ASSOCIATION OF IRON REGULATING GENES WITH IRON DEFICIENCY ANAEMIA (IDA) AND IRON REFRACTORY ANAEMIA

By

LIEW JIN ROU

A dissertation submitted to the Department of Allied Health Sciences,
Faculty of Science,
Universiti Tunku Abdul Rahman,
in partial fulfilment of the requirements for the degree of
Master of Science
November 2023

ABSTRACT

ASSOCIATION OF IRON REGULATING GENES WITH IRON DEFICIENCY ANAEMIA (IDA) AND IRON REFRACTORY ANAEMIA

Liew Jin Rou

Anaemia is a condition of haemoglobin level below the established cut-off level with reduction of erythrocytes and lead to insufficient to meet body's oxygen demands. This may lead to poor motor, mental performance, low work productivity in adults and poor pregnancy outcome. Many genetic variants in iron regulatory genes have been associated with impairment of iron metabolism, which in leading to iron deficiency anaemia. Malaysia had been reported with high prevalence rate of anaemia up to 13.8% and mostly resulted of iron deficiency. This had led to enormous impact on health quality of life and economic burden due to the healthcare cost. Hence, this had arisen to the attention of this study to elucidate the underlying associated factors including genetic variants in iron regulatory genes to iron metabolising parameters and its predisposition to anaemia. A total of 183 young adult subjects were recruited at Universiti Tunku Abdul Rahman, consisting of 83 males and 100 females. Demographic data, family history, current physiological conditions and clinical history were self-declared by respondents and anthropometric and haemoglobin level were measured. A volume of 6 mL of venous blood was collected for genotyping of genetic variants and measurement of hepcidin and serum iron concentrations. Eight genetic variants from 4 genes (rs10414846, rs10421768,

rs855791, rs4820268, rs12769, rs1799852, rs1799945 and rs1800562) were genotyped. Statistical analyses were done using SPSS version 22. This study reported high prevalence of anaemia with 14.75% (27/183) of the population were anaemic. Women who are currently on menstruation and having the experience of menorrhagia in the past 3 months were found to have lower haemoglobin, hepcidin and serum iron level compared to men due to excessive iron loss. Hepcidin expression was downregulated to increase iron absorption to replenish iron loss through menstruation. High BMI was found with higher haemoglobin level. Family history of anaemia shows lower haemoglobin level as inherited blood disorder may contribute to the development of anaemia. Both ethnicity and experience of bleeding problem in the past 3 months were found affect the hepcidin concentration. *HAMP* gene variant rs10421768 was found with lower serum iron level while *TF* gene variant rs12769 was found with significantly lower hepcidin and serum iron level. Genetic variants on the same gene were found to show significant difference but no significant difference was found in between genetic variants on different iron regulatory genes. In conclusion, gender, ethnicity, BMI, physiological conditions and genetic factors contributed to the development of anaemia in this study. The significant genetic variants could be potentially using as biomarkers to diagnose on the predisposition to anaemia.

Keywords: anaemia, single nucleotide polymorphisms, *TMPRSS6*, *HAMP*, *TF*, *HFE*

Subject Area: QH301-705.5 Biology (General)

ACKNOWLEDGEMENT

First and foremost, I would like to express my gratitude to my supervisors, Dr. Teh Lai Kuan and Dr. Loo Keat Wei for providing me an opportunity to participate in this study, providing tremendous guidance, encouragement, motivation and knowledge throughout my master's study. I would also like to acknowledge the laboratory personnel, Mr Saravanan, Ms Fatin Masturah, Madam Natrah, Madam Farhanah, Madam Nurhanisah, Ms Luke Choi May and Ms Nurul Illiana for their assistance during the bench work. I would like to thank my friends, Natalia Shania, Xin Yen and Li Fang for the motivational support and guidance throughout this study and made my life in Kampar a wonderful time. Finally, my sincere appreciation extends to my parents and my brother. Without their tremendous understanding and encouragement in the past few years, it would be impossible for me to complete my study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALK	Activin receptor-like kinase
ARMS-PCR	Amplification refractory mutation system polymerase chain reaction
BMI	Body mass index
BMP	Bone morphogenic protein
bp	Base pair
CBC	Complete blood count
CRP	C-reactive protein
CUB	Urchin embryonic growth factor and morphogenic protein
DctB	Duodenal cytochrome B
DMT-1	Divalent metal transporter 1
ESA	Erythropoiesis stimulating agent
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
F_I	Inner reverse
FKBP	FK506 binding protein
F_O	Outer forward
FPN1	Ferroportin-1
G6PD	Glucose-6-phosphate dehydrogenase deficiency
HAMP	Hepcidin antimicrobial peptide
Hb	Haemoglobin
Hct	Haematocrit
HFE	Homeostatic iron regulator
HO-1	Haem-oxygenase 1
HRG 1	Haem-transporting permease
IDA	Iron deficiency anaemia
IL	Interleukin
IRIDA	Iron refractory iron deficiency anaemia
LDLR	Low-density lipoprotein receptor
MAF	Minor allele frequency
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
NRAMP 1	Natural resistance associated macrophage protein 1
OD	Optical density
RDW	Red cell distribution width
RFLP	Restriction fragment length polymorphism
R_I	Inner reverse
R_O	Outer reverse
ROS	Reactive oxygen species
SEA	Sea urchin sperm protein, enteropeptidase and argin
SMAD	Sons of mothers against decapentaplegic
SNP	Single nucleotide polymorphism
TE	Tris-EDTA
TF	Transferrin
TFR	Transferrin receptor

TIBC	Total iron binding capacity
TMPRSS6	Transmembrane serine protease 6
TSAT	Transferrin saturation
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Background

Anaemia is one of the global public health problems that affecting both developing and developed countries. It was defined as a condition where the haemoglobin level lower than the established cut-off level and lead to insufficient oxygen to meet individual's physiological demands (Chaparro and Suchdev, 2019; World Health Organisation, 2017). In year 2008, WHO declared that anaemia is a common disease that affects about 1.6 billion people worldwide which is approximately 24.8% of the global population. Recent year, anaemia has been found affecting approximately 1.2 billion people worldwide (Jallow et al., 2020; Warner and Kamra, 2020).

Anaemia could occur at any stages of life. However, it is more prevalent in preschool-age children, pregnant women as well as women of reproducing age. Anaemia caused numerous challenges to the society including major consequences on human health, social development as well as economic development. Severe anaemia in pregnant women had increased the risk of maternal and child mortality, negative consequences on cognitive and physical development in children and work productivity of adults due to nutritional anaemia become one of the major concerns of the society. Moreover, the risk of postoperative morbidity and mortality were greatly elevated due to the high prevalence of anaemia in surgical patients (World Health Organisation, 2008).

1.2 Aetiology of Anaemia

Anaemia could develop when there is an imbalance in erythrocyte loss relative to its production. Development of this condition could be due to blood loss, deficient in erythropoiesis, excessive destruction of red blood cell as well as genetic. Both acute and chronic blood loss could lead to the development of anaemia. However, anaemia would not develop until several hours after acute blood loss as the remaining erythrocyte mass in the intravascular spaces were not diluted by the diffused interstitial fluid (Braunstein, 2020).

Deficient in erythropoiesis may arise due to nutritional insufficiency, inflammation and chronic diseases (Li et al., 2020). Deficient in iron, folate or vitamin B can impair red cell production as the minerals and vitamins is insufficient to support the production of red blood cell. This will lead to impair production of red blood cell that could not function normally and eventually in leading to anaemia (Brazier and Martinez, 2019).

Anaemia of chronic disease which is also known as anaemia of inflammation. It is a condition which associated with multiple underlying disorders such as cancer, infection, autoimmune disease as well as inflammatory diseases including rheumatoid arthritis or lupus (National Organisation for Rare Disorders, 2018). Anaemia of inflammation is a consequence of decreased in red blood cell survival in a combination with impaired erythrocyte or erythropoietin production. For instances, cancer cells may secrete certain substances which in turn causes damages or destroy the immature erythrocytes. Cancer cells may also infiltrate the bone marrow which resulted in impaired red

cell production. Other than that, in the state of chronic inflammation, the release of large number of inflammatory cytokines activates macrophages which in turn increased the destruction of erythrocytes. Moreover, renal excretion of hepcidin decreased during the state of severe inflammation. This contributed to accumulation of hepcidin and iron restriction, thus leads to anaemia. In some cases, the production of erythropoietin was affected due to cytokines released and exert their effects on renal cells that produce erythropoietin and eventually lead to decreased red cell production and progress to state of anaemia (Nemeth and Ganz, 2014).

Several glitches in genes are the factors that resulted in anaemia. Thalassaemia causing body unable to produce enough haemoglobin due to defective globin gene (Scott, 2010). Besides that, some defective globin genes increased destruction of circulating red blood cell leading to have inherited haemolytic anaemia. The major causes of intrinsic haemolytic anaemia included defective in haemoglobin (Hb), red cell membrane and red blood cell enzymes which referred to haemoglobinopathy, membranopathy and enzymopathy. more commonly these genetic disorders are α- and β-haemoglobinopathy, glucose-6-phosphate dehydrogenase deficiency (G6PD) as well as hereditary spherocytosis. Patients with these disorders experienced haemolysis more easily as their erythrocytes are more fragile than normal healthy erythrocytes. As such, extensive haemolysis may eventually lead to anaemia (Kim et al., 2017).

1.3 Signs and Symptoms of Anaemia

Signs and symptoms of anaemia varies according to age, concurrent clinical history as well as severity of anaemia. Mild anaemia is asymptomatic and easy to be overlooked by the practicing physicians. When it left untreated, mild anaemia could proceed to more severe stage and may be fatal (Maakaron et al., 2019). The most common physical finding in patients with anaemia is pallor due to shunting of blood from skin and peripheral tissues to permit enhanced blood flow to vital organs such as brain, heart, lungs, kidneys and liver. This condition could also lead to cold extremities as there is lesser blood flow to extremities (Cecil, 2012). People who are anaemic will also often experience fatigue, lethargy and tiredness. This could because of haemoglobin insufficiency where oxygen transportation throughout the body is highly affected and thus cause the body tissues to be hypoxic and patients will experience these symptoms (Handzel, 2020). In more severe cases, patients could experience symptoms such as chest pain and irregular heartbeat. This is because severe anaemia causes a reduction in oxygen amount that delivered to tissues, which could lead to myocyte dysfunction in heart. To compensate the oxygen demands to tissues, the heart tends to increase in cardiac output to maintain adequate oxygen delivery (Hedge et al., 2006).

1.4 Problem Statement

Many studies shows that genetic variation on iron regulating genes such as *TMPRSS6*, *HAMP*, *HFE* as well as *TF* genes were significantly associated with anaemia. However, most of the studies were targeted among children, pregnant

women, child-bearing age women and elderly. Until now, there is still lack of study conducted in Asia region especially on young adults who are at the age between 18 to 35 years old. Besides that, the most significant single nucleotide polymorphisms (SNPs) on iron regulating genes with iron metabolism parameters that contribute to anaemia among Malaysian young adults were not elucidated. Thus, this study will focus on Malaysian-young adults on iron regulating genes with their iron metabolism parameters. Currently, DNA direct sequencing and restriction fragment length polymorphism (RFLP) on iron regulating gene variants analysis were found costly, time consuming and labour intensive (Mahdieh and Rabbani, 2013). This study develops the method of tetra primer-amplification refractory mutation system polymerase chain reaction (tetra primer-ARMS PCR) which is more cost- and time-effective in SNPs and polymorphism detection.

1.5 Objectives

Main objective:

- To investigate the underlying associated factors (gender, age, ethnicity, BMI, family history, clinical history, SNPs on iron regulating genes) to iron metabolising parameters and its predisposition to anaemia.

Specific objectives:

- To identify the most significant SNPs on iron regulating genes in contributing to anaemia among Malaysian young adults.

- To determine the prevalence of anaemia among young adults in Malaysia.
- To elucidate the association of demographic, anthropometric measurements, family history and clinical history with iron profile.
- To determine the relationship between iron metabolising parameters as well as gene-gene interaction among the iron regulating genes.
- To design allele-specific PCR primers for the SNPs that are lined to the targeted iron regulating genes.

CHAPTER 2

LITERATURE REVIEW

2.1 Iron Deficiency Anaemia

Red blood cell or erythrocyte is the specialised cell in human body that function to carry oxygen from the lungs to every part of the body. The functional component that took part in this process is an oxygen-binding protein known as haemoglobin. Haemoglobin is composed of 4 polypeptide globin chains. Each globin subunit contains a haem moiety that is composed of an organic protoporphyrin ring and a ferrous iron (Fe^{2+}). Each Fe^{2+} in haem plays a major role in binding and unbinding oxygen molecule, for oxygen transport action in the body. thus, iron is an essential element in erythropoiesis (Farid et al., 2022). As mentioned by Meynard et al. (2014), most of the iron present in human body were used for haem synthesis and is incorporated in haemoglobin as well as myoglobin. Reduction iron level in the body would limit haemoglobin production and decrease the synthesis of red blood cells in bone marrow. As the consequences, this may result in anaemia (Nagababu et al., 2009).

According to World Health Organisation (2011), anaemia was defined as a condition with inadequate number of erythrocytes in leading to insufficiency oxygen-carrying capacity to meet body's physiological demands. Several factors can contribute to anaemia including nutritional deficiency such as folate, vitamin B12 or vitamin A, occurrence of inflammation, parasite infection as well as inherited or acquired disorders that affect haemoglobin production.

Among many factors that contribute to anaemia, iron deficiency is the most common leading contributors to anaemia worldwide which known as iron deficiency anaemia (Parischa et al., 2021).

Iron deficiency anaemia (IDA) mainly occur in two forms, absolute and functional IDA. Absolute IDA was characterised by low or depleted total body iron stores which can be due to increased iron demand, decreased iron intake or absorption as well as chronic blood loss. While functional IDA was characterised by normal or increased total body iron stores but reduced iron supply to the bone marrow. Many physiological, pathological and genetic factors such as occurrence of inflammation, erythropoiesis stimulating agent (ESA) therapy as well as iron refractory iron deficiency anaemia (IRIDA) could be the factors contribute to functional IDA (Cappellini et al., 2019; Lopez et al., 2016).

According to Warner and Kamran (2021), there is approximately 25% of the people worldwide was suffering from anaemia and 50% of it were accounted by iron deficiency. IDA affects both developed and developing countries. Iron deficiency anaemia was found at higher prevalence rate in developing countries compared to developed country. The most common cause contributed to the high prevalence of IDA in developing countries was due to the low iron bioavailability of the diet (Stoltzfus, 2008).

In a study conducted by Abdullah and co-authors in 2020, the prevalence of anaemia among Malaysian adults were 13.8% and more than half of the cases

(59.7%) were hypochromic microcytic anaemia which can be seen in thalassaemia or IDA. Hypochromic microcytic anaemia is a type of anaemia where the circulating erythrocytes has a smaller size (microcytic) and decreased red colour (hypochromic) compared to the normal healthy erythrocytes. One of the most common causes that led to this type of anaemia was due to the decreased in iron stores in the body. Among all the anaemia cases in Malaysia, there have been reported more than half of it were iron deficiency anaemia (Abdullah et al., 2020; Chaudhry and Kasarla, 2022).

2.2 Clinical Findings and Diagnosis of Iron Deficiency Anaemia

2.2.1 Clinical Manifestation of Iron Deficiency Anaemia

The clinical manifestation for mild or moderate iron deficiency anaemia (IDA) was asymptomatic. IDA will get worsen and symptoms will be manifested as the iron storage in the body continuously depleted. Patients with IDA can present with symptoms including pale skin, fatigue, dyspnoea, dizziness as well as headache. These symptoms were due to hypoxia as there is a reduction of oxygen carry to all body parts. As IDA progressed into severe IDA, patients might develop dyspnoea at rest, tachycardia, angina pectoris as well as haemodynamic instability (Lopez et al., 2016; National Heart, Lung and Blood Institute, 2022; Norhafiah et al., 2020).

According to Lopez et al. (2016), skin roughness and dryness, alopecia and koilonychia, characterised by a spoon-shaped fingernail might develop among IDA patients as iron deficiency predominantly affect the epithelial cells with

rapid turnover especially epithelium lines on skin surface hair and nail. Atrophic glossitis, which is the absence of papillae on the dorsal surface of tongue can be presented in severe IDA cases. Chiang et al. (2019) reported 26.7% of IDA patients were suffering from atrophic glossitis and this is due to the reduced capacity of red blood cell to carry oxygen to the dorsal surface mucosa od the tongue and lead to this symptom. Plummer-Vinson syndrome or Kelly-Paterson syndrome is a rare symptom that associated with IDA where there is less than 0.1% of IDA patients develop this symptom. It was characterised by dysphagia, iron deficiency anaemia and oesophageal webs. Apart from carrying oxygen in haemoglobin and myoglobin, iron is also vital in DNA synthesis as well as some enzymatic reactions that involved in the normal functioning, proliferation and repair of epithelial cells (Abbaspour et al., 2014; Chiang et al., 2020; Lopez et al., 2016).

There is 24% of IDA patients develop neurological complication of restless leg syndrome which is also known as Willis-Ekbom disease (Lopez et al., 2016; Zhu et al., 2020). It is a syndrome that cause an unpleasant and uncomfortable sensation in the legs and lead to an irresistible urge to move the legs during rest due to reduced brain iron level (National Institute of Neurological Disorders and Stroke, 2022). Another neurological sequela that found to be associated with IDA is pica, which is a dietary complication where an individual compulsively eats non-food item. Studies revealed that pica was found to be associated with majority of IDA pregnant women in Africa region (Njiru et al., 2011).

2.2.2 Laboratory Findings and Diagnosis of Iron Deficiency Anaemia

To diagnose IDA, clinical manifestation is insufficient and laboratories findings including complete blood count (CBC), peripheral blood smear, reticulocyte count as well as serum iron indices are mandatory (Johnson-Wimbley and Graham, 2011). The most common parameter used in diagnosis of anaemia is the haemoglobin (Hb) and haematocrit (Hct) level of an individual. The cut off level of Hb and Hct level to diagnose a person as anaemia are varies in gender, age, pregnancy, altitude as well as smoking (Lopez et al., 2016). Based on World Health Organisation (WHO), anaemia was defined when the haemoglobin level is less than 13.0 g/dL in men and 12.0 g/dL in women at sea level. It was further categorised into mild, moderate and severe anaemia in both men and women based on the Hb level. In mild anaemia, the Hb level between 11.0 g/dL to 12.9 g/dL were categorised as mild anaemia in men and 11.0 g/dL to 11.9 g/dL in women. The Hb level for both men and women were 8.0 g/dL to 10.9 g/dL in moderate anaemia in moderate anaemia and below 8 g/dL for severe anaemia (World Health Organisation, 2011). Haematocrit level refereed to the percentage of red blood cells compared to the total blood volume (Billet, 1990). According to William and Melissa (2022), the normal reference range for haematocrit level in a healthy male and female was 42% to 54% and 36% to 46%, respectively and IDA individuals presented with low haematocrit level. Haemoglobin and haematocrit level revealed anaemia, but the underlying root cause need to rule out for treatment. Hence, differential diagnosis is essential in identifying iron deficiency anaemia from other types of anaemia.

Other than Hb and Hct level, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were used to define the size and haemoglobin content of red blood cells. MCV is used to define the size of erythrocytes, MCH reveals the amount of haemoglobin per erythrocyte and MCHC indicates the amount of haemoglobin per unit volume (Sarma, 1990). These three diagnostic parameters are able to rule out iron deficiency anaemia from anaemia due to nutritional deficiency such as folate and vitamin B12 deficiency (Johnson-Wimbley and Graham, 2011). Patients with iron deficiency anaemia presented with low MCV, MCH and MCHC which correspond to red cell in microcytosis and hypochromatosis (Lopez et al., 2016). Apart from red cell indices, peripheral blood smear of IDA individuals shows microcytic-hypochromic erythrocytes where the red blood cells are smaller and have decreased red colour compared to usual erythrocytes. Elevated level of platelets may appear in severe IDA (Chaudhry and Kasaria, 2022; Lopez et al., 2016).

Iron profiles included diagnostic tests such as serum iron, serum ferritin, total iron binding capacity (TIBC) as well as transferrin saturation are important in the diagnosis of IDA. Serum iron reveals the amount of circulating iron, ferritin is the protein that stores iron. Thus, measuring serum iron level is equivalent to assess the status of iron storage in the body. transferrin is the carrier protein for iron in systemic circulation. The ability of transferrin saturation to bind and transport iron is known as total iron binding capacity. Transferrin saturation help to determine the percent saturation of transferrin with iron in the blood. In a normal healthy individual, the serum iron, serum ferritin, TIBC and transferrin

saturation lies between 23 to 27 μmol/L, 15 to 300 μg/L, 45 to 72 μmol/L and 15 to 50%, respectively (Williams et al., 2010). When iron studies show a decreased serum iron, serum ferritin and transferrin saturation but increased TIBC, it can be diagnosed as iron deficiency anaemia (Bermejo and García-López, 2009). In the absent of inflammation, serum ferritin is widely used in the diagnosis of iron deficiency anaemia. When there is a reduced haemoglobin or haematocrit level as well as in red cell indices, serum ferritin level was measured to rule out the underlying cause of anaemia. A reduced serum ferritin, serum iron as well as transferrin saturation but elevated total iron binding capacity, this is sufficient to rule out the cause of anaemia is due to iron deficiency (Killip et al., 2007).

Moreover, a protein named hepcidin plays a significant role in controlling iron homeostasis. Measuring the level of hepcidin is a promising tool in diagnosis of IDA and able to distinguish acquired iron deficiency anaemia and familial iron deficiency anaemia (Girelli et al., 2016). This is because by regulating hepcidin expression able to regulate iron absorption in human body. In acquired iron deficiency anaemia, there will be a significantly low or undetectable serum and urinary hepcidin concentration. Hepcidin appear to be a diagnostic marker as it can be detected in serum or urine. Decreased in hepcidin level associated with reduced transferrin saturation as well as serum ferritin could be a sensitive indicator for iron deficiency. To distinguish acquired IDA and familial IDA, iron refractory iron deficiency anaemia (IRIDA), IRIDA patients presented with increased hepcidin level but low serum iron and transferrin saturation together

with a normal or slight elevated serum ferritin (D'Angelo, 2013; de Falco et al., 2013).

2.3 Iron Metabolism

Iron is a mineral that required in small amount and it is essential to human body for growth and development. According to Institute of Medicine (2001), a normal healthy adult man and woman absorbed approximately 1 mg and 1.5 mg of iron per day, respectively. Fuqua et al. (2012) mentioned that iron can enter into human body through two routes, which are across the placenta during fetal life and through intestinal absorption of dietary in postnatal life.

Dietary iron that will be absorbed by body are mainly presence in two forms which are haem and non-haem iron. Haem iron (Fe^{2+}) chelates in a complex organic structure (Hathcock, 2013). While non-haem iron encompasses both ferrous (Fe^{2+}) and ferric iron (Fe^{3+}). Haem iron was found abundantly in meat as part of haemoglobin as well as myoglobin. While non-haem iron was mainly found in plants food and some animal source food (Fuqua et al., 2012; Buzala et al., 2016). Haem irons are derived from animal source food and was found to be more bioavailable than non-haem iron that derived from plant source food. This is because non-haem iron is more likely to exist in many complexes with other digestion products in the intestinal tract that will greatly impair the absorption of non-haem iron while haem iron absorption was less likely to be influenced by other dietary constituent (Gulec et al., 2014). For instance, the bioavailability of non-haem iron was compromised when it is concurrently

ingested with tannins and phytates that reduced its solubility in the intestinal lumen and thus sequestered the bioavailability of non-haem iron (Schulze and Dreyfuss, 2005; Fuqua et al., 2012).

Intestinal iron absorption occurs in duodenum and proximal jejunum (Waldvogel-Abramowski et al., 2014). Haem and non-haem iron were absorbed through different ways. Haem iron was hypothesised to be absorbed through receptor mediated endocytosis. Internalised haem form endosomes within enterocytes, iron from haem will be liberalised within the vesicle by haem oxygenase-1 (HO-1) (Abbaspour et al., 2014; Gulec et al., 2014; West and Oates, 2008).

At physiological pH, non-haem iron predominantly existed in the oxidised form, which is the insoluble ferric iron (Fe^{3+}) form (Abbaspour et al., 2014). However, the acidic gastric acid from stomach lowers the pH in proximal duodenum, thereby rapidly reduce insoluble Fe^{3+} into soluble Fe^{2+} in the intestinal lumen by ferric reductases such as duodenal cytochrome B (DctB) and Steap2 and thus permitting the transport of Fe^{2+} across apical membrane of enterocytes (Abbaspour et al., 2014; Fuqua et al., 2012). According to Yanatori and Kishi (2019), divalent metal transporter 1 (DMT-1) is the key iron transport protein that contributed to the transport of non-haem iron across apical membrane of enterocytes. Iron derived from different source was then form an intracellular iron pool. The intracellular iron pool is either being exported across the basolateral membrane by ferroportin-1 (FPN1) depending on body iron demands or being stored as ferritin inside the enterocytes (Gulec et al., 2014).

Meanwhile, the intracellular iron needs to be exported out to the circulation and transport to the specific locations such as basolateral membrane of enterocytes as well as macrophages. A specific protein FPN1 is an iron transporter that was identified in exportation of intracellular iron into the blood circulation (Waldvogel-Abramowski et al., 2014; Ward and Kaplan, 2012). Donovan and co-workers proved this in which there is a complete block of iron exportation in *FPN1*-knockout animal models and was associated with iron accumulation in enterocytes as well as macrophages. According to Knutson (2017), FPN1 export only Fe^{2+} while the carrier protein for iron, transferrin will bind only Fe^{3+}. Thus, there is a need of reoxidised the Fe^{2+} into Fe^{3+} to allow the binding to their carrier protein, transferrin. The membrane-bound hephaestin and its plasma homologue, ceruloplasmin are the intestinal ferroxidase that play a major role in reoxidation of Fe^{2+}. The exported Fe^{3+} will load to transferrin that present in bloodstream and delivered to the tissues or cells that needed it. The ferric iron will maintain in a redox-inert form due to physiological pH (7.4) in the bloodstream. When the iron-bound transferrin binds to a transferrin receptor, the molecule will be endocytosed and bring into the cell, forming endosome. The lower pH in endosome facilitates the release of iron from transferrin (Mayle et al., 2012). The apotransferrin was then being recycled and released to cell surface for further usage. Iron freed from transferrin will be released into cytosol through DMT-1 (Wallace, 2016).

Apart from iron loss due to bleeding, pregnancy and menstruation, iron is highly conserved in human body and it is not readily to be excreted by the body. Iron loss was replenished by daily iron uptake from iron-containing food. The iron

pool that presents in human body that was not utilised will be stored in the form of ferritin and haemosiderin in the cell, primarily liver cells (Ems et al., 2022; Saito, 2014).

There is approximately 2 to 5 g of iron present in a healthy adult human. Most of the iron (~ 2 to 3 g) were found incorporated in haem which present in the form of haemoglobin and myoglobin. A well-balanced diet contains about 10 to 20 mg of iron. However, there is only about 10% (~ 1 to 2 mg) of the ingested iron were absorbed by human body and the iron absorbed from alimentation were used to compensate the iron loss through bleeding, menstruation or sloughing of mucosa (Meynard et al., 2014). Thus, for a normal healthy adult, the daily iron demands were contributed by the recycled iron from senescent erythrocytes (Abbaspour et al., 2014; Fuqua et al., 2012).

According to Franco (2012), the lifespan of erythrocytes is approximately 120 days. When the erythrocytes reach the end of their lifespan, the senescent erythrocytes will be endocytosed and the available iron can be reused for the production of new erythrocytes in bone marrow. Senescent or damaged red blood cells will be engulfed by reticuloendothelial macrophages in a process of erythrophagocytosis was then being digested in phagolysosome. Through this process, iron was liberated from haem and transported into cytosol via the paralogue of DMT-1, natural resistance associated macrophage protein 1 (NRAMP 1) for storage or recycling. At the same time, haem also can be directly transported across the phagolysosomal membrane into cytosol via a conserved haem-transporting permease, HRG 1 that was highly expressed on

reticuloendothelial macrophage. Once the haem was exported into cytosol, HO-1 plays its role in liberate iron from the haem. The resultant iron released through the breakdown of erythrocyte can be either released back to systemic circulation through ferroportin-mediated transport across the plasma membrane or being stored as ferritin in the macrophage (Korolnek and Hamza, 2015; Wallace, 2016).

2.4 The Role of Hepcidin Antimicrobial Peptide (*HAMP*) Gene in Iron Metabolism

Hepcidin was encoded by hepcidin antimicrobial peptide (*HAMP*) gene which located at the long arm (q arm) of chromosome 19 (National Centre for Biotechnology Information, 2022). Hepcidin was synthesised as an 84-amino acid preprohormone. This 84-amino acid preprohormone consisted of a N-terminal that was 24-amino acid endoplasmic reticulum-targeting signal sequence and a 60-amino acid prehormone that consisted of consensus from cleavage motif and some proprotein convertases. The preprohormone will undergo several cleavages and become a mature hormone which consisting of 25 amino acid peptides. The prehormone will then become mature hormone and will then be secreted from the cell into circulation (Nemeth and Ganz, 2009; Pandey et al., 2018; Parajes et al., 2010).

According to Rishi et al. (2015), hepcidin is the negative regulator for iron stores in the body. It regulates the cellular iron export through iron exporter ferroportin (FPN1) into plasma and extracellular fluid. FPN1 was identified as the only iron exporter in the body and it is expressed on duodenal enterocytes and function to

absorb dietary iron. It is also expressed on macrophages in liver and spleen which play its role in recycling of senescent red blood cell, hepatocytes that serves as the reservoir for iron in the body as well as placenta trophoblast which take part in iron transfer from the mother to the foetus during pregnancy. The binding of hepcidin to its receptor, FPN1 that present on the aforementioned cells trigger the internalisation and degradation of the receptor-ligand complex, hepcidin-ferroportin complex. Once internalised, degradation of the complex occur in lysosome and cellular iron exportation will be ceased (Nemeth and Ganz, 2009).

The expression of hepcidin is regulated by iron in the body as well as the erythropoietic activity. High iron level will stimulate production of hepcidin and increased hepcidin concentration will in turn block the dietary iron absorption and release, subsequently prevent further iron loading. Conversely, when the iron level in the body is decreased, expression of hepcidin will be upregulated, allowing more dietary iron to be absorbed by the enterocytes to replenish iron stores. Increased activity of erythropoiesis will supress hepcidin production. This is to enhance duodenal iron absorption and enable rapid release of iron from macrophages and hepatocytes to enhance the iron supply for erythropoiesis. Moreover, hepcidin expression is upregulated during inflammation and infection. It is believed that this regulation is a host defence mechanism to reduce the iron bioavailability to the pathogens that invaded into the host (Nemeth and Ganz, 2009; Rishi et al., 2015).

Expression of hepcidin should be tightly regulated to maintain iron homeostasis. Over expression of hepcidin resulted in iron deficiency anaemia while hepcidin deficiency causes iron overload in association with iron deposition in liver and other parenchyma. A completely absent of hepcidin resulted in juvenile haemochromatosis which is the most severe form of hereditary iron overload, characterised by increased serum iron and iron deposition in parenchyma (Nemeth and Ganz, 2009; Wallace and Subramaniam, 2007). Elevated hepcidin concentration may lead to excessive internalisation and degradation of ferroportin. This causes a reduction in intestinal iron absorption and iron trapping in macrophages as well as hepatocytes, resulted in drastically reduce in serum iron, leading to reduced iron bioavailability for erythropoiesis and thus leads to anaemia. Conversely, when the hepcidin concentration is abnormally low, leading to an excessive ferroportin mediated iron influx, resulted in iron overload or haemochromatosis (Nemeth and Ganz, 2009; Pandey, et al., 2018).

2.5 The Role of Transmembrane Serine Protease 6 (*TMPRSS6*) Gene in Iron Metabolism

Transmembrane serine protease 6 (*TMPRSS6*) gene is located at the long arm (q arm) of chromosome 22. It has a function to regulate iron homeostasis by encoding matriptase-2, a member in type II transmembrane serine protease family that is essential in regulating hepcidin expression. Matriptase-2 is predominantly expressed in liver and low level of matriptase-2 is expressed in other organs such as kidney, brain, lungs, mammary gland as well as reproductive organ. Matriptase-2 is an 811 amino acid inactive proenzyme comprising of a short N-terminal cytoplasmic transmembrane domain, a sea

urchin sperm protein, enteropeptidase and argin (SEA) domain, 2 complement factor (C1r/C1s), urchin embryonic growth factor and morphogenic protein (CUB) domain, 3 low-density lipoprotein receptor (LDLR) class A repeats and a C-terminal trypsin like serine protease domain. The inactive proenzyme undergoes proteolytic cleavage by itself at the arginine residue and transform into active matriptase-2 enzyme (Finberg et al., 2008; National Centre for Biotechnology Information, 2022; Wang et al., 2014).

Matriptase-2 regulates iron homeostasis by regulating the master iron regulator, hepcidin. Wang et al. (2014) reported that matriptase-2 had been recognised to have enzymatic activity to degrade fibronectin, fibrinogen as well as type I collagen. The membrane bound haemojuvelin was found to be the substrate for matriptase-2. Haemojuvelin was found to be correlated with hepcidin expression as it acts as an essential coreceptor for some bone morphogenic proteins (BMPs). BMP6 participates in the activation of bone morphogenic (BMP) / sons of mothers against decapentaplegic (SMAD) signalling pathway with the purpose to upregulate hepcidin gene transcription. Matriptase-2 serve as the biological cutter to cleave the membrane bound haemojuvelin. Without the presence of membrane bound haemojuvelin, the BMP/SMAD signalling will be ceased, in turn downregulate hepcidin transcription. A reduction in hepcidin concentration resulted in increased duodenal iron absorption as well as release of iron stores from macrophages and hepatocytes. While to upregulate hepcidin transcription, matriptase-2 expression will be supressed to promote hepatic BMP signalling and increased hepcidin expression to downregulate iron level (Lee, 2009; Nemeth and Ganz, 2009; Wang et al., 2014).

According to de Falco et al. (2013), defects in *TMPRSS6* gene that encoded for matriptase-2 causes iron refractory iron deficiency anaemia (IRIDA). IRIDA is characterised with microcytic-hypochromic anaemia, associated with very low serum iron indices but a normal or slightly elevated serum ferritin. The major difference of IRIDA with acquired IDA was the iron unresponsiveness and partial responsive to parenteral iron of IRIDA (Sal et al., 2016). These was shown by the study carried out by Buchanan and Sheehan in early of 1980s. In the study, there were 3 siblings without any history of inadequate dietary iron intake or massive gastrointestinal bleeding exhibited iron deficiency anaemia and were not responsive to oral ferrous sulphate therapy but were partially responsive to intramuscular iron dextran treatment (Buchanan and Sheehan, 1981). Similar findings were seen in study conducted by Heeney and Finberg using a *TMPRSS6* knockout (*TMPRSS6*$^{-/-}$) mice. The *TMPRSS6*$^{-/-}$ mice presented with iron deficiency anaemia phenotype even when maintained with a standard iron diet. Furthermore, the *TMPRSS6*$^{-/-}$ mice presented with a defect hepcidin regulation under an iron deficient diet in which the *HAMP* mRNA remains elevated. With this evidence, scientist suggested that the mutation in *TMPRSS6* resulted in IDA associated with elevated hepcidin concentration as well as unresponsive to oral iron treatment and partially responsive to parenteral iron (Heeney and Finberg, 2014).

Mutation in *TMPRSS6* gene leads to iron deficiency anaemia. In the study carried out by An and co-workers, *TMPRSS6* polymorphisms include rs855791 (A736V) and rs4820268 (D521D) were significantly correlated with a lower haemoglobin and serum iron level which increased the risk of iron deficiency

anaemia (An et al., 2012). Heeney and Finberg (2014) reported that the variants rs855791 with a substitution of an alanine to valine at the position of 736 (A736V) within the matriptase-2 serine protease domain were found to be less efficient in supressing hepcidin which in turns caused a reduction in serum iron level. As reported by Pelusi and colleagues, the *TMPRSS6* variants A736V and D521D was reported to be significantly associated with haemoglobin and serum ferritin level in Chinese Han population. Iron lowering alleles in A736V and D521D were associated with lower haemoglobin and serum ferritin level and associated with higher risk of developing iron deficiency anaemia (Gan et al., 2012; Pinto et al., 2017).

2.6 The role of Transferrin (*TF*) Gene in Iron Metabolism

Transferrin protein which encoded by the homonymous transferrin (*TF*) gene. Transferrin protein is the major cargo that function to transport iron in systemic circulation (Waldvogel-Abramowski et al., 2014). It is an 80 kD monomeric glycoprotein, consisted of two homologous N-lobes and C-lobes that connected by a short peptide. Both N- and C-lobes were made up of one aspartic, two tyrosine, one histidine and one arginine. In between each lobe, there is a cleft that act as the binding site for ferric iron (Fe^{3+}), controlled by the opening and closing of sub-domains. When there is Fe^{3+} bound to transferrin molecule, sub-domains will close to hold the Fe^{3+} and open again when the iron-bound transferrin binds to transferrin receptor to release the Fe^{3+} (Ogun and Adeyinka, 2022).

Before ferric iron (Fe^{2+}) is released to systemic circulation, ferroxidases such as hephaestin and ceruloplasmin play a role in re-oxidise Fe^{2+} to Fe^{3+} as transferrin only binds Fe^{3+} (Knutson, 2017). At physiological pH, iron existed in Fe^{3+} form. The free Fe^{3+} that present in circulation is toxic to human body as it can form free radicals that can lead to tissue damage. Thus, once released from the cells, it must be bound to the transferrin protein and 20 to 40% of the binding site of transferrin will be occupied by Fe^{3+}. Transferrin not only serve as the carrier protein to carry Fe^{3+} to whole parts of the body, it also functions to chelate the iron, making the insoluble Fe^{3+} to be soluble and prevent the formation of reactive oxygen species (ROS) that destroy body's cell (Abbaspour et al., 2014; Ems et al., 2022; Waldvogel-Abramowski et al., 2014). Iron-transferrin complex or iron-bound transferrin is formed after Fe^{3+} bound to the transferrin molecule. This complex will circulate in systemic circulation until it bound to the cells that presented with transferrin receptor, predominantly transferrin receptor 1 (TFR1). Once the iron-transferrin complex bound to transferrin receptor, internalisation of the complex together with the receptor will occur within the cell by endosomal recycling vesicles. The lower pH in endosome facilitates the release of iron from transferrin and the transferrin-transferrin receptor complex will be recycled to the cell surface. At the cell surface, where the pH is neutral, transferrin will be dissociated from the transferrin receptor and used for iron transportation in systemic circulation (Waldvogel-Abramowski et al., 2014).

TF gene located at the long arm (q arm) of chromosome 3. Mutation at the *TF* gene (3q21) resulted in a very rare disease known as congenital

atransferrinaemia or also being known as hypotransferrinaemia which leads to iron deficiency anaemia. To date, there is only 16 reported cases for congenital atransferrinaemia (Chandra et al., 2017; National Centre for Biotechnology Information, 2022). According to Chandra et al. (2017), patients with congenital atransferrinaemia characterised with microcytic hypochromic anaemia which is unresponsive to iron therapy associated with iron overload in parenchyma of main iron storing organs such as liver and spleen which can in turn lead to liver cirrhosis and splenomegaly. The onset of the disease is usually at the early childhood or during infancy. Patients will be presented with common symptoms of IDA, anorexia, irritability, recurrent infections as well as growth retardation. Previous study shows that congenital atransferrinaemia was inherited in autosomal recessive mode. In the reported study, the transferrin protein of patients and the family were being analysed and concluded that the patients were homozygous with the missense mutation at codon 394 of *TF* gene while the two healthy siblings were heterozygous with a paternal "variant" and maternal "null" allele (Asada-Senju et al., 2002). Mutations on *TF* gene lead to extremely low or absent of transferrin protein. SNPs on *TF* gene were also found to be associated with serum transferrin level. According to Blanco-Rojo et al. (2011), minor allele of rs1799852 (T allele) was found to be associated with lower serum transferrin level. While the wildtype allele (C allele) presented with significantly higher serum transferrin level compared to CT genotype. Individuals with GG (wildtype) genotype in variant rs12769 were found to have significant higher iron level compared to GA (heterozygous) genotype (Fujihara et al., 2019).

2.7 The Role of Homeostatic Iron Regulator (*HFE*) Gene in Iron Metabolism

Homeostatic iron regulator (HFE) protein is encoded by *HFE* gene which located at the short arm (p arm) of chromosome 6. HFE protein regulates iron homeostasis through regulating the interaction between transferrin and transferrin receptor (National Centre for Biotechnology Information, 2022). HFE protein is a 343-amino acid protein consisted of a signal peptide, an extracellular transferrin receptor-binding region known as alpha-1 (α1) and alpha-2 (α2) domain, a transmembrane region and a short cytoplasmic tail. The HFE protein associated with a beta-2 (β2) microglobulin at the alpla-3 (α3) domain, forming a heterodimer and expressed at the cell surface (Barton et al., 2015).

HFE protein allows binding of transferrin receptor-1 (TFR1) which is bound by circulating iron. Iron homeostasis is regulated through the competition between HFE protein and iron-bound transferrin binding to the transferrin receptor, regardless TFR1 or TFR2. When iron is at homeostatic state, HFE protein is partially localised at the cell surface, bound with TFR1. However, transferrin saturation increased when iron concentration is high in the body. Comparing the binding affinity of holo-transferrin (transferrin bounded to two iron atoms) and HFE protein to TFR1, holo-transferrin has a higher binding affinity towards TFR1 with a stronger affinity towards TFR1. Thus, when iron saturation is high, holo-transferrin binds to TFR1 with a stronger affinity, leading to HFE protein dissociated from TFR1 and become available to associate with TFR2 or remain free on cell surface. Holo-transferrin will first bound with TFR1 and when TFR1

is saturated, the excess holo-transferrin will bind to TFR2 (Kleven et al., 2018; Li et al., 2020; Sangkhae and Nemeth, 2017; Silva and Faustino, 2015).

On the other hand, the HFE protein that remains freely on cell surface is likely to interact with a BMP receptor type 1 which is activin receptor-like kinase (ALK) 2. HFE protein increase the expression of ALK 3 protein by stabilising it, prevent the ubiquitination and proteasomal degradation of ALK 3 protein. The formation of the holo-transferrin-TFR2-HFE (Fe-Tf-TFR2-HFE) complex and the interaction of free HFE protein with ALK 3 on cell surface trigger the signalling pathways and hence stimulate the expression of hepcidin, thereby reduce intestinal iron absorption and the iron release from iron stores. Whereas, when iron saturation decreases, HFE protein will be able to associate with TFR1, leaving the unbound TFR2. This weakens the effect of stimulation and expression of hepcidin decreased to augment intestinal iron absorption (Kleven et al., 2018; Li et al., 2020; Sangkhae and Nemeth, 2017; Silva and Faustino, 2015).

Mutation in *HFE* gene leads to iron overload disease, which is known as type 1 hereditary haemochromatosis, also termed as HFE-associated hereditary haemochromatosis (Alexander and Kowdley, 2005; Alexander and Kowdley, 2009). HFE-associated hereditary haemochromatosis is characterised by excessive intestinal iron absorption, leading to highly elevated iron storage in liver, pancreas, heart, joint, anterior pituitary gland as well as skin. These can lead to liver cirrhosis, diabetes mellitus, cardiomyopathy, arthropathy, hypogonadism and progressive skin pigmentation. Clinical findings of type 1

hereditary haemochromatosis included elevated serum transferrin saturation as well as serum ferritin concentration due to excessive iron present in circulation. Mutation in *HFE* gene disrupted the production of hepcidin where expression of hepcidin will be downregulated, thus resulted in abnormally iron absorption and iron deposition in parenchyma (Barton and Edwards, 2018).

Several studies reported that two missense mutations of *HFE* gene, rs1800562 (C282Y) and rs1799945 (H63D) were significantly associated with hereditary haemochromatosis especially in European ancestry (Juzėnas et al., 2016; Nandar and Connor, 2011). C282Y mutation is a missense mutation where the cysteine at amino acid position 282 was replaced by tyrosine, resulted in a disruption of an intramolecular disulphide bond which in turns interfere the binding of β2 macroglobulin to the α3 domain of HFE protein (Levy et al., 1999). According to Jones et al. (2015), C282Y mutation prevent HFE protein from reaching the cell surface, resulted in the inability to sense body iron level accurately. While for the H63D mutation, a histidine at amino acid position of 63 was substituted by an aspartic acid (Kelley et al., 2014). There are more than 80% of the hereditary haemochromatosis patients were carrying C282Y mutant allele (Coppin et al., 2003; Rossi and Jeffrey, 2004). Homozygous for C282Y and H63D were significantly associated with hereditary haemochromatosis. Carrier for the two variants were both resulted in an increment of cellular iron uptake. Heterozygous for both variants were rarely to develop haemochromatosis but were associated to have elevated mean transferrin saturation and serum ferritin level. Coppin et al. (2003) also reported that individuals who are heterozygous for C282Y were less likely to develop iron

deficiency anaemia as there is a slightly but significant higher serum iron level and transferrin saturation. Juzėnas et al. (2016), concluded that, carrier for C282Y and H63D allele is a risk factors to develop iron overload.

2.8 Interactions between Iron Regulating Genes

Iron regulating genes consisting of *HAMP* gene and *TMPRSS6* gene encoded for hepcidin and matriptase-2. While *TF* gene and *HFE* gene encoded for the homonymous protein, transferrin and HFE protein, respectively. The encoded proteins interact with each other to regulate body iron level. Hepcidin is well known as the master regulator for iron homeostasis with matriptase-2 serves as its regulator to regulate production of hepcidin in response to body iron level. While transferrin and HFE protein were involved in iron sensing mechanism. Several pathways were involved in the regulation of hepcidin protein and the main pathway is through the bone morphogenic (BMP) / sons of mothers against decapentaplegic (SMAD) signalling pathway. There were two branches of BMP/SMAD signalling pathway took part in hepcidin regulation which are activin receptor-like kinase (ALK) 3, HFE protein dependent BMP/SMAD signalling pathway and ALK2 dependent BMP/SMAD signalling pathway. The former is signalling through bone morphogenic (BMP) 6 and inhibited by FK506 binding protein (FKBP) 12. While the latter is activated by BMP 2 and upregulated by haemojuvelin, HFE protein and transferrin receptor (TFR) 2 (Sangkhae and Nemeth, 2017; Silvestri et al., 2019.)

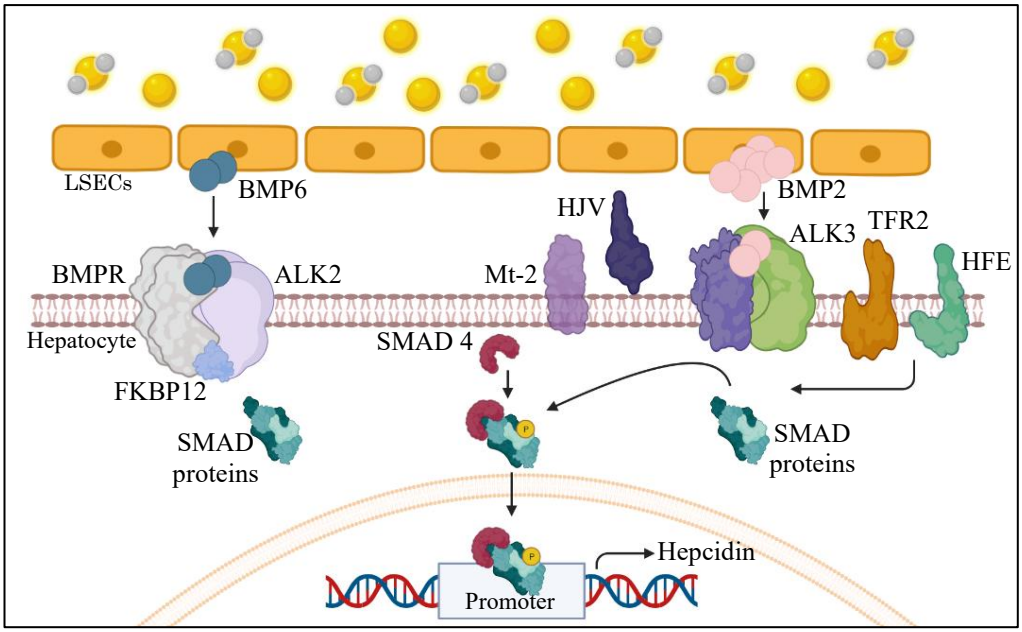


Figure 2.1: Branches of BMP/SMAD signalling pathway (Silvestri et al., 2019).

BMP/SMAD signalling will be activated when body is at the state of iron overload and supressed at the state of iron deficient. During the state of iron overload, the expression of BMP ligands that involves in iron regulation which are BMP6 and BMP2 will be transcriptionally upregulated. Through the binding of BMP6 to its BMP receptor which is ALK2 and the displacement of FKBP 12 from ALK2, ALK2 dependent signalling will be activated. At the same time, the interaction between HFE protein with ALK3 as well as the interaction between HFE protein and TFR2 in sensing the level of holo-transferrin both activate the BMP/SMAD signalling pathway. Moreover, matriptase-2 protein that encoded by *TMPRSS6* gene will be down-regulated to activate BMP2-dependent ALK3 signalling pathway. The binding of BMP ligands to BMP receptors induces the phosphorylation of SMAD 1, 5 and 8. The phosphorylated SMAD proteins will form a complex with SMAD 4. This SMAD complex was then translocated into the nucleus and binds to a specific sequence in *HAMP* promoter region. The SMAD complex serves as the transcription factor to

activate the transcription of hepcidin mRNA. Elevated hepcidin concentration will supress intestinal iron absorption to counter the iron overload state. (Canali et al., 2016; Silvestri et al., 2019).

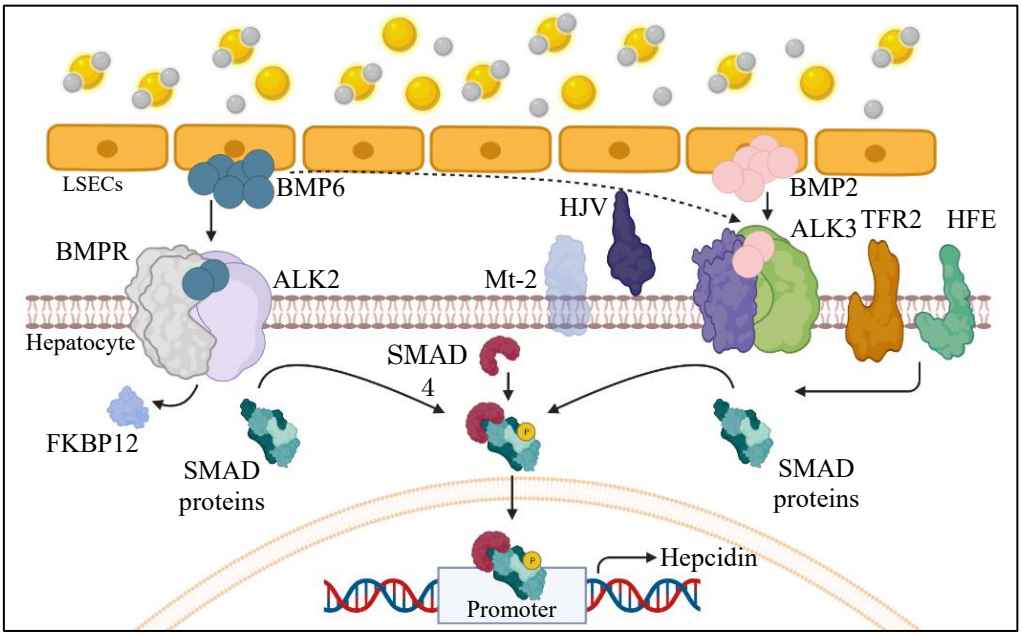


Figure 2.2: BMP/SMAD signalling pathway in iron overload state (Silvestri et al., 2019).

On the other hand, when body is at the state of iron deficient, TFR2 on the cell surface is reduced through cleavage or increased endocytosis. HFE protein is attached to TFR1 instead of TFR2 as the iron bound transferrin level significantly decreased during iron deficiency, leaving unsaturated TFR1 for HFE protein to bind to it. At the same time, as HFE protein is attached to TFR1, there will be reduced level of free HFE protein to stabilise the ALK3 protein. Thus, ALK protein will be ubiquitinated and degraded. *TMPRSS6* expression will be upregulated to increase the production of matriptase-2. Matriptase-2 was then functions to cleave the membrane-bound haemojuvelin that serve as BMP co-receptor in ALK3-mediated BMP/SMAD signalling pathway. BMP6 protein expression will also decrease. Without the binding of BMP ligands to BMP

receptors nor the modulation of BMP co-receptor, the downstream activation processes will not occur or only occur at the basal level. thus, hepcidin expression will be down-regulated, intestinal iron absorption will increase to elevate the systemic iron level (Rishi et al., 2015; Silva and Faustino, 2015; Silvestri et al., 2019). Hence, the four iron regulating genes (*HAMP*, *TMPRSS6*, *TF* and *HFE* gene) were important to be included in this study to investigate their roles in iron regulation.

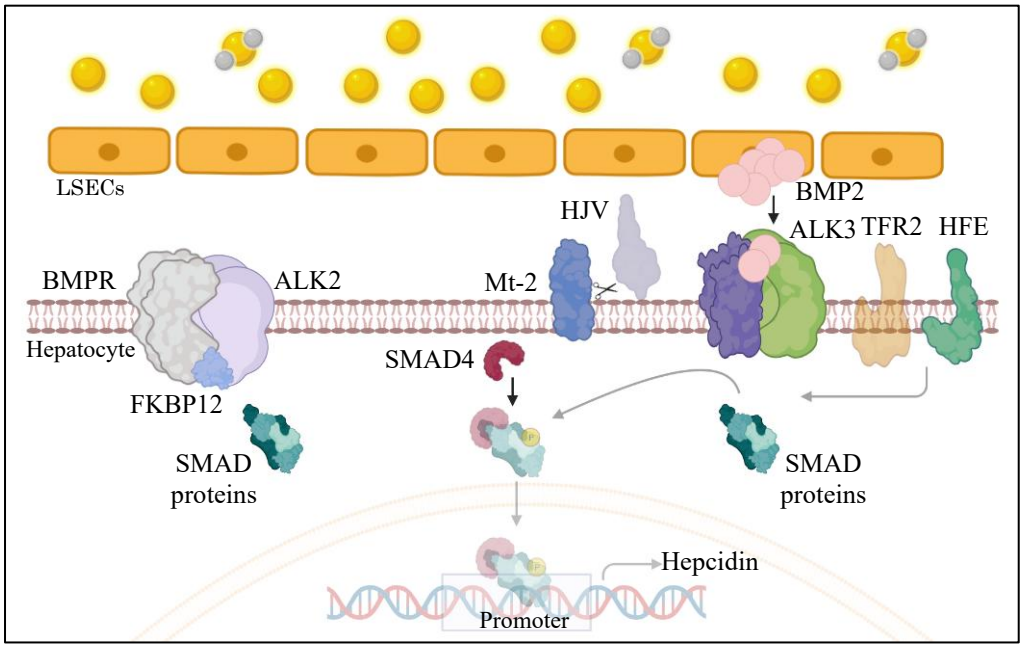


Figure 2.3: BMP/SMAD signalling pathway in iron deficiency state (Silvestri et al., 2017).

CHAPTER 3

METHODOLOGY

3.1 Chemicals, Reagents and Instruments

The chemicals and reagents used in this study were listed in Table 3.1. The instruments and consumables used were listed in Table 3.2.

Table 3.1: Chemicals and reagents used in the study.

Chemical / Reagents	Manufacturer
100 bp DNA ladder	BIO-HELIX Co., LTD., Taiwan
50 bp DNA ladder	BIO-HELIX Co., LTD., Taiwan
Agarose powder	Vivantis Technologies Sdn. Bhd., Malaysia
DNA extraction kit	FAVORGEN Biotech Corp., Taiwan
DNA purification kit	FAVORGEN Biotech Corp., Taiwan
Tri-Color 6× DNA loading dye	Axil Scientific Pte Ltd., Singapore
Midori Green Advance DNA stain	NIPPON Genetics Co., Ltd., Japan
Human Hepc (Hepcidin) ELISA kit	Elabscience Biotechnology Co., Ltd., USA
Iron colorimetric assay kit	Elabscience Biotechnology Co., Ltd., USA
Primers	Integrated DNA Technologies, Malaysia
2× PCR Master Mix	Axil Scientific Pte Ltd., Singapore
50× Tris-acetate-EDTA (TAE) buffer, pH 8.0	Axil Scientific Pte Ltd., Singapore
10× Tris-EDTA (TE) buffer, pH 8.0	Axil Scientific Pte Ltd., Singapore

Table 3.2: Instruments and consumables used in the study.

Instruments / Consumables	Manufacturer
Syringes	Terumo Corporation, Japan
Hypodermic needle	Nipro Corporation, Japan
EDTA vacutainer	Becton Dickinson Sdn. Bhd., USA
Plain vacutainer	Becton Dickinson Sdn. Bhd., USA
Centrifuge machine	Eppendorf, Germany
Microcentrifuge machine	Thermo Fisher Scientific, USA
Microcuvette	HemoCue AB, Sweden
Haemoglobinometer	HemoCue AB, Sweden
ACCU-CHEK single-use lancing device	Roche Holding AG, Switzerland
Micropipette	Eppendorf, Germany
Water bath incubator	Memmert, Germany
PCR thermal cycler	BioRad laboratories Inc., USA
UV transilluminator	BioRad laboratories Inc., USA
Microplate reader	BMG Labtech, Malaysia
Heat block shaker	Q Instruments, Germany
Analytical balance	Kern and Sohn, Germany
Autoclave machine	Hiramaya, Japan
Freezer (-20 °C)	Panasonic Holdings Corporation, Japan
Fridge (4 °C)	Panasonic Holdings Corporation, Japan
Gel tank	Major Science, USA
Measuring cylinder	Favorite, Malaysia
Microwave	Panasonic Holdings Corporation, Japan
Power supply	Major Science, USA
Schott bottle	Duran, Germany
Nanodrop	Thermo Fisher Scientific, USA
Vortex machine	Stuart, United Kingdom

3.2 Experimental Design

Figure 3.1 present the experimental flow for this study. The sampling was done after obtaining the ethical approval and ended with statistical analysis.

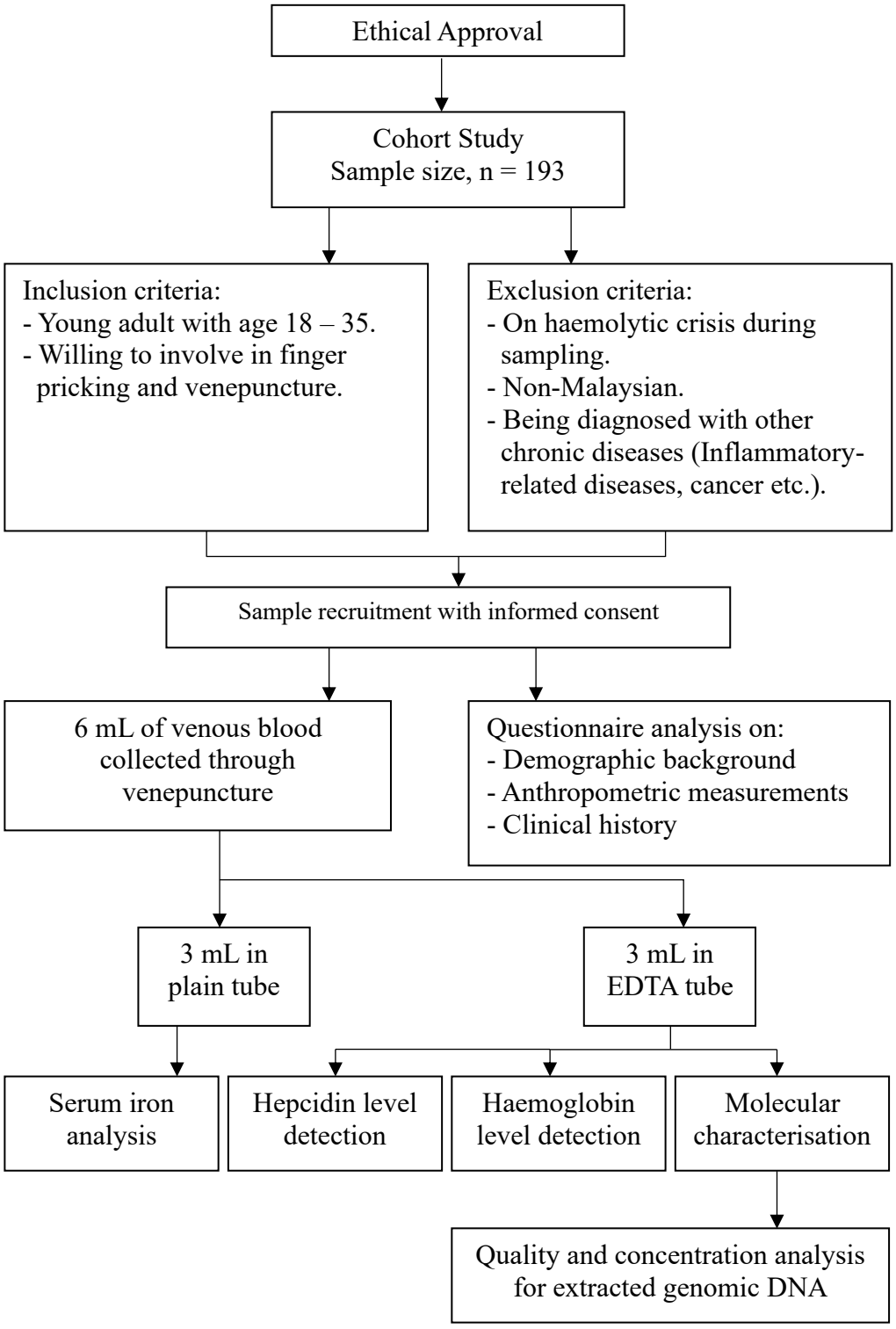


Figure 3.1: Overview of the experimental flow for this study.

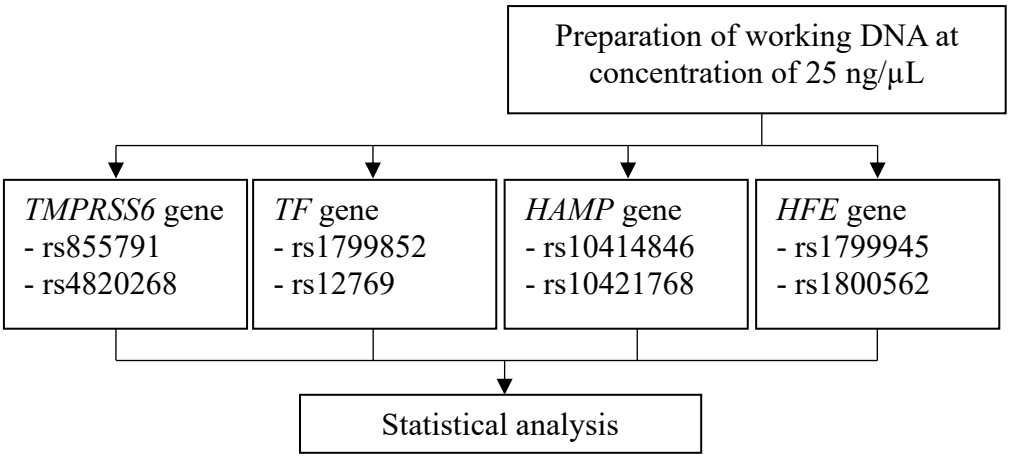


Figure 3.1 (cont.): Overview of the experimental flow for this study.

3.3 Sample Recruitment

Ethical approval from UTAR Scientific and Ethical Review Committee (SERC) was obtained for human sampling prior commencement of the study. Convenient sampling was performed to recruit study subjects from UTAR (Kampar, Perak Campus). Subjects were recruited on voluntary basis with informed consents before data and blood sample collection Subjects were recruited based on the inclusion and exclusion criteria as listed below.

Inclusion criteria:

- Young adult aged between 18 to 35 years old.
- Willing to involve in finger pricking and venepuncture.

Exclusion criteria:

- Non-Malaysian.
- Suffering of other chronic diseases (thalassaemia, Hb variants, inflammatory-related diseases, cancer, etc.)

Based on calculated sample size in formula below, a minimum of 193 study subjects were recruited for this study. Sample size was calculated to meet 95% confidence level with 5% drop off rate. According to Abdullah et al. (2020), the prevalence rate of anaemia was 13.8%. The calculated sample size for this study was 183. In addition to 5% drop off rate, the sample size was 193 study subjects.

$$n = \frac{Z^2 P(1-P)}{d^2}$$

$$= \frac{(1.96)^2 0.138(1-0.138)}{0.05^2}$$

$$n = 183$$

Adding 5% drop off rate,

$n = 183 \times 105\%$

$= 192.15$

$n \approx 193$

n = sample size

Z = statistics level for level of confidence

= 1.96

P = expected prevalence

= 0.138

d = allowable error

= 0.05

3.4 Anthropometric and Haemoglobin Level Measurements

Body height and weight of study subjects were measured using stadiometer and electronic weighing scale. Haemoglobin level was measure by using haemoglobinometer HemoCue 201+ system. Capillary blood was taken through finger pricking method. First drop of blood was removed as this drop of blood might contaminated with tissue fluid or debris. Second drop of capillary blood was collected into HemoCue microcuvette. The microcuvette was placed into the aforementioned system and measurements were done within 5 minutes.

3.5 Data Sampling through Google Form

Anthropometric measurements and haemoglobin level were recorded using a Google Form. The google form also filled by participants for family history of anaemia and clinical history of study subjects. The data were self-declared by study subjects.

3.6 Blood Sample Collection

A volume of 6 mL of venous blood was collected through venepuncture by authorised personnel into plain and EDTA vacutainer. The collected blood samples were centrifuged at 3300 ×g for 15 minutes to separate the whole blood into 3 distinct layers which are packed red cells, buffy coat layer and plasma in EDTA tube while serum in plain tube. Plasma and serum were isolated and stored in -20 °C for hepcidin level and serum iron level detection using Human

Hepc (Hepcidin) ELISA kit and Iron colorimetric assay kit. A volume of 200 µL of buffy coat layer was isolated for genomic DNA extraction.

3.7 Hepcidin Assay

Before conducting hepcidin analysis, wash buffer, standard working solution, biotinylated detection antibody working solution and HRP conjugate working solution was prepared by diluting the concentrated solution with diluent following the instruction provided in the Human Hepc (Hepcidin) ELISA kit (Elabscience Biotechnology, United States of America).

Wash buffer was prepared by diluting the 25 times concentrated wash buffer with sterile distilled water (sdH_2O) into 1 times working solution. Biotinylated detection antibody and HRP conjugate working solution was prepared by diluting the 100 times concentrated biotinylated antibody and 100 times concentrated HRP conjugate with respective diluents into 1 times working solution. Standard working solution with concentration of 4000 pg/mL was produced by adding 1 mL of Reference Standard & Sample Diluent into Reference Standard. Serial dilution was done to produce 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL,125 pg/mL and 63.5 pg/mL from 4000 pg/mL standard working solution. Blank was prepared with sdH_2O.

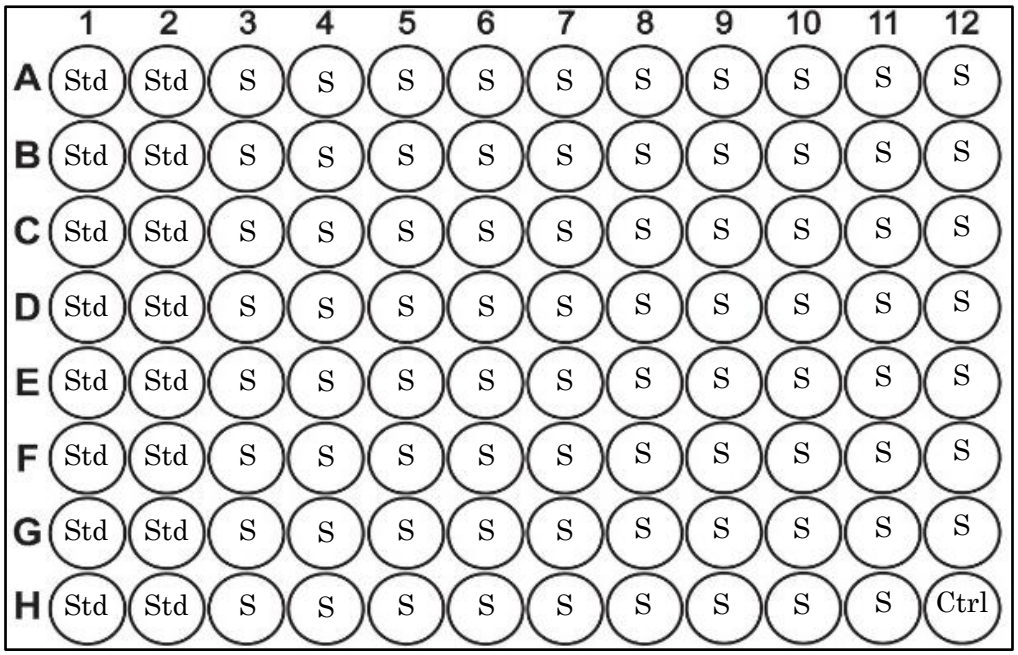


Figure 3.2: Plate layout for hepcidin assay. (Std = Standard; S = Sample; Ctrl = Control)

A volume of 100 µL of prepared standard working solution of different concentration were added into well A1 to H1 of antibody-precoated microplate. Duplication was run in well A2 to H2. Well H12 was served as control with random concentration of standard working solution was chosen and added into it. A volume of 100 µL of plasma samples were added into the remaining wells. The plate was sealed and incubated at 37 °C for 90 minutes. Hepcidin protein from samples were interacted with primary antibody. After 90 minutes of incubation, the solutions were removed and added with 100 µL of 1 times biotinylated detection antibody working solution, followed by 1 hour of incubation at 37 °C.

After 1-hour of incubation, the solutions were removed and the wells were washed for 3 times with 1 times wash buffer working solution. Each washing step was done by adding 350 µL of 1 times wash buffer working solution,

followed by 1 to 2 minutes of soaking and the solution was discarded. One hundred microlitre of 1 times HRP conjugate working solution were added to each well after washing steps and the plate was sealed and incubated at 37 °C for 30 minutes. The solutions were removed and the wells were washed for 5 times following the aforementioned washing steps. A volume of 90 µL substrate reagent were added into each well, followed by 15 minutes incubation at 37 °C. The plate was sealed and covered with aluminium foil to avoid direct light exposure.

Fifty microlitres of stop solution were added into each well after 15 minutes of incubation. Optical density (OD) value for each standard and sample were measured with microplate reader at 450 nm. Duplicate results were obtained for each sample by repeating the assay. Average OD value of standard working solutions and samples were obtained. Standard curve was plotted using the average OD value of standard working solutions and hepcidin level for each sample was calculated from the standard curve.

3.8 Serum Iron Assay

Serum iron was detected on serum sample using Iron colorimetric assay kit (Elabscience Biotechnology, United States of America). Chromogenic agent working solution was prepared by dissolving Reagent 2 and Reagent 3 with 20 mL of reagent 4, respectively. Iron standard working solutions were prepared by serial dilute 10 mg/L iron standard stock solution with sterile distilled water

into the recommended dilution gradient as follows: 10 mg/L, 8 mg/L, 6 mg/L, 5 mg/L, 4 mg/L, 2 mg/L, 1 mg/L and 0 mg/L which served as blank.

A volume of 75 µL of iron standard working solution at different dilution and serum sample were added into a 1.5 mL Eppendorf tube, respectively. Three hundred microlitres of chromogenic agent working solution were added into each tube and mixed thoroughly with vortex mixer. The tubes were incubated in water bath at 100 °C for 5 minutes, followed by cooled with running water. The tubes were centrifuged at 3000 ×g for 10 minutes and 200 µL if supernatant were taken into respective wells of microplate as the plate layout shown in Figure 3.3. then, the plates were run for OD measurements at 520 nm.

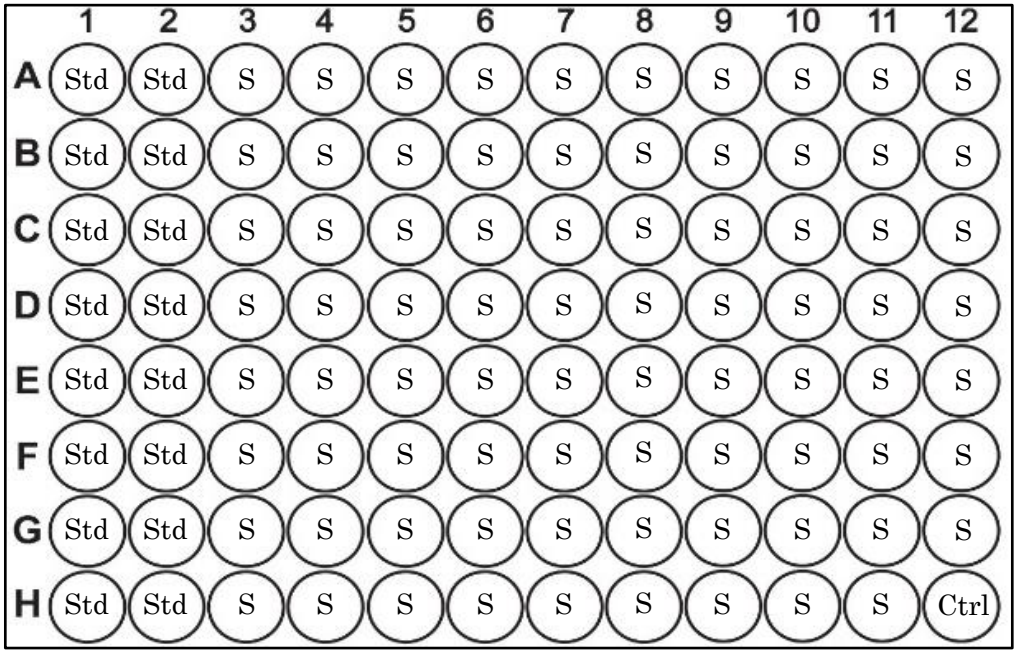


Figure 3.3: Plate layout for serum iron assay. (Std = Standard; S = Sample; Ctrl = Control)

The assay was repeated twice for obtaining duplicate result. OD value of iron standard working solutions and samples were calculated. Standard curve was

plotted using the average OD value of standard working solutions and serum iron level for each sample was calculated from the standard curve.

3.9 Genomic DNA Extraction

The genomic DNA was extracted from buffy coat layer using FavorPrep™ Blood Genomic DNA Extraction Mini Kit (FAVORGEN Biotech, Taiwan). A volume of 200 µL of buffy coat layer was added with 20 µL of proteinase K and 200 µL of FABG buffer. The mixture was mixed thoroughly by pulse-vortexing using vortex mixer, followed by incubation in water bath at 60 °C for 15 minutes. During the incubation, the mixture was mixed thoroughly every 3 to 5 minutes with a vortex mixer.

The mixture in the tubes were spun down. Two hundred microlitres of absolute ethanol was added into the tube, followed by through mixing by pulse vortexing. All the mixture was transferred to FABG mini column in collection tube and was centrifuged at 6000 ×g for 1 minute. The FABG mini column was transferred into a new collection tube and 400 µL of W1 buffer was added. The column was centrifuged at 18000 ×g for 30 seconds. The filtrate was discarded, followed by adding of 750 µL wash buffer to the column. The column was centrifuged again at 18000 ×g for 30 seconds and the filtrate was discarded. Additional 3 minutes of centrifugation at 18000 ×g was done. First elution was done by adding 30 µL of pre-heated elution buffer to the column and incubated at room temperature for 30 minutes. Second elution was done with another 30 µL of elution buffer. The column was centrifuged at 18000 ×g for 1 minute to

elute all the extracted DNA. The extracted DNA was collected into the elution tube and stored at -20 °C.

3.9.1 DNA Concentration and Purity Analysis

The concentration and purity of the extracted DNA was examined using nanodrop spectrophotometer and gel electrophoresis. One microlitre of extracted DNA was placed onto the nanodrop spectrophotometer to measure its concentration and purity by checking the A_{260}/A_{280} ratio. A pure DNA would have the A_{260}/A_{280} ratio between 1.7 – 2.0.

Genomic gel electrophoresis was run to check the purity of the extracted DNA. Extracted DNA was loaded into the agarose gel together with loading dye. The gel was ran using 100 V power supply for 30 minutes. The gel was viewed using Biorad UV transilluminator for the presence of any unspecific band or smeared band. The DNA that was without any unspecific band was proceeded with single nucleotide polymorphisms (SNPs) genotyping.

3.9.2 Working DNA Preparation

The stock DNA samples were used to prepare working DNA at concentration of 25 ng/µL by diluting the sample with sdH_2O. All the working DNA were prepared at a final volume of 30 µL after calculated with formula below.

$$M_1V_1 = M_2V_2$$

M_1 = Concentration of extracted stock DNA

V_1 = Volume of extracted stock DNA required

M_2 = Concentration of working DNA [25 ng]

V_2 = Volume of working DNA [30 µL]

3.10 Primers Design

Tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) was used in the detection of eight (8) SNPs in four genes including rs855791, rs4820268, rs1799852, rs12769, rs10414846, rs10421768, rs1799945 and rs1800562. A total of 8 sets of primer were designed. Each set of primer consisted of 4 primers which are outer forward (F_O), outer reverse (R_O), inner forward (F_I) and inner reverse (R_I) primer. Nucleotide sequence for the targeted gene and SNP point were obtained from National Centre for Biotechnology Information (NCBI) database. Primer sequences were designed using PRIMER1 software. The designed primer sequences were aligned to the target gene to check for alignment. The selected sequences were sent to Integrated DNA Technologies for primer synthesis. The primer sequences were listed in Table 3.3.

Table 3.3: Primer sequences, targets and amplicon size for genotyping.

Gene	SNP	Primer	Primer sequence 5' – 3'	Amplification	Amplicon size (bp)
TMPRSS6	**rs855791**	Inner forward (F_I)	ATGCGTGGCGTCACCTGGTAGCGATCGA	IF-OR	233
		Inner reverse (R_I)	TGATCCCACAGGACCTGTGCAGCGATGC	OF-IR	171
		Outer forward (F_O)	AAGGGCCAGACCCTGGTGATGTGGGCAG	OF-OR	348
		Outer reverse (R_O)	GCGACCCTAGCAGGGGTAAGGGTGGGCA		
	rs4820268	Inner forward (F_I)	CCCTACCTTCCTGGCACTGCTCTGCA	IF-OR	208
		Inner reverse (R_I)	GCAGCCTGATTGTCTCAACGGCAGCTAC	OF-IR	263
		Outer forward (F_O)	TCAATGGACAGACGGTGATTGGAGACCA	OF-OR	417
		Outer reverse (R_O)	TACAGCACACCTTCTACAGGCATCGCCA		
TF	**rs1799852**	Inner forward (F_I)	GACAGGGACCAGTATGAGCTGCTTTTCC	IF-OR	211
		Inner reverse (R_I)	ATCTACCGGCTTCCGGGTGTTGTCAAA	OF-IR	267
		Outer forward (F_O)	TGCTACATGGGCATCAGAAGCCTTTACC	OF-OR	423

Table 3.3 (cont.): Primer sequences, targets and amplicon size for genotyping.

		Outer reverse (R_O)	AATCCAAGCAGGGAGCACTAAGCTGGAG	OF-OR	423
	rs12769	Inner forward (F_I)	ACCCTTAACCAATACTTCGGCTACGCA	IF-OR	198
		Inner reverse (R_I)	GCAGACATCTCACTCACTTGAAGGCTACC	OF-IR	252
		Outer forward (F_O)	GTTTTAGTCCCCTCTGTTCCCTGATGG	OF-OR	394
		Outer reverse (R_O)	CTAGGAGTCCCAGGTTGTGCTTCTGACT		
HAMP	**rs10414846**	Inner forward (F_I)	TCAACATATGTGCCATCGTATAAAATGGAC	IF-OR	209
		Inner reverse (R_I)	AGTAATAGCTAACATCTACAGTCCGATTAA	OF-IR	252
		Outer forward (F_O)	TTTTCTGTAAAGTGAAGGAAATGAGTGTC	OF-OR	401
		Outer reverse (R_O)	ACACATCTGTAGTCCCAGCTACTTGG		
	rs10421768	Inner forward (F_I)	TCTCAAGGGTCTGACACTGGGAAAACAACG	IF-OR	220
		Inner reverse (R_I)	CATCAGCGTGTGCCCGATCCGCAAGT	OF-IR	171
		Outer forward (F_O)	GCCTTGGCCTCCCAAATTGCTGGGATTA	OF-OR	335

Table 3.3 (cont.): Primer sequences, targets and amplicon size for genotyping.

HFE	**rs1800562**	Outer reverse (R_O)	TCCACTCACTTCCCCATCGCCTACATGC	OF-OR	335
		Inner forward (F_I)	CCCTGGGGAAGAGCAGAGATATACTTG	IF-OR	215
		Inner reverse (R_I)	GATCCAGGCCTGGGTGCTCCACCTTGT	OF-IR	182
		Outer forward (F_O)	CTACCCCCAGAACATCACCATGAAGTGG	OF-OR	343
		Outer reverse (R_O)	GTTAAAGCCTTTGATTGCCACCCTCAGC		
	rs1799945	Inner forward (F_I)	GATGACCAGCTGTTCGTGTTCTATGTTC	IF-OR	195
		Inner reverse (R_I)	TTCGGGGCTCCACACGGCGACTCTCTTC	OF-IR	219
		Outer forward (F_O)	GAGTTGATGCAGGTGTGTGGAGCCTCAA	OF-OR	358
		Outer reverse (R_O)	CCTCTCCACGTACCCTTGCTGTGGTTGT		

The synthesized primers were received in lyophilised form and were reconstituted with 1 times tris-EDTA (TE) buffer to obtain 100 µM of primer concentration.

3.11 Genotyping

Genotyping of SNPs were run in final volume of 10 µL PCR reaction. The PCR reaction was prepared consisting of PCR master mix, working primers at different concentration and 75 ng of working DNA as shown in Table 3.4 (a), Table 3.4 (b), Table 3.4 (c) and Table 3.4 (d).

Table 3.4 (a): PCR mixture preparation for rs855791 and rs4820268.

Reagents	Initial concentration	Final concentration	Volume (µL)
Master mix	2 × [ab]	1 × [ab]	5
F_O	4 µM [a] / 8 µM [b]	0.4 µM [a] / 0.8 µM [b]	1
R_O	4 µM [a] / 8 µM [b]	0.4 µM [a] / 0.8 µM [b]	
F_I	4 µM [a] / 8 µM [b]	0.4 µM [a] / 0.8 µM [b]	
R_I	4 µM [a] / 8 µM [b]	0.4 µM [a] / 0.8 µM [b]	
Working DNA	25 ng [ab]	75 ng [ab]	3
sdH_2O	-	-	1
Total	-	-	10

[a] PCR mixture preparation of rs85579; [b] PCR mixture preparation of rs482268.

Table 3.4 (b): PCR mixture preparation for rs1799852 and rs12769.

Reagents	Initial concentration	Final concentration	Volume (µL)
Master mix	2 × [ab]	1 × [ab]	5
F_O	8 µM [a] / 2 µM [b]	0.8 µM [a] / 0.2 µM [b]	1
R_O	8 µM [a] / 2 µM [b]	0.8 µM [a] / 0.2 µM [b]	
F_I	4 µM [a] / 2 µM [b]	0.4 µM [a] / 0.2 µM [b]	
R_I	4 µM [a] / 2 µM [b]	0.4 µM [a] / 0.2 µM [b]	
Working DNA	25 ng [ab]	75 ng [ab]	3
sdH_2O	-	-	1
Total	-	-	10

[a] PCR mixture preparation of rs1799852; [b] PCR mixture preparation of rs12769.

Table 3.4 (c): PCR mixture preparation for rs10414846 and rs10421768.

Reagents	Initial concentration	Final concentration	Volume (µL)
Master mix	2 × [ab]	1 × [ab]	5
F_O	6 µM [a] / 4 µM [b]	0.6 µM [a] / 0.4 µM [b]	1
R_O	4 µM [ab]	0.4 µM [ab]	
F_I	4 µM [a] / 3 µM [b]	0.4 µM [a] / 0.3 µM [b]	
R_I	6 µM [a] / 3 µM [b]	0.6 µM [a] / 0.3 µM [b]	
Working DNA	25 ng [ab]	75 ng [ab]	3
sdH_2O	-	-	1
Total	-	-	10

[a] PCR mixture preparation of rs10414846; [b] PCR mixture preparation of rs10421768.

Table 3.4 (d): PCR mixture preparation for rs1800562 and rs1799945.

Reagents	Initial concentration	Final concentration	Volume (µL)
Master mix	2 × [ab]	1 × [ab]	5
F_O	4 µM [ab]	0.4 µM [ab]	1
R_O	4 µM [ab]	0.4 µM [ab]	
F_I	4 µM [ab]	0.4 µM [ab]	
R_I	4 µM [a] / 6 µM [b]	0.4 µM [a] / 0.6 µM [b]	
Working DNA	25 ng [ab]	75 ng [ab]	3
sdH_2O	-	-	1
Total	-	-	10

[a] PCR mixture preparation of rs1800562; [b] PCR mixture preparation of rs1799945.

Optimisation was performed by running gradient PCR to check for the most optimum temperature and primer concentration for genotyping. PCR was performed using Biorad 96-well T100 PCR thermal cycler with cycling condition of initial denaturation, denaturation, annealing, extension and final extension as in Table 3.5 (a), Table 3.5 (b), Table 3.5 (c) and Table 3.5 (d).

Table 3.5 (a): Cycling condition for PCR of rs855791 and rs4820268.

Event	Temperature (°C)	Time/Cycle	No. of cycles
Initial denaturation	95 [ab]	3 minutes [ab]	1
Denaturation	95 [ab]	30 seconds [ab]	30 [a] / 35 [b]
Annealing	69 [a] / 67 [b]	30 seconds [a] / 40 seconds [b]	30 [a] / 35 [b]
Extension	72 [ab]	30 seconds [a] / 40 seconds [b]	30 [a] / 35 [b]
Final extension	72 [ab]	3 minutes [a] / 5 minutes [b]	1

[a] cycling condition of rs855791; [b] cycling condition of rs4820268.

Table 3.5 (b): Cycling condition for PCR of rs1799852 and rs12769.

Event	Temperature (°C)	Time/Cycle	No. of cycles
Initial denaturation	95 [ab]	3 minutes [ab]	1
Denaturation	95 [ab]	30 seconds [ab]	35 [ab]
Annealing	64 [a] / 64.6 [b]	30 seconds [ab]	35 [ab]
Extension	72 [ab]	30 seconds [ab]	35 [ab]
Final extension	72 [ab]	5 minutes [ab]	1

[a] cycling condition of rs1799852; [b] cycling condition of rs12769.

Table 3.5 (c): Cycling condition for PCR of rs10414846 and rs10421768.

Event	Temperature (°C)	Time/Cycle	No. of cycles
Initial denaturation	95 [ab]	3 minutes [ab]	1
Denaturation	95 [ab]	30 seconds [ab]	35 [ab]
Annealing	56 [a] / 66 [b]	30 seconds [ab]	35 [ab]
Extension	72 [ab]	30 seconds [ab]	35 [ab]
Final extension	72 [ab]	5 minutes [ab]	1

[a] cycling condition of rs10414846; [b] cycling condition of rs10421768.

Table 3.5 (d): Cycling condition for PCR of rs1800562 and rs1799945.

Event	Temperature (°C)	Time/Cycle	No. of cycles
Initial denaturation	95 [ab]	3 minutes [ab]	1
Denaturation	95 [ab]	30 seconds [ab]	30 [a] / 35 [b]
Annealing	59 [a] / 65 [b]	30 seconds [ab]	30 [a] / 35 [b]
Extension	72 [ab]	30 seconds [ab]	30 [a] / 35 [b]
Final extension	72 [ab]	5 minutes [ab]	1

[a] cycling condition of rs1800562; [b] cycling condition of rs1799945.

PCR products were electrophoresed using 2% agarose gel. The 2% agarose gel was prepared using the formula below.

$$\frac{\text{weight}}{\text{volume}} = 2\%$$

A small gel was casted by dissolving 0.4 g of agarose powder in 20 mL of 1 times TAE buffer. A big gel was casted by dissolving 0.6 g of agarose powder in 30 mL of 1 times TAE buffer. Pre-staining method was used to stain the gel. A volume of 0.5 µL and 1 µL of GelRed was added into a 20 mL and 30 mL melted agarose gel during preparation.

PCR products were mixed with 1 µL of 1 times loading dye and loaded into the respective well of solidified agarose gel together with first well loaded with 50 base pair (bp) DNA ladder. The loaded gel was electrophoresed at 100 V for 30 minutes. The gel was viewed using Biorad UV transilluminator. Genotypes were interpreted based on the amplicon size listed in Table 3.6. Internal control band served as validation of amplification of specific target site.

Table 3.6: Amplicon size for targeted SNPs.

		Wildtype	Heterozygous	Mutant	Internal control
rs855791 (A > G)	**Genotype**	AA	AG	GG	
	Amplicon size (bp)	233	171 and 233	171	348
rs4820268 (G > A)	**Genotype**	GG	GA	AA	
	Amplicon size (bp)	263	208 and 263	208	417
rs1799852 (C > T)	**Genotype**	CC	CT	TT	
	Amplicon size (bp)	211	211 and 267	267	423
rs12769 (G > A))	**Genotype**	GG	GA	AA	
	Amplicon size (bp)	252	198 and 252	198	394
rs10414846 (C > T)	**Genotype**	CC	CT	TT	
	Amplicon size (bp)	209	209 and 252	252	401
rs10421768 (A > G)	**Genotype**	AA	AG	GG	
	Amplicon size (bp)	171	171 and 220	220	335
rs1800562 (G > A)	**Genotype**	GG	GA	AA	
	Amplicon size (bp)	215	182 and 215	182	343
rs1799945 (C > G)	**Genotype**	CC	CG	GG	
	Amplicon size (bp)	195	195 and 219	219	358

3.12 PCR Products Purification and DNA Sequencing

For validation of genotypes, two samples were randomly selected for nucleotide direct sequencing. PCR products were purified prior sequencing service. Purification of PCR products were done using FavorPrep™ GEL/PCR purification kit. The sample was first amplified with outer primers only following same cycling condition listed in Table 3.5 (a), Table 3.5 (b), Table

3.5 (c), and Table 3.5 (d). Each sample was prepared in 50 µL of PCR volume and in duplicates to reach total PCR products in 100 µL.

The 100 µL of PCR products was transferred into 2 microcentrifuge tubes (50 µL each). A volume of 250 µL FADF buffer was added to 50 µL of PCR product and mixed thoroughly by vortexing. FADF column was placed into a collection tube and the sample mixture were transferred to the FADF column. Column was centrifuged at 11000 ×g for 30 seconds and the flow-through was discarded. A volume of 750 µL of ethanol added-wash buffer was added to the column, followed by centrifugation at 11000 ×g for 30 seconds and flow-through was discarded. Additional 3 minutes of centrifugation at 18000 ×g to dry the column. FADF column was placed into a new microcentrifuge tube and 40 µL of elution buffer was added to the membrane centre of the column. Column was centrifuged at 18000 ×g for 1 minute to elute the DNA after stand at room temperature for 1 minute. The concentration and purity of the purified DNA was measured using nanodrop spectrophotometer. Sample with concentration above 20 ng and purity with the A_{260}/A_{280} ratio between 1.7 – 2.0 was used to send for sequencing.

3.13 Statistical Analysis

Statistical analysis was done using SPSS version 22.0 at the confidence interval of 95%, with p-value < 0.05. Kolmogorov-Smirnov and Shapiro-Wilk test were used to test for normality. As the data was not normally distributed, non-parametric test was conducted and median (interquartile range) was determined

in this study. Spearman's Rank Order Correlation Test was used to identify the relationship between haemoglobin, hepcidin and serum iron level. Mann-Whitney U Test and Kruskal-Wallis H Test were used to assess the distribution of demographic distribution, anthropometric measurements, family history, clinical history and SNPs with haemoglobin, hepcidin and serum iron level. Chi-Square (χ^2) Test was used to identify the relationship between the eight SNPs on four iron regulating genes.

CHAPTER 4

RESULTS

4.1 Study Subjects and Prevalence of Anaemia

A total of 183 samples were successfully recruited based on the inclusion and exclusion criteria. This fulfilled the calculated sample size and fitted into 95% confidence interval. The haemoglobin (Hb) level for all the study subjects were measured using HemoCue haemoglobinometer (HemoCue 201+ System) The Hb level at 13.0 g/dL and 12.0 g/dL was used as cut-off value to diagnose anaemia for male and female, respectively (World Health Organisation, 2017). The mean haemoglobin level among the study subjects was 13.80 g/dL with 43.36% of male and 56.64% of female study subjects. In this study, there was twenty-seven out of 183 subjects (27/183) were found as anaemic with the calculated prevalence rate at 14.75%. this prevalence rate was slightly higher than the prevalence rate reported by Abdullah et al. (2020) as at 13.80%.

4.2 Genotyping of Genetic Variants in Iron Regulatory Genes

4.2.1 Genomic DNA Analysis

The whole blood samples for all 183 study subjects were spun down and the buffy coat layer was obtained for DNA extraction using FavorPrep™ Blood Genomic DNA Extraction Kit. The extracted genomic DNA were quantitated and checked for purity using NanoDrop spectrophotometer and genomic gel electrophoresis. The mean concentration and purity for all extracted DNA was

103.92 ng/μL and 1.82 for the A_{260}/A_{280} ratio, respectively. Figure 4.1 shows the representative genomic gel electrophoresis for some of the extracted genomic DNA. All the extracted samples showed only a band in the gel image, indicating the intactness of extracted genomic DNA. There was no smearing of DNA, showing the DNA was pure.

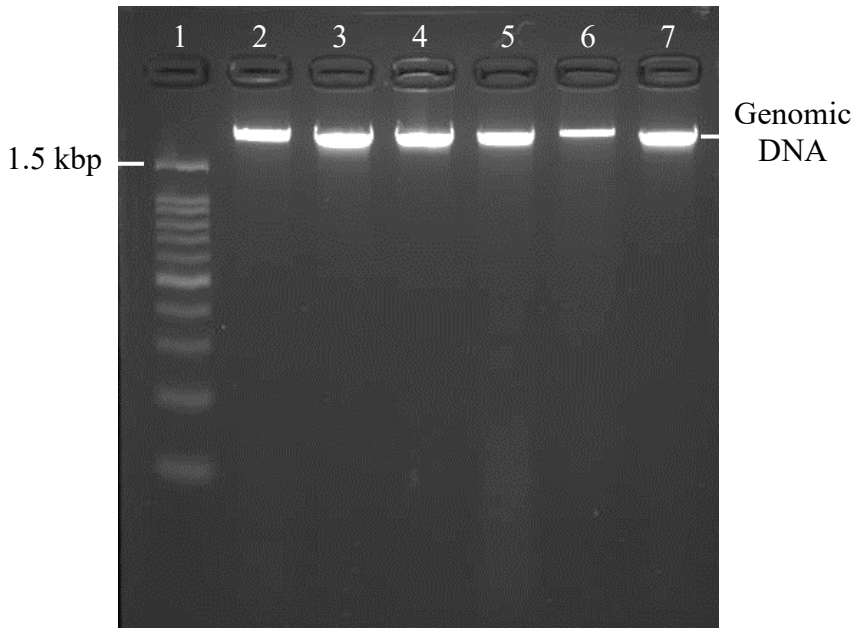


Figure 4.1: Representative genomic gel electrophoresis for extracted genomic DNA.
Lane 1: 100 bp DNA ladder; Lane 2 to Lane 7: genomic DNA samples.

4.2.2 Single Nucleotide Polymorphisms (SNPs) Detection for *HAMP* gene (rs10414846 and rs10421768)

A total of eight SNPs were included into this study, including two SNPs at hepcidin antimicrobial peptide (*HAMP*), transferrin (*TF*), homeostatic iron regulator (*HFE*) and transmembrane serine protease 6 (*TMPRSS6*) gene, respectively. The *HAMP* gene variants that being selected for genotyping were rs10414846 and rs10421768. Figure 4.2 (a) shows the representative gel image

for genotyping of rs10414846. Three bands were shown with internal control band at 401 bp to validate the targeted gene while 209 bp and 252 bp amplicons represented C and T allele, respectively. For rs10421768, the 3 bands that being amplified were the internal control band with molecular size of 335 bp, A allele with molecular size of 171 bp and G allele with molecular size of 220 bp. Figure 4.2 (b) shows the representative gel image for the genotyping of rs10421768.

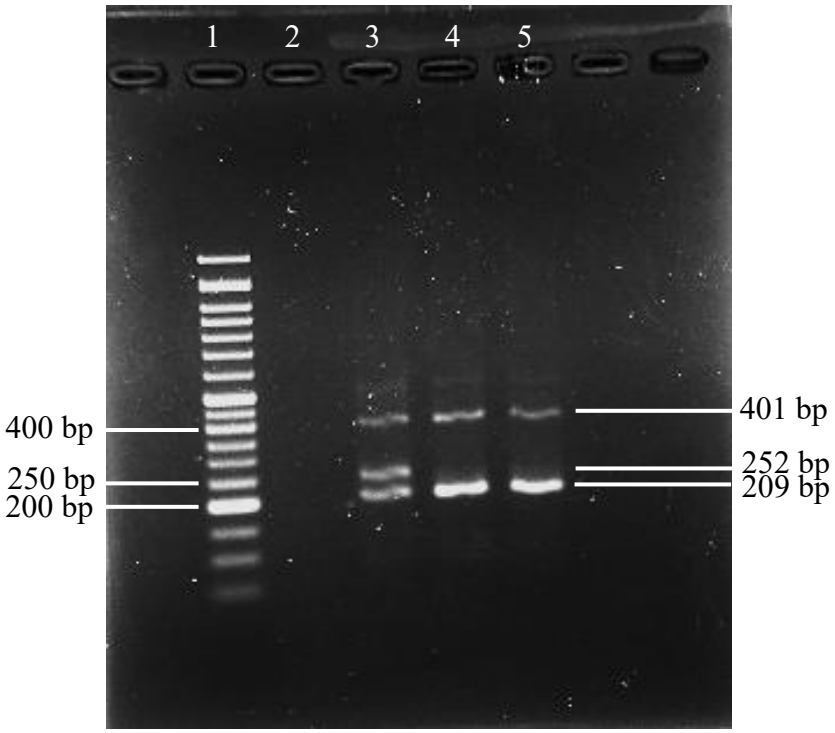


Figure 4.2 (a): Representative agarose gel electrophoresis for genotyping of rs10414846.
Lane 1: 50 bp DNA ladder; Lane 2: Non-template control (NTC); Lane 3: CT genotype sample; Lane 4: CC genotype sample; Lane 5: CC genotype sample.

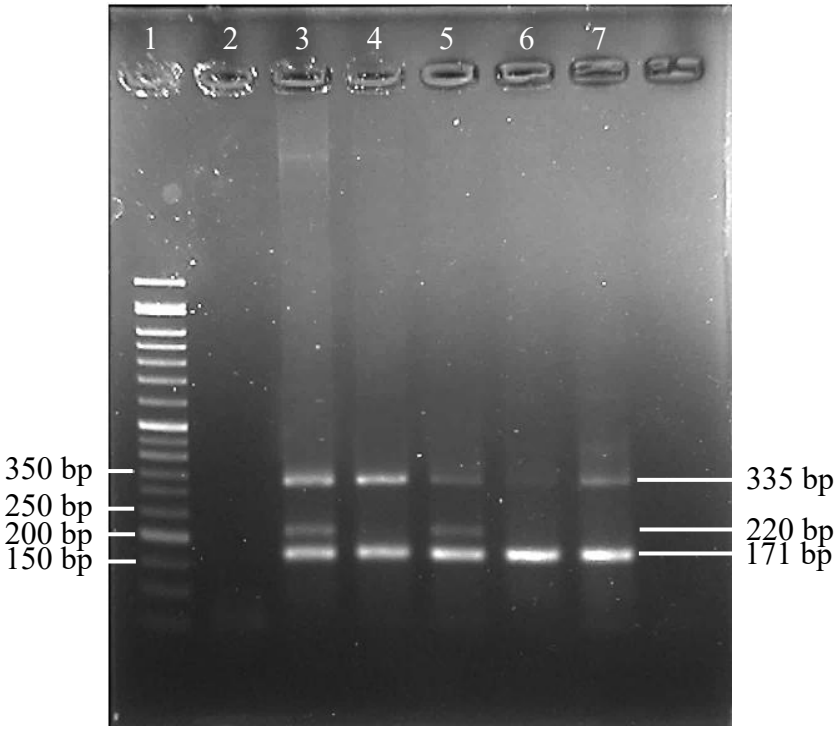


Figure 4.2 (b): Representative agarose gel electrophoresis for genotyping of rs10421768.
Lane 1: 50 bp DNA ladder; Lane 2: Non-template control (NTC); Lane 3: AG genotype sample; Lane 4: AA genotype sample; Lane 5: AG genotype sample; Lane 6: AA genotype sample; Lane 7: AA genotype sample.

4.2.3 Single Nucleotide Polymorphisms (SNPs) Detection for *TMPRSS6* gene (rs855791 and rs4820268)

For transmembrane serine protease 6 (*TMPRSS6*) gene, rs855791 and rs4820268 were selected to genotype. Figure 4.2 (c) shows the genotyping for rs855791. Three bands were amplified in rs855791, including the internal control band with amplicon size of 348 bp, A allele with amplicon size of 233 bp and G allele with amplicon size of 171 bp. Figure 4.2 (d) shows the genotyping result for rs4820268. Internal control, G allele and A allele were amplified as the band size of 417 bp, 263 bp and 208 bp, respectively.

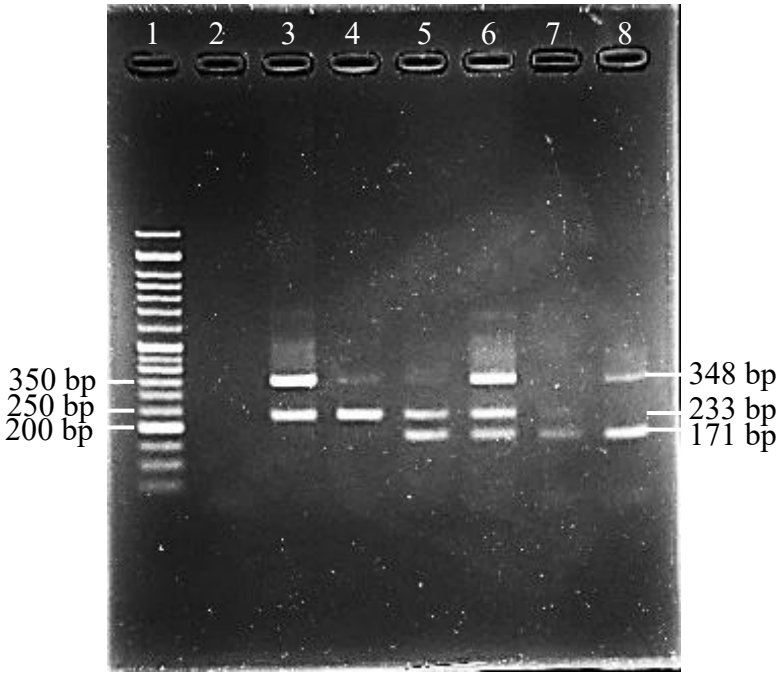


Figure 4.2 (c): Representative agarose gel electrophoresis for genotyping of rs855791.
Lane 1: 50 bp DNA ladder; Lane 2: Non template control (NTC); Lane 3: AA genotype sample; Lane 4: AA genotype sample; Lane 5: AG genotype sample; Lane 6: AG genotype sample; Lane 7: GG genotype sample; Lane 8: GG genotype sample.

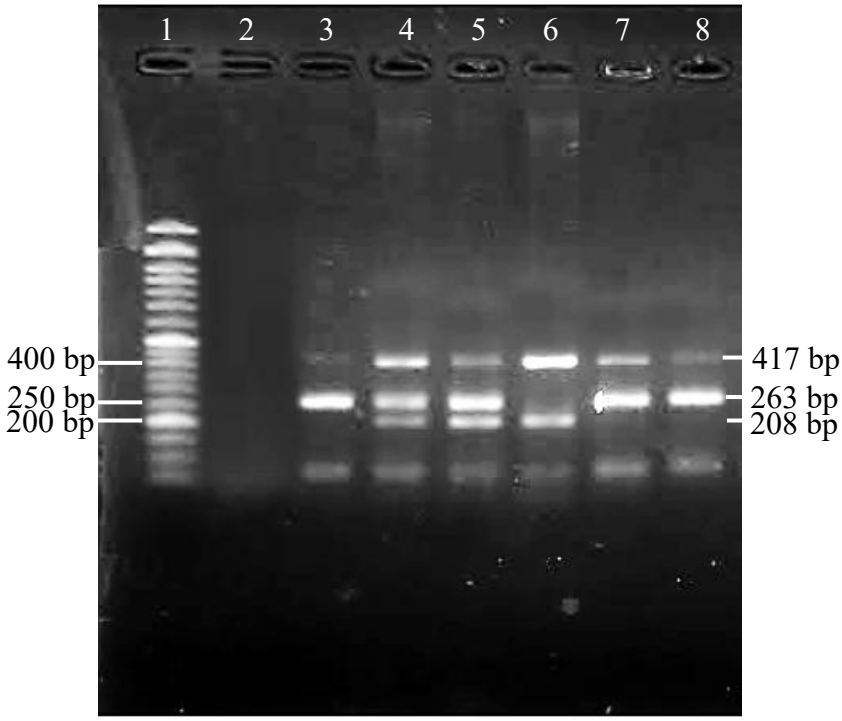


Figure 4.2 (d): Representative agarose gel electrophoresis for genotyping of rs4820268.
Lane 1: 50 bp DNA ladder; Lane 2: Non template control (NTC); Lane 3: GG genotype sample; Lane 4: GA genotype sample; Lane 5: GA genotype sample; Lane 6: AA genotype sample; Lane 7: GG genotype sample; Lane 8: GG genotype sample.

4.2.4 Single Nucleotide Polymorphisms (SNPs) Detection for *TF* gene (rs1799852 and rs12769)

Two SNPs on transferrin (*TF*) gene, rs1799852 and rs12769 were genotyped. For rs1799852, three amplicons including internal control with amplicon size of 423 bp, C allele with amplicon size of 211 bp and T allele with amplicon size of 267 bp. While for rs12769, there were 3 amplicons with molecular size of 394 bp, 252 bp and 198 bp indicating internal control band, G allele and A allele, respectively. Figure 4.2 (e) and Figure 4.2 (f) shows the representative gel electrophoresis for genotyping of rs1799852 and rs12769, respectively.

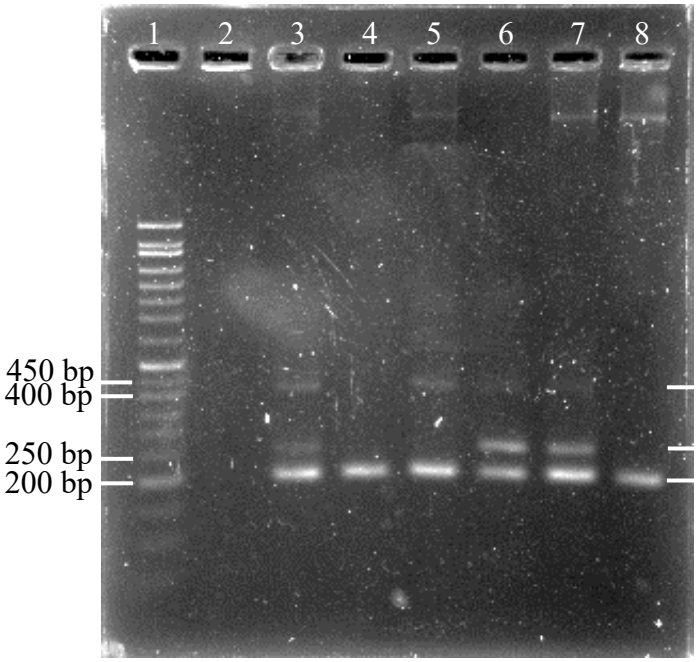


Figure 4.2 (e): Representative agarose gel electrophoresis for genotyping of rs1799852.
Lane 1: 50 bp DNA ladder; Lane 2: Non template control (NTC); Lane 3: CT genotype sample, Lane 4: CC genotype sample; Lane 5: CC genotype sample; Lane 6: CT genotype sample; Lane 7: CT genotype sample; Lane 8: CC genotype sample.

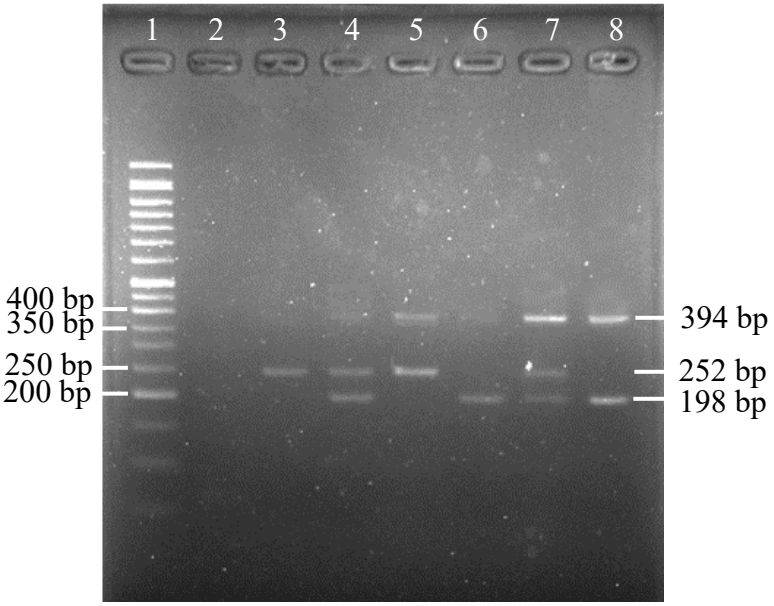


Figure 4.2 (f): Representative agarose gel electrophoresis for genotyping of rs12769.
Lane 1: 50 bp DNA ladder; Lane 2: Non template control (NTC); Lane 3: GG genotype sample; Lane 4: GA genotype sample; Lane 5: GG genotype sample; Lane 6: AA genotype sample; Lane 7: GA genotype sample; Lane 8: AA genotype sample.

4.2.5 Single Nucleotide Polymorphisms (SNPs) Detection for *HFE* gene (rs1799945 and rs1800562)

SNP located at homeostatic iron regulator (*HFE*) gene, rs1799945 was genotyped. Figure 4.2 (g) represents the genotyping result for rs1799945. The internal control band was presented at the amplicon size of 358 bp; C allele presented at the amplicon size of 195 bp while G allele at 219 bp. The other SNP of *HFE* gene, rs1800562 was genotyped. Lane 3 to 8 in Figure 4.2 (h) showed 2 amplicons with the internal control band at 343 bp and the G allele band at 215 bp.

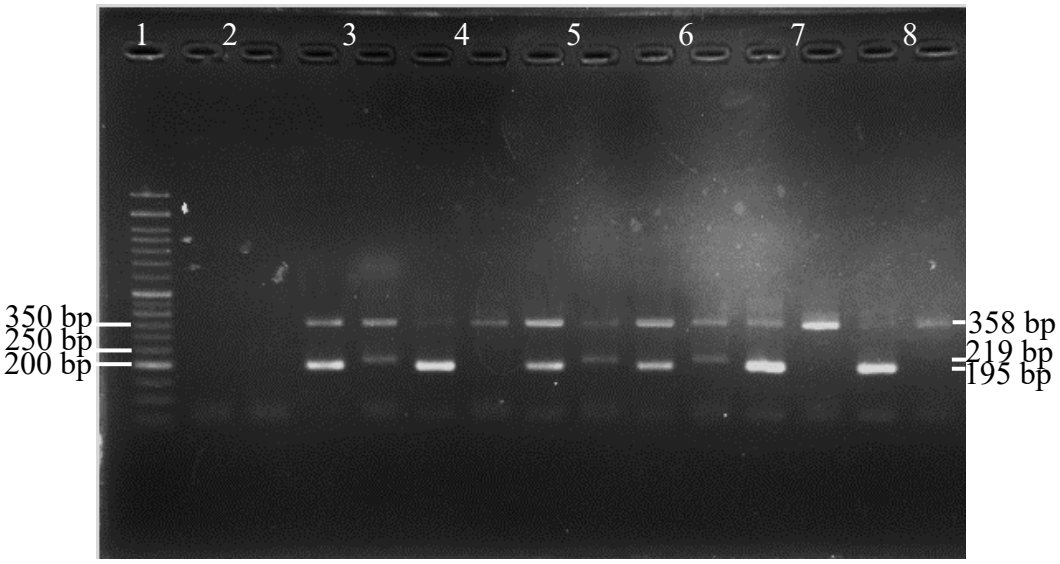


Figure 4.2 (g): Representative agarose gel electrophoresis for genotyping of rs1799945.
Lane 1: 50 bp DNA ladder; Lane 2: Non template control (NTC); Lane 3: CG genotype sample; Lane 4: CC genotype sample; Lane 5: CG genotype sample; Lane 6: CG genotype sample; Lane 7: CC genotype sample; Lane 8: CC genotype sample.

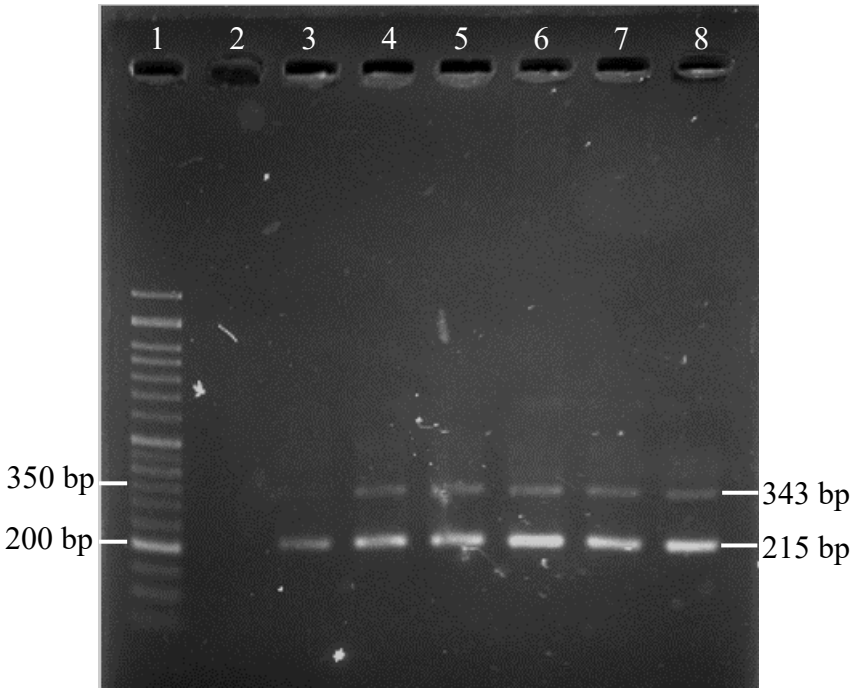


Figure 4.2 (h): Representative agarose gel electrophoresis for genotyping of rs1800562.
Lane 1: 50 bp DNA ladder; Lane 2: Non template control (NTC); Lane 3 – Lane 8: GG genotype samples.

4.2.6 Nucleotide Sequencing

To validate the genotyping results, samples were randomly selected for each SNP and sent for DNA sequencing. After sequencing, the nucleotide sequence was aligned using DNA Baser software. The sequences obtained were compared with the nucleotide sequence on NCBI database. The sequencing results were consistent with the sequence from the NCBI and hence validated the genotyping results were accurate using T-ARMS-PCR.

4.2.7 Genotypic and Allelic Frequencies for Genetic Variants

Genotypic and allelic frequencies for all the genotyped SNPs were presented in Table 4.1. Two genotypes were identified in rs10414846 which are CC and CT genotypes, respectively. C allele was the major allele with allelic frequency of 0.97 while T allele was the minor allele with allelic frequency of 0.03. CC genotype was the predominant genotype with genotypic frequency of 93.99% (n = 172), followed by CT genotype with genotypic frequency of 6.01% (n =11).

For rs10421768, two genotypes (AA and AG genotype) were determined. A total of 178 samples were identified as AA genotype, presenting as dominant genotype. This was followed by AG genotype with n = 5 (2.73%). Thus, A allele was the major allele and G allele was the minor allele with allelic frequencies of 0.99 and 0.01, respectively.

For variant rs855791, AG genotype was identified as dominant genotype with genotypic frequency of 45.90% (n = 84), followed by AA genotype at 37.70% (n = 69) and GG genotype at 16.40% (n = 30). A allele was the major allele with allelic frequency of 0.61 and G allele was the minor allele with allelic frequency of 0.39.

For rs4820268, GA genotype was identified as the dominant genotype which expressed at 45.36% (n = 83), followed by GG genotype which expressed at 36.07% (n = 33) and AA genotype at 18.57% (n = 34). The major allele was G allele with allelic frequency of 0.59 and the minor allele was A allele with allelic frequency of 0.41.

For rs1799852 at *TF* gene, CC genotype was expressed as dominant genotype at 37.76% (n = 124) and CT genotype at 32.34% (n = 59). The C allele was found as the major allele with major allele frequency of 0.84 and T allele was the minor allele with minor allele frequency of 0.16. The predominant genotype for rs12769 is GA genotype which expressed at 48.63% (n = 89), followed by GG genotype at 32.79% (n = 60) and AA genotype at 18.58% (n = 34). The calculated allelic frequency for the major allele (G allele) and minor allele (A allele) were 0.57 and 0.43, respectively.

There were two genotypes, CC and CG genotypes were identified for rs1799945. The CC genotype was found as dominant genotype which was at 89.07% (n = 163) while CG allele was the genotype expressed at 10.93% (n = 20). The allelic frequency for major allele (C allele) was 0.95 while minor allele (G allele) was 0.05. There was only one genotype (GG genotype) was identified for *HFE* variant rs1800562.

Table 4.1: Genotypic and allelic frequencies for genetic variants.

		Genotypic Frequency, n (%)			Allelic Frequency	
***HAMP* gene**	**rs10414846 (C>T) n = 183**	**CC** 172 (93.99%)	**CT** 11 (6.01%)	**TT** 0 (0%)	**C** 0.97	**T** 0.03
	rs10421768 (A>G) n = 183	**AA** 178 (97.27%)	**AG** 5 (2.73%)	**GG** 0 (0%)	**A** 0.99	**G** 0.01
***TMPRSS6* gene**	**rs855791 (A>G) n = 183**	**AA** 69 (37.70%)	**AG** 84 (45.90%)	**GG** 30 (16.40%)	**A** 0.61	**G** 0.39
	rs4820268 (G>A) n = 183	**GG** 66 (36.07%)	**GA** 83 (45.36%)	**AA** 34 (18.57%)	**G** 0.59	**A** 0.41
***TF* gene**	**rs1799852 (C>T) n = 183**	**CC** 124 (67.76%)	**CT** 59 (32.34%)	**TT** 0 (0%)	**C** 0.84	**T** 0.16
	rs12769 (G>A) n = 183	**GG** 34 (18.58%)	**GA** 89 (48.63%)	**AA** 60 (32.79%)	**G** 0.43	**A** 0.57
***HFE* gene**	**rs1799945 (C>G) n = 183**	**CC** 163 (89.07%)	**CG** 20 (10.93%)	**GG** 0 (0%)	**C** 0.95	**G** 0.05
	rs1800562 (G>A) n = 183	**GG** 183 (100%)	**GA** 0 (0%)	**AA** 0 (0%)	**G** 1.00	**A** 0.00

4.3 Analysis of Iron Metabolising Parameters and the Relationship

4.3.1 Analysis of Iron Metabolising Parameters

In this study, Human Hepc (Hepcidin) ELISA assay kit and Iron Colorimetric assay kit were used to analyse hepcidin concentration and serum iron level in this study. The concentration of all the samples were calculated based on the standard curve obtained from the assays. Figure 4.3 (a) and Figure 4.3 (b) shows the standard curve obtained from the assays for hepcidin concentration and serum iron level, respectively.

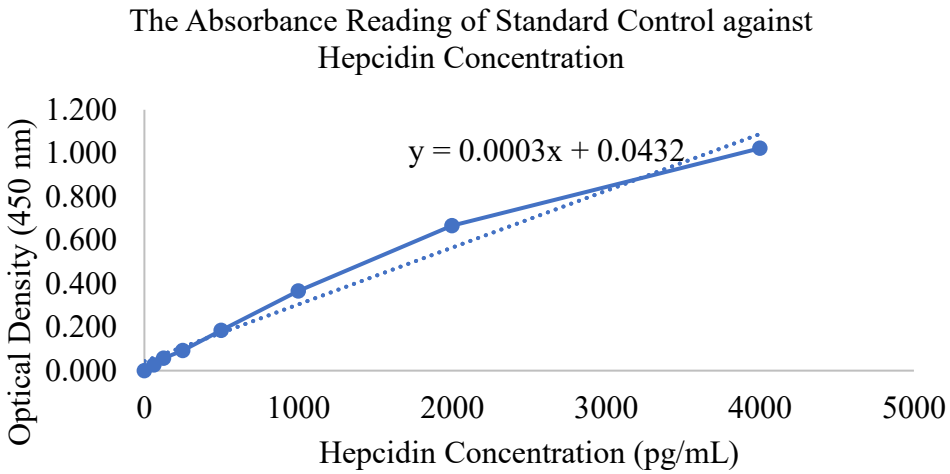


Figure 4.3 (a): The hepcidin concentration for a standard control.

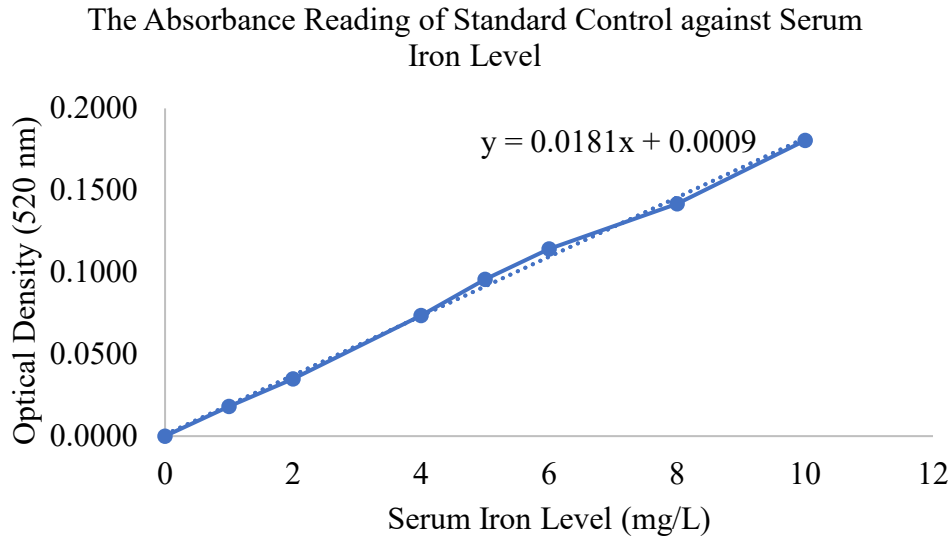


Figure 4.3 (b): The serum iron concentration for a standard control.

4.3.2 Relationship between Iron Metabolising Parameters

Spearman's Rank Order Correlation Test was conducted to determine the relationship between haemoglobin hepcidin and serum iron level among 183 study subjects at 95% confidence interval ($p<0.05$). There was significant positive correlation between haemoglobin level with hepcidin concentration and

serum iron level, respectively with the Spearman's correlation at 0.459 and 0.220. Besides, there was significant positive correlation between serum iron level and hepcidin concentration. Table 4.2 and Figure 4.4 present the Spearman's rank order correlation and distribution of iron metabolising parameter, respectively.

Table 4.2: Spearman's rank order correlation of iron metabolising parameters.

	Haemoglobin Level		Hepcidin Concentration	
	R_s	p-value	R_s	p-value
Hepcidin Concentration	0.459	0.000*		
Serum Iron Level	0.220	0.003*	0.247	0.001*

* Correlation is significant with p-value less than 0.05.

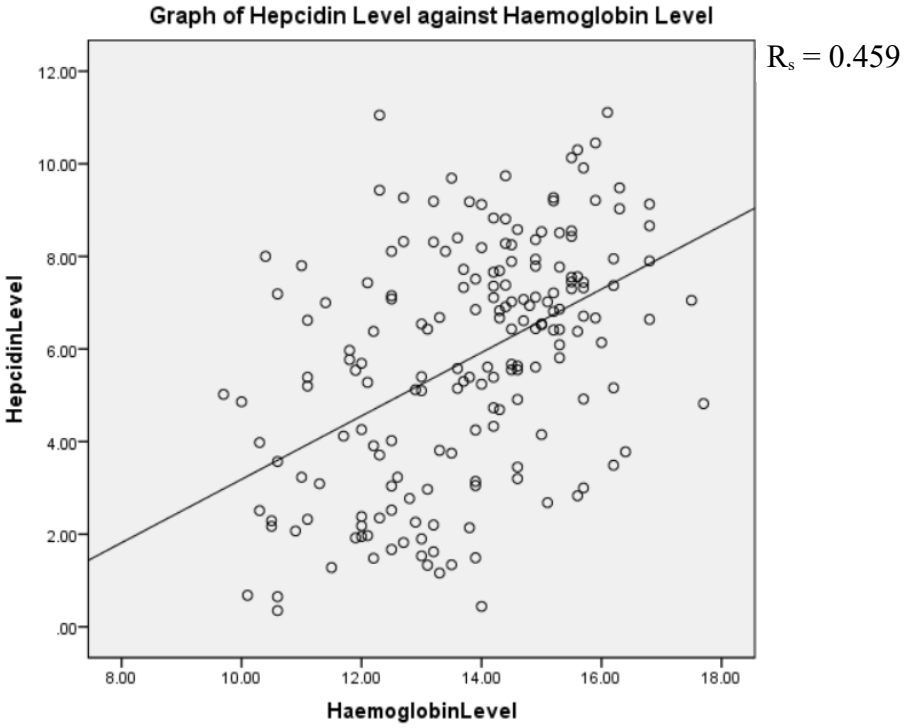
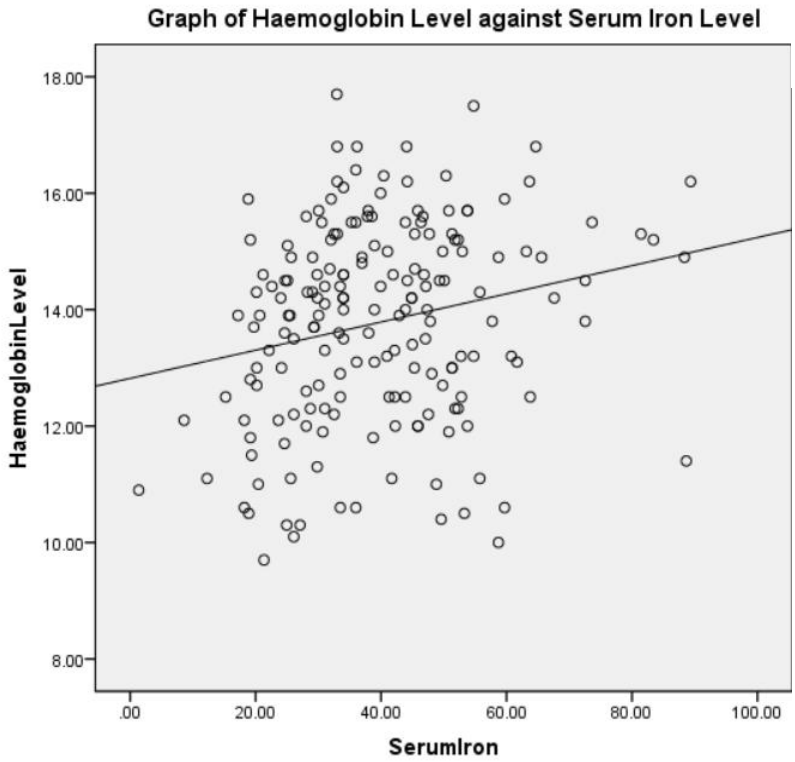
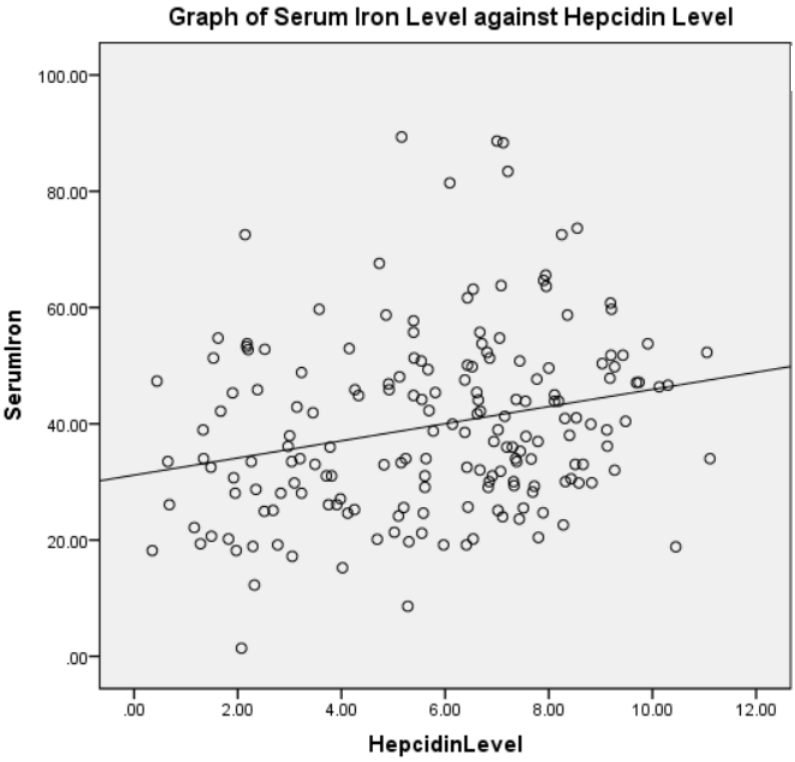


Figure 4.4 (a): Graph for hepcidin concentration against haemoglobin level.



$R_s = 0.220$

Figure 4.4 (b): Graph for haemoglobin level against serum iron level.



$R_s = 0.247$

Figure 4.4 (c): Graph for serum iron level against hepcidin concentration.

4.4 Demographic Distribution with Iron Metabolising Parameters

Kruskal-Wallis analysis was run to analyse the demographic distribution of 183 study subjects with iron metabolising parameters. Demographic information including gender, age and ethnicity were self-declared by study subjects into a Google Form. Table 4.3 shows the demographic distribution with haemoglobin level, hepcidin concentration and serum iron level among 183 study subjects. Out of 183 study subjects, 45.36% (n = 83) were male and 54.64% (n = 100) were female. The Kruskal-Wallis test revealed that there was significant difference between haemoglobin level, serum iron level and hepcidin concentration with gender at $p = 0.000$. Between both gender, male exhibited markedly higher hepcidin, haemoglobin and serum iron level compared to female with median of 15.10 g/dL, 7.33 ng/mL and 41.91 μmol/L, respectively.

The age of study subjects was ranged between 18 to 35 years old and was being categorised into three age group, which are 18 – 23, 24 – 29 and 30 – 35 years old. 77.60% (n = 142) of the study subjects were at the age range of 18 – 23 years old, 15.58% (n = 29) aged between 24 – 29 years old and the remaining 6.55% (n = 12) were aged between 30 – 35 years old. There was no significant difference in Kruskal-Wallis test between age and haemoglobin level, serum iron level and hepcidin concentration at $p = 0.334$, $p = 0.399$ and $p = 0.061$, respectively.

This study consisted of ethnic group including Chinese, Malays, Indians and indigenous population. The predominant ethnic group in this study was Chinese (84.15%; 154/183), followed by Malays (7.10%; 13/183), Indians (7.10; 13/183)

and only three with indigenous (1.65%). There were significant differences in Kruskal-Wallis test between ethnicity and hepcidin concentration at $p = 0.006$ ($p < 0.05$). Indians presented with the lowest hepcidin concentration (median = 2.29 ng/mL), followed by indigenous (median = 2.51 ng/mL), Chinese (median = 6.41 ng/mL) and Malays (median = 7.72 ng/mL).

Table 4.3: Demographic distribution with haemoglobin level, hepcidin concentration and serum iron level.

Gender	Male (n = 83)	Female (n = 100)	p-value
Haemoglobin level (g/dL) Median (IQR)	15.10 (1.30)	12.75 (1.95)	0.000*
Hepcidin concentration (ng/mL) Median (IQR)	7.33 (2.01)	4.61 (4.24)	0.000*
Serum iron level (µmol/L) Median (IQR)	41.91 (19.27)	33.39 (22.57)	0.000*

Age	18 – 23 (n = 142)	24 – 29 (n = 29)	30 – 35 (n = 12)	p-value
Haemoglobin level (g/dL) Median (IQR)	14.05 (2.63)	13.80 (5.40)	14.55 (2.92)	0.344
Hepcidin concentration (ng/mL) Median (IQR)	6.38(4.10)	5.39 (4.29)	7.67 (1.87)	0.061
Serum iron level (µmol/L) Median (IQR)	36.05 (20.39)	38.94 (24.62)	43.34 (14.48)	0.399

Ethnicity	Malays (n = 13)	Chinese (n = 154)	Indians (n = 13)	Indigenous (n = 3)	p-value
Haemoglobin level (g/dL) Median (IQR)	13.70 (3.50)	14.20 (2.53)	12.30 (2.60)	13.00	0.087
Hepcidin concentration (ng/mL) Median (IQR)	7.72 (3.81)	6.41 (3.70)	2.29 (5.12)	2.51	0.006*
Serum iron level (µmol/L) Median (IQR)	29.85 (12.85)	38.84 (20.18)	36.96 (43.39)	42.89	0.115

* Significant difference at p-value < 0.05.

4.5 Comparison between BMI with Haemoglobin Level, Hepcidin Concentration and Serum Iron Level

Anthropometric measurements including height and weight of each each study subjects were used to calculate their body mass index (BMI) using the formula below:

$$\text{BMI (kg/m}^2\text{)} = \frac{\text{Weight (kg)}}{(\text{Height})^2(\text{m}^2)}$$

The study subjects were further categorised into four different categories which are underweight (BMI < 18.50 kg/m²), normal weight (BMI between 18.50 to 22.99 kg/m²), overweight (BMI between 23.00 to 27.49 kg/m²) and obese (BMI ≥ 25.50 kg/m²) based on the WHO Experts Consultation (2004) for BMI categories in Asian population. In this study, study subjects were predominantly normal weight with 24 out of 183 (45.90%), followed by overweight (45/183; 24.60%), underweight (29/183; 15.85%) and obese (25/183; 13.65%). The Kruskal-Wallis test revealed that there was significant difference between BMI and haemoglobin level. subjects with higher BMI manifested higher haemoglobin level where overweight and obese group presented higher haemoglobin level compared to underweight and normal weight subjects. Table 4.4 shows the distribution of BMI with iron metabolising parameters.

Table 4.4: Distribution of BMI with haemoglobin level, hepcidin concentration and serum iron level.

Body Mass Index (BMI)	Haemoglobin level (g/dL) Median (IQR)	p-value	Hepcidin concentration (ng/mL) Median (IQR)	p-value	Serum Iron level (µmol/L) Median (IQR)	p-value
Underweight (n = 29)	13.70 (2.00)		6.41 (4.38)		35.98 (21.49)	
Normal weight (n = 84)	13.45 (2.47)		5.39 (4.30)		37.88 (21.99)	
		0.002*		0.060		0.901
Overweight (n = 45)	14.40 (1.90)		6.71 (3.32)		39.93 (16.08)	
Obese (n = 25)	15.00 (2.15)		6.85 (3.09)		33.30 (26.14)	

* Significant difference at p-value < 0.05.

** Categorisation of BMI is based on the BMI cut points defined by WHO Expert Consultation (2004).

4.6 Family and Clinical History of Anaemia with Iron Metabolising Parameters

4.6.1 Family History of Anaemia with Iron Metabolising Parameters

Out of the 183 study subjects, 154 (84.15%) participants reported no family history of anaemia, 16 (8.74%) participants have family history of anaemia and the remaining 13 participants (7.11%) were uncertain on their family history of anaemia. The Kruskal-Wallis analysis revealed that family history of anaemia shows significant difference with haemoglobin level. Study subjects with family history of anaemia exhibited significantly lower haemoglobin level compared to study subjects without family history of anaemia with median of 11.45 g/dL and 14.05 g/dL, respectively. Table 4.5 shows the distribution of family history of anaemia with haemoglobin level, hepcidin concentration and serum iron level.

Table 4.5: Distribution of family history of anaemia with iron metabolising parameters.

	Family History of Anaemia			
	No (n = 154)	**Yes (n = 16)**	**Uncertain (n = 13)**	**p-value**
Haemoglobin level (g/dL) Median (IQR)	14.05 (2.50)	11.45 (3.55)	14.26 (3.05)	0.002*
Hepcidin concentration (ng/mL) Median (IQR)	6.48 (4.34)	5.03 (3.19)	5.69 (2.97)	0.161
Serum iron level (µmol/L) Median (IQR)	38.28 (19.99)	37.47 (28.99)	38.05 (23.3)	0.509

* Significant difference at p-value < 0.05.

4.6.2 Clinical History with Iron Metabolising Parameters

Clinical history including menstruation, experience of menorrhagia (heavy menstruation), bleeding problem and blood donation in the past three months were recorded and analysed with iron metabolising parameters.

Study subjects were categorised to currently having menstruation and not having menstruation. The Kruskal-Wallis test presented significant different between menstruation with haemoglobin level, hepcidin concentration and serum iron level. Individual who are currently having menstruation presented with remarkably lower haemoglobin, hepcidin and serum iron level with median of 12.50 g/dL, 4.69 ng/mL and 29.08 μmol/L, respectively.

The experience of menorrhagia in the past three months shows significant difference with haemoglobin level, hepcidin concentration as well as serum iron concentration. Individual with experience of menorrhagia in the past three months showed significantly lower haemoglobin level, hepcidin concentration and serum iron level compared to individual without experiencing menorrhagia in the past three months with the medians of 12.50 g/dL, 3.12 ng/mL and 25.52 μmol/L, respectively.

Majority of the study subjects (92.90%; 170/183) do not experience bleeding problem in the past three months, while only 13 study subjects (7.10%) have the experience of bleeding problem in past three months. Kruskal-Wallis analysis revealed significant difference between the experience of bleeding problem in the past three months with hepcidin concentration (p-value = 0.039).

Individual with experience of bleeding problem in the past three months manifested higher hepcidin concentration with median of 7.32 ng/mL.

Active blood donor was defined as donating blood at least three times in a year, while moderate donor was defined as donating blood once or twice in a year. In this study, 111 (60.66%) study subjects were non-donor, 65 (35.52%) were moderate donor and the remaining 7 (3.82%) were active donor. No significant difference was found between blood donation with haemoglobin, hepcidin and serum iron level. Table 4.6 shows the distribution of clinical history of study subject with iron metabolising parameters.

Table 4.6: Clinical history of study subjects with haemoglobin level, hepcidin concentration and serum iron level in Kruskal-Wallis analysis.

Currently Having Menstruation	**No (n = 160)**	**Yes (n = 23)**	**p-value**
Haemoglobin level (g/dL) Median (IQR)	14.20 (2.55)	12.50 (2.60)	0.000*
Hepcidin concentration (ng/mL) Median (IQR)	6.54 (3.77)	4.69 (3.35)	0.007*
Serum iron level (µmol/L) Median (IQR)	38.64 (19.85)	29.08 (29.19)	0.038*

* Significant difference at p-value < 0.05.

Table 4.6 (cont.): Clinical history of study subjects with haemoglobin level, hepcidin concentration and serum iron level in Kruskal-Wallis analysis.

Experience of Menorrhagia in Past 3 Months	No (n = 167)	Yes (n = 16)	p-value
Haemoglobin level (g/dL) Median (IQR)	14.20 (2.50)	12.50 (2.25)	0.001*
Hepcidin concentration (ng/mL) Median (IQR)	6.43 (3.75)	3.12 (4.27)	0.004*
Serum iron level (µmol/L) Median (IQR)	38.94 (19.76)	25.52 (22.83)	0.005*

Experience of Bleeding Problem in Past 3 Months	No (n = 170)	Yes (n = 13)	p-value
Haemoglobin level (g/dL) Median (IQR)	14.00 (2.63)	13.60 (2.95)	0.615
Hepcidin concentration (ng/mL) Median (IQR)	6.12 (4.18)	7.32 (3.16)	0.039*
Serum iron level (µmol/L) Median (IQR)	36.56 (20.78)	38.94 (18.83)	0.292

Blood Donation	Non-donor (n = 111) [Male: 47; Female: 64]	Moderate donor (n = 65)	Active donor (n = 7)	p-value
Haemoglobin level (g/dL) Median (IQR)	13.90 (2.55)	14.00 (2.80)	(13.10 (2.70)	0.615
Hepcidin concentration (ng/mL) Median (IQR)	6.41 (4.19)	6.38 (3.75)	4.15 (4.95)	0.544
Serum iron level (µmol/L) Median (IQR)	38.02 (19.47)	36.11 (21.25)	53.27 (32.62)	0.145

* Significant difference at p-value < 0.05.

** Active donor: Donating blood at least three times per year; Moderate donor: Donating blood once or twice per year.

4.7 Genetic Variants with Iron Metabolising Parameters

A total of eight single nucleotide polymorphisms (SNPs) on four iron regulating genes (*HAMP*, *TMPRSS6*, *TF* and *HFE* genes) were included in this study. For *HAMP* gene, rs10414846 and rs10421768 were genotyped. For rs10414846, 93.99% (n = 172) were found with CC genotype and 6.01% (n = 11) were CT genotype. No significant difference was shown between rs10414846 and iron metabolising parameters. While for rs10421768, 178 (97.27%) were found with AA genotype and only 5 (2.73%) were AG genotype. There was significant difference between rs10421768 genotypes with serum iron level where AG genotype presented with significantly lower serum iron compared to AA genotype with the median of 29.08 µmol/L and 38.28 µmol/L, respectively.

For *TMPRSS6* gene, rs855791 and rs4820268 were genotyped with three genotypes were presented. For rs855791, AA genotype (37.70%; 69/183), AG genotype (45.90%; 84/183) and GG genotype (16.40%; 30/183) were determined. While for rs4820268, GG genotype (36.07%; n = 66), GA genotype (45.36%; n = 83) and AA genotype (18.57%; n = 34) were identified. There was no significant difference between both variants with haemoglobin level, hepcidin concentration and serum iron level.

In genotyping of *TF* gene, only CC and CT genotype were identified for rs1799852. One hundred and twenty-four (67.76%) were CC genotype and the remaining 59 (32.24%) were CT genotype. While for *TF* gene variant rs12769, three genotypes, GG, GA and AA genotype were identified. Eighty-nine (89/183; 48.62%) were found with GA genotype, followed by 60 (32.79%) of

AA genotype and 34 (8.58%) of GG genotype. No significant difference was found between rs1799852 with iron metabolising parameters but rs12769 shows significant difference with hepcidin concentration and serum iron level. AA genotype presented with lower hepcidin and serum iron level compared to GG and GA genotypes with median of 5.49 ng/mL and 33.01 µmol/L, respectively.

Two *HFE* variants, rs1799945 and rs1800562 were genotyped. Two genotypes (CC and CG genotype) were identified for rs1799945 with percentage of 89.07% (163/183) for CC genotype and 10.93 (20/183) for CG genotype, Kruskal-Wallis revealed no significant difference between rs1799945 and iron metabolising parameters. While for variant rs1800562, homogenous genotype (GG genotype) was shown. Table 4.7 shows the Kruskal-Wallis analysis for genetic variants with haemoglobin level, hepcidin concentration and serum iron level.

Table 4.7 (a): *HAMP* gene variants with iron metabolising parameters in Kruskal-Wallis analysis.

rs10414846	CC (n = 172)	CT (n = 11)	TT (n = 0)	p-value
Haemoglobin level (g/dL) Median (IQR)	14.00 (2.60)	14.20 (3.20)	-	0.988
Hepcidin concentration (ng/mL) Median (IQR)	6.40 (3.96)	6.14 (5.43)	-	0.900
Serum iron (µmol/L) Median (IQR)	38.28 (20.28)	29.26 (26.04)	-	0.093

rs10421768	AA (n = 178)	AG (n = 5)	GG (n = 0)	p-value
Haemoglobin level (g/dL) Median (IQR)	14.00 (2.63)	13.70 (4.15)	-	0.732
Hepcidin concentration (ng/mL) Median (IQR)	6.42 (3.97)	5.61 (4.80)	-	0.362
Serum iron (µmol/L) Median (IQR)	38.28 (20.53)	29.08 (16.04)	-	0.032*

* Significant difference at p-value < 0.05.

Table 4.7 (b): *TMPRSS6* gene variants with iron metabolising parameters in Kruskal-Wallis analysis.

rs855791	AA (n = 69)	AG (n = 84)	GG (n = 30)	p-value
Haemoglobin level (g/dL) Median (IQR)	14.20 (2.55)	14.05 (2.78)	13.75 (2.52)	0.107
Hepcidin concentration (ng/mL) Median (IQR)	6.81 (3.21)	5.57 (4.48)	5.83 (3.40)	0.112
Serum iron (µmol/L) Median (IQR)	34.00 (19.06)	40.18 (20.14)	33.02 (21.65)	0.427

rs4820268	GG (n = 66)	GA (n = 83)	AA (n = 34)	p-value
Haemoglobin level (g/dL) Median (IQR)	14.20 (2.42)	14.00 (2.90)	13.75 (2.52)	0.443
Hepcidin concentration (ng/mL) Median (IQR)	6.66 (3.97)	5.69 (3.97)	5.81 (4.18)	0.567
Serum iron (µmol/L) Median (IQR)	38.45 (19.74)	38.94 (20.98)	30.02 (21.65)	0.114

* Significant difference at p-value < 0.05.

Table 4.7 (c): *TF* gene variants with iron metabolising parameters in Kruskal-Wallis analysis.

rs1799852	CC (n = 124)	CT (n = 59)	TT (n = 0)	p-value
Haemoglobin level (g/dL) Median (IQR)	13.95 (2.47)	14.20 (3.10)	-	0.616
Hepcidin concentration (ng/mL) Median (IQR)	6.43 (3.84)	6.14 (4.31)	-	0.945
Serum iron (µmol/L) Median (IQR)	38.75 (21.41)	35.98 (18.28)	-	0.328

rs12769	GG (n = 34)	GA (n = 89)	AA (n = 60)	p-value
Haemoglobin level (g/dL) Median (IQR)	13.96 (1.68)	14.20 (2.75)	13.70 (3.07)	0.702
Hepcidin concentration (ng/mL) Median (IQR)	5.58 (4.16)	6.71 (4.15)	5.49 (3.94)	0.033*
Serum iron (µmol/L) Median (IQR)	46.08 (18.90)	38.53 (18.60)	33.01 (21.95)	0.027*

* Significant difference at p-value < 0.05.

Table 4.7 (d): *HFE* gene variants with iron metabolising parameters in Kruskal-Wallis analysis.

rs1799945	**CC (n = 163)**	**CG (n = 11)**	**GG** (n = 0)	**p-value**
Haemoglobin level (g/dL) Median (IQR)	14.00(2.60)	14.05(3.10)	-	0.720
Hepcidin concentration (ng/mL) Median (IQR)	6.40(3.96)	6.14(5.43)	-	0.336
Serum iron (µmol/L) Median (IQR)	38.28(20.28)	29.26(26.04)	-	0.058

rs1800562	**GG (n = 183)**	**GA (n = 0)**	**AA (n = 0)**	**p-value**
Haemoglobin level (g/dL) Median (IQR)	-	-	-	-
Hepcidin concentration (ng/mL) Median (IQR)	-	-	-	-
Serum iron (µmol/L) Median (IQR)	-	-	-	-

* Significant difference at p-value < 0.05.
** No comparison can be made on rs1800562 as it shows homogenous genotype.

4.8 Relationship between Genetic Variants

The interaction between the eight genetic variants on four different iron regulating genes were identified using Chi-Square (χ^2) analysis. There were significant association between rs855791 with rs4820268 of *TMPRSS6* gene ($\chi^2(4, N = 183) = 138.9$, $p = .000$), rs1799852 with rs12769 of *TF* gene (χ^2 (2, $N = 183) = 21.551$, $p = .000$) and rs10414846 with rs10421768 of *HAMP* gene

(χ^2 (1, N = 183) = 80.378, p = .000). On the other hand, no association between genetic variants present on different gene with $p > 0.05$. Interaction between different genetic variants with variant rs1800562 of *HFE* gene were unable to analyse due to the single genotype shown by rs1800562. Table 4.8 shows the Chi-Square (χ^2) analysis between the eight variants on four different iron regulating genes.

Table 4.8: Chi-Square analysis between genetic variants on four iron regulating genes.

***TMPRSS6* gene**	***TMPRSS6* gene**	**rs4820268 (G > A)**						
		Wildtype (GG)	**Heterozygous (GA)**	**Mutant (AA)**	**Total**	**X^2**	***df***	**p-value**
rs855791 (A > G)	**Wildtype (AA)**	49	17	3	69	113.387	4	0.000*
	Heterozygous (AG)	14	60	10	84			
	Mutant (GG)	3	6	21	30			
	Total	66	83	34	183			
	***TF* gene**	**rs1799852 (C > T)**						
		Wildtype (CC)	**Heterozygous (CT)**		**Total**	**X^2**	***df***	**p-value**
	Wildtype (AA)	47	22		69	0.021	2	0.990
	Heterozygous (AG)	57	27		84			
	Mutant (GG)	20	10		30			
	Total	124	59		183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

***TMPRSS6* gene**	***TF* gene**	**rs12769 (G > A) Wildtype (GG)**	**Heterozygous (GA)**	**Mutant (AA)**	**Total**	**X^2**	***df***	**p-value**
rs855791 (A > G)	**Wildtype (AA)**	19	38	12	69	6.246	4	0.181
	Heterozygous (AG)	32	33	19	84			
	Mutant (GG)	9	18	3	30			
	Total	60	89	34	183			

	***HFE* gene**	**rs1799945 (C > G) Wildtype (CC)**	**Heterozygous (CG)**	**Total**	**X^2**	***df***	**p-value**
	Wildtype (AA)	62	7	69	0.226	2	0.893
	Heterozygous (AG)	75	9	84			
	Mutant (GG)	26	4	30			
	Total	163	20	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

***TMPRSS6* gene**		**rs10414846 (C > T)**					
	***HAMP* gene**	**Wildtype (CC)**	**Heterozygous (CT)**	**Total**	**X^2**	***df***	**p-value**
	Wildtype (AA)	65	4	69			
	Heterozygous (AG)	79	5	84	0.029	2	0.986
	Mutant (GG)	28	2	30			
	Total	172	11	183			

rs855791 (A > G)		**rs10421768 (A > G)**					
	***HAMP* gene**	**Wildtype (AA)**	**Heterozygous (AG)**	**Total**	**X^2**	***df***	**p-value**
	Wildtype (AA)	67	2	69			
	Heterozygous (AG)	81	3	84	1.072	2	0.585
	Mutant (GG)	30	0	30			
	Total	178	5	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

***TMPRSS6* gene**		**rs1799852 (C > T)**					
	***TF* gene**	**Wildtype (CC)**	**Heterozygous (CT)**	**Total**	**X^2**	***df***	**p-value**
	Wildtype (GG)	48	18	66	1.390	2	0.499
	Heterozygous (GA)	55	28	83			
	Mutant (AA)	21	13	34			
	Total	124	59	183			

rs4820268 (G > A)		**rs12769 (G > A)**						
	***TF* gene**	**Wildtype (GG)**	**Heterozygous (GA)**	**Mutant (AA)**	**Total**	**X^2**	***df***	**p-value**
	Wildtype (GG)	19	35	12	66	4.425	4	0.351
	Heterozygous (GA)	29	35	19	83			
	Mutant (AA)	12	19	3	34			
	Total	60	89	34	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

TMPRSS6 gene	*HFE* gene	rs1799945 (C > G) Wildtype (CC)	Heterozygous (CG)	Total	X^2	*df*	p-value
rs4820268 (G > A)	Wildtype (GG)	59	7	66	0.269	2	0.874
	Heterozygous (GA)	73	10	83			
	Mutant (AA)	31	3	34			
	Total	163	20	183			

	HAMP gene	rs10414846 (C > T) Wildtype (CC)	Heterozygous (CT)	Total	X^2	*df*	p-value
	Wildtype (GG)	62	4	66	0.685	2	0.710
	Heterozygous (GA)	79	4	83			
	Mutant (AA)	31	3	34			
	Total	172	11	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

***TMPRSS6* gene**	***HAMP* gene**	**rs10421768 (A > G)**					
		Wildtype (AA)	**Heterozygous (AG)**	**Total**	**X^2**	***df***	**p-value**
rs4820268 (G > A)	**Wildtype (GG)**	65	1	66	0.617	2	0.735
	Heterozygous (GA)	80	3	83			
	Mutant (AA)	33	1	34			
	Total	178	5	183			

***TF* gene**	***TF* gene**	**rs12769 (G > A)**						
		Wildtype (GG)	**Heterozygous (GA)**	**Mutant (AA)**	**Total**	**X^2**	***df***	**p-value**
rs1799852 (C > T)	**Wildtype (CC)**	29	63	32	124	21.551	2	0.000*
	Heterozygous (CT)	31	26	2	59			
	Total	60	89	34	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

TF gene	*HFE* gene	rs1799945 (C > G) Wildtype (CC)	Heterozygous (CG)	Total	X^2	*df*	p-value
rs1799852 (C > T)	Wildtype (CC)	112	12	124	0.619	1	0.431
	Heterozygous (CT)	51	8	59			
	Total	163	20	183			
	HAMP gene	rs10414846 (C > T) Wildtype (CC)	Heterozygous (CT)	Total	X^2	*df*	p-value
	Wildtype (CC)	119	5	124	2.665	1	0.103
	Heterozygous (CT)	53	6	59			
	Total	172	11	183			

* Significant difference at p-value < 0.05.
** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

***TF* gene**	***HAMP gene***	**rs10421768 (A > G)**					
		Wildtype (AA)	**Heterozygous (AG)**	**Total**	**X^2**	***df***	**p-value**
rs1799852 (C > T)	**Wildtype (CC)**	122	2	124	1.813	1	0.178
	Heterozygous (CT)	56	3	59			
	Total	178	5	183			

***TF* gene**	***HFE* gene**	**rs1799945 (C > G)**					
		Wildtype (CC)	**Heterozygous (CG)**	**Total**	**X^2**	***df***	**p-value**
rs12769 (G > A)	**Wildtype (GG)**	53	7	60	1.110	2	0.574
	Heterozygous (GA)	78	11	89			
	Mutant (AA)	32	2	34			
	Total	163	20	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

TF gene	*HAMP* gene	rs10414846 (C > T) Wildtype (CC)	Heterozygous (CT)	Total	X^2	*df*	p-value
rs12769 (G > A)	Wildtype (GG)	56	4	60	0.697	2	0.706
	Heterozygous (GA)	83	6	89			
	Mutant (AA)	33	1	34			
	Total	172	11	183			

TF gene	*HAMP* gene	rs10421768 (A > G) Wildtype (AA)	Heterozygous (AG)	Total	X^2	*df*	p-value
rs12769 (G > A)	Wildtype (GG)	57	3	60	2.033	2	0.362
	Heterozygous (GA)	88	1	89			
	Mutant (AA)	33	1	34			
	Total	178	5	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

***HFE* gene**		**rs10414846 (C > T)**					
	***HAMP* gene**	**Wildtype (CC)**	**Heterozygous (CT)**	**Total**	**X^2**	***df***	**p-value**
	Wildtype (CC)	153	10	163	0.041	1	0.840
	Heterozygous (CG)	19	1	20			
	Total	172	11	183			

rs1799945 (C > G)		**rs10421768 (A > G)**					
	***HAMP* gene**	**Wildtype (AA)**	**Heterozygous (AG)**	**Total**	**X^2**	***df***	**p-value**
	Wildtype (CC)	158	5	163	0.631	1	0.427
	Heterozygous (CG)	20	0	20			
	Total	178	5	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

Table 4.8 (cont.): Chi-Square analysis between genetic variants on four iron regulating genes.

***HAMP* gene**	***HAMP* gene**	**rs10421768 (A > G)**		**Total**	**X^2**	***df***	**p-value**
		Wildtype (AA)	**Heterozygous (AG)**				
rs10414846 (C > T)	**Wildtype (CC)**	172	0	172	80.378	1	0.000*
	Heterozygous (CG)	6	5	11			
	Total	178	5	183			

* Significant difference at p-value < 0.05.

** No statistics are computed for rs1800562 because rs1800562 is a constant.

CHAPTER 5

DISCUSSION

5.1 Prevalence of Anaemia among the Study Subjects

The prevalence rate of anaemia among the 183 study subjects reported in this study (haemoglobin level < 12.0 g/dL for female and <13.0 g/dL for male) (McLean et al., 2009) was 14.75% This reported prevalence rate was found higher than the prevalence rate reported by Abdullah and coworkers at 13.80% in year 2020 (Abdullah et al., 2020). This higher anaemia prevalence rate could be due to the study population was found with more female than male. Female tends to have lower haemoglobin level compared to male due to their physiological condition (Murphy et al., 2014). The mean haemoglobin level was found to be approximately 12% lower than age- and race-match male. Consistent finding was seen in most of the adult mammals, reptiles as well as birds including non-menstruating and non-placental species (Murphy, 2014).

5.2 Genotyping of Genetic Variants in Iron Regulating Genes

A total of eight single nucleotide polymorphisms (SNPs) on four genes (*HAMP*, *TMPRSS6*, *TF* and *HFE* genes) were genotyped using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). In this genotyping assay, two allele-specific amplicons were generated using the outer and inner primers for either wildtype or mutant allele detection (Medrano and de Oliveira, 2014). The inner reverse (R_I) primer was designed to carry a mutated 3' end

allele in genotyping for mutant allele but not the wildtype allele. While the inner forward (F_I) primer carries wildtype allele at the 3' end binds and amplify the wildtype allele.

In this study, our reported MAF allele was found significant for rs855791 (MAF = 0.39), rs4820268 (MAF = 0.41) in *TMPRSS6* gene, rs1799852 (MAF = 0.16), rs12769 (MAF = 0.43) in *TF* gene and rs1799945 (MAF = 0.05) in *HFE* gene with MAF more than 0.05. The other three SNPs were reported with low MAF (MAF < 0.05) and rs1800562 in *HFE* gene was found with wildtype allele (A allele) only.

Our study reported consistent MAF for rs10414846 (T allele) as in National Centre for Biotechnology Information (NCBI) (2022) with MAF among Asian population as at 0.03. However, the MAF for rs10421768 (G allele) in this study (0.01) was found lower as NCBI among Asian was found as at 0.3. For *TMPRSS6*, this study reported the MAF value for rs855791 (G allele) as at 0.39 and MAF value for rs4820268 (A allele) as at 0.41. this was found consistent as reported by NCBI which MAF for rs855791 at 0.45 and rs4820268 at 0.40. MAF for rs1799852 (T allele) (0.16) and rs12769 (A allele) (0.43) reported in this study was found slightly varies with the MAF reported by NCBI where the MAF for rs1799852 and rs12769 of Asian population was at 0.22 and 0.49, respectively. For *HFE* gene, the reported MAF in NCBI of Asian population for variant rs1799945 (G allele) as at 0.04 and rs1800562 (A allele) at 0.00 which is consistent with the MAF reported in our study which MAF for rs1799945 as at 0.04 and rs1800562 as at 0.00.

In overall, some of our reported MAF was found lower than the MAF reported in NCBI. This can be attributed to the different population in the Southeast Asian population mat present different frequencies on the genetic variants (Runcharoen et al., 2021). Moreover, the difference in allele frequencies among population in African population (Jallow wet al., 2021). This postulates that different population may present with different allelic frequency due to the geographical variation as well as the flow of genetic information.

5.3 Relationship among Iron Metabolising Parameters

There is a positive monotonic relationship between hepcidin concentration and haemoglobin level with $R_s = 0.459$. this could be attributed as haemoglobin is the main component in erythrocytes that function to transport gases to whole part of the body. High haemoglobin level represents high red blood cell count in the body. According to Jones et al. (2015), regulation of hepcidin is also affected by erythropoiesis in red blood cell production. Active erythropoiesis would supress hepcidin production in order to increase iron absorption and to release stored iron for synthesis of red blood cells. Thus, when the body's haemoglobin level is high, the needs of erythropoiesis would decrease and body mechanism would tend to upregulate hepcidin production to supress intestinal iron absorption and release form both enterocytes and reticuloendothelial macrophages which is to maintain systemic iron level in homeostatic (Ganz and Nemeth, 2012).

A weak positive correlation was shown between serum iron and haemoglobin level at R_s of 0.220. Most of the iron in body will be incorporated into haemoglobin for red blood cell production (Abbaspour et al., 2014). Haemoglobin in being synthesised in the later stage of erythropoiesis with incorporation of heme and globin chains to produce red blood cells that carry oxygen in the body. During active erythropoiesis, iron absorption and iron release will be highly increased, allowing the synthesis of haemoglobin as well as normal erythrocytes. Thus, this contributed to positive correlation between serum iron level and haemoglobin level (Coates, 2014). Moreover, when body is in the state of iron deficiency or iron depletion, haemoglobin level will decrease drastically as there is insufficient amount of iron to be incorporated in the production of haemoglobin nor erythrocytes, leading to iron deficiency anaemia. This explained the haemoglobin level was uses as the main parameter in diagnosis of iron deficiency anaemia. Iron deficiency anaemia individual presented notably low haemoglobin level but also low serum iron level (Johnson-Wimbley and Graham, 2011).

A weak positive correlation (R_s = 0.247) was presented between hepcidin and serum iron. This was consistent with the findings by Sato and co-authors in 2020 in which hepcidin level was significantly elevated when serum iron level is higher. Hepcidin is the master regulator involving in systemic iron homeostasis. It regulates systemic iron level by regulating cellular iron export through ferroportin of ferroportin-targeting cells including enterocytes, macrophages and hepatocytes (Nemeth and Ganz, 2009). Hepcidin blocks the iron flows into plasma, inhibit intestinal iron absorption, inhibit the release of iron from

macrophages and inhibit the movement of iron stored in hepatocytes (Girelli et al., 2016). When the systemic iron level is high, the expression of hepcidin was stimulated via bone morphogenic protein (BMP) / sons of mothers against decapentaplegic (SMAD) signalling pathway to produce more hepcidin to maintain the balance of body iron (Sangkhae and Nemeth, 2017).

5.4 Demographic Distribution with Iron Metabolising Parameters

Gender with male was found with significantly higher haemoglobin, hepcidin and serum iron level compared to female. According to Garvin (2010), the haemoglobin level for both male and female were equivalent since infancy until childhood. The haemoglobin level would be changed in expression when reaching to the puberty. The haemoglobin level for female start to reach plateau when reaching puberty while the haemoglobin level for male will continue rising throughout puberty until adulthood due to the stimulatory effect of androgen in stimulating erythropoiesis. Androgen would upregulate the mRNA synthesis in the nucleus and lead to the differentiation of bone marrow cell from erythropoietin-non-responsive to erythropoietin-responsive.

Moreover, the primary androgen which is testosterone that highly secreted in male has an inhibitory effect on the secretion of hepcidin. This will result in an increment of iron bioavailability to enhance the incorporation of iron into erythrocytes. Our body mechanisms will tend to increase hepcidin production when a high level of systemic iron is detected to ensure an appropriate systemic iron concentration (Al-Sharefi et al., 2019; Collins et al., 2008; Shanani et al.,

2009). This explained why the hepcidin concentration is higher in male compared to female. Furthermore, androgen plays its role in raising the production of insulin-like-growth factor 1 (IGF-1). The elevation of IGF-1 was suggested to drive the proliferation and differentiation of erythroid progenitor cells (Al-Sharefi et al., 2019) and hence leading to higher haemoglobin level.

Female was found to have significant lower haemoglobin, hepcidin and serum iron level. this could be attributed to menstruation that only occur in childbearing age women. Since the haemoglobin level is consistent for male and female until they reached puberty, the difference in haemoglobin and serum iron level could only be seen after the onset of menstruation. The loss of blood from the body during menstruation will cause a drop in serum iron as well as haemoglobin level in female. The drop of systematic iron level will inhibit the expression of hepcidin and allow more dietary iron to be absorbed through duodenal enterocytes and more iron could be released from the iron store (D'Angelo, 2013; Garvin et al., 2010; Ofojekwu et al., 2013; Rushton et al., 2001; Yang et al., 2012).

Besides gender, ageing is another factor that leading to anaemia in which children and elderly were prone to develop anaemia. The predominant cause of anaemia in children and elderly were due to iron deficiency (Le, 2016). This could be attributed as children need higher iron demand for body growth and development compared to a healthy adult. Thus, when the iron supply is insufficient to meet the body's demand, it will ultimately predisposition to anaemia with low haemoglobin and serum iron but elevated hepcidin

concentration (Djokic et al., 2010). Meanwhile, chronic diseases and nutritional deficiency especially iron deficiency could lead to anaemia in older age adults (Smith, 2000). This study showed no significant difference between age with haemoglobin level in this study population as the study subjects in this study were within the age ranged between 18 to 35 years old and were predominant to be young adult (Petry, 2002). Hence, this study presented age would not modulate haemoglobin and iron profiles among the young adult population as consistent with study by Raisinghani and co-authors in which no significant difference between age and haemoglobin level (Raisinghani et al., 2019).

Ethnicity was found significant difference with hepcidin concentration but no difference with haemoglobin and serum iron level. Malaysia is a multi-racial country and this study recruited four ethnicities including Chinese, Malay, Indian and other indigenous population. Each ethnicity group has their own cultures, beliefs, dietary choices as well as the lifestyle. The findings in this study showed Indians having the lowest haemoglobin level and hepcidin concentration. This may be attributed of insufficient dietary iron intake among Indian respondents. Due to different cultural beliefs and religion prohibitions among different ethnicities, majority of the Indian respondents were reported to be vegetarians or they are prohibited from consuming red meat especially beef that highly rich in iron. Thus, Indians presented with lower haemoglobin and hepcidin level compared to the other ethnic groups as consistent with earlier study by Dutta in 2021 and Yusof in 2018. Tan and co-authors (2013) reported higher incidence rate of moderate to severe anaemia among Indian with the highest prevalence rate of anaemia among Malaysian population. (Tan et al.,

2013). Loh and Khor (2010) reported that there is significant higher prevalence of iron deficiency anaemia in Indian population. In this study, Indian population were also found to show significant higher serum iron level. Development of iron deficiency anaemia can be developed into several stages. During the first stage of iron deficiency, the laboratory findings were characterised by a decreased in bone marrow iron stores, leading to low haemoglobin level but a significant low serum ferritin level. At this stage, serum iron was remains normal or slight elevated. This is due to the compensatory effect of body mechanisms. When there is decreased of iron stores in body, a compensatory mechanism will increase iron absorption, resulted in normal or slight elevated serum iron level (Braunstein, 2022). Thus, a higher serum iron level can be seen in populations presented with lower haemoglobin and hepcidin level.

5.5 Body Mass Index (BMI) with Iron Metabolising Parameters

Body mass index (BMI) of the study subjects were categorised into underweight, normal weight, overweight and obese using the evaluated anthropometric measurements (body weight and height). Our findings revealed significant difference between BMI with haemoglobin level. Subjects with underweight and normal weight were presented with a lower haemoglobin level compared to overweight and obese population. This could be indicating underweight person with thin body features are often associated with undernutrition and there is a deficiency in energy and nutrient intake including iron that is essential for haemoglobin production, hence resulted in low haemoglobin level (World Health Organisation, 2021). This finding is concordance as Marcin (2017)

whose reported underweight were associated with several health issues including anaemia due to imbalance diet. Thus, a relatively higher hepcidin concentration were seen in overweight and obese subjects compared to underweight and normal weight subjects. Moreover, overweight and obese population was believed to have more variety of food intake, allowing them able to absorb more dietary iron from food compared to those underweight and normal weight population (Chang et al., 2014; Kamruzzaman, 2021; Qin et al., 2013). Erythropoiesis is one of the suppressive factors for hepcidin expression. Hepcidin level will be downregulated during active erythropoiesis. This is to enhance the exportation of cellular iron into circulation and transportation to bone marrow to support haemoglobin and red cell production (Parischa et al., 2016). However, erythropoiesis will remain at the basal level when there is sufficient number of erythrocytes and haemoglobin in the body. This is to ensure a balance between erythrocyte production and removal of senescent red blood cells (Conley and Schwartz, 2022). Conversely, hepcidin production will be upregulated to decrease iron export when red blood cell is maintained at homeostasis. Hence, a higher hepcidin level can be seen among the overweight and obese population due to sufficient of iron level. In this study, obese population was found to be the lowest followed by underweight and normal weight population. Obesity was found associated with comorbidities and complications such as diabetes mellitus, cardiovascular diseases and hypertension. Study reported obesity can disrupt iron homeostasis and is closely linked with higher risk to develop low-grade chronic inflammation. The pro-inflammatory cytokines including interleukin-6 (IL-6) secreted by adipose tissues disrupt iron homeostasis by upregulating hepcidin expression. With

there is elevation of hepcidin level, duodenal iron absorption and release of iron from macrophages was being inhibited, resulting in reduced serum iron level (Mohammad Alshwaiyat, 2021; Kamruzamman, 2021; Lee, 2005). This explained why the obese population in this study was found with the lowest level of serum iron level while the hepcidin concentration was the highest.

5.6 Family History of Anaemia with Iron Metabolising Parameters

Anaemia is a multifactorial disease. Several factors including nutritional intake, physical condition, lifestyle as well as family history can contribute to anaemia. Multiple types of anaemia can be inherited from parents to offspring including sickle-cell anaemia, Fanconi anaemia, iron refractory iron deficiency anaemia and thalassaemia (Djokic et al., 2010; Scott, 2010). However, when an individual is the carrier of certain anaemic disorder such as thalassaemia carrier or G6PD carrier who carries only one mutated gene, the individual would not develop into severe anaemia but will be at risk to develop mild anaemia (National Centre on Birth Defects and Developmental Disabilities, 2022; National Health Service, 2019).

Family history of anaemia was found to show significant difference with haemoglobin level. Study group with family history of anaemia was found to have lower haemoglobin level than those without family history. The group with family history of anaemia presented with lower mean hepcidin concentration and serum iron level. Similar findings were shown in a study carried out in Saudi Arabia in which presence of personal and family history of iron deficiency is

associated with increased prevalence rate of anaemia. This suggested that blood-related genetic abnormalities can be inherited by the offspring from their parents (AlQuaiz at al., 2013).

5.7 Clinical History with Iron Metabolising Parameters

5.7.1 Menstruation, Experience of Menorrhagia and Experience of Bleeding Problem in the Past Three Months

Iron loss through menstruation and bleeding were one of the factors contributed to iron deficiency anaemia. In this study, individuals with menstruation and experience of menorrhagia (excessive menstrual bleeding) were found significant with lower haemoglobin, hepcidin and serum iron level. Women in reproductive age experience menstruation every month and up to one-third of the women in reproductive age experienced menorrhagia which may lead to anaemia (Kocaoz, 2019; Mansour et al., 2020). Menstrual bleeding especially heavy menstrual bleed led to a loss of iron from the body and a prolonged heavy blood loss will result in decreased of iron level in the body. Iron plays key role in red blood cell production by incorporating with haem to form haemoglobin. An extended declination of body iron will diminish haemoglobin level and impair the red cell production in giving rise to iron deficiency anaemia (Mansour et al., 2020). Systemic iron level in the body was closely related with hepcidin concentration. When body detects a drop in iron level, the BMP/SMAD signalling pathway that stimulate production of hepcidin will be switched off. This is an adaptation mechanism that stimulate dietary and pharmacological iron absorption to replenish iron loss from the body. When severe anaemia coexists with hypoxia condition, kidneys were triggered to

release erythropoietin. Erythropoietin activity has a suppressive effect on hepcidin transcription, in turn to increase iron absorption (Pagani et al., 2019). Thus, this explained why this study showed lower hepcidin concentration among participants who are having menstruation.

Participants with experience of bleeding problem in the past three months presented significant difference between hepcidin concentration but no significant different on serum iron and haemoglobin level. Experience of bleeding problem in the past three months will cause the body to experience iron loss. Thus, to replenish the iron loss through bleeding and to increase the red cell production, hepcidin production will be downregulated to allow more iron to be reabsorbed from the intestine (Bergamaschi and Villani, 2009). In this study, those experience with bleeding problem presented with higher hepcidin and serum iron but a lower haemoglobin level. Participants who experience bleeding problem in the past three months presented higher hepcidin and serum iron level may be attributed by body's compensatory mechanism during blood loss. Blood loss will cause drop in body's iron level and thus body's corrective mechanism tends to restock the lost iron by permitting enhanced duodenal iron absorption and iron release from macrophages. This would significantly increase the serum iron level in the body. At the same time, when iron level is replenished, hepcidin production will be stimulated to avoid excessive iron present in the circulation (Larson and Coyne, 2013).

5.7.2 Blood Donation with Iron Metabolising Parameters

Blood donation could be one of the risk factors in leading to iron deficiency anaemia (Pehlic et al., 2021). There is approximately 220 to 250 mg of iron loss in every blood donation, leads to a fall in body's iron level which in turns diminished haemoglobin and red cell production and lead to iron deficiency anaemia. Recurrent blood donation will contribute to more severe iron deficiency anaemia as it takes time for the body to replace the iron that had lost through blood donation and the time for this replenishment can be varies based on an individual's physiological condition and eating habit (Armstrong, 2016; The American National Red Cross, 2022). This explained why active blood donors in this study showed lower haemoglobin level and the lowest hepcidin concentration in this study compared to population of moderate donors. As active donors loss more iron compared to moderate donors and non-donors, thus the hepcidin level tends to be the lowest to avoid iron deficiency. Low hepcidin level to permit more duodenal iron absorption as well as iron release from stores (Collins et al., 2008). While non-donors in this study presented lower haemoglobin level compared to moderate blood donors. As there are more female in non-donor group, female tends to have lower haemoglobin level compared to male.

5.8 Genetic Variants with Iron Metabolising Parameters

Earlier studies reported presence of genetic variants in iron regulating genes (*HAMP*, *TMPRSS6*, *TF* and *HFE* genes) has been reported influence the iron and haemoglobin level. In this study, a total of eight variants were studied.

Presence of G allele in rs10421768 at *HAMP* gene was associated with a reduced hepcidin transcription and enhanced iron absorption. Parajes and co-authors concluded that rs10421768 variant decreases the transcription activity of hepcidin gene promoter but do not significantly affect on serum iron, serum ferritin, transferrin level as well as transferrin saturation (Parajes et al., 2010; Stachowska et al., 2022). In this study, there was significant difference between *HAMP* variant rs10421768 with serum iron level but no significant difference with hepcidin concentration. However, a slight reduction in hepcidin level could be observed in heterozygous genotype (AG genotype) compared to wildtype (AA genotype). This is because, the SNP was located at the upstream enhancer box region of *HAMP* gene which tends to affect the binding of enhancers and then affect the activation of transcriptional activity of hepcidin (National Centre for Biotechnology Information, 2022; Pennacchio et al., 2015). In the later year, this variant has been reported to associated with higher iron and ferritin level in β-thalassaemia patient. A slight reduction in hepcidin level might contribute to elevation in serum iron level as hepcidin function to increase iron absorption and iron release from cellular storage. However, rs10421768 was found to show significant difference with reduced serum iron level in this study. This might be due to the presence of other variants with iron-lowering effect which exist on other genes that able to counter the iron lifting effect of rs10421768 (Andreani et al., 2009).

Previous study showed that *TMPRSS6* variants (rs855791 and rs4820268) were significantly associated with the risk of iron deficiency anaemia and rs855791 and rs4820268 were significantly associated with higher hepcidin level.

However, presence of genetic variants in *TMPRSS6* were less likely to affect serum iron level (Jallow et al., 2021; Poggiali et al., 2015). In this study, presence of G allele in rs855791 and A allele in rs4820268 presented with relatively lower haemoglobin level. GG genotype in rs855791 also presented with reduced serum iron level compared to AA and AG genotypes. While AA genotype presented similar result when comparing the serum iron level to GG and GA genotypes. However, there was no significant difference between *TMPRSS6* variants and the iron metabolising parameters. Finding in this study was supported by the work carried out by An and co-authors as their finding shows that lower serum iron and haemoglobin level were associated with *TMPRSS6* polymorphisms (An et al., 2012).

In this study, presence of major allele (G allele) in rs12769 was found with significant higher hepcidin concentration and serum iron level. This finding is supported by Fujihara and co-authors where they reported that the GG genotype in variant rs12769 was associated with higher serum iron level. Variants rs12769 is a SNP that located in exon 7 of *TF* gene, yielding a synonymous mutation which may alter the translation rate as well as half-life of *TF* gene. This may affect the transferrin production. Transferrin serves as the major transporter in transporting iron in the circulation as well as one of the major components in activating the signalling pathway for hepcidin expression. Defective or diminished transferrin production may significantly affect hepcidin transcription. However, when transferrin production was unaffected, higher iron can be presented in circulation, signalling the hepcidin expression in order to diminished dietary iron absorption as well as to inhibit iron release from iron

stores (Fujihara et al., 2019; Rishi et al., 2015; Robert and Pelletier, 2018; Silva and Faustino, 2015; Silvestri et al., 2019).

Single nucleotide polymorphisms (SNPs) that present in *HAMP* (rs10414846), *TF* (rs1799852) and *HFE* gene (rs1799945 and rs1800562) were found affect the iron parameter. Presence of T allele in rs1799852, A allele in rs1800562 and G allele in rs1799945 presented a reduced transferrin level. Iron regulation might be affected by the presence of SNPs in *TF* and *HFE* gene as the transferrin-transferrin receptor-HFE complex is critical in regulating hepcidin expression. Mutation in *TF* and *HFE* gene were suggested to have a protective effect against iron deficiency anaemia. The H63D (rs1799945) were found to be associated with type 1 haemochromatosis and characterised by reduced hepcidin expression (Blanco-Rojo et al., 2011). In this study, no significant difference presented between rs1799945 with hepcidin concentration, but there is a slight reduction in hepcidin concentration presented by CG genotype compared to CC genotype. At the same time, serum iron level in CG genotype was lower than CC genotype which is contradicted with the protective effect of rs1799945 against iron deficiency anaemia. The reduction in serum iron level co-exist with reduced hepcidin concentration may contribute to the compensatory effect in iron regulation. According to Silvestri and co-authors (2019), two branches of BMP/SMAD signalling pathway took part in hepcidin regulation which in turn regulating body iron level. The increased serum iron level due to reduction in hepcidin may activate the ALK2 dependent signalling of BMP/SMAD signalling pathway whereby BMP6 protein was transcriptionally upregulated in order to activate ALK2 dependent signalling

pathway, thereby activate transcription and translation of hepcidin in order to supress duodenal iron absorption, to counter the iron overload state (Canali et al., 2016; Silvestri et al., 2019).

5.9 Interaction between Genetic Variants

In this study, there is no association between variants from two different genes. Association of genetic variants with rs1800562 were unable to identified due to the single genotype of rs1800562 presented in this study. It was believed that a slight changes in nucleotide base with or without changing the encoded amino acid might contribute to an alteration in downstream nucleotide bases. Moreover, a substitution of nucleotide bases at the upstream region of *HAMP* gene might not affect the protein coding structure. However, the substitution may affect the binding of transcription factors which specifically bind to the upstream region of the gene, resulted in reduced transcription and translation activity (National Centre for Biotechnology Information, 2022; Stachowska et al., 2022). In *TMPRSS6* gene, variant rs4820268 is significantly associated with variant rs855791. Genetic variant rs4820268 (G > A) mutation is locate at position 1536. While variant rs855791 (A > G) mutation is located at position 2207 (Lone et al., 2021; Parisinos et al., 2020). According to Mohd Atan and co-authors (2022), there is high linkage disequilibrium between rs4820268 and rs855791, causing the alteration in matriptase-2 serine protease domain that further resulted in iron imbalance effect. Moreover, both variant rs1799852 and variant rs12769 were located in the exon of *TF* gene. Variant rs12769 (G > A) mutation located at the position 624 while variant rs1799852 (C > T) mutation

located at the position 739. It was believed that both genetic variants were involved in the synthesis of same protein. Thus, significant association was found between the two variants on *TF* gene (Blanco-Rojo et al., 2011; National Centre for Biotechnology Information, 2022). Genetic variants rs10421768 located within the upstream enhancer box region of *HAMP* gene. While variant rs10414846 was located at the upstream promoter region of *HAMP* gene, approximately 450 bp upstream of rs10421768. Both variants may involve in regulating the transcriptional activity of *HAMP* gene where enhancers bind to the enhancer box region of the gene and activate the transcription of it whereas transcription factors bound to it, inducing expression of the gene. This could justify the significant association of variant rs10421768 and rs10414846 (Dao and Spicuglia, 2018; Pennacchio et al., 2015).

5.10 Limitations

This study was confronted with some limitations. As convenient sampling was adopted in sampling of study subjects, thus most of the study subjects were found with younger population. Moreover, the population in Kampar, Perak were mostly made up of Malaysian Chinese and this may not be able to fully represent the whole Malaysian community which consisted of various ethnicity.

5.11 Future Recommendations

This study was using HemoCue to determine haemoglobin level. this is recommended that using of haematological analyser to measure the

haemoglobin level will provide more red cell indices including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell count, reticulocyte count and red cell distribution width (RDW). Furthermore, serum ferritin, total iron binding capacity (TIBC), transferrin saturation (TSAT), interleukin 6 (IL-6) and C-reactive protein (CRP) level was suggested to be included to better rule out the cause of anaemia among Malaysian young adult population. A greater sample size was also suggested to clearly elucidate the underlying factors as a larger sample size able to better represent the population and provide more reliable association.

CHAPTER 6

CONCLUSION

In conclusion, the prevalence rate of anaemia among the young adult population in Malaysia was found to be as high to 14.75% in this study. Anaemia can be concluded as a multifactorial disorder where the associated factors included gender, body mass index (BMI), ethnicity, family history of anaemia, menstruation as well as the experience of menorrhagia and bleeding problem. Single nucleotide polymorphisms (SNPs) on transferrin (*TF*) gene (rs12769) and rs10421768 on hepcidin antimicrobial peptide (*HAMP*) gene were found to play a role in modulating iron metabolising parameters such as hepcidin and serum iron level. The interaction between genetic variants with other genes including rs10414846, rs855791, rs4820268, rs1799852, rs1799945 and rs1800562 was not able to be clearly rule out due to low sample size. Lastly, the gene-gene interaction between the four iron regulating genes were elucidated where there is significant association between the genetic variants present on the same gene, but no association was found between genetic variants on different genes due to the location of each gene were at different chromosome.

REFERENCES

Abbaspour, N., Hurrell, R. and Kelishadi, R., 2014. Review on iron and its importance for human health. *Journal of Research in Medical Sciences*, 19(2), pp.164–174.

Abdullah, N., Ismail, N., Abd Jalal, N., Mohd Radin, F., Othman, R., Kamalul Arifin, A. S., Kamaruddin, M. A. and Jamal, R., 2020. Prevalence of anaemia and associated risk factors amongst the Malaysian cohort participants. *Annals of Haematology*, 99, pp.2521 – 2527.

Alexander, J. and Kowdley, K.V., 2005. Hereditary hemochromatosis: Genetics, pathogenesis and clinical management. *Annals of Hepatology*, 4(4), pp.240 – 247.

Alexander, J. and Kowdley, K.V., 2009. HFE-associated hereditary haemochromatosis. *Genetics in Medicine*, 11, pp.307 – 313.

AlQuaiz, A. M., Mohamed, A. G., Khoja, T. A. M., AlSharif, A., Shaikh, S. A., Al Mane, H., Aldiris, A., Kazi, A. and Hammad, D., 2013. Prevalence of anaemia and associated factors in childbearing age women in Riyadh, Saudi Arabia. *Journal of Nutrition and Metabolism*.

Al-Sharefi, A., Mohammed, A., Abdalaziz, A. and Jayasena, C. N., 2019. Androgens and anaemia: Current trends and future prospects. *Frontiers in Endocrinology*, 10(754).

An, P., Wu, Q., Guan, Y., Mu, M.D., Liao, Y.J., Zhou, D.Z., Song, P.K., Wang, C., Meng, L.P., Man, Q.Q., Li, L.X., Zhang, J. and Wang, F.D., 2012. TMPRSS6, but not TF, TFR2 or BMP2 variants are associated with increased risk of iron-deficiency anaemia. *Human Molecular Genetics*, 20(9), pp.2124 – 2131.

Armstrong, K. L., 2016. Blood donation and anaemia. *Canadian Family Physician*, 62(9), pp. 730 – 731.

Asada-Senju, M., Naeda, T., Sakata, T., Hayashi, A. and Suzuki, T., 2002. Molecular analysis of the transferrin gene in a patient with hereditary hypotransferrinaemia. *American Journal of Human Genetics*, 47, pp.355 – 359.

Barton, J.C. and Edward, C.Q., 2018. HFE Haemochromatosis. In Adam, M.P., Everman, D.B., Mirzaa, G.M., Pagon, R. A. and Wallace, S.E. eds. 1993 – 2022. *Gene Reviews®*. Seattle: University of Washington.

Barton, J.C., Edwards, C.Q. and Acton, R.T., 2015. HFE gene: structure, function, mutations and associated iron abnormalities. *Gene*, 574(2), pp.179 – 192.

Bergamaschi, G. and Villani, L., 2009. Serum hepcidin: a novel diagnostic tool in disorders of iron metabolism. *Haematologica*, 94(12), pp.1631 – 1633.

Bermejo, F. and García-López, S., 2009. A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases. *World Journal of Gastroenterology*, 15(37), pp.4638–4643.

Billett, H.H., 1990. Haemoglobin and Haematocrit. In: Walker, H.K., Hall, W.D.and Hurst, J.W., eds. 1990. *Clinical Methods: The History, Physical, and Laboratory Examinations*. 3rd edition. Boston: Butterworths. Chapter 151.

Blanco-Rojo, R., Baeza-Richer, C., López-Parra, A.M., Pérez-Granados, A.M., Brichs, A., Bertoncini, S., Buil, A., Arroyo-Pardo, E., Soria, J.M. and Vaquero, M.P., 2011. Four variants in transferrin and HFE genes as potential markers of iron deficiency anaemia risk: an association study in menstruating women. *Nutrition and Metabolism*, 8(69).

Braunstein, E. M., 2022. *Aetiology of Anaemia*. [online] Available at: < https://www.msdmanuals.com/professional/hematology-and-oncology/approach-to-the-patient-with-anemia/etiology-of-anemia> [Accessed 9 October 2022].

Braunstein, E. M., 2022. *Iron Deficiency Anaemia (Anaemia of Chronic Blood Loss; Chlorosis)*. [online] Available at:< https://www.msdmanuals.com/professional/hematology-and-oncology/anemias-caused-by-deficient-erythropoiesis/iron-deficiency-anemia> [Accessed 20 September 2022].

Brazier, Y. B. and Martinez, K., 2019. *What is nutritional-deficiency anaemia?* [online] Available at: < https://www.medicalnewstoday.com/articles/188770> [Accessed 9 October 2022].

Buchanan, G.R. and Sheehan, R.G., 1981. Malabsorption and defective utilisation of iron in three siblings. *The Journal of Paediatrics*, 98(5), pp.723 – 728.

Buerkli, S., Pei, S. N., Hsiao, S. C., Lee, C. T., Zeder, C., Zimmermann, M. B. and Moretti, D., 2021. The TMPRSS6 variant (SNP rs855791) affects iron metabolism and oral iron absorption – a stable iron isotope study in Taiwanese women. *Haematologica*, 106(11), pp.2897 – 2905.

Buzała, M., Słomka, A. and Janicki, B., 2016. Heme iron in meat as the main source of iron in the human diet. *Journal of Elementology*, 21(1), pp.303–314.

Canali, S., Vecchi, C., Garuti, C., Montosi, G., Babitt, J. L and Pietrangelo, A., 2016. The SMAD pathway is required for hepcidin response during endoplasmic reticulum stress. *Endocrinology*, 157(10), pp.3935 – 3945.

Cappellini, M.D., Musallam, K.M. and Taher, A.T., 2019. Iron deficiency anaemia revisited. *Journal of Internal Medicine*, 287(2), pp.153 – 170. Available at: <https://doi.org/10.1111/joim.13004>.

Chandra, D., Dhingra, B., Seth, T., Mishra, P., Bansal, D., Mahapatra, M. and Pati, H.P., 2017. Congenital hypotransferrinaemia, an unusual cause of iron deficiency anaemia: report of two cases. *Journal of Haematology and Blood Transfusion*, 33(3), pp.402 – 404.

Chaparro, C. M. and Suchdev, P. S., 2019. Anaemia epidemiology, pathophysiology, and etiology in low- and middle-income countries. *Annals of the New York Academy of Sciences*, 1450(1), pp.15 – 31.

Chaudhry, H.S. and Kasarla, M.R., 2022. *Microcytic Hypochromic Anaemia*. [e-book] Florida: Treasure Island (FL): StatPearls Publishing. Available at: Google Books <https://www.ncbi.nlm.nih.gov/books/NBK470252/> [Accessed 9 August 2022].

Chiang, C.P., Chang, J.Y., Wang, Y.P., Wu, Y.H., Wu, Y.C. and Sun, A., 2020. Atrophic glossitis: aetiology, serum autoantibodies, anaemia, hematinic deficiencies, hyperhomocysteinemia, and management. *Journal of the Formosan Medical Association*, 119, pp.774 – 780.

Coates, T. D., 2014. Physiology and pathophysiology of iron in haemoglobin-associated diseases. *Free Radical Biology and Medicine*, 72, pp.23 – 40.

Collins, J. F., Wessling-Resnick, M. and Knutson, M. D., 2008. Hepcidin regulation of iron transport. *The Journal of Nutrition*, 138(11), pp.2284 – 2288.

Conley, C. L. and Schwartz, R. S., 2022. *Production of Red Blood Cells (Erythropoiesis)*. [online] Available at: < https://www.britannica.com/science/blood-biochemistry/Production-of-red-blood-cells-erythropoiesis> [Accessed 21 September 2022].

Coppin, H., Bensaid, M., Fruchon, S., Borot, N., Blanché, H. and Roth, M.P., 2003. Longevity and carrying the C282Y mutation for haemochromatosis on the HFE gene: case control study of 492 French centenarians. *BMJ*, 327(7407), pp.132 – 133.

D'Angelo, G., 2013. Role of hepcidin in the pathophysiology and diagnosis of anaemia. *Blood Research*, 48(1), pp.10 – 15.

Dao, L. T. M. and Spicuglia, S., 2018. Transcriptional regulation by promoters with enhancer function. *Transcription*, 9(5), pp.307 – 314.

de Falco, L., Sanchez, M., Silvestri, L., Kannengiesser, C., Muckenthaler, M.U., Lolascon, A., Gouya, L., Camaschella, C. and Beaumont, C., 2013. Iron refractory iron deficiency anaemia. *Haematologica*, 98(6), pp.845 – 853.

Djokic, D., Drakulovic, M. B., Radojicic, Z., Radovic, L. C., Rakic, L., Kocic, S. and Davidovic, C., 2010. Risk factors associated with anaemia among Serbian school-age children 7 – 14 years old: results of the first national health survey. *Hippokratia*, 14(4), pp.252 – 260.

Donova, A., Lima, C. A., Pinkus, J. L., Pinkus, G. S., Zon, L. I., Robine, S. and Andrews, N. C., 2005. The iron exporter ferroportin/SLc40a1 is essential for iron homeostasis. *Cell Metabolism*, 1(3), pp.191 - 200.

Dutta, S., Karkada, I. R., Sengupta, P. and Chinni, S. V., 2021. Anthropometric markers with specific cut-offs can predict anaemia occurrence among Malaysian young adults. *Frontiers in Physiology*, 12.

Ems, T., St Lucia, K. and Huecker, M.R., 2022. *Biochemistry, Iron Absorption*. [e-book] Florida: Treasure Island (FL): StatPearls Publishing. Available at: Google Books <https://www.ncbi.nlm.nih.gov/books/NBK448204/> [Accessed 4 August 2022].

Farid, Y., Bowman, N.S. and Lecat, P., 2022. *Biochemistry, Haemoglobin Synthesis*. [e-book] Florida: Treasure Island (FL): StatPearls Publishing. Available at: Google Books < https://www.ncbi.nlm.nih.gov/books/NBK536912/> [Accessed 6 August 2022].
Finberg, K.E., Heeney, M.M., Campagna, D.R., Ayinok, Y., Pearson, H.A., Hartman, K.R., Mayo, M.M., Samuel, S.M.,Strouse, J.J., Markianos, K., Andrews, N.C. and Fleming, M.D., 2008. Mutation in TMPRSS6 cause iron-refractory iron deficiency anaemia (IRIDA), Nature Genetics, 40, pp.569 – 571.

Franco, R. S., 2012. Measurement of red cell lifespan and aging. *Transfusion Medicine and Hemotherapy*, 39(5), pp.302 - 307.

Fujihara, J., Yasuda, T., Kimura-Kataoka, K. and Takeshita, H., 2019. Association of SNPs in transferrin and transferrin receptor genes with blood iron levels in human. *Legal Medicine (Tokyo, Japan)*, 36, pp.17 – 20.

Fuqua, B.K., Vulpe, C.D. and Anderson, G.J., 2012. Intestinal iron absorption. *Journal of Trace Elements in Medicine and Biology*, 26(2–3), pp.115–119. Available at: <http://dx.doi.org/10.1016/j.jtemb.2012.03.015>.

Gan, W., Guan, Y., Wu, Q., An, P., Zhu, J.W., Lu, L., Jing, L., Yu, Y., Ruan, S., Xie, D., Makrides, M., Gibson, R.A., Anderson, G.J., Li, H.X., Lin, X. and Wang, F.D., 2012. Association of TMPRSS6 polymorphisms with ferritin, haemoglobin, and type 2 diabetes risk in a Chinese Han population. *The American Journal of Clinical Nutrition*, 95, pp.626 – 632.

Ganz, T. and Nemeth, E., 2012. Hepcidin and iron homeostasis. *Biochimica et Biophysica Acta (BBA) – Molecular Cell Research*, 1823(9), pp.1434 – 1443.

Garvin, J. H., 2010. Gender-specific aspects of paediatric haematology and oncology. In Legato, M. J. ed. 2009. *Principles of Gender-Specific Medicine* (Second Edition). Massachusetts: Academic Press. Chapter 4, pp.51 – 61.

Girelli, D., Nemeth, E. and Swinkels, D. W., 2016. Hepcidin in the diagnosis of iron disorders. *Blood*, 127(23), pp.2809 – 2813.

Gulec, S., Anderson, G.J. and Collins, J.F., 2014. Mechanistic and regulatory aspects of intestinal absorption. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 307(4), pp.397 – 409. Available at: <https://doi.org/10.1152/ajpgi.00348.2013>.

Hathcock, J.N., 2013. *Vitamin and Mineral Safety 3rd Edition*. [pdf] Washington DC: Council for Responsible Nutrition. Available at: < https://www.crnusa.org/sites/default/files/files/resources/CRN-SafetyBook-3rdEdition-2014-fullbook.pdf> [Accessed 9 July 2022].

Heeney, M.H. and Finberg, K.E., 2014. Iron-refractory iron deficiency anaemia (IRIDA). *Haematology/Oncology Clinics of North America*, 28(4), pp.637 – 652.

Hegde, N., Rich, M. W. and Gayomali, C., 2006. The cardiomyopathy of Iron Deficiency. *Texas Heart Institute Journal*, 33(3), pp.340 – 344.

Institute of Medicine, 2001. *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. Washington, DC: The National Academies Press.

Jallow, M. W., Campino, S., Saidykhan, A., Prentice, A. M. and Cerami, C., 2021. Common variants in the TMPRSS6 gene alter hepcidin but not plasma iron in response to oral iron in healthy Gambian Adults: A recall-by-genotype study. *Current Developments in Nutrition*, pp. 5(3).

Jallow, M. W., Cerami, C., Clark, T. C., Prentice, A. M. and Campino, S., 2020. Differences in the frequency of genetic variants associated with iron imbalance among global population. *Plos One*, 15(7).

Johnson Wimbley, T.D. and Graham, D.Y., 2011. Diagnosis and management of iron deficiency anemia in the 21st century. *Therapeutic Advances in Gastroenterology*, 4(3), pp.177–184.

Jones, E., Pasricha, S. R., Allen, A., Evans, P., Fisher, C. A., Wray, K., Premawardhena, A., Bandaram D., Perera, A., Webster, C., Sturges, P., Olivieri, N. F., St. Pierre, T., Armitage, A. E., Porter, J. P., Weatherall, D. J. and Drakesmith, H., 2015. Hepcidin is supressed by erythropoiesis in haemoglobin E β-thalassaemia and β-thalassaemia trait. *Blood*, 125(5), pp.873 – 880.

Jones, N. R., Ashmore, J. H., Lee, S. Y., Richie, J.P., Lazarus, P. and Muscat, J. E., 2015. Association studies of HFE C282Y and H63D variants with oral cancer risk and iron homeostasis among whites and blacks. *Cancers*, 7(4), pp.2386 – 2396.

Juzėnas, S., Kupčinskas, J., Valantienė, I., Šumskienė, J., Petrenkienė, V., Kondrackienė, J., Kučinskas, L., Kiudelis, G., Skiecevičienė, J. and Kupčinskas, L., 2016. Association of HFE gene C282Y and H63D mutations with liver cirrhosis in the Lithuanian population. *Medicina*, 52(5), pp.269 – 275.

Kamruzzaman, M., 2021. Is BMI associated with anaemia and haemoglobin level of women and children in Bangladesh: a study with multiple statistical approaches. *Plos One*, 16(10).

Kelley, M., Joshi, N., Xie, Y.G. and Borgaonkar, M., 2014. Iron overload is rare in patients homozygous for the H63D mutation. *Gastroenterology and Hepatology*, 28(4), pp.198 – 202.

Killip, S., Bennett, J.M. and Chambers, M.D., 2007. Iron deficiency anemia. *American Family Physician*, 75(5), pp.671–678.

Kim, Y. G., Park, J. H. and Kim, M. S., 2017. Diagnostic approaches for inherited haemolytic anaemia in the genetic era. *Blood Research*, 52(2), pp.84 – 94.

Kleven, M.D., Jue, S. and Enns, C.A., 2018. The transferrin receptors, TfR1 and TfR2 bind transferrin through differing mechanisms. *Biochemistry*. 57(9), pp.1552 – 1559.

Knutson, M.D., 2017. Iron transport proteins: gateways of cellular and systemic iron homeostasis. *Journal of Biological Chemistry*, 292(31), pp.12735 – 12743.

Kocaoz, S., Cirpin, R. and Degirmencioglu, A. Z., 2019. The prevalence and impacts heavy menstrual bleeding on anaemia, fatigue and quality of life in women of reproductive age. *Pakistan Journal of Medical Sciences*, 35(2), pp.365 – 370.

Korolnek, T. and Hamza, I., 2015. Macrophages and iron trafficking at the birth and death of red cells. *Blood*, 125(19), pp.2893–2897.

Larson, D. S. and Coyne, D. W., 2013. Understanding and exploiting hepcidin as an indicator of anaemia due to chronic kidney disease. *Kidney Research and Clinical Practice*, 32(1), pp.11 – 15.

Le, C. H. H., 2016. The prevalence of anaemia and moderate-severe anaemia in the US population (NHANES 2003 – 2012). *Plos One*, 11(11).

Lee, P., 2009. Role of matriptase-2 (TMPRSS6) in iron metabolism. *Acta Haematologica*, 122(2-3), pp.87 – 96.

Lee, P., Peng, H. F., Gelbart, T., Wang, L. and Beutler, E., 2005. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *PNAS*, 102(6), pp.1906 – 1910.

Lescano, M.A., Tavares, L.C. and Santos, P.C.J.L., 2017. Juvenile hemochromatosis: HAMP mutation and severe iron overload treated with phlebotomies and deferasirox. *World Journal of Clinical Cases*, 5(10), pp.381–383.

Levy, J. E., Montross, L. K., Cohen, D. E., Fleming, M. D. and Andrews, N. C., 1999. The C282Y mutation causing hereditary haemochromatosis does not produce a null allele. *Blood*, 94(1), pp.9 – 11.

Li, H. X., Xiao, J., Liao, M. H., Huang, G. W., Zheng, J. F., Wang, H., Huang, Q. and Wang, A. H., 2020. Anaemia prevalence, severity and associated factors among children aged 6 – 71 months in rural Hunan province, China: a community-based-cross-sectional study. *BMC Public Health*, 21(1).

Li, Y.K., Huang, X.L., Wang, J.J., Huang, R.L. and Wan, D., 2020. Regulation of iron homeostasis and related diseases. *Mediators of Inflammation*.

Loh, S. P. and Khor, G. L., 2010. Iron intake and iron deficiency anaemia among oung women in Kuala Lumpur. *Malaysian Journal of medicine and Health Sciences*, 6(1), pp.63 – 70.

Lone, N. M., Sajjad Shah, S. H., Farooq, M., Arif, M., Younis, S. and Riaz, S., 2021. Role of TMPRSS6 rs855791 (T>C) polymorphism in reproductive age women with iron deficiency anaemia from Lahore, Pakistan. *Saudi Journal of Biological Sciences*, 28(1), pp.748 – 753.

Lopez, A., Cacoub, P., Macdougall, I.C. and Peyrin-Biroulet, L., 2016. Iron deficiency anaemia. *The Lancet*, 387(10021), pp.907–916.

Mahdieh, N. and Rabbani, B., 2013. An overview of mutation detection methods, in genetic disorders. *Iran Journal of Paediatrics*. 23(4), pp.375 – 388.

Mansour, D., Hofmann, A. and Gemzell-Danielsson, K., 2020. A review of clinical guidelines on the management of iron deficiency and iron-deficiency anaemia in women with heavy menstrual bleeding. *Advances in Therapy*, 38, pp.201 – 225.

Marcin, A., 2017. 6 *Health Risks of Being Underweight*. [online] Available at: <https://www.healthline.com/health/underweight-health-risks#outlook> [Accessed 21 September 2022].

Mayle, K.M., Le, A.M. and Kamei, D.T., 2012. The intracellular trafficking pathway of transferrin. *Biochimica et Biophysica Acta - General Subjects*, 1820(3), pp.264–281.

McLean, E., Cogswell, M., Egli, I., Wojdyla, D. and de Benoist, B., 2009. Worldwide prevalence of anaemia, WHO vitamin and mineral nutrition information system, 1993-2005. *Public Health Nutrition*, 12(4), pp.444 – 454.

Medrano, R. F. V. and de Oliveira, C. A., 2014. Guidelines for the tetra-primer ARMS-PCR technique development. *Molecular Biotechnology*, 56(7), pp.599 – 608.

Meynard, D., Babitt, J.L. and Lin, H.Y., 2014. The liver: Conductor of systemic iron balance. *Blood*, 123(2), pp.168–176.

Mohammad Alshwaiyat, N., Ahmad, A., Wan Hassan, W. M. R. and Al-Jamal, H. A. N., 2021. Association between obesity and iron deficiency (Review). *Experimental and Therapeutic Medicine*, 22(5), pp.1268.

Mohd Atan, F. N. E., Wan Mohd Saman, W. A., Kamsani, Y. S., Khalid, Z. And Abdul Rahman, A., 2022. TMPRSS6 gene polymorphisms associated with iron deficiency anaemia among global population. *Egyptian Journal of Medical Human Genetics*, 23(1447).

Murphy, W.G., 2014. The sex difference in haemoglobin levels in adult – Mechanisms, causes, and consequences. *Blood Reviews*, 28(2), pp.41 – 47.

Nagababu, E., Gulyani, S., Earley, G.J., Cutler, R.G., Mattson, M.P. and Rifkind, J.M., 2009. Iron-deficiency anaemia enhances red blood cell oxidative stress. *Free Radical Research*, 42(9), pp.824 – 829. Available at: < https://doi.org/10.1080/10715760802459879>.

Nalado, A. M., Dickens, C., Dix-Peek, T., Mahlangu, J. N., Olorunfemi, G., Paget, G., Duarte, R. and Naicker, S., 2019. TMPRSS6 rs855791 polymorphism and susceptibility to iron deficiency anaemia in non-dialysis chronic kidney disease patients in South Africa. *International Journal of Molecular Epidemiology and Genetics*, 10(1), pp.1 – 9.

Nandar, W. and Connor, J. R., 2011. HFE gene variants affect iron in the brain. *The Journal of Nutrition*, 141(4), pp. 729 – 739.

National Centre for Biotechnology Information, 2022. *HAMP hepcidin antimicrobial peptide [Homo sapiens (human)]*. Bethesda: National Institute of Health.

National Centre for Biotechnology Information, 2022. *HFE homeostatic iron regulator [Homo sapiens (human)]*. Bethesda: National Institute of Health.

National Centre for Biotechnology Information, 2022. *TF transferrin [Homo sapiens (human)]*. Bethesda: National Institute of Health.

National Centre for Biotechnology Information, 2022. *TMPRSS6 transmembrane serine protease 6 [Homo sapiens (human)]*. Bethesda: National Institute of Health.

National Centre on Birth Defects and Developmental Disabilities, 2022. *What is Thalassaemia?* [online] Atlanta: Centres for Disease Control and Prevention. Available at: <https://www.cdc.gov/ncbddd/thalassemia/facts.html> [Accessed 21 September 2022].

National Health Service, 2019. *Thalassaemia Carriers*. [online] Leeds: Department of Health and Social Care. Available at: < https://www.nhs.uk/conditions/thalassaemia/carriers/ > [Accessed 21 September 2022].

National Heart, Lung and Blood Institute, 2022. *Iron-Deficiency Anaemia*. Bethesda: National Institute of Health.

National Institute of Neurological Disorders and Stroke, 2022. *Restless Legs Syndrome Fact Sheet*. Bethesda: National Institute of Health.

National Organisation for Rare Disorders, 2018. *Anaemia of Chronic Diseases*. [online] Available at: < https://rarediseases.org/rare-diseases/anemia-of-chronic-disease/> [Accessed 9 October 2022].

Nemeth, E. and Ganz, T., 2009. The role of hepcidin in iron metabolism. *Acta Haematologica*, 122(2-3), pp.78 – 86.

Nemeth, E. and Ganz, T., 2014. Anaemia of Inflammation. *Haematology/Oncology Clinics of North America*, 28(4), pp.671 – 681.

Njiru, H., Elchalal, U. and Paltiel, O., 2011. Geophagy during pregnancy in Africa: A literature review. *Obstetrical and Gynaecological Survey*, 66(7), pp.452 – 459.

Norhafiah, A., Tan, K.K. and Jubaida, P., 2020. *Iron Deficiency Anaemia*. Putrajaya: Ministry of Health Malaysia.

Ofojekwu, M. J. N., Nnanna, O. U., Okolie, C. E., Odewumi, L. A., Isiguzoro, I. O. U. and Lugos, M. D., 2013. Haemoglobin and serum iron concentrations in menstruating Nulliparous women in Jos, Nigeria. *Laboratory Medicine*, 44(2), pp.121 – 124.

Ogun, A.S. and Adeyinka, A., 2022. *Biochemistry, Transferrin*. [e-book] Florida: Treasure Island (FL): StatPearls Publishing. Available at: Google Books < https://www.ncbi.nlm.nih.gov/books/NBK532928/> [Accessed 20 August 2022].

Pagani, A., Nai, A., Sivestri, L. and Camaschella, C., 2019. Hepcidin and anaemia: A tight relationship. *Frontiers in Physiology*, 10(1294).

Pandey, S., Pandey, S.K. and Shah, V., 2018. Role of HAMP Genetic Variants on Pathophysiology of Iron Deficiency Anemia. *Indian Journal of Clinical Biochemistry*, 33(4), pp.479–482.

Parajes, S., González-Quintela, A., Campos, J., Quinteiro, C., Domínguez, F. and Loidi, L., 2010. Genetic study of the hepcidin gene (HAMP) promoter and functional analysis of the c.-582 A > G variant. *BMC Genetics*, 11.

Parisinos, C. A., Wilman, H. R., Thomas, E. L., Kelly, M., Nicholls, R. C., McGonigle, J., Neubauer, S., Hingorani, A. D., Patel, R. S., Hemingway, H., Bell, J. D., Banerjee, R. and Yaghootkar, H., 2020. Corrigendum to: "Genome-wide and Mendelian randomisation studies of liver MRI yield insights into the pathogenesis of steatohepatitis". *Journal of Hepatology*, 73(6), pp.1594 – 1595.

Pasricha, S. Y., McHugh, K. and Drakesmith, H., 2016. Regulation of hepcidin by erythropoiesis: The story so far. *Annual Review of Nutrition*, 17(36), pp.417 – 434.
Pasricha, S.R., Tye-Din, J., Muckenthaler, M.U. and Swinkels, D.W., 2021. Iron deficiency. *The Lancet*, 397(10270), pp.233–248. Available at: >http://dx.doi.org/10.1016/S0140-6736(20)32594-0>.

Pehlic, V., Volken, T., Holbro, A., Jirout, Z., Drexler, B., Buser, A. and Infanti, L., 2021. Long-term course of haemoglobin and ferritin values in high-frequency donors of whole blood and double erythrocyte apheresis. *Transfusion Medicine and Haemotherapy*, 48(2), pp.71 – 78.

Pelusi, S., Girelli, D., Rametta, R., Campostrini, N., Alfieri, C., Traglia, M., Dongiovanni, P., Como, G., Toniolo, D., Camaschella, C., Messa, P., Fargion, S. and Valenti, L., 2013. The A736V TMPRSS6 polymorphism influences hepcidin and iron metabolism in chronic hemodialysis patients: TMPRSS6 and hepcidin in hemodialysis. *BMC Nephrology*, 14(48).

Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A. and Bejerano, G., 2015. Enhancer: five essential questions. *Nature Reviews Genetic*, 14(4), pp.288 – 295.

Petry, N. M., 2002. A comparison of young, middle-aged, and older adult treatment – seeking pathological gamblers. *The Gerontologist*, 42(1), pp.92 – 99.

Pinto, J., de Jesus, G.N., Anselmo, M.P., Gonçalves, L., Brás, D., Lopez, J.M., Meneses, J., Victorino, R. and Faustino, P., 2017. Iron refractory iron deficiency anaemia in dizygotic twins due to a novel TMPRSS6 gene mutation in addition to polymorphisms associated with high susceptibility to develop ferropenic anaemia. *Journal of Investigative Medicine High Impact Case Reports*, 5(2).

Raisinghani, N., Kumar, S., Acharya, S., Gadegone, A. and Pai, V., 2019. Does aging have an impact on haemoglobin? Study in elderly population at rural teaching hospital. *Journal of Family Medicine and Primary Care*, 8(10), pp.3345 – 3349.

Rishi, G., Wallace, D. F. and Subramaniam, V.N. 2015. Hepcidin: regulation of the master iron regulator. *Bioscience Reports*, 35(3).

Rossi, E. and Jeffrey, G. P., 2004. Clinical penetrance of C282Y homozygous HFE haemochromatosis. *The Clinical Biochemist Reviews*, 25(3), pp.183 – 190.

Runcharoen, C., Fukunaga, K., Sensorn, I., Iemwimangsa, N., Klumsathian, S., Tong, H., Vo, N. S., Le, L., Hlaing, T. M., Thant, M., Mohd Zain, S., Mohamed, Z., Pung, Y. F., Capule, F., Nevado, J., Silao, C. L., Al-Mahayri, Z. N., Ali, B. R., Yuliwulandari, R., Prayuni, K., Zahroh, H., Mohamed Moor, D. A., Xangsayarath, P., Xayavong, S., Kounnavong, S., Sayasone, S., Kordou, Z., Liopetas, I., Tsikrika, A., Tsermpini, E., Koromina, M., Mitropoulou, C., Patrinos, G. P., Kesornsit, A., Charoenyingwattana, A., Wattanapokayakit, S., Mahasirimongkol, S., Mushiroda, T. and Chantratita, W., 2021. Prevalence of pharmacolgenomic variants in 100 pharmacogenes among Southeast Asian populations under the collaboration of the Southeast Asian Pharmacogenomics Research Network (SEAPharm). *Human Genome Variation*, 8(7).

Rushton, D. H., Dover, R., Sainsbury, A. W., Norris, M. J., Gilkes, J. J. H. and Ramsay, I. D., 2001. Why should women have lower reference limits for haemoglobin and ferritin concentrations than men? *BMJ*, 322(7298), pp.1355 – 1357.

Saito, H., 2014. Metabolism of iron stores. *Nagoya Journal of Medical Science*, 76(3-4), pp.235 – 254.

Sal, E., Keskin, E.Y., Yenicesu, I., Bruno, M. and De Falco, L., 2016. Iron-refractory iron deficiency anaemia (IRIDA) cases with 2 novel TMPRSS6 mutations. *Paediatric Haematology and Oncology*, 33(3), pp.226 – 232.

Sangkhae, V. and Nemeth, E., 2017. Regulation of the iron homeostatic hormone hepcidin. *Advances in Nutrition*, 8(1), pp.126 – 136.

Sarma, P.R., 1990. Red Cell Indices. In: Walker, H.K., Hall, W.D.and Hurst, J.W., eds. 1990. *Clinical Methods: The History, Physical, and Laboratory Examinations*. 3rd edition. Boston: Butterworths. Chapter 152.

Sato, H., Takai, C., Kazama, J. J., Wakamatsu, A., Hasegawa, E., Kobayashi, D., Kondo, N., Nakatsue, T., Abe, A., Ito, S., Ishikawa, H., Kuroda, T., Suzuki, Y. and Narita, I., 2020. Serum hepcidin level, iron metabolism and osteoporosis in patients with rheumatoid arthritis. *Scientific Reports*, 10(9882), pp.1 – 12.

Schulze, K.J. and Dreyfuss, M.L., 2005. Anemia | Iron-deficiency anemia. *Encyclopedia of Human Nutrition*, pp.101–109.

Scott, J. A., 2010. *Anaemia and Your Genes*. [online] Available at: < https://www.everydayhealth.com/anemia/anemia-and-heredity.aspx> [Accessed 9 October 2022].

Shanani, S., Braga-Basaria, M., Maggio, M. and Basaria, S., 2009. Androgens and erythropoiesis: past and present. *Journal of Endocronological Investigation*, 32(8), pp.704 – 716.

Shinta, D., Asmarinah, Adhiyanto, C., Htet, M. K. and Fahmida, U., 2019. The association of TMPRSS6 gene polymorphism and iron intake with iron status among under-two-year-old children in Lombok, Indonesia. *Nutrients*, 11(4), pp.878.

Silva, B. and Faustino, P., 2015. An overview of molecular basis of iron metabolism regulation and the associated pathologies. *Biochimica et Biophysica Acta-Molecular Basis of Disease*. 1852(7), pp.1347 – 1359.

Silvestri, L., Nai, A., Dulja, A. and Pagani, A., 2019. Hepcidin and the BMP-SMAD pathway: An enexpected liaison. *Vitamins and Hormones*, 110, pp. 71-99.

Smith, D. L., 2000. Anaemia in elderly. *American Family Physician*, 62(7), pp.1565 – 1572.

Stachowska, L., Kaziarska, D., Karakiewicz, B., Kotwas, A., Knyszyńska, A., Folwarski, M., Dec, K., Stachowska, E., Hawryłkowicz, V., Kulaszyńska, M., Sołek-Pastuszka, J. and Skonieczna-Żydecka, K., 2022. Hepcidin (rs10421768), transferrin (rs3811647, rs1049296) and transferrin receptor 2 (rs7385804) gene polymorphism might be associated with the origin of multiple sclerosis. *International Journal of Environmental Research and Public Health*, 19(11), pp.6875.

Stoltzfus, R.J., 2008. Research needed to strengthen science and programs for the control of iron deficiency and its consequences in young children. *Journal of Nutrition*, 138(12), pp.2542–2546.

Tan, A. C. C., Leonh, E. W. K., Chua, A. C. and Moy, F M., 2013. Racial variations in booking haemoglobin of primigravidae in Malaysia: a prospective study. *BMC Research Notes*, 6(17).

The American National Red Cross, 2022. *Frequent Blood Donors and the Importance of Iron.* [online] Available at: <https://www.redcrossblood.org/donate-blood/blood-donation-process/before-during-after/iron-blood-donation/iron-informationforfrequentdonors.html#:~:text=Each%20time%20you%20donate%20blood,lost%20through%20a%20blood%20donation.> [Accessed 24 September 2022].

Waldvogel-Abramowski, S., Waebar, G., Gassner, C., Buser, A., Frey, B.M., Favrat, B. and Tissot, J., 2014. Physiology of iron metabolism. *Transfusion Medicine and Hemotherapy*, 41, pp.213 – 221. Available at: <https://doi.org/10.1159/000362888>.

Wallace, D.F. and Subramaniam, V.N., 2007. Non-HFE haemochromatosis. *World Journal of Gastroenterology*, 13(35), pp.4690 – 4698.

Wallace, D.F., 2016. The Regulation of Iron Absorption and Homeostasis. The Clinical biochemist. *Reviews*, 37(2), pp.51–62. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/28303071%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5198508>.

Wang, C.Y., Meynard, D. and Lin, H.Y., 2014. The role of TMPRSS6/matriptase-2 in iron regulation and anaemia. *Frontiers in Pharmacology*, pp.1 – 6.

Ward, D.M. and Kaplan, J., 2012. Ferroportin-mediated iron transport: Expression and regulation. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1823(9), pp.1426–1433. Available at: http://dx.doi.org/10.1016/j.bbamcr.2012.03.004.

Warner, M.J. and Kamran, M.T., 2021. *Iron Deficiency Anaemia*. [e-book] Florida: Treasure Island (FL): StatPearls Publishing. Available at: Google Books <https://www.ncbi.nlm.nih.gov/books/NBK448065/> [Accessed 8 August 2022].

West, A.R. and Oates, P.S., 2008. Mechanisms of heme iron absorption: Current questions and controversies. *World Journal of Gastroenterology*, 14(26), pp.4101–4110.

WHO Expert Consultation, 2004. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet*, 363, pp.157 - 163.

WHO, 2008. *Worldwide Prevalence of Anaemia 1993 – 2005 WHO Global Database on Anaemia*. [pdf] Geneva: World Health Organisation. Available at: < https://www.who.int/publications/i/item/9789241596657> [Accessed 9 October 2022].

WHO, 2017. *Nutritional anaemias: Tools for Effective Prevention and Control*. [pdf] Geneva: World Health Organisation. Available at: <https://www.who.int/publications/i/item/9789241513067?sequence=1> [Accessed 9 October 2022].

William, C.S. and Melissa, C.S., 2022. *Haematocrit Ranges (Normal, High, Low)*. MeficineNet, [online] Available at: < https://www.medicinenet.com/hematocrit/article.htm> [Accessed 11 August 2022].

World Health Organisation, 2011. *Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity*. Geneva, Switzerland: World Health Organization, pp.1 – 6. Available at: <http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Haemoglobin+concentrations+for+the+diagnosis+of+anaemia+and+assessment+of+severity#1>.

World Health Organisation, 2021. *Malnutrition*. [online] Geneva: World Health Organisation. Available at: <https://www.who.int/news-room/fact-sheets/detail/malnutrition> [Accessed 21 September 2022].

Yanatori, I. and Kishi, F., 2019. DMT1 and iron transport. *Free Radical Biology and Medicine*, 133, pp.55–63. Available at: https://doi.org/10.1016/j.freeradbiomed.2018.07.020.

Yang, H. Y., Zhou, B., Prinz, M. and Siegel, D., 2012. Proteomic analysis of menstrual blood. *Molecular & Cellular Proteomics*, 11(10), pp.1024 – 1035.

Yusof, M., Awaluddin, S. M., Omar, M., Ahmad, N. A., Abdul Aziz, F. A., Jamaluddin, R., Aris, T. and Tan, M. P., 2018. Prevalence of anaemia among elderly in Malaysia and its associated factors: Does ethnicity matter? *Journal of Environmental and Public Health*.

Zhu, X.Y., Wu, T.T., Wang, H.M., Li, X., Ni, L.Y., Chen, T.J., Qiu, M.Y., Shen, J., Liu, T., Ondo, W.G. and Wu, Y.C., 2020. Correlates of nonanemic iron deficiency in restless legs syndrome. *Frontiers in Neurology*, 11(298), pp.1 – 9. Available at: < https://doi.org/10.3389/fneur.2020.00298 >.

APPENDIX A

UNIVERSITI TUNKU ABDUL RAHMAN
Wholly Owned by UTAR Education Foundation (Company No. 578227-M)

Re: U/SERC/06/2021

11 January 2021

Dr Teh Lai Kuan
Department of Allied Health Sciences
Faculty of Science
Universiti Tunku Abdul Rahman
Jalan Universiti
Bandar Baru Barat
31900 Kampar, Perak

Dear Dr Teh,

Ethical Approval For Research Project/Protocol

We refer to your application which was circulated for consideration of the UTAR Scientific and Ethical Review Committee (SERC). We are pleased to inform that your application for ethical approval of your research project involving human subjects has been approved by SERC.

The details of the project are as follows:

Research Title	Case-control Study: Anemia and Its Association Factors and Regulating Genes
Investigator(s)	Dr Teh Lai Kuan Liew Jin Rou
Research Area	Science
Research Location	UTAR, Kampar Campus
No of Participants	200 participants
Research Costs	UTAR Research Fund 2020 Cycle 1
Approval Validity	11 January 2021 - 10 January 2022

The conduct of this research is subject to the following:

(1) The participants' informed consent be obtained prior to the commencement of the research.

(2) Confidentiality of participants' personal data must be maintained; and

(3) Compliance with procedures set out in related policies of UTAR such as the UTAR Research Ethics and Code of Conduct, Code of Practice for Research Involving Humans and other related policies/guidelines.

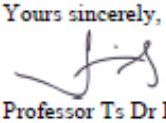
Kampar Campus : Jalan Universiti, Bandar Barat, 31900 Kampar, Perak Darul Ridzuan, Malaysia
Tel: (605) 468 8888 **Fax**: (605) 466 1313
Sungai Long Campus : Jalan Sungai Long, Bandar Sungai Long, Cheras, 43000 Kajang, Selangor Darul Ehsan, Malaysia
Tel: (603) 9086 0288 **Fax**: (603) 9019 8868
Website: www.utar.edu.my

Should you collect personal data of participants in your study, please have the participants in the research signed the attached Personal Data Protection Statement for your records.

The University wishes you all the best in your research.

Thank you.

Yours sincerely,



Professor Ts Dr Faidz bin Abd Rahman
Chairman
UTAR Scientific and Ethical Review Committee

c.c Dean, Faculty of Science
Director, Institute of Postgraduate Studies and Research

Kampar Campus : Jalan Universiti, Bandar Barat, 31900 Kampar, Perak Darul Ridzuan, Malaysia
Tel: (605) 468 8888 **Fax**: (605) 466 1313
Sungai Long Campus : Jalan Sungai Long, Bandar Sungai Long, Cheras, 43000 Kajang, Selangor Darul Ehsan, Malaysia
Tel: (603) 9086 0288 **Fax**: (603) 9019 8868
Website: www.utar.edu.my