CLINICAL UTILITY OF EXTENDED RED BLOOD CELL PARAMETERS IN THE DIAGNOSIS OF LATENT IRON DEFICIENCY AND THALASSAEMIA TRAIT IN HOSPITAL UNIVERSITI SAINS MALAYSIA

DR SIVANESAN A/L SOCKALINGAM

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PATHOLOGY (HAEMATOLOGY)

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

%HYPO	Percentage of hypochromic erythrocytes
APC	Article processing fee
ARMS	Amplification refractory mutation system
AUC	Area under the curve
CE	Capillary electrophoresis
CHr	Reticulocyte hemoglobin content
CI	Confidence interval
CMIA	Chemiluminescent Microparticle Immunoassay
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBC	Full blood count
FS	Forward scatter
HCMC	Hypochromic microcytic
HCT	Haematocrit
HFR	High fluorescence ratio
HGB	Haemoglobin concentration
HGVS	Human Genome Variation Society
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HS	Hereditary spherocytosis
HYPO-He	Hypochromic RBC <17pg
IDA	Iron deficiency anaemia
IQR	Interquartile range

IRF	Immature reticulocyte fraction
JePEM	Human Research Ethics Committee USM
LFR	Low fluorescence ratio
LHD	Low Hemoglobin Density
LID	Latent iron deficiency
MAF	Microcytic Anaemia Factor
MARMS	Multiplex amplification refractory mutation system
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MFR	Medium fluorescence ratio
MicroR	Microcytic RBC <60fL
NTDT	Non-transfusion-dependent thalassaemia
OHRP	Office for Human Research Protections
PCR	Polymerase chain reaction
PLT	Platelet
RBC	Red blood cell
RCPA	Royal College of Pathologist of Australasia
RDW	Red cell distribution width
RDW-CV	RDW in coefficient of variation
RDW-SD	RDW in standard deviation
RET#	Reticulocyte count
RET%	Reticulocyte percentage
RET-He	Reticulocyte haemoglobin equivalent
RLU	Relative light units

ROC	Receiver operating curve
RSf	Red Blood Cell Size Factor
SAO	Southeast Asian Ovalocytosis
SD	Standard deviation
SDG	Sustainable Development Goals
SF	Serum ferritin
SI	Serum iron
SLS	Sodium lauryl sulfate
SS	Side scatter
sTfR	Soluble transferrin receptor
TDT	Transfusion-dependent thalassaemia
TIBC	Total iron binding capacity
TT	Thalassaemia trait
USM	Universiti Sains Malaysia
UTR	Untranslated region
α	Alpha
β	Beta

ABSTRAK

Pengenalan: Penggunaan ujian kiraan darah keseluruhan adalah sukar untuk mengenal pasti kes kekurangan zat besi tanpa anemia dan pembawa talasemia. Diagnosis yang tepat adalah penting kerana kaedah pengurusan dan rawatan adalah berbeza untuk kedua-dua jenis penyakit ini. Oleh hal yang demikian, keputusan tambahan ujian kiraan darah keseluruhan seperti kandungan setara hemoglobin dalam retikulosit (RET-He), peratus sel darah merah mikrosit <60fL (MicroR) dan peratus sel darah merah hipokromia <17pg (HYPO-He) mungkin dapat membantu mengenal pasti kekurangan zat besi dan pembawa talasemia. Tujuan utama kajian ini adalah untuk mengenal pasti kebolehan RET-He, MicroR dan HYPO-He dalam mendiagnosis dua keadaan ini.

Metodologi: Kajian kes-kawalan prospektif ini dijalankan di Hospital Universiti Sains Malaysia dari Ogos 2021 sehingga Jun 2022. Seratus enam puluh dua daripada 212 penderma darah memenuhi kriteria penyertaan. Peserta digolongkan dalam kumpulan kekurangan zat besi, kumpulan pembawa talasemia dan kumpulan kawalan berdasarkan nilai parameter RBC, nilai ferritin dalam serum dan status talasemia mereka. Penilaian statistik deskriptif dibuat terhadap parameter-parameter biasa dan tambahan ujian kiraan darah keseluruhan. Data diuji untuk kenormalan taburan, perbandingan antara kumpulan, keluasan di bawah lengkung, sensitiviti dan spesifisiti. Ambang optimal parameter tambahan ini dikenal pasti menggunakan indeks Youden. Perbandingan keluasan di bawah lengkung juga dibuat.

Keputusan: Seramai 17 (10.5%) peserta digolongkan dalam kumpulan kekurangan zat besi, 26 (16.0%) peserta dalam kumpulan pembawa talasemia dan 119 (73.5%) peserta dalam kumpulan kawalan. Semua parameter RBC biasa adalah dalam julat normal kecuali

RBC (di kalangan peserta perempuan), MCV and MCH dalam kumpulan pembawa talasemia. Dalam kumpulan kekurangan zat best dan pembawa talasemia, RET-He adalah lebih rendah daripada julat normal manakala MicroR adalah lebih tinggi daripada julat normal. HYPO-He adalah dalam julat normal dalam semua kumpulan. Dengan mengambil kira julat normal, RET-He adalah parameter terbaik untuk mengenal pasti kekurangan zat besi (AUC 0.723, 95% CI 0.608-0.839; 76.47% sensitiviti dan 73.95% spesifisiti dengan ambang 30.1pg) dan pembawa talasemia (AUC 0.832, 95% CI 0.749-0.914; 84.62% sensitiviti dan 73.11% spesifisiti dengan ambang 30.2pg) daripada kawalan. MicroR merupakan parameter terbaik untuk mengenal pasti pembawa talasemia daripada kumpulan kekurangan zat besi (AUC 0.742, 95% CI 0.591-0.893; 65.38% sensitiviti dan 76.47% spesifisiti dengan ambang 6.5%). Walaupun perbezaan keluasan di bawah lengkung bagi RET-He dan MicroR adalah tidak signifikan untuk mengenal pasti setiap kumpulan, hanya MicroR yang mempunyai perbezaan nilai median yang signifikan antara pembawa talasemia (9.1%, IQR 17.5) dan kumpulan kekurangan zat besi (4.0%, IQR 6.1).

Kesimpulan: Antara keputusan tambahan ujian darah keseluruhan, RET-He merupakan parameter terbaik untuk mengenal pasti kekurangan zat besi dan pembawa talasemia, manakala MicroR sesuai untuk mengenalpasti pembawa talasemia di kalangan kekurangan zat besi. Ujian tambahan ini adalah kos-berkesan, mempunyai keupayaan celusan tinggi dan senang untuk diperolehi daripada penganalisis hematologi moden. Ciri-ciri ini boleh membantu dalam pengurusan kekurangan zat besi dan saringan talasemia di kalangan penduduk.

ABSTRACT

Introduction: Recognition of latent iron deficiency (LID) and thalassemia trait (TT) are challenging with the use of standard red blood cell (RBC) parameters. Accurate diagnosis is vital as the management of iron deficiency and haemoglobinopathy are different. The use of extended RBC parameters - reticulocyte haemoglobin equivalent (RET-He), percentage of microcytic RBC <60fL (MicroR), and percentage of hypochromic RBC <17pg (HYPO-He) may aid in the detection of LID and TT. The study aims to determine the diagnostic ability of these extended RBC parameters to distinguish LID and TT.

Methodology: This was a prospective, case-control study conducted in Hospital USM from August 2021 to June 2022. 162 out of 212 blood donors who fulfilled the inclusion criteria were classified into the control group, LID group and TT group based on RBC parameters, serum ferritin and thalassaemia status. Between-group comparisons, receiver operating characteristic (ROC) curve analysis and comparison of area under the curve (AUC) for the RBC parameters were determined. The optimal cut-off was determined using the best Youden index.

Result: There were 17 (10.5%) subjects with LID, 26 (16.0%) subjects with TT and 119 (73.5%) subjects in the control group. Standard RBC parameters were within the reference interval for all groups except for RBC (in females), MCV and MCH in the TT group. In LID and TT, RET-He and MicroR were lower and higher than the reference interval respectively, while HYPO-He was within the reference interval in all the groups. Taking into consideration the reference interval, RET-He was the best parameter to distinguish LID (AUC 0.723, 95% CI 0.608-0.839; 76.47% sensitivity and 73.95% specificity at 30.1pg cut-off) and TT (AUC 0.832, 95% CI 0.749-0.914; 84.62%

sensitivity and 73.11% specificity at 30.2pg cut-off) from control. MicroR was the best parameter to distinguish TT from LID (AUC 0.742, 95% CI 0.591-0.893; 65.38% sensitivity and 76.47% specificity at 6.5% cut-off). Although the AUC of RET-He and MicroR were not significantly different in all the case comparisons, only MicroR was significantly higher in TT (median 9.1%, IQR 17.5) than LID (median 4.0%, IQR 6.1).

Conclusion: Among the extended RBC parameters, RET-He was the best parameter for the detection of LID and TT, while MicroR performed well in differentiating TT from LID. These accessible parameters through modern automated haematology analyzers are low cost, with high throughput and rapid turn-around time can optimize the management of iron deficiency and thalassemia screening in the population.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Anaemia is a key public health problem globally with slightly more than a fifth of the population afflicted by it (Gardner and Kassebaum, 2020). It is defined as a lower-than-normal haemoglobin level, which is associated with the decreased oxygen-carrying capacity of the blood to the tissue (Beutler and Waalen, 2006). Despite numerous causes of anaemia ranging from excessive loss of red blood cells (RBC) to disorders in erythropoiesis, the most prevalent causes of anaemia are iron deficiency, haemoglobinopathies and hemolytic anaemia according to the Global Burden of Disease Study 2019. Iron deficiency forms a significant proportion affecting 66.1% of males and 56.8% of females with anaemia, while haemoglobinopathies and haemolytic anaemia affect 13.6% of males and 16.1% of females (Safiri et al., 2021).

These disorders are equally prevalent in Malaysia. Several studies have looked at the prevalence of iron deficiency in selected populations. 31.6 to 34.6% of pregnant women and 5.2% of children between the ages of 3 to 12 years old have iron deficiency anaemia (IDA) (Abd Rahman et al., 2022; Shanita et al., 2018). As for latent iron deficiency (LID), it is found in 2% of first-time donors and 13.3-24.5% of regular donors (Amir et al., 2019; V. Nadarajan et al., 2008).

The development of IDA occurs progressively as the iron is depleted. As iron continues to be lost or not replenished adequately, the iron store decreases. During this iron store depletion stage, haemoglobin concentration (HGB) and serum ferritin (SF) remains within the reference interval. This will progress to iron-deficient erythropoiesis,

also known as LID. Iron store at this stage will be below normal. However, HGB and other standard RBC parameters are still within the reference interval. The gradual decrease of HGB eventually develops into anaemia (Higgs et al., 2016).

As for haemoglobinopathy, this heterogeneous genetic disorder due to mutations in the globin gene leads to reduced production of globin chains or structural haemoglobin variants. Broadly, haemoglobinopathies are grouped by the afflicted globin chain. The common α-thalassaemia and β-thalassaemia refer to reduced production of α and β globin chains, respectively. Mutations that result in reduced production are denoted as α^+ and β^+, while the complete absence of globin chain production is denoted as α^0 and β^0. Clinically, thalassaemia is classified based on blood transfusion therapy requirements. Thalassaemia traits (TT) are generally asymptomatic with normal HGB or mild anaemia. Thalassaemia intermedia or non-transfusion-dependent thalassaemia (NTDT) consists of a more severe phenotype with moderate anaemia that may require red cell transfusion. The most severe form is thalassaemia major or transfusion-dependent thalassaemia (TDT) with severe anaemia and requires red cell transfusion for survival (Cappellini et al., 2021).

Thalassaemia is primarily found in tropical zones stretching from Africa, the Mediterranean region, the Middle East, South Asia, East Asia to Southeast Asia (Williams and Weatherall, 2012). Due to immigration, these disorders have become a global concern rather than regionally restricted (Vichinsky, 2005). In Malaysia, 9.25% have α-thalassaemia trait while 3.5-4.0% have β-thalassaemia trait (Elizabeth and Ann, 2010; Rosnah et al., 2012). For those requiring blood transfusion therapy, 34.37% have haemoglobin E/ β-thalassaemia, 33.52% have β-thalassaemia major, 18.26% with haemoglobin H disease (HbH) and 9.37% with β-thalassaemia intermedia (Mohd Ibrahim et al., 2020).

Due to the high prevalence of these disorders, the diagnosis and management of iron deficiency and haemoglobinopathies have important public health implications. Iron deficiency is associated with cognitive impairment, a decline in productivity, maternal mortality, child mortality and perinatal mortality (Stoltzfus, 2003). From the public health perspective, the importance of iron deficiency is highlighted in the United Nations supported Sustainable Development Goals' (SDG) Goal 3 (Good Health and Well-being) (Binns et al., 2017). Clinical identification of LID is challenging due to non-specific symptoms. Early recognition and the benefits of early treatment with iron are evident in studies among pregnant women, surgical patients, and patients with chronic diseases (Al-Naseem et al., 2021). Iron supplementation also showed a reduction in subjectively measured fatigue in a systemic review on the treatment of LID (Houston et al., 2018).

Likewise, the importance of diagnosing haemoglobinopathy is in recognition of the costly management of thalassaemia major and intermedia. Management of the complications of the disease, regular blood transfusion therapy, iron overload complications and iron chelating agents and loss of productivity have a high socioeconomic burden. From a societal perspective, USD 606,665 is the total estimated cost over the lifetime of a TDT patient in Malaysia. Iron chelating agents account for most of the total cost (56.9%) followed by blood transfusion therapy (13.1%). An estimated 1% of the Ministry of Health's budget in 2018 was used for the management of TDT (Shafie et al., 2021). The National Thalassaemia Prevention and Control Programme was set up in 2004 to reduce the disease burden by increasing awareness of the disease, targeted screening programmes, prenatal diagnosis, the establishment of genetic counselling and improving laboratory support services. The screening programme consists of cascade screening of index cases, screening in adolescents, young adults, and pregnant women (Ministry of Health Malaysia, 2009).

Full blood count (FBC) is the primary screening test for the investigation of anaemia. Corresponding to the morphology in IDA with anisopoikilocytosis and hypochromic microcytic (HCMC) RBC, the FBC features are low RBC, HGB, haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and high red cell distribution width (RDW). However, FBC is a poor test to screen for LID. Except for RDW in coefficient of variation (RDW-CV), there were no significant differences for RBC, HGB, HCT, MCV, MCH, MCHC and RDW in standard deviation (RDW-SD) between the control group and LID (Ambayya et al., 2019). MCV, MCH and MCHC also have low sensitivity of between 6.1-12.8% in detecting LID (Tiwari et al., 2018).

On the other hand, FBC features for TT are HCMC RBC with normal to high RBC count. HGB is often normal with some having mild anaemia. RBC cells are uniform as signified by normal RDW-SD and RDW-CV. The more severe type of haemoglobinopathy has moderate to severe anaemia, HCMC RBC with anisopoikilocytosis. The National Thalassaemia Screening Programme incorporates FBC as the initial screening test. Cases with MCH≤ 27pg are investigated further with haemoglobin analysis (Ministry of Health Malaysia, 2009). FBC however, is not an ideal screening test as cases can be missed. Among those with normal HGB and normochromic normocytic cells, 15.8% have Hb E trait, 2.0% have Hb Constant Spring and 0.6% have β-thalassaemia trait (Insiripong et al., 2014). In another study, 1 out of 13 thalassaemic donors has normal HGB with MCH>27pg (Rosline et al., 2006).

To establish the proper diagnosis, additional laboratory tests are required. Iron profile in the form of serum iron (SI), total iron binding capacity (TIBC) and serum ferritin (SF) would often establish the diagnosis of iron deficiency. However, there are pertinent limitations that need to be considered. SI has a high diurnal variation with a 2-

4 µmol/L difference (Burtis et al., 2012). Preanalytical variables that increase SI are premenstrual phase, pregnancy, oral contraceptives, iron therapy, hepatitis, and iron overload conditions. SI is reduced during menstruation, in inflammatory conditions, and iron deficiency state. TIBC, on the other hand, is elevated by oral contraceptives and iron deficiency, while being reduced by inflammatory state and iron overload conditions. With SF as the gold standard, the sensitivity and specificity for SI were 63.5% and 38.6% respectively, while the sensitivity and specificity for TIBC were 64.5% and 42.8% respectively (Asif et al., 2016). Thus, the low specificity and high variability of SI and TIBC hamper their use in iron status assessment.

As the main iron store, the soluble protein ferritin is confined mostly intracellularly with small amounts in the serum. The serum concentration is proportional to the total body iron stores. The sensitivity and specificity of this test were determined using bone marrow samples which were considered the gold-standard to evaluate iron deficiency. With a cut-off of <16µg/L, the sensitivity and specificity were 75% and 98% respectively, while a higher cut-off of <30µg/L had a sensitivity and specificity of 93% and 75% respectively (Daru et al., 2017). Pre-analytical consideration needs to be given for this test as ferritin is an acute phase reactant. Inflammations, renal disease, liver disease and neoplastic disorders can elevate the concentration of ferritin in the serum (Burtis et al., 2012). In the absence of inflammation, SF is a good test to determine iron storage (Fletcher et al., 2021).

The presumptive diagnosis of haemoglobinopathy may be ascertained with haemoglobin analysis. This is based on the separation and identification of haemoglobin based on its physicochemical properties. Reduction of the globin gene can change the proportion of haemoglobin A, A_2 and F while mutations that produce variant haemoglobin may have different physicochemical properties. Commonly used methods

are high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). HPLC and CE are automated with high throughput and require a small volume of samples. Definitive diagnosis is obtained using molecular methods. This is advantageous to confirm diagnosis after haemoglobin analysis, to identify α^0, α^+ mutations and silent β-thalassaemia. Several targeted methods which are cost-effective are available based on the mutations. Deletional forms of α and β thalassaemia utilize gap polymerase chain reaction (PCR) technique while non-deletional forms utilize allele-specific PCR technique. The PCR products are then separated using electrophoresis and identified (Greene et al., 2014).

Considering the shortcomings of FBC and the divergent nature of confirmatory tests to differentiate LID and TT, extended RBC parameters from Sysmex automated haematology analyzers (Sysmex, Kobe, Japan) – reticulocyte haemoglobin equivalent (RET-He), percentage of microcytic RBC (MicroR) and percentage of hypochromic RBC (HYPO-He) are being studied to improve the diagnosis of these disorders. RET-He was introduced in the XE5000 haematology analyzer as a novel parameter to assess the haemoglobin content in reticulocytes. Previously in the XE2100 haematology analyzer, it was known as RET-Y. The mean value of the forward scatter of the reticulocyte population is used to algorithmically derive RET-He (Franck et al., 2004). MicroR refers to the percentage of RBC that is below 60fL while HYPO-He refers to the percentage of RBC that is below 17pg (Eloísa Urrechaga et al., 2009). MicroR is obtained from the RBC histogram that is generated by the impedance method while HYPO-He is derived from RET channel (Matsushita, 2011).

The extended RBC parameters have been explored in several studies focusing on its characteristics in iron deficiency, chronic kidney disease, haemoglobinopathy and the ability of these parameters to distinguish conditions with restricted erythropoiesis (E

Urrechaga et al., 2013; Eloísa Urrechaga et al., 2009, 2011). Since reticulocytes take 1-2 days to mature into RBC in peripheral blood, RET-He also serves as a direct assessment of recent functional iron availability for erythropoiesis (Brugnara et al., 2006). MicroR and HYPO-He provide insight into the haemoglobinization of mature RBC which has a lifespan of 120 days. This serves as a proxy for the availability of iron in the past 3 months (Eloísa Urrechaga et al., 2009). These parameters are not influenced by inflammatory conditions (Agarwal et al., 2021; Chinudomwong et al., 2020). In Malaysia, the reference intervals for RET-He, MicroR and HYPO-He were established in 2014 (Ambayya et al., 2014). Comparatively, only one study locally explored latent iron deficiency and these three parameters, however, the study population was limited to females (Ambayya et al., 2019). Other studies have focused on RET-Y in LID, RET-He in IDA, HYPO-He in LID, and all three parameters with IDA and haemoglobinopathy (Amir et al., 2019; Bakri et al., 2020; V. Nadarajan et al., 2008; S. Y. Wee et al., 2020). There are limited studies on the features of extended RBC parameters in LID and thalassaemia trait.

To fill this knowledge gap, this study aims to determine the characteristics of RET-He, MicroR and HYPO-He in the healthy, those with LID and TT in the local adult population. This study would also evaluate the diagnostic ability and optimal cut-off of these parameters to diagnose LID and TT. This would shine a light on the possibility of using these novel parameters to detect and differentiate between these two common disorders.

During the study period, 400 subjects have been recruited. FBC, SF and DNA analysis for α-thalassaemia have been performed on all the subjects while DNA analysis β-thalassaemia is still ongoing. Currently, DNA analysis for 8 β-thalassaemia mutations has been performed for 212 subjects. One hundred and sixty-two subjects fulfilled the inclusion criteria. This dissertation is based on the analysis of these 162 subjects.

1.2 General objective

To identify the clinical utility of RET-He, MicroR and HYPO-He in the diagnosis of LID and TT.

1.3 Specific objectives

1. To compare the level of RET-He, MicroR and HYPO-He between control, LID and TT.
2. To determine the sensitivity and specificity of RET-He, MicroR and HYPO-He in the diagnosis of LID and TT.
3. To compare the ROC curve between RET-He, MicroR and HYPO-HE in distinguishing LID and TT from normal.

The manuscript covers objectives for clinical utility of RET-He, MicroR and HYPO-He in the diagnosis of LID. The remaining objectives are covered in Chapter 4.

CHAPTER 2

STUDY PROTOCOL

2.1 Study protocol

Research Title:

Clinical utility of MicroR and Hypo-He in the diagnosis of latent iron deficiency and thalassemia trait in Hospital Universiti Sains Malaysia.

Principal investigator:

Dr. Sivanesan A/L Sockalingam

Supervisor:

Dr. Zefarina Binti Zulkafli

Co-supervisor:

Associate Professor Dr. Rosnah Bahar

Introduction

Anemia is a major burden globally. It is defined as a haemoglobin concentration level that is 2 standard deviations below the mean for age, gender, pregnancy, and altitude. The World Health Organization (WHO) defines anemia in males who are aged 15 and above with having hemoglobin concentration below 13 g/dL, while in non-pregnant females aged 15 years and above with hemoglobin concentration below 12 g/dL (WHO, 2011). A systematic analysis of global prevalence in 2010 shows anemia afflicting 32.9% of the global population, with developing countries sharing the bulk of the burden. The primary causes of anemia are deficiencies of iron (iron deficiency anemia (IDA), schistosomiasis, hookworm infestation), hemoglobinopathies (sickle cell disorders and thalassemia) and malaria (Kassebaum et al., 2014). In Malaysia, there is a lack of prevalence studies among the general population. Prevalence studies are primarily targeting specific groups. A cross-sectional study found 35% of pregnant women to be anemic, while another study found latent iron deficiency (LID) in 2-7.4%% of first-time blood donors and 17.4-24.5% among regular donors (Amir et al., 2019; Haniff et al., 2007; V. S. Nadarajan and Eow, 2002; V. Nadarajan et al., 2008).

As a very prevalent form of nutritional deficiency, lack of iron is associated with increased mortality and morbidity across all age groups, especially in contributing to maternal and perinatal mortality. The socioeconomic impact of the disease is difficult to overlook as it is also associated with loss of productivity and cognition impairment (Ross and Horton, 1998; Stoltzfus, 2003). The spectrum of iron-deficient states encompasses LID with normal hemoglobin concentration which progresses to anemia when iron store is depleted. Treatment using iron therapy is targeted towards restoring the hemoglobin level and replenishing iron store, which takes up to 1 year or more (Goddard et al., 2011). With a prolonged treatment period, the risk of non-compliance to treatment increases,

especially towards oral iron therapy. The cause of non-compliance to iron therapy is multifactorial with fear of side-effects being one of its more prominent reasons (R and J, 1994). Thus, identification and early treatment of latent iron deficiency can reduce the treatment duration and improve compliance rates.

Thalassemia and hemoglobinopathies are a heterogeneous group of hereditary disorders relating to the reduced synthesis or synthesis of abnormal globin chain, respectively. Globally, it is a growing public health burden primarily due to transfusion dependence in severe forms of the disease. The Mediterranean region is commonly associated with β-thalassemia, the Far East region is associated with α-thalassemia, HbS in the sub-Saharan region and HbE in the Southeast Asia region. However, with changing demographics due to migration, these disorders are no longer geographically restricted but is now a global public health concern. Hb Eβ-thalassemia and Hb H are projected to contribute to most of the increase (Vichinsky, 2005). In Malaysia, 3.5–4% of the population have β-thalassemia trait (Elizabeth and Ann, 2010). 9.25-15.8% have α-thalassemia trait (Rosnah et al., 2012; Y. C. Wee et al., 2005). Overall, a study among blood donors found 16.25% have thalassemia in Malaysia (Rosline et al., 2006).

The genotype of β-thalassemia varies with race. In Malays, 73.1% of β-thalassemia composed of HbE [CD 26 (CAG→AAG)], IVS 1-5(G→C), and IVS1-1 (G→T). Among the Chinese, 90% of β-thalassemia are composed of CD 41/42 (-TCCT), IVS2-654 (C→T), IVS2-28 (A→G), CD 17 (A→T) and CD 71/72 (+A) (Elizabeth and Ann, 2010). For α-thalassemia, 8% are heterozygous for α3.7 deletion, 0.5% are heterozygous for SEA type deletion and 0.25% are heterozygous for α4.2 deletion (Rosnah et al., 2012).

The first step in the diagnostic workup of anemia is the full blood count (Short and Domagalski, 2013). The reportable red blood cell parameters which are offered by

hematology analyzers are hemoglobin concentration (HGB), RBC count (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width, expressed in standard deviation and percentage (RDW-SD and RDW-CV, respectively). Additional parameters can be obtained using the reticulocyte channel for quantification of reticulocytes in percentage (RET%) or absolute (RET#), immature reticulocyte fraction (IRF) and reticulocyte hemoglobin equivalent (Ret-He). By reviewing the results of this initial FBC, anemia is broadly categorized into microcytic (2 standard deviations below mean MCV), normocytic and macrocytic (2 standard deviations above mean MCV). This categorization can narrow down the differential diagnosis of anemia. A second sample is then taken to identify the etiology of microcytic anemia. This usually involves the investigation of iron status (e.g., serum iron, total iron-binding capacity (TIBC), serum ferritin), hemoglobin analysis and other tests based on the history and physical examination of the patient, as anemia of chronic disease and sideroblastic anemia can also cause microcytosis. Iron deficiency anemia, thalassemia and hemoglobinopathies which constitute a major proportion of the global anemia burden have primarily microcytic red blood cell morphologically.

Problem Statement and Study Rationale

Diagnosis of iron deficiency and/or thalassemia trait in the population is difficult with just the use of currently reportable parameters provided in the full blood count, as both conditions are associated with hypochromic microcytic RBCs. In routine clinical practice, hemoglobin concentration is often used to screen patients for the presence of iron deficiency, however, this marker has low sensitivity in the early stages (V. Nadarajan et al., 2008). If iron deficiency or thalassemia is suspected, then secondary testing

involving measurement of serum iron, total iron-binding capacity (TIBC) and serum ferritin are done. Serum ferritin is a good marker for the diagnosis of iron deficiency. Both serum iron and TIBC are highly variable and more suited to the investigation of iron overload (Marshall et al., 2017). Serum ferritin is a good marker, however being an acute phase protein, it is affected by infectious or non-infectious inflammations and malignancies (Wang et al., 2010).

Thalassemia screening has also relied on FBC as the primary method of investigation. Increased RBC count, MCV < 80fL, MCH < 27pg and normal RDW are associated with thalassemia. Secondary testing involves hemoglobin analysis using high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) or gel electrophoresis and confirmed using mutation analysis. However, in individuals with normal hemoglobin concentration and normal MCV, 15.8% have HbE trait, 2.0% have Hb-CS trait and 0.6% have β-thalassemia trait, while another study showed 13.75% have thalassemia with normal hemoglobin concentration (Insiripong et al., 2014; Rosline et al., 2006). Secondary tests can contribute to delay in diagnosis, and possible mistreatment if the microcytosis is considered as iron deficiency instead of thalassemia, especially in heterozygotes (Ohene-Frempong and Schwartz, 1980).

Ret-He is being progressively included in FBC and has good performance in identifying iron-deficient states, with a cut-off value of 27.2pg (Brugnara et al., 2006). However, reticulocyte evaluation is not usually done in the initial FBC. Cost is a concern as reticulocyte evaluation entails the use of a reagent that stains the RNA content of immature erythrocytes. Nadarajan et al. reported that FBC without reticulocyte analysis is USD1.13, and USD1.30 with reticulocyte analysis. Serum ferritin on the other hand costs USD1.60 (V. Nadarajan et al., 2008).

Extended RBC parameters are introduced in the Sysmex XE series and XN series automated hematology analyzers (Sysmex Corporation, Kobe, Japan). They are percentages of microcytic RBC with less than 60fL volume (%MicroR), percentage of macrocytic RBC with more than 120fL volume (%MacroR), percentage of hypochromic RBCs with less than 17pg hemoglobin content equivalent (%Hypo-He) and percentage of hyperchromic RBCs with more than 49pg hemoglobin content equivalent (%Hyper-He). %MicroR and %MacroR are both derived from the RBC histogram, obtained via RBC/platelet channel using the impedance method. Hypo-He and Hyper-He are derived using high-angle forward scatter of mature RBC in the reticulocyte channel (Eloísa Urrechaga et al., 2009).

Thus, by focusing this study on the use of extended RBC parameters, specifically %MicroR and %HypoHe in the initial diagnostic process of identifying LID and thalassemia trait, it is hoped that correct test for confirmation can be ordered and treatment instituted promptly while reducing the risk of mistreatment. The performance of %MicroR is especially important since reticulocyte analysis is not required to derive this parameter, hence can be obtained from the initial FBC. Hence, the potential for cost savings exists by reducing unnecessary reticulocyte analysis and secondary testing. Another advantage in utilizing %MicroR is minimizing additional training cost for laboratory personnel as the same automated hematology analyzer and workflow process are employed.

Apart from the excellent work of Ambayya et al. in determining the reference intervals of the extended RBC parameters, there are very limited studies that explore the use of %MicroR and %HypoHe in the determination of LID and thalassemia trait in our local population. This study can provide additional data to construct a reference interval for these extended parameters.

Blood donors are chosen as the study population for this study as data obtained from this population, especially first-time donors can give a reasonably good estimate for the population as a whole, while taking into account differences in age, gender, and ethnicity (Mast et al., 2012). A Dutch study comparing blood donors to the general population found that donors exhibited a healthier lifestyle by consuming more fruit and vegetables, smoking less and physically more active (Atsma et al., 2011). The rigorous selection process that consists of self-deferral, application of pre-determined deferral criteria, and medical check-up before donation aim to select healthy donors. These factors can reduce the incidence of infections, inflammations, and chronic diseases in the study population.

As a prospective study that uses whole blood samples from participants for analysis, recruitment of blood donors would reduce the number of venepunctures. Samples for this study would be obtained during the process of bleeding for blood donation. Thus, no additional venepunctures are required specifically for this study.

By studying the characteristics of %MicroR and %Hypo-He among blood donors, diagnosis of latent iron deficiency can be improved. Donor deferrals is a major concern in the procurement of blood products. 5.6% of donors were deferred in a study conducted in our center in 2006, with a majority of them (40.7%) due to low hemoglobin concentration (Rabeya et al., 2008). As mentioned in the introduction, 2-7.4% of first-time blood donors and 24.5% of regular blood donors have latent iron deficiency. These findings mirror results of the REDS-II Donor Iron Status Evaluation study whereby iron deficiency is a prevalent issue, strongly associated with frequent donations, gender, menstrual status, weight, and age (Cable et al., 2011). Therefore, temporary deferrals due to low hemoglobin concentration reduces return rate especially among first-time donors, increase in the time to return for a donation, reduction in the number of donations, and

more likely to drop out compared to non-deferred donors (Hillgrove et al., 2011). This represents a significant opportunity cost to the blood service.

In routine practice, FBC analysis has a faster turn-around time compared with serum ferritin level. In this center, FBC results can be obtained in less than 1.5 hours. Thus, the use of extended RBC parameters to predict iron deficiency and counsel donors during the same visit could improve understanding of the condition, and increase the donor return rate. These parameters can also be used as a surrogate to monitor iron levels during subsequent donations, with appropriate treatment. Apart from deficiency of iron, the possibility of thalassemia from the RBC parameters can aid the blood transfusion service to provide counselling and assist in sending samples for subsequent hemoglobin analysis.

Research Questions

1. How does the value of MicroR, Hypo-He and Ret-He change between normal population, latent iron deficiency and thalassemia trait?
2. What is the specificity and sensitivity of MicroR, Hypo-He and Ret-He to distinguish latent iron deficiency and thalassemia trait from normal?
3. Is there a difference in the AUC of MicroR, Hypo-He and Ret-He to distinguish latent iron deficiency and thalassemia trait from normal?

Hypothesis

1. There is a mean MicroR, Hypo-He and Ret-He difference between normal, latent iron deficiency and thalassemia trait.
2. There is a difference in AUC of MicroR, Hypo-He and Ret-He to distinguish latent iron deficiency and thalassemia trait from normal.

Objectives

General:

To identify the clinical utility of MicroR, Hypo-He and Ret-He in the diagnosis of latent iron deficiency and thalassemia trait.

Specific:

1. To compare the level of MicroR, Hypo-He and Ret-He between normal, latent iron deficiency and thalassemia trait.
2. To determine the sensitivity, specificity of MicroR, Hypo-He and Ret-He in the diagnosis of latent iron deficiency and thalassemia trait.
3. To compare the ROC curve between MicroR, Hypo-He and Ret-He to distinguish latent iron deficiency and thalassemia trait from normal.

Literature review

MicroR and Hypo-He have been investigated for their clinical utility in the differential diagnosis of microcytic anemia. Urrechaga et al in 2009 conducted a study to establish reference ranges for MicroR, MacroR, Hyper-He and Hypo-He and evaluated their role in discerning different types of anemia. The parameters are normally distributed with no difference in values between genders. The difference in mean of these parameters

between healthy participants (%MicroR with mean 1.1, standard deviation (SD) 0.44, %Hypo-He 0.3(0.16)), thalassemia group (%MicroR 37.6(11.5), %Hypo-He 14.2(9.4)), iron deficiency anemia group (%MicroR 16.5(13.1), %Hypo-He 16.2(15.8)) and those with chronic kidney disease (%MicroR 2.5(2.4), %Hypo-He 1.7(1.8)) are statistically significant, except for Hypo-He for iron deficiency and thalassemia. However, Hypo-He among restricted erythropoiesis (iron deficiency and thalassemia) was significantly different from normal and those with chronic kidney disease (Eloísa Urrechaga et al., 2009).

A case-control study in 2011 by the same author among healthy subjects, iron deficiency and β-thalassemia carriers found similar findings. Iron deficiency is characterized by anisocytosis and hypochromic cells, while thalassemia is characterized by uniform microcytic cells. %Hypo-He and %MicroR are proportional to the severity of the anemia. MicroR was better than RBC (AUC 0.917, sensitivity 93.7%, specificity 72.5% for cut-off 5.1) in differentiating β-thalassemia trait from a mild iron deficiency with AUC 0.938, 93.7% sensitivity and 75.4% specificity for cut-off 20% (Eloísa Urrechaga et al., 2011)

Latent iron deficiency was explored in 2016 by the same author in a study conducted among non-anemic premenopausal women. Serum ferritin <30μg/L was used as a marker to diagnose latent iron deficiency with a normal hemoglobin concentration of >12g/dL. RBC, Hb, MCV, MCH and MCHC show an overlap between non-iron deficient and iron-deficient groups, with Hb MCV and MCHC showing no statistical difference. Ret-He, with a mean of 33.8pg (SD 2.1pg) and %Hypo-He, 0.3(0.15) in the non-iron deficient group is significantly different from Ret-He, 27.8(2.4) and Hypo-He, 6.5(5.5) in the iron-deficient group. In distinguishing LID from normal, Ret-He with cut-off 29.9pg has a sensitivity of 86.8% and specificity of 85.7%, AUC 0.914 while Hypo-He

showed AUC 0.934, with cut-ff 1.6%, sensitivity 85.7% and specificity 92.1% (Eloísa Urrechaga et al., 2016).

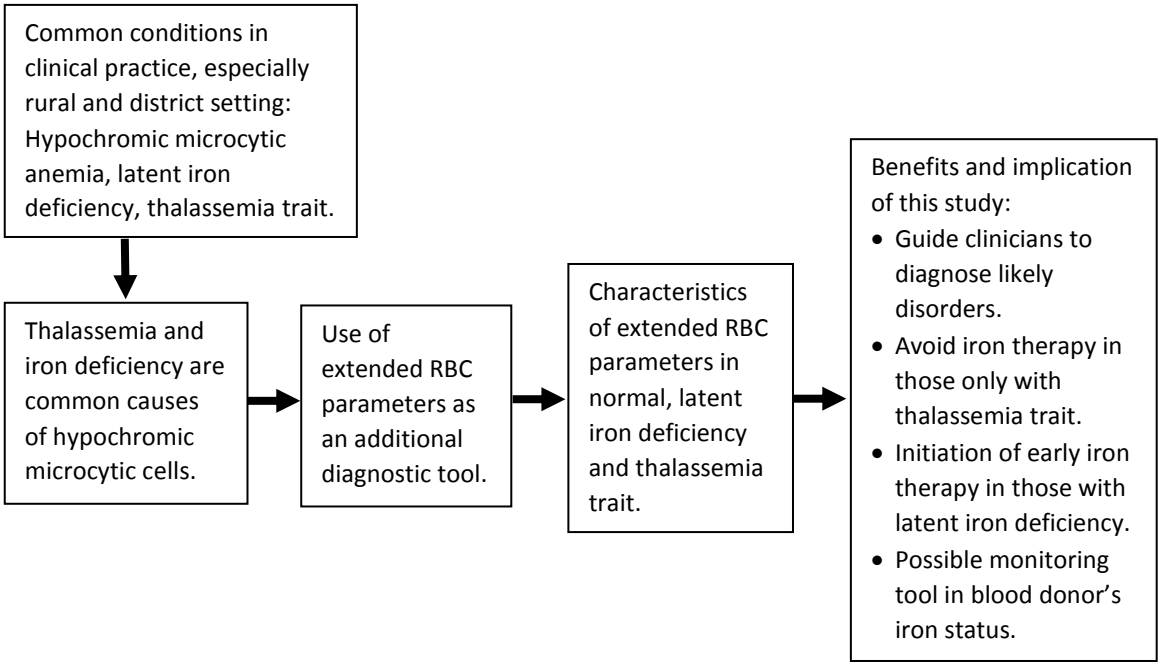
In Malaysia, 4 studies explored the use of extended RBC parameters. Reference interval for full blood count, serum ferritin, serum soluble transferrin receptor (sTfR), Hb A2, %Hypo-He, %Hyper-He, %MicroR and %MacroR are established by using data from 2725 healthy adults. There is no significant difference between age, gender and race for the extended RBC parameters. Reference interval for parameters that are relevant for this study are as follows: MicroR 0-3.8%, Hypo-He 0-1.3%, RetHe 30.7-38.9pg and serum ferritin 55.7-173.48ng/mL for males and 14.45-87.75ng/mL for premenopausal females (Ambayya et al., 2014). Another study focused on the normal population, latent iron deficiency, and iron deficiency anemia among non-pregnant women. MicroR and Hypo-He are significantly different ($p<0.001$) between healthy (1.7(1.07) and 0.5(0.45)), IDA (18.7(14.02) and 18.8(17.35)) and LID (4.3(3.02) and 2.2(2.37)). MicroR was also the best parameter to distinguish IDA from LID (AUC 0.915, sensitivity 85.57%, specificity 86.58% for the cut-off value of 0.655%). Other parameters that have AUC > 0.8 are Ret-He, Hypo-He and sTfR (Ambayya et al., 2019). Among donors, Amir et al. demonstrated that Hypo-He with AUC 0.906, cut-off 0.6%, 74.51% sensitivity and 88.24% specificity was a good marker to detect iron deficiency (Amir et al., 2019). MicroR: Hypo-He ratio (M: H ratio) demonstrated to be a useful tool in discriminating thalassemia minor and IDA with AUC of 0.83, sensitivity 80.8%, specificity of 71.6% using cut-off value 2.25 (S. Y. Wee et al., 2020)

Serum ferritin was chosen as a diagnostic tool to determine the presence of iron deficiency as it is the most powerful test with an AUC of 0.95, in comparison with transferrin saturation, MCV, RDW and red cell protoporphyrin (Wang et al., 2010). The

soluble transferrin receptor is sensitive and accurate however this is an expensive test with limited availability (Eloísa Urrechaga et al., 2016).

These studies on extended parameters have focused on providing additional clues in the diagnosis of iron deficiency – in latent and anemic stage, and thalassemia. However, studies on exploring the utility of MicroR and Hypo-He in the differentiation of LID and thalassemia trait are lacking.

Conceptual framework



Research Design

A prospective, case-control design is used to determine the characteristics and diagnostic accuracy of MicroR, Hypo-He and Ret-He.

Study area

1. Blood Bank, Hospital USM.
2. Mobile blood donation sites organized by Blood Bank, Hospital USM.

Study population

1. Reference population/Target populations are adults in Kelantan.
2. Source population is blood donors in Kelantan.
3. Study population is blood donors in Kota Bharu.
4. Sampling frame is those that fulfilled the study criteria.

Subject criteria

Inclusion:

1. All donors who are eligible for blood donation based on guideline published by National Blood Center, Malaysia and Transfusion Unit, Hospital USM.

Exclusion:

1. Postmenopausal.
2. On iron therapy.

Withdrawal:

1. Hemoglobin concentration based on FBC results
    a. Men: <13 g/dL or >18 g/dL
    b. Women: <12 g/dL or >18 g/dL
2. Participants with combined latent iron deficiency and thalassemia trait.
3. Reactive donors.

Recruitment and assessment of inclusion and exclusion criteria will be reviewed by the principal investigator, and medical officers in the Transfusion Unit, Hospital USM.

Sample size estimation

Specific objective 1

To compare the level of MicroR, Hypo-He and Ret-He between normal, latent iron deficiency and thalassemia trait.

Method: Sample size calculator for one-way analysis of variance using Stata/MP 14.0 for Windows.

$\alpha = 0.05$

Power = 0.8

Number of groups = 3

Variance = Largest variance in the group is used for each parameter

The prevalence of LID among blood donors with Hb>12.5g/dL is 21.8%, and the prevalence of thalassemia trait among blood donors with Hb≥13.0g/dL is 13.75% (Amir et al., 2019; Rosline et al., 2006). Approximately, group weight of 4:2:1 is used for normal: latent iron deficiency: thalassemia trait. The sample size is corrected for a 10% dropout rate.

RBC parameters	Normal, Mean (SD)	LID, Mean (SD)	Thalassemia trait, Mean (SD)	Calculated sample size, n	Corrected sample size, n_c
MicroR	1.8 (1.02) (Ambayya et al., 2014)	4.3 (3.02) (Ambayya et al., 2019)	37.5 (12.4) (E. Urrechaga et al., 2011)	14 (8:4:2)	16
Hypo-He	0.46 (0.43) (Ambayya et al., 2014)	2.2 (2.37) (Ambayya et al., 2019)	13.2 (9.9) (E. Urrechaga et al., 2011)	56 (32:16:8)	63
Ret-He	34.8 (2.09) (Ambayya et al., 2014)	31.4 (2.73) (Ambayya et al., 2019)	21.8 (3.8) (Canals et al., 2005)	14 (8:4:2)	16

For specific objective 1, 56 samples are required with the distribution of 32 normal samples, 16 latent iron deficiency samples and 8 thalassemia trait samples. 63 samples are required assuming a 10% drop-out rate.

Specific objective 2

To determine the sensitivity, specificity of MicroR, Hypo-He and Ret-He in the diagnosis of latent iron deficiency and thalassemia trait.

Method: Sample size calculator for sensitivity and specificity written by Dr Lin Naing and Mohd. Ayub Sadiq, School of Dental Sciences, USM, dated 23 March 2004.

Desired precision = 0.1, Confidence interval = 95%

Prevalence of LID among blood donors 21.8% (Amir et al., 2019).

Parameters	**Sensitivity**	**Specificity**	**N_{Sn}**	**N_{Sp}**	**Corrected sample size, n_c**
MicroR (Ambayya et al., 2019)	73.17	81.92	362	73	402
Hypo-He (Ambayya et al., 2019)	78.05	84.12	315	66	350
Ret-He (Ambayya et al., 2019)	75.61	78.05	339	84	377

Based on my literature review, there are incomplete data regarding prevalence, sensitivity, and specificity of MicroR, Hypo-He and Ret-He in distinguishing thalassemia trait from normal and in distinguishing latent iron deficiency from normal to complete sample size calculation. For specific objective 2, 362 samples are required. If a 10% drop-out rate is assumed, 402 samples are required.

Specific objective 3

To compare the ROC curve between MicroR, Hypo-He and Ret-He to distinguish latent iron deficiency and thalassemia trait from normal.

Method: Sample size calculation for comparison of two ROC curves using Medcalc, Version 19.4.1.

Type I error = 0.05

Type II error = 0.20

Correlation in the positive and negative group assumed to be 0.5.

In distinguishing LID from normal, AUC for MicroR, Hypo-He and Ret-He are 0.844, 0.891 and 0.852 respectively (Ambayya et al., 2019). There are no published data for AUC of MicroR, Hypo-He and Ret-He in distinguishing thalassemia trait from normal, and thalassemia trait from latent iron deficiency.

Distinguishing latent iron deficiency from normal

a. The ratio of sample sizes in negative and positive groups = 2
b. Expected difference between AUC to be 10%.
c. Sample size corrected for a 10% dropout rate.

	n	n_c
MicroR and Ret-He	174	194
Hypo-He and Ret-He	126	140
MicroR and Hypo-He	174	194

For specific objective 3, 17 samples are required. If a 10% drop-out rate is assumed, 194 samples are required. Thus, to satisfy the requirement of all three specific objectives and a 10% drop-out rate, 402 samples are required, with the distribution of 230 normal samples, 115 latent iron deficiency samples and 57 thalassemia trait samples.