84.6	94.34	84.3 - 98.8	13.90	0.23
≤24.3	79.88	73.0 - 85.6	94.34	84.3 - 98.8	14.11	0.21

≤24.4	81.66	75.0 - 87.2	94.34	84.3 - 98.8	14.43	0.19
≤24.6	82.84	76.3 - 88.2	94.34	84.3 - 98.8	14.64	0.18
≤24.7	83.43	77.0 - 88.7	94.34	84.3 - 98.8	14.74	0.18
≤24.9	85.21	78.9 - 90.2	94.34	84.3 - 98.8	15.05	0.16
≤25.6	86.39	80.3 - 91.2	94.34	84.3 - 98.8	15.26	0.14
≤25.7	86.39	80.3 - 91.2	92.45	81.8 - 97.9	11.45	0.15
≤25.8	86.98	81.0 - 91.7	92.45	81.8 - 97.9	11.53	0.14
≤25.9	87.57	81.6 - 92.1	92.45	81.8 - 97.9	11.60	0.13
≤26	88.17	82.3 - 92.6	92.45	81.8 - 97.9	11.68	0.13
≤26.1	88.76	83.0 - 93.1	92.45	81.8 - 97.9	11.76	0.12
≤26.3	89.35	83.7 - 93.6	92.45	81.8 - 97.9	11.84	0.12
≤26.4	89.94	84.4 - 94.0	92.45	81.8 - 97.9	11.92	0.11
≤26.6	90.53	85.1 - 94.5	92.45	81.8 - 97.9	12.00	0.10
≤26.7	90.53	85.1 - 94.5	90.57	79.3 - 96.9	9.60	0.10
≤26.8	91.12	85.8 - 94.9	88.68	77.0 - 95.7	8.05	0.10
≤27	91.72	86.5 - 95.4	88.68	77.0 - 95.7	8.10	0.093
≤27.5	91.72	86.5 - 95.4	86.79	74.7 - 94.5	6.94	0.095
≤27.6	92.90	87.9 - 96.3	86.79	74.7 - 94.5	7.03	0.082
≤27.8	94.08	89.4 - 97.1	84.91	72.4 - 93.3	6.23	0.070
≤27.9	94.08	89.4 - 97.1	81.13	68.0 - 90.6	4.99	0.073
≤28.1	94.67	90.1 - 97.5	81.13	68.0 - 90.6	5.02	0.066
≤28.6	95.27	90.9 - 97.9	81.13	68.0 - 90.6	5.05	0.058
≤28.7	95.86	91.7 - 98.3	75.47	61.7 - 86.2	3.91	0.055
≤28.9	95.86	91.7 - 98.3	73.58	59.7 - 84.7	3.63	0.056
≤29	95.86	91.7 - 98.3	71.70	57.7 - 83.2	3.39	0.058
≤29.5	95.86	91.7 - 98.3	66.04	51.7 - 78.5	2.82	0.063
≤29.6	95.86	91.7 - 98.3	64.15	49.8 - 76.9	2.67	0.065
≤29.7	96.45	92.4 - 98.7	64.15	49.8 - 76.9	2.69	0.055
≤29.9	97.04	93.2 - 99.0	64.15	49.8 - 76.9	2.71	0.046
≤30	97.63	94.1 - 99.4	64.15	49.8 - 76.9	2.72	0.037
≤30.1	97.63	94.1 - 99.4	60.38	46.0 - 73.5	2.46	0.039
≤30.2	97.63	94.1 - 99.4	58.49	44.1 - 71.9	2.35	0.040
≤30.3	98.22	94.9 - 99.6	56.60	42.3 - 70.2	2.26	0.031
≤30.5	98.22	94.9 - 99.6	54.72	40.4 - 68.4	2.17	0.032
≤30.6	98.22	94.9 - 99.6	52.83	38.6 - 66.7	2.08	0.034
≤30.7	98.22	94.9 - 99.6	50.94	36.8 - 64.9	2.00	0.035
≤31	98.22	94.9 - 99.6	49.06	35.1 - 63.2	1.93	0.036
≤31.2	98.22	94.9 - 99.6	47.17	33.3 - 61.4	1.86	0.038
≤31.4	98.22	94.9 - 99.6	45.28	31.6 - 59.6	1.80	0.039
≤31.6	98.82	95.8 - 99.9	45.28	31.6 - 59.6	1.81	0.026
≤32	98.82	95.8 - 99.9	43.40	29.8 - 57.7	1.75	0.027
≤32.1	98.82	95.8 - 99.9	39.62	26.5 - 54.0	1.64	0.030
≤32.3	98.82	95.8 - 99.9	37.74	24.8 - 52.1	1.59	0.031
≤32.4	98.82	95.8 - 99.9	35.85	23.1 - 50.2	1.54	0.033
≤32.6	98.82	95.8 - 99.9	33.96	21.5 - 48.3	1.50	0.035
≤32.8	98.82	95.8 - 99.9	32.08	19.9 - 46.3	1.45	0.037

≤32.9	98.82	95.8 - 99.9	30.19	18.3 - 44.3	1.42	0.039
≤33	98.82	95.8 - 99.9	28.30	16.8 - 42.3	1.38	0.042
≤33.1	98.82	95.8 - 99.9	24.53	13.8 - 38.3	1.31	0.048
≤33.2	98.82	95.8 - 99.9	22.64	12.3 - 36.2	1.28	0.052
≤33.3	98.82	95.8 - 99.9	20.75	10.8 - 34.1	1.25	0.057
≤33.4	98.82	95.8 - 99.9	18.87	9.4 - 32.0	1.22	0.063
≤33.5	98.82	95.8 - 99.9	16.98	8.1 - 29.8	1.19	0.070
≤33.6	98.82	95.8 - 99.9	13.21	5.5 - 25.3	1.14	0.090
≤33.7	98.82	95.8 - 99.9	11.32	4.3 - 23.0	1.11	0.10
≤33.8	98.82	95.8 - 99.9	9.43	3.1 - 20.7	1.09	0.13
≤33.9	98.82	95.8 - 99.9	7.55	2.1 - 18.2	1.07	0.16
≤34.1	98.82	95.8 - 99.9	5.66	1.2 - 15.7	1.05	0.21
≤34.2	99.41	96.7 - 100.0	5.66	1.2 - 15.7	1.05	0.10
≤34.6	99.41	96.7 - 100.0	3.77	0.5 - 13.0	1.03	0.16
≤36.1	99.41	96.7 - 100.0	1.89	0.05 - 10.1	1.01	0.31
≤36.2	99.41	96.7 - 100.0	0.00	0.0 - 6.7	0.99	
≤36.4	100.00	97.8 - 100.0	0.00	0.0 - 6.7	1.00	



Graph 1. ROC analysis of RET-He using conventional criteria of Iron Deficiency

ROC analysis in determining sensitivity and specificity of RET-He in diagnosis of Functional Iron Deficiency in chronic kidney disease.

Sample size	59

Positive group [a]	18 (30.51%)
Negative group [b]	41 (69.49%)

[a] diagnosis = 1
[b] diagnosis = 2, 3, 4

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	0.562
Standard Error [a]	0.0840
95% Confidence interval [b]	0.427 to 0.691
z statistic	0.742
Significance level P (Area=0.5)	0.4582

[a] DeLong et al., 1988
[b] Binomial exact

Youden index

Youden index J	0.1829
95% Confidence interval [a]	0.1152 to 0.2371
Associated criterion	≤29.6
95% Confidence interval [a]	≤24 to ≤34.2
Sensitivity	50.00
Specificity	68.29

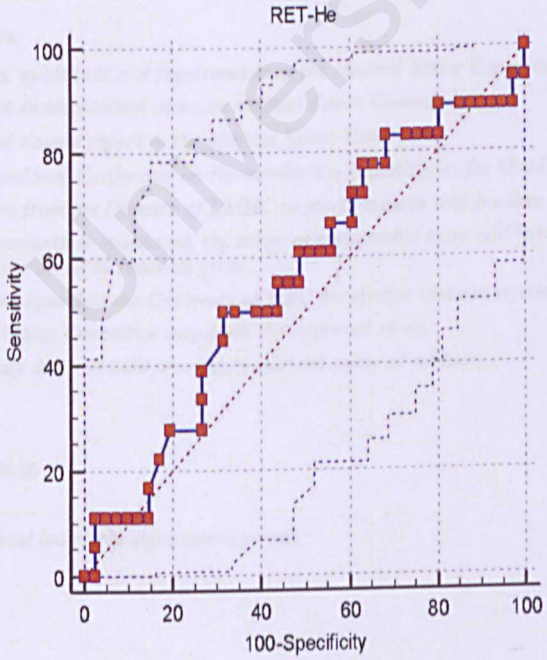
[a] BC_a bootstrap confidence interval (1000 iterations; random number seed: 978).

Criterion values and coordinates of the ROC curve [Hide]

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
<15.1	0.00	0.0 - 18.5	100.00	91.4 - 100.0		1.00
≤15.1	0.00	0.0 - 18.5	97.56	87.1 - 99.9	0.00	1.03
≤18.9	5.56	0.1 - 27.3	97.56	87.1 - 99.9	2.28	0.97
≤24	11.11	1.4 - 34.7	97.56	87.1 - 99.9	4.56	0.91
≤24.2	11.11	1.4 - 34.7	95.12	83.5 - 99.4	2.28	0.93
≤24.6	11.11	1.4 - 34.7	92.68	80.1 - 98.5	1.52	0.96
≤25.7	11.11	1.4 - 34.7	90.24	76.9 - 97.3	1.14	0.98
≤26.7	11.11	1.4 - 34.7	87.80	73.8 - 95.9	0.91	1.01
≤26.8	11.11	1.4 - 34.7	85.37	70.8 - 94.4	0.76	1.04
≤27.5	16.67	3.6 - 41.4	85.37	70.8 - 94.4	1.14	0.98
≤27.8	22.22	6.4 - 47.6	82.93	67.9 - 92.8	1.30	0.94

≤27.9	27.78	9.7 - 53.5	80.49	65.1 - 91.2	1.42	0.90
≤28.7	27.78	9.7 - 53.5	73.17	57.1 - 85.8	1.04	0.99
≤28.9	33.33	13.3 - 59.0	73.17	57.1 - 85.8	1.24	0.91
≤29	38.89	17.3 - 64.3	73.17	57.1 - 85.8	1.45	0.84
≤29.5	44.44	21.5 - 69.2	68.29	51.9 - 81.9	1.40	0.81
≤29.6	50.00	26.0 - 74.0	68.29	51.9 - 81.9	1.58	0.73
≤30	50.00	26.0 - 74.0	65.85	49.4 - 79.9	1.46	0.76
≤30.1	50.00	26.0 - 74.0	60.98	44.5 - 75.8	1.28	0.82
≤30.2	50.00	26.0 - 74.0	58.54	42.1 - 73.7	1.21	0.85
≤30.3	50.00	26.0 - 74.0	56.10	39.7 - 71.5	1.14	0.89
≤30.5	55.56	30.8 - 78.5	56.10	39.7 - 71.5	1.27	0.79
≤30.6	55.56	30.8 - 78.5	53.66	37.4 - 69.3	1.20	0.83
≤30.7	55.56	30.8 - 78.5	51.22	35.1 - 67.1	1.14	0.87
≤31	61.11	35.7 - 82.7	51.22	35.1 - 67.1	1.25	0.76
≤31.2	61.11	35.7 - 82.7	48.78	32.9 - 64.9	1.19	0.80
≤31.4	61.11	35.7 - 82.7	46.34	30.7 - 62.6	1.14	0.84
≤31.6	61.11	35.7 - 82.7	43.90	28.5 - 60.3	1.09	0.89
≤32	66.67	41.0 - 86.7	43.90	28.5 - 60.3	1.19	0.76
≤32.1	66.67	41.0 - 86.7	39.02	24.2 - 55.5	1.09	0.85
≤32.3	72.22	46.5 - 90.3	39.02	24.2 - 55.5	1.18	0.71
≤32.4	72.22	46.5 - 90.3	36.59	22.1 - 53.1	1.14	0.76
≤32.6	77.78	52.4 - 93.6	36.59	22.1 - 53.1	1.23	0.61
≤32.8	77.78	52.4 - 93.6	34.15	20.1 - 50.6	1.18	0.65
≤32.9	77.78	52.4 - 93.6	31.71	18.1 - 48.1	1.14	0.70
≤33	83.33	58.6 - 96.4	31.71	18.1 - 48.1	1.22	0.53
≤33.1	83.33	58.6 - 96.4	26.83	14.2 - 42.9	1.14	0.6

						2
≤33.2	83.33	58.6 - 96.4	24.39	12.4 - 40.3	1.10	0.68
≤33.3	83.33	58.6 - 96.4	21.95	10.6 - 37.6	1.07	0.76
≤33.4	83.33	58.6 - 96.4	19.51	8.8 - 34.9	1.04	0.85
≤33.5	88.89	65.3 - 98.6	19.51	8.8 - 34.9	1.10	0.57
≤33.6	88.89	65.3 - 98.6	14.63	5.6 - 29.2	1.04	0.76
≤33.7	88.89	65.3 - 98.6	12.20	4.1 - 26.2	1.01	0.91
≤33.8	88.89	65.3 - 98.6	9.76	2.7 - 23.1	0.98	1.14
≤33.9	88.89	65.3 - 98.6	7.32	1.5 - 19.9	0.96	1.52
≤34.1	88.89	65.3 - 98.6	4.88	0.6 - 16.5	0.93	2.28
≤34.2	88.89	65.3 - 98.6	2.44	0.06 - 12.9	0.91	4.56
≤34.6	94.44	72.7 - 99.9	2.44	0.06 - 12.9	0.97	2.28
≤36.1	94.44	72.7 - 99.9	0.00	0.0 - 8.6	0.94	
≤36.2	100.00	81.5 - 100.0	0.00	0.0 - 8.6	1.00	



Graph 2. ROC analysis of RET-He for Functional Iron Deficiency

UNIVERSITY OF MALAYA MEDICAL CENTRE

MEDICAL ETHICS COMMITTEE
UNIVERSITY MALAYA MEDICAL CENTER
ADDRESS : LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEPHONE : 03-79493209/2251 FAXIMILE : 03-79492030

NAME OF ETHICS COMMITTEE/IRB Medical Ethics Committee, University Malaya Medical Center	**MECID.NO**: 20161-2008
ADDRESS : LEMBAH PANTAI, 59100 KUALA LUMPUR	
PROTOCOL.NO(if applicable) :	
TITLE: Reticulocyte Haemoglobin Equivalent (Ret-He) in iron deficiency anaemia and anaemia of chronic disease.	
PRINCIPAL INVESTIGATOR : Dr. ERMI NEIZA BINTI MOHD SAHID	**SPONSOR** -

The following item [✓] have been received and reviewed in connection with the above study to conducted by the above investigator.

[✓] Application to Conduct Research Project(form)	Ver.No :	Ver.Date : 06-01-2016
[✓] Study Protocol	Ver.No :	Ver.Date :
[ ]	Ver.No :	Ver.Date :
[ ]	Ver.No :	Ver.Date :
[ ] Questionnaire	Ver.No :	Ver.Date :
[✓] Investigator's CV / GCP (Dr. ERMI NEIZA BINTI MOHD SAHID,HEMALATHA A/P SHANMUGAM,)	Ver.No :	Ver.Date :
[ ] Insurance certificate	Ver.No :	Ver.Date :
[✓] Other Attachments		
1) PURSUE FORM HOD	Ver.No : -	Ver.Date :

and the decision is [✓]

[✓] Approved

[ ] Rejected(reasons specified below or in accompanying letter)

Coinments:

Retrospective study

Investigator are required to:

1) *follow instructions, guidelines and requirements of the Medical Ethics Committee.*
2) *report any protocol deviations/violations to Medical Ethics Committee.*
3) *provide annual and closure report to the Medical Ethics Committee.*
4) *comply with International Conference on Harmonization – Guidelines for Good Clinical Practice (ICH-GCP) and Declaration of Helsinki.*
5) *obtain a permission from the Director of UMMC to start research that involves recruitment of UMMC patient.*
6) *ensure that if the research is sponsored, the usage of consumable items and laboratory tests from UMMC services are not charged in the patient's hospital bills but are borne by research grant.*
7) *note that he/she can appeal to the Chairman of MEC for studies that are rejected.*
8) *note that Medical Ethics Committee may audit the approved study.*
9) *ensure that the study does not take precedence over the safety of subjects.*

Date of approval : 12-02-2016

This is a computer generated letter. No signature required.