A COMPARISON STUDY OF RED BLOOD CELL INDICES IN SOUTHEAST ASIAN OVALOCYTOSIS (SAO), IRON DEFICIENCY ANAEMIA (IDA) AND SAO WITH CONCOMITANT IDA

BY

NIK SHAIDA SHAMIM BINTI NIK GHAZALI

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (TRANSFUSION SCIENCE)

ADVANCED MEDICAL AND DENTAL INSTITUTE
UNIVERSITI SAINS MALAYSIA

2019

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DECLARATION

I hereby declare that this research was sent to Universiti Sains Malaysia (USM) for the degree of Master of Science in Transfusion Science. It has not been sent to other universities. With that, this research can be used for consultation photocopied as reference.

Sincerely,

…….

NIK SHAIDA SHAMIM BINTI NIK GHAZALI

(P-IPM0045/18)

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In the name of Allah the Most Gracious and the Most Merciful

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LIST OF SYMBOLS

α	Alpha
β	Beta
μ	Micro
±	Plus-minus sign
Δ	Precision
>	More than
<	Less than
mm	Millimetre
fL	Femtolitre
pg	Picograms
μg/L	Micrograms per litre
ng/mL	Nanograms per millilitre
mg	Milligrams
μL	Microlitre
g/dL	Grams per decilitre

LIST OF ABBREVIATIONS AND ACRONYMS

AE1	Anion Exchanger-1
β-TT	Beta Thalassaemia Trait
bp	Base Pair
cdb3	Cytoplasmic Domain of Band 3
DNA	Deoxyribonucleic Acid
dRTA	Distal Renal Tubular Acidosis
Fe	Iron
FBP	Full Blood Picture
Hb	Haemoglobin
HE	Hereditary Elliptocytosis
HPP	Hereditary Pyropoikilocytosis
HS	Hereditary Spherocytosis
ID	Iron Deficiency
IDA	Iron Deficiency Anaemia
IPPT	Institut Perubatan dan Pergigian Termaju
IQR	Interquartile Range
JEPeM	Human Research Ethics Committee
LIS	Laboratory Information System
MCV	Mean Cell Volume
MCH	Mean Cell Haemoglobin
MCHC	Mean Cell Haemoglobin Concentration
MREC	Medical Research Ethics Committee
MSCV	Mean Sphered Cell Volume
PBS	Peripheral Blood Smear
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
RBC	Red Blood Cell
RDW	Red Distribution Width
Ret-He	Reticulocyte Haemoglobin Equivalent
SAO	Southeast Asian Ovalocytosis
SD	Standard Deviation

SPSS	Statistical Package for the Social Sciences
TIBC	Total Iron Binding Capacity
TSAT	Transferrin Saturation
USM	Universiti Sains Malaysia
WBC	White Blood Cell
WHO	World Health Organization

KAJIAN PERBANDINGAN INDEKS SEL DARAH MERAH DALAM KALANGAN INDIVIDU PENGHIDAP OVALOSITOSIS ASIA TENGGARA (SAO), ANEMIA AKIBAT KEKURANGAN ZAT BESI (IDA) DAN SAO DENGAN IDA

ABSTRAK

Pengenalan: Ovalositosis Asia Tenggara (SAO) dan anemia akibat kekurangan zat besi (IDA) merupakan penyakit yang biasa berlaku di Malaysia. Kedua-duanya mempunyai corak parameter sel darah merah (RBC) tertentu namun mempunyai ciri-ciri morfologi yang saling bertindih antara satu sama lain. Apabila pesakit SAO mempunyai IDA, ia memberikan satu cabaran untuk membezakan keadaan ini. Oleh itu, kajian ini bertujuan untuk mengkaji parameter RBC dalam tiga kumpulan individu dengan SAO sahaja, IDA sahaja dan SAO dengan IDA dan mengenal pasti parameter yang berpotensi dalam mendiskriminasi ketiga-tiga keadaan ini.

Metodologi: Kajian rentas keratan retrospektif ini dijalankan dalam kalangan 79 subjek dari Institut Perubatan dan Pergigian Termaju (IPPT), USM (30 kes dengan SAO sahaja dan IDA sahaja dan 19 kes SAO dengan IDA). Data diperoleh dari sistem maklumat makmal (LIS) di Makmal Diagnostik Lanjutan dari Januari 2010 hingga Mei 2019. Analisis univariable (Ujian ANOVA atau Kruskal-Wallis) digunakan untuk membandingkan parameter sel darah merah antara ketiga-tiga kumpulan ini, iaitu parameter RBC, Hb, MCV, MCH, MCHC dan RDW. Kepentingan statistik telah ditetapkan pada nilai $P<0.05$.

Keputusan: SAO dengan IDA menunjukkan purata RBC, Hb, MCV, MCH, MCHC dan RDW sebanyak 3.5 x 10^6/μL, 9.2 g/dL, 74.6 fL, 25.7 pg, 33.7 g/dL dan 46.0 fL. Tiga parameter sel darah merah iaitu Hb, MCV dan MCH dalam kumpulan SAO dengan IDA menunjukkan nilai yang lebih rendah berbanding dengan kumpulan SAO sahaja (P<0.001). Walau bagaimanapun, parameter RBC, MCHC dan RDW menunjukkan nilai yang tidak ketara antara kedua-dua kumpulan (P>0.05).

Kesimpulan: Tiga parameter sel darah merah iaitu Hb, MCV dan MCH, mempunyai potensi untuk mendiskriminasi antara pesakit yang mempunyai SAO dengan IDA dan SAO sahaja. Oleh itu, bagi pusat-pusat hematologi yang tidak mempunyai kemudahan untuk menganalisis sel darah merah menggunakan teknologi mesin terkini, ketiga-tiga parameter asas ini berpotensi menjadi panduan kepada pemilihan ujian profil besi atau analisis Hb bagi pengesahan diagnosis IDA atau talasemia (di mana secara klinikal ditunjukkan) bagi mengoptimumkan pengurusan pesakit. Walau bagaimanapun, ini merupakan kajian awal dan saiz sampel yang lebih besar diperlukan dalam penyelidikan masa depan.

Kata Kunci: SAO, IDA, SAO dengan IDA dan parameter sel darah merah

A COMPARISON STUDY OF RED BLOOD CELL INDICES IN SOUTHEAST ASIAN OVALOCYTOSIS (SAO), IRON DEFICIENCY ANAEMIA (IDA) AND SAO WITH CONCOMITANT IDA

ABSTRACT

Introduction: Southeast Asian ovalocytosis (SAO) and iron deficiency anaemia (IDA) are common in Malaysia. Both have specific red blood cells (RBC) parameters pattern but overlapping morphological features. When SAO patients having concomitant IDA, it is quite challenging to differentiate them. Thus, the study aims to study RBC parameters in three groups of individuals with SAO alone, IDA alone and SAO with concomitant IDA and identifying potential parameters in discriminating them.

Methodology: A retrospective cross-sectional study was conducted among 79 subjects from Institut Perubatan dan Pergigian (IPPT), USM (30 cases with SAO alone and IDA alone and 19 cases SAO with IDA). The data were obtained from the laboratory information system (LIS) of Advanced Diagnostic Laboratory from January 2010 to May 2019. Univariable analysis (One-way ANOVA or Kruskal-Wallis test) was used to compare the red blood cell parameters between the three groups, which include RBC, Hb, MCV, MCH, MCHC and RDW. Statistical significance was set at a *P*-value of <0.05.

Results: SAO with concomitant IDA showed mean of RBC, Hb, MCV, MCH, MCHC and RDW of 3.5 x 10^6/μL, 9.2 g/dL, 74.6 fL, 25.7 pg, 33.7 g/dL and 46.0 fL, respectively. It was found that the Hb, MCV and MCH of SAO with concomitant IDA group were lower compared to the SAO alone group with significance *P*-value

(P<0.001). However, the RBC count, MCHC and RDW parameters were not significant between both groups (P>0.05).

Conclusion: Three RBC parameters, namely Hb, MCV and MCH, were shown to be potential parameters to discriminate between patients SAO with concomitant IDA and SAO alone. Therefore, in a centre where advance haematology analyser is not available, these three basic parameters may be potentially used to guide ordering of iron profile or Hb analysis for confirmatory diagnosis of IDA or thalassaemia trait (where clinically indicated) to optimise patients management. However, this is a preliminary study, and larger sample size is needed in future research.

Keywords: SAO, IDA, SAO with concomitant IDA, Red blood cell parameters

CHAPTER 1

INTRODUCTION

1.1 Study Background

Hereditary Elliptocytosis (HE) characterised by the presence of elongated or oval shape of erythrocytes that is due to red blood cell membrane defects. It is rare in the United States and Europe but high in malaria endemic-region, such as African and Southeast Asia (Naeim et al., 2013). In a full blood picture, the most prominent finding is the oval shape and elongated red blood cells. HE is occurred due to defects in various membrane proteins resulting in weakness of the skeleton membrane of erythrocytes. Most of the HE cases affecting the spectrin dimer-to-dimer lateral bonds or the spectrin-ankyrin-protein 4.1 junction complex. It is classified into three subtypes according to morphology, which are Common Elliptocytosis, Spherocytic Elliptocytosis and Southeast Asian ovalocytosis (SAO) (Figure 1.1) (Hoffbrand et al., 2011).

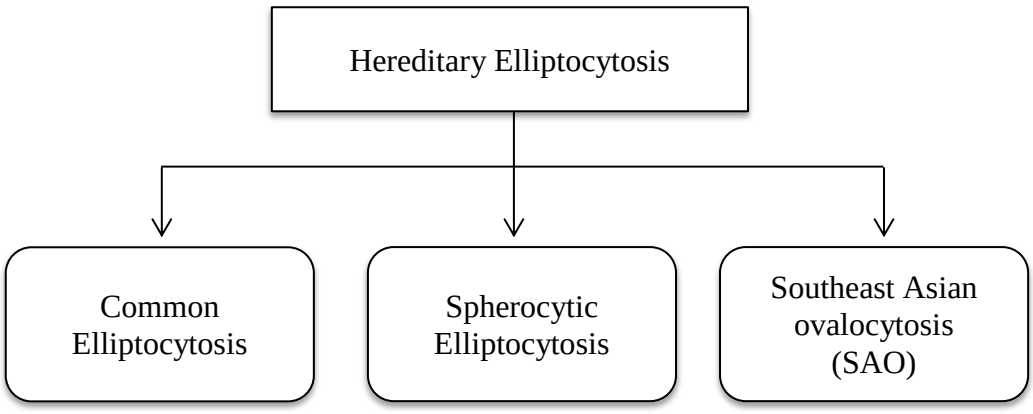

Figure 1.1 Classification of Hereditary Elliptocytosis

Common HE, as the name itself, it is the most common form of elliptocytosis. About 5 to 10% cases present with haemolytic anaemia with mostly elliptical shape (ellipse shape), some ovalocytes, and rod-shaped (Figure 1.2) (Da Costa *et al.*, 2013; Fathima and Sitalakshmi, 2013). Mutation in α or β chains are the leading causes of typical HE, and it has wide distribution, most commonly affected persons from African origin, but it is also present in other populations as well (Conboy *et al.*, 1991). Spherocytic elliptocytosis is a rare disease, an elliptocytosis and spherocytosis phenotypic hybrid with the presence of variable numbers of elliptocytes and some spherocytes. Hereditary elliptocytosis and hereditary spherocytosis are the most prevalent red cell membrane diseases in the area of North America, and Northern European countries with an incidence of about 0.05% (one out of 2000) affected cases. Because of underdiagnosis of asymptomatic forms, it is also probable to be even higher.

Southeast Asian ovalocytosis is characterised by oval-shaped red cells, tend to appear as stomatocytes and knizocytes (Figure 1.3). The distribution of SAO is more restricted than common elliptocytosis, which appears to be restricted to Southeast Asia and individual Western Pacific islands (Nurse et al., 1992). It has a high prevalence in the area where malaria is endemic such as Melanesia, Indonesia and Southern Thailand and thus, it is giving advantage to the individual with SAO phenotypes with the protection against both *Plasmodium falciparum* and *Plasmodium vivax* (Mohandas and An, 2012; Rosanas-Urgell et al., 2012). In this study, we are going to focus on SAO patients specifically.

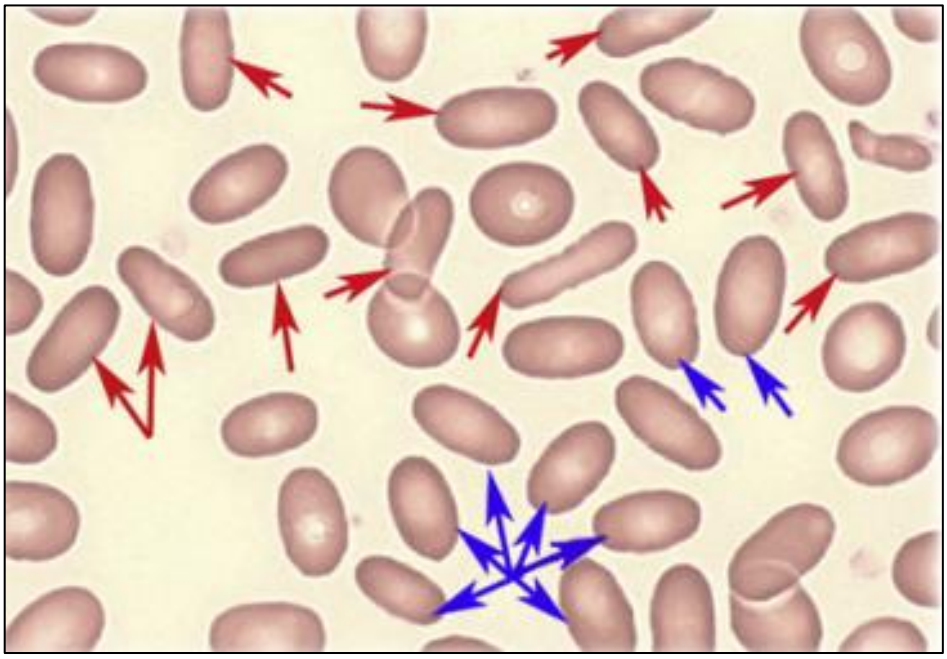

Figure 1.2 Hereditary elliptocytosis with ellipse shape (red arrows) and more ovalocytic red cells (blue arrows). Figure adapted from Da Costa et al. (2013).

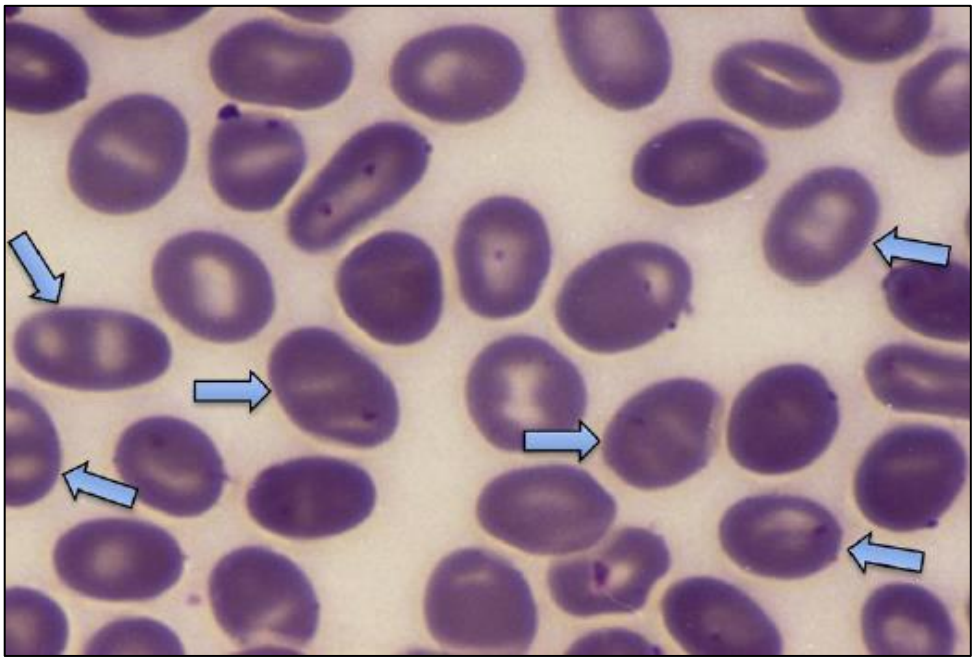

Figure 1.3 Morphologic consequence of SAO with the presence of ovalocytosis along with stomatocytes and knizocytes (arrow) on peripheral blood smears. Figure adapted from Nixon et al. (2018).

1.2 The Rationale of the Study

If there is more than 25% stated cells in the full blood picture (FBP), diagnosis ovalocytosis can be made. The incidence is one case in 2000 to 4000 individuals worldwide, but the exact true incidence is not known since the majority of the patients are asymptomatic (Gallagher, 2004). In Malaysia, the prevalence of SAO in the Malay population in Kelantan was reported as high as 4% (Yusoff *et al.*, 2003).

Iron deficiency is one of the significant nutritional deficiencies in industrialised and developing nations, including Malaysia. The World Health Organization (WHO) estimated that around 50% of the two billion people suffering from anaemia are due to an iron deficiency (World Health Organization, 2007). For iron deficiency anaemia (IDA), the prevalence reported ranged from 7.4% in the town area to 34% in a rural area (Al-Mekhlafi *et al.*, 2013; Nadarajan and Eow, 2002).

In FBP of IDA, pencil-shaped cells, cigar cells and elliptocytes were commonly found in IDA patients. However, through these FBP findings, it is difficult to tell whether patients with SAO having concomitant IDA or not as the morphology in SAO can be confusing, and some are overlapping. The status of iron deficiency is usually be confirmed by doing serum ferritin. Bone marrow aspirate becomes the gold standard to assess the iron storage; however, this technique not routinely done due to the invasive technique. The concentration of serum ferritin is used as an iron store index as the quantity of ferritin relative to iron concentration in the body: about 8 mg of storage iron is equal to 1 ng/mL of ferritin.

Although most of the hospitals are using the latest full blood count machine such as Sysmex XE5000, where iron status can be predicted from the reading of particular

parameter, namely retic haemoglobin (RET-He), however, due to budget constraint, some district hospitals do not have these privileges and still using the old, outdated machine. This old machine has only provided the primary parameter such as MCV, MCH and MCHC. Therefore, a haematologist who works in these district hospitals may encounter difficulty differentiating SAO with concomitant IDA. It is because the morphology of SAO red blood cells is not the same as in a typical IDA RBC morphology. In SAO, the RBC usually are macroovalocytes with stomatocyte and knizocytes while in pure IDA, the RBC morphology consists of pencil shape, cigar shape and hypochromic microcytic RBC. In an event where these two diseases co-exist together, we do not know how it may affect the primary RBC parameters.

To date, no study has been conducted to study these haematological parameters in SAO patients with concomitant IDA and thus to differentiate it from SAO with no iron deficiency cases. From the literature review, many studies are only focusing on differentiating IDA with thalassaemia trait. A study from Liao *et al.* (2014) shows that the measurement of mean cell volume (MCV) and mean sphered cell volume (MSCV) able to discriminate between an individual with hereditary spherocytosis and thalassaemia rapidly. In 2016, related research by January F Matos in Sao Paulo developed a new parameter, known as Matos & Carvalho Index, with a cut-off point of 23.85 to differentiate between IDA and thalassaemia. IDA is categorised as <23.85, while values >23.85 classify the patient as a carrier of thalassaemia (Matos *et al.*, 2016). Another study by Bose and Maimoon (2018) assess the accuracy of Mentzer index in IDA and thalassaemia differentiation. The result shows that Mentzer index of equal to or more than 13 (≥13) indicating iron deficiency anaemia and less than 13 indicating thalassaemia was found to be a reliable screening tool to differentiate between these two diseases.

Therefore, in this study, we are focusing on RBC indices between patients of IDA and those with SAO co-inheritance. It is hoped that from this study, we can get haematological parameter values that can rapidly discriminate SAO patients having concomitant with IDA with SAO patients without IDA. A haematologist may rapidly screen and identify SAO patients with possible iron deficiency based on the RBC, Hb, MCV, MCH, MCHC and RDW parameter index and thus suggest for iron deficiency workup to the clinicians.

1.3 Objectives of the Study

1.3.1 General Objectives

To study red blood cell indices, namely RBC, Hb, MCV, MCH, MCHC and RDW values in three groups of individuals with SAO alone, IDA alone and SAO with concomitant IDA.

1.3.2 Specific Objectives

1) To determine the latest prevalence and demographic characteristics of SAO disease patients in Institut Perubatan & Pergigian Termaju (IPPT), USM Bertam.

2) To compare red blood cell indices, namely RBC, Hb, MCV, MCH, MCHC and RDW in individuals with SAO alone, IDA alone and SAO with concomitant IDA.

CHAPTER 2

LITERATURE REVIEW

2.1 Southeast Asian Ovalocytosis (SAO)

2.1.1 Red Blood Cell Membrane Structure

Mature red blood cells (RBC) have unique morphology that makes it easy to recognise. RBC have a biconcave-disc shape that is essential for normal function and with 120 days of survival in the peripheral circulation. It has a diameter of approximately 6 to 8 millimetre (mm), 2 mm thickness and an average volume of approximately 90 femtolitres (fL) with a surface area of about 136 mm (Figure 2.1). It has lacks of nucleus or mitochondria, and about 30% of its content is made up of haemoglobin. The plasma membrane is a lipid bilayer embedded in proteins and linked to the underlying protein cytoskeleton, and it is the key to maintaining the shape (Kohaine *et al.*, 2016).

The biconcave shape of RBC depends on vertical and horizontal interactions between integral membrane proteins and cytoskeletal protein. Ankyrin and protein 4.1 are the two major transmembrane protein complexes, whereas the two major cytoskeleton protein is α-spectrin and β-spectrin. The transmembrane protein complexes, which are ankyrin complex and protein 4.2 complex form the vertical interaction (Figure 2.2). Ankyrin complex links transmembrane protein and band 3 to the cytoskeleton while protein 4.1 complex links transmembrane proteins, glycophorin C to the cytoskeleton, and adducin links transmembrane proteins, band 3 and glucose transport (Kohaine *et al.*, 2016). α-spectrin and β-spectrin formed flexible rod-like heterodimers that form tetramers together with other spectrin heterodimers (Kohaine *et*

al., 2016; Tse and Lux, 1999). The spectrin heterodimers also form a spectrin-actin-protein 4.1 junctional complex with accessory proteins such as tropomyosin, tropomodulin and adducin, resulting in the horizontal interaction stability.

Any defects in the interaction between components of the red cell can affect the structure and function of the membrane, which can be further divided into those affects the vertical interactions and horizontal interactions. Changes in vertical and horizontal interactions result in changes in the density of the spectrum network, which invariably creates modifications in cell morphology, membrane instabilities and RBC deformability for many RBC hereditary disorders (Diez-Silva *et al.*, 2010). Hereditary elliptocytosis or inherited pyropoikilocytosis have been related to deficiencies in horizontal interaction, while deficiencies in vertical interactions can lead to hereditary spherocytosis (Morris and Lux, 1995).

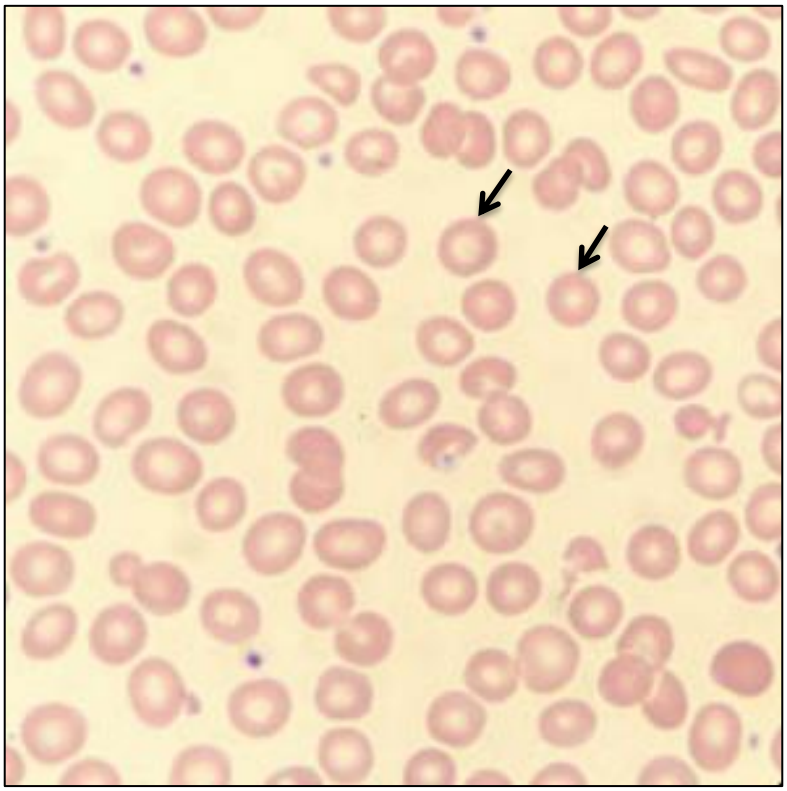

Figure 2.1 Morphology of healthy red blood cells (arrow) with the presence of normochromic and normocytic from peripheral blood smear. Figure adapted from Ford (2013).

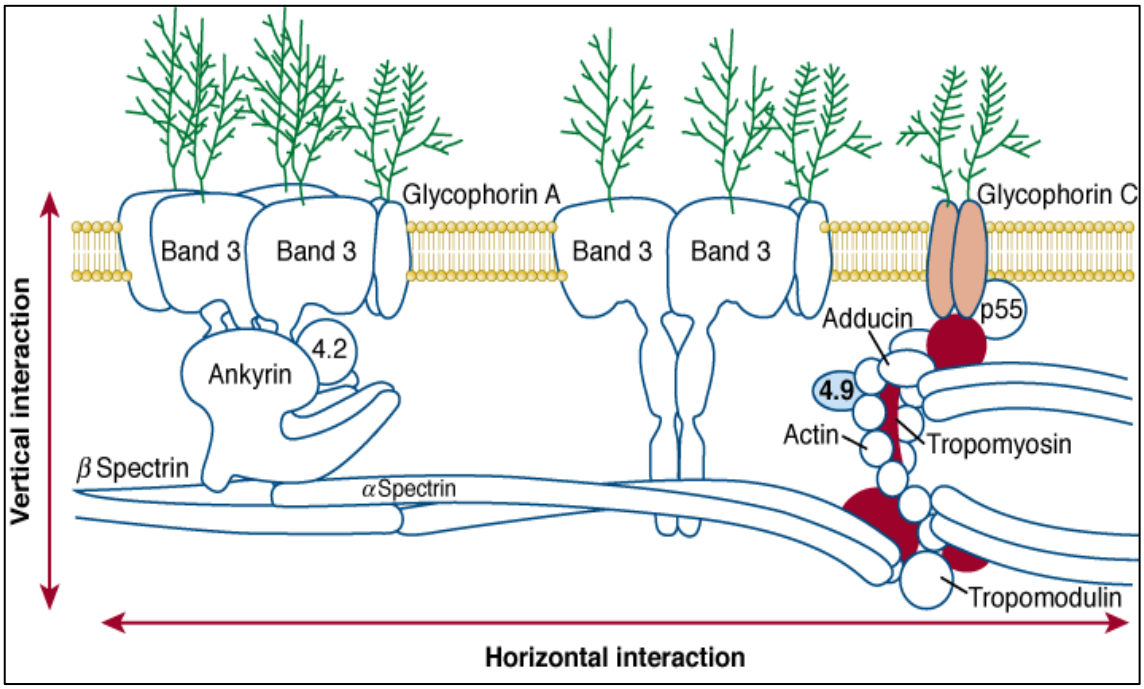

Figure 2.2 The schematic diagram of the red blood cell membrane, specifying the vertical and horizontal interaction of its components. Figure adapted from Tse and Lux (1999).

2.1.2 Aetiology of SAO

Southeast Asian ovalocytosis (SAO) is a unique hereditary type of elliptocytosis commonly found in Southeast Asian countries, including Malaysia, Indonesia, and the Philippines (Tse and Lux, 1999; Yusoff *et al.*, 2003). It is an autosomal-dominant red blood cell abnormality that results from the removal of 27 base pairs on chromosome 17 in exon 11 of the band 3 protein gene (Jarolim *et al.*, 1991). Band 3 is the main transmembrane protein, also known as anion exchanger 1. This transmembrane protein is essential for maintaining both intracellular environments, by passive anion transport and its shape through attachment via ankyrin to spectrin in the cytoskeleton protein (Jay, 1996).

The molecular genetic defect in SAO is the result of the deletion in SLC4A1 of 27 base pairs, the gene that encodes for band 3 membrane proteins. This gene defect results in the removal of nine amino acids (codon 400 to 408) in the edge of the band 3 cytoplasmic and membrane domains that are closely related to the Lys 56-to-Glu substitution (Jarolim *et al.*, 1991; Liu *et al.*, 1995). In the RBC membrane, band 3 is the most abundant protein which consists of two domains; N-terminal 41-kD cytoplasmic domain of band 3 (cdb3) and C-terminal 56-kD membrane domain. Several research revealed the biochemical, biophysical and ultrastructural implications of this mutation, including a marked reduction in lateral membrane mobility of band 3, enhanced extensional membrane rigidity, enhanced association of band 3 with membrane skeleton, enhanced tendency to form oligomers and linear membrane aggregates, and reduced anion transport activity (Liu *et al.*, 1995; Mohandas *et al.*, 1992; Schofield *et al.*, 1992).

Besides, the inheritance of these mutations provides a selective advantage over the survival of the severe complications of malaria diseases endemic to Southeast Asia (Jeng and Vichinsky, 2004). These ovalocytes were indeed resistant to invasion by several strains of the plasmodium malaria, including *Plasmodium falciparum* and *Plasmodium knowlesi* following research in vitro (Jarolim *et al.*, 1991). A study by Mohandas et al. (1984) revealed that the marked rise in ovalocyte membrane rigidity resulted in resistance to parasite invasion. The changes in the RBC membrane and anion transport activity induced by SAO could affect the development of malaria parasites in several ways, including altering the parasite ability to grow within the erythrocyte, inhibiting the growth of parasites within the RBC of SAO and impairing the parasite's ability to remodel the RBC surface (Aikawa, 1988; Bruce, 2008; Rosanas-Urgell et al., 2012).

2.1.3 Clinical Description of SAO

SAO is also called Melanesian elliptocytosis or elliptocytosis stomatocyte. Like the name itself, it is due to the existence of rounded elliptocytes or ovalocytes and distinctive longitudinal slit stomatocytes on the peripheral blood smear of individuals with SAO (Figure 2.3) (Golafshan *et al.*, 2014). Deletion of codons 400 to 408 in the SLC4A1 gene encoding the band 3 erythrocyte membrane protein responsible for an abnormal erythrocyte stiffness and oval shape on the blood smear. Most people with SAO heterozygosity are asymptomatic with no clinically detectable haemolysis while homozygous deletion of SAO is considered to be fatal in the embryonic state and is not feasible without extensive antenatal care (Amato and Booth, 1977; Moulin *et al.*, 2017). SAO can occur at any age. SAO usually asymptomatic in adults; however, SAO may be expressed in neonates as haemolytic anaemia and needs phototherapy (Da Costa *et al.*, 2013).

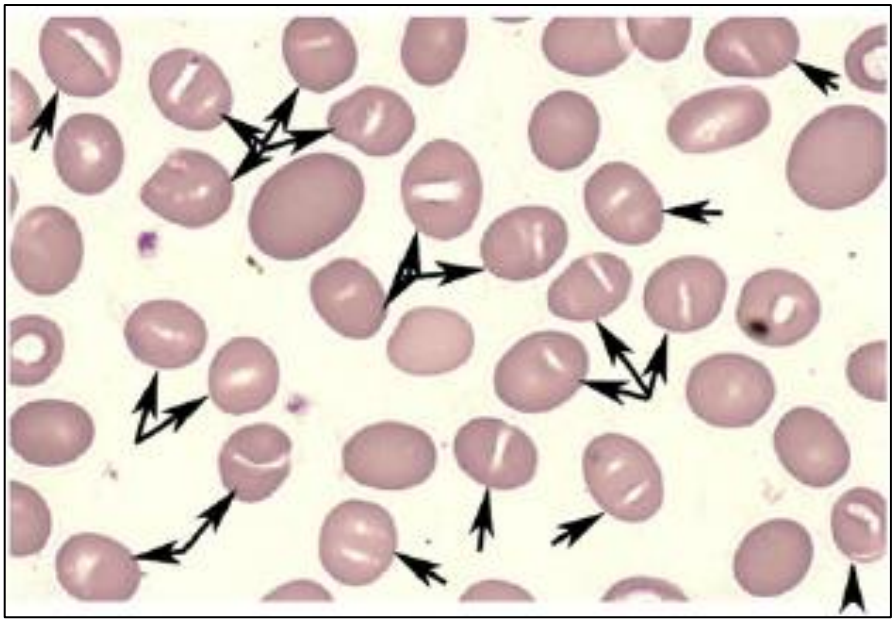

Figure 2.3 Morphology of red blood cells in Southeast Asian ovalocytosis with one to two transverse ridges (arrows). Figure adapted from Da Costa et al. (2013).

2.2 Prevalence

2.2.1 Prevalence of SAO

As mentioned earlier, SAO is an autosomal dominant form of inherited elliptocytosis prevalent in Southeast Asian communities, including those of Papua New Guinea with elevated coastal and lowland incidence (Amato and Booth, 1977; O'Donnell *et al.*, 1998). The incidence ranges from 5 to 25% in Indonesia, Philippines, Melanesia and Southern Thailand, where it mostly affected the regions of malaria-endemic (Fathima and Sitalakshmi, 2013). A study by Mgone *et al.* (1996) showed the incidence of erythrocyte band 3 deletion range from zero in both the lowland coastal region of Wosera, East Sepik Province and the Goroka (highland areas), Eastern Highlands Province to 35% on the west shore of Madang Province.

The incidence mostly associated well with altitude where malaria transmission is high at the highest on the shoreline, intermediate in the lowlands and lowest in the non-malarious highlands. In Indonesia, Kimura *et al.* (2002) recorded an incident of SAO around 12.6%. The incidence of SAO is about 4% in the Malaysian population (Yusoff *et al.*, 2003). Although there is no knowledge of the accurate distribution in distinct ethnic groups in Malaysia, study by Eng (1965) showed that the prevalence of inherited ovalocytosis was 12.3% in the Senoi tribe populations of Malaysia. SAO was found in 18 of the 22 patients with distal renal tubular acidosis (dRTA) (81.8%), but only two of the 50 healthy volunteers (4%) in Malays in Kelantan, Malaysia. This research by Yusoff *et al.* (2003) showed a significantly high incidence of SAO in those with dRTA, indicating a dysfunctional role of band 3 protein/AE1 in the development of dRTA.

2.2.2 Prevalence of IDA

In developing countries, the prevalence of anaemia is three to four times greater than in industrialised countries. The World Health Organization (WHO) indicated in 2002 that IDA becomes one of the ten most significant contributing factors to the global disease burden that raises morbidity and mortality in children and pregnant ladies. Of the two billion anaemic individuals, about 50% are due to iron deficiencies (World Health Organization, 2007).

In Malaysia, for those aged 15 years and above, the overall prevalence of anaemia in 2015 was 24.6% (Institute for Public Health, 2015). It is estimated that 30% of the population of the world is influenced by IDA and mostly by those living in developing countries (Vehapoglu *et al.*, 2014). The group of peoples who are at risk to develop IDA are young children, adolescent females and pregnant women due to their high iron demands (ACC/SCN, 2000; Al-Mekhlafi *et al.*, 2013). Approximately 50% of East Asian school-aged children and 60% of children under the age of five were revealed to have IDA (Stoltzfus, 2003).

2.3 SAO with Other Complication

2.3.1 SAO with distal Renal Tubular Acidosis (dRTA)

In the same patient, familial distal renal tubular acidosis (dRTA) and SAO may coexist. It is due to the mutations of the AE1 gene, which codes the bicarbonate or chloride exchanger for band 3. Band 3 protein is not only expressed in the membrane of red blood cells, but also distal renal tubules (Wrong *et al.*, 2002). In Malays of Kelantan, Malaysia, the incidence of SAO was examined in dRTA, which is about 81.8% in 22 dRTA patients and 4% in 50 healthy individuals (Yusoff *et al.*, 2003). Failure to produce a normal minimum urine pH (pH<5.5) is the characteristic of dRTA patients even in the presence of severe systemic acidosis (Wrong *et al.*, 2002; Yusoff *et al.*, 2003). The result from the impaired secretion of hydrogen ions from the distal nephron, leading to metabolic acidosis, frequently with nephrocalcinosis, hypokalaemia and metabolic bone disease (Yusoff *et al.*, 2003).

2.3.2 SAO with Thalassaemia trait

Thalassaemia is a genetic disease triggered by haemoglobin chains synthesis defects. These syndromes are among the world's most common genetic disorders, carrying thalassaemic mutations with approximately 1.7% of the world's population (Urrechaga *et al.*, 2011). β-thalassaemia is one of the types of thalassaemia that caused by deficient or absent synthesis of β-globin chains, leading to excess α-chains. Synthesis of β-globin is controlled by one gene on each chromosome 11. The production of the β-globin chain can range from near normal to completely absent, depends on the varying degrees of excess α-globin to β-globin chain production.

If one gene defect, it can lead to β-thalassaemia trait (minor). Individuals are known to have β-thalassaemia major when the synthesis from both genes is severely reduced or absent. While, the person has β-thalassaemia intermedia when the synthesis of β-chains is less severely reduced (Herbert and James, 2009). Among those three, β-thalassaemia trait (β-TT) is the most common type of haemoglobinopathy transmitted by heredity (Vehapoglu *et al.*, 2014). Individuals with β-TT are generally asymptomatic, and individuals are unaware of their carrier status unless diagnosed by screening. It is estimated that around 50% of the world's β-TT population are in Southeast Asia, the Mediterranean area, the Middle East, Southwest Europe and Central Africa (Urrechaga *et al.*, 2011). Thalassaemia is a public health issue in Malaysia, with approximately 4.5% of Malays, and Chinese are carriers of β-thalassaemia (George, 2001).

SAO and β-TT are relatively common genetic disorders in Malaysia. Despite the relatively high incidence of these two diseases in Malaysia, SAO and β-TT co-inheritance is rarely stated because the RBC indices are ignored and peripheral blood

smear tests are not routine. (Raman *et al.*, 2015). When SAO and β-thalassaemia separately inherited as heterozygous, conditions are usually clinically mild (Chen *et al.*, 2017). The study from Fucharoen *et al.* (2007) showed that the peripheral blood film examinations in the patient of SAO with β-thalassaemia repeatedly revealed moderate ovalocytosis.

2.3.3 SAO with Iron Deficiency Anaemia (IDA)

Anaemia is a condition where there are inadequate red blood cells to meet human physiological needs and is usually confirmed by looking at the haemoglobin level. It can be caused by many factors, the most common being deficiency of vital components for haemoglobin synthesis (iron, vitamin B_{12} and folic acid), blood loss, reproductive age-female with repeated pregnancies, worm infestation, haemolysis and conditions of bone marrow causing suppression of red cell synthesis. The most common contributing factor to anaemia is iron deficiency anaemia (IDA) due to the absence of adequate iron to synthesise haemoglobin.

SAO and IDA seem to be the most common haematological disorder in Southeast Asia, including in Malaysia. The prevalence of SAO among Malaysian population is 4% (Yusoff *et al.*, 2003) and the prevalence of IDA ranged from 7.4% in town area to 34% in a rural area (Al-Mekhlafi *et al.*, 2013; Nadarajan and Eow, 2002). Therefore, the chances of both disorders occur together may be high and could have some effects on RBC parameters. However, the data and research posed in patients with the association of these two disorders are still limited. Therefore, in this study, we are focusing on red blood cells indices between patients of IDA and those with SAO co-inheritance.

2.4 Diagnostic Methods

2.4.1 Peripheral Blood Smear

Initially, SAO is most often suspected based on a routine peripheral blood smear (PBS). PBS is the first line of investigation as abnormalities observed in some red cell morphology may indicate that patients can be suspected of having such particular haematological disorder. It is often the first indication of this form of red cell disorder, including hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and inherited pyropoikilocytosis (HPP) disorder (Delaunay, 2007). The criteria for diagnosing SAO in older children and adults are the presence of RBC with two or more linear or irregularly shaped pale regions and 20% of ovalocytes present in PBS (O'Donnell *et al.*, 1998). It is crucial to detect SAO heterozygosity by careful examination of RBC morphology in PBS in countries where DNA analysis is not available (Laosombat *et al.*, 2005).

PBS is pathognomonic in a patient with pure SAO, characterising with macro-ovalocytes with stomatocytes more than 25%. Usually, these ovalocytes have double Y- or V-shaped central pallors without polychromasia (Raman *et al.*, 2015). Characteristic of alterations in red blood cell morphology of SAO can be identified by the presence of ovalocytes (red cells with a ratio of width: length >1:1 and <2:1), stomatocytes (central region of pallor with single slit-like) and knizocytes (red cell with two or more pale region with narrow separated) (Amato and Booth, 1977). Cook *et al.* (2009) noted that both P. *falciparum* and P. *vivax* parasite levels in vivo were considerably lower in individuals with ovalocyte compared to those with normal red cells, while parasite invasion of rigid ovalocyte membranes was lowered in vitro. Unlike SAO with marked

ovalocytic red cells, both IDA and β-TT commonly presented with microcytosis and hypochromia (Jameel *et al.*, 2017). It is difficult to distinguish between the morphological results in both IDA and β-TT. Thalassaemia generally characterises a mild to moderate microcytic anaemia, sometimes presumptively diagnosed as IDA. Therefore, differentiating between these two anaemias is clinically relevant because both have a completely different cause, therapy and prognosis (Eldibany *et al.*, 1999).

Microcytosis usually occurs due to quantitative lack of synthesis of haemoglobin resulting from defects in either haem or globin chains production comprising the molecule of haemoglobin. Patient diagnosis of IDA by PBS often reveals anisopoikilocytosis with the presence of codocytes (target cells), elliptocytes (cigar-shaped) and bizarre RBC. Pencil cells are also commonly seen, and it is more numerous in IDA compare than in β-thalassaemia (Harrington *et al.*, 2008). A study by Urrechaga *et al.* (2011) reported that RBC tends to be more hypochromic and less microcytic in IDA than in thalassaemia patients (Figure 2.4).

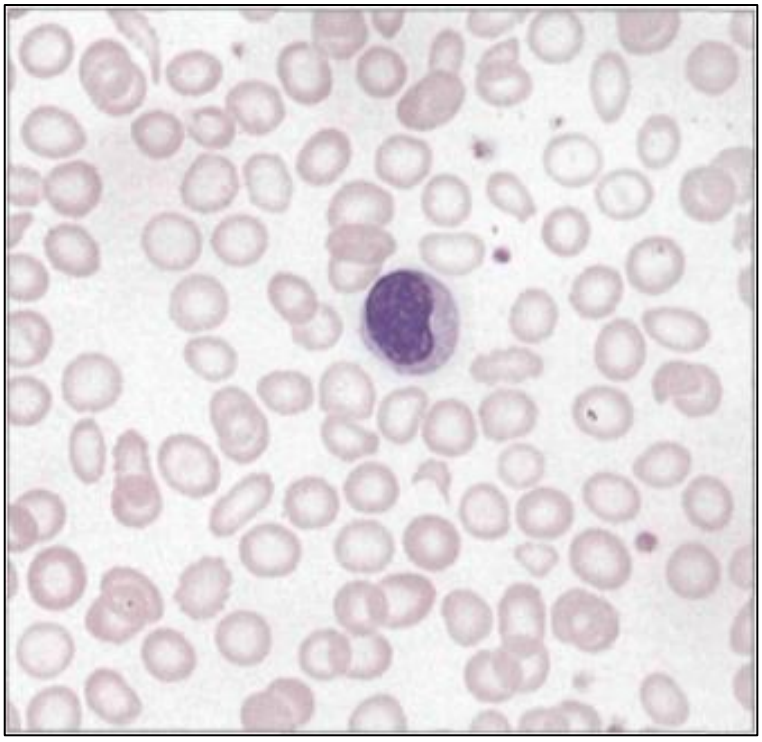

Figure 2.4 Patient with iron deficiency anaemia with microcytic and hypochromic red blood cell, (Wright stain, original magnification x1000). Figure adapted from Meredith and Rosenthal (1999).