Sudan University of Science and Technology

College of Graduate Studies

Association between Trans-Membrane Protease Serine 6 Gene (C1795T) Polymorphism and Risk of Iron Deficiency Anemia in Khartoum State

العلاقة بين تعدد الأشكال الجيني لانزيم البروتينى سيرين 6 عابر الغشاء (سي1795 تى) وخطر فقر الدم بسبب نقص الحديد في ولاية الخرطوم

Dissertation Submitted for Partial Fulfillment of the Requirements M.Sc. Degree in Medical Laboratory Sciences (Hematology and Immunohematology).

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

الآية:

﴿وَ فِى ٱلْأَرْضِ ءَايَاتٌ لِّلْمُوقِنِينَ* وَفِى أَنفُسِكُمْ أَفَلَا تُبْصِرُونَ *﴾

صدق الله العظيم

سورة الذاريات (20-21)

Dedication

To my father who taught me the mean of life

To my mother who lactating me the mean of patience

To my brothers and sisters who always encouraged me

To my lovely friends who participated with me in this study.

Acknowledgements

All thank and praise to Allah to give me the health and strength, and patient to achieving this research. Great thanks to Dr. Selma Elmalieh Abdalla for her effort, support and supervision. Great thanks to teaching staff of Sudan University for Science and Technology. Voice thanks to my teachers Fayad Osman, Khnsaaa Ibrahim, Osama and Safaa Dafalla for their technical support and encouragement. Thank you so much Ms. AL Romisa Ahmed for help me in collection of the samples moral support. Grateful thanks to all individuals who participated in this study.

Abstract

This analytical study (case and control) aimed to investigate the association between TMPRSS6 gene C1795T on uncoded region intron15 polymorphism and risk of iron deficiency anemia. Two hundred subjects were recruited for this study, 100 patients affected with iron deficiency anemia and 100 healthy volunteers as a control group. Patients data (age, gender, hemoglobin, mean corpuscular volume and red cell distribution width) was collected from patient's medical files by questionnaire, three ml of blood were collected from all participants in EDTA anticoagulant containers, genomic DNA was extracted by salting out method and the TMPRSS6 C/T polymorphism was analyzed using polymerase chain reaction. Amplified fragments separated on 2% agarose gel stained with ethidium promide and demonstrated by gel documentation system, which produce single band at 249 bp represented C homozygous (CC). The data analyzed by computer program SPSS version16. TMPRSS6 gene investigated by PCR, The TT genotype of TMPRSS6 C/T polymorphism was higher frequent in IDA patients (71.4%). The results showed low hemoglobin concentration and MCV while RDW was increase significant difference ($P \leq 0.00$) in IDA patients when compere with normal individuals. But the well gene and mutant gene within the IDA patients had no interaction with the CBC parameters (Hb, MCV and RDW). In conclusion, there were statistically significant association between TMPRSS6 C/T polymorphism and risk of IDA among Sudanese patients in Khartoum state. The Further studies should be use other techniques (RFLP and DNA sequencing) to detect TMPRSS6 gene.

الخلاصة

هدفت الدراسة التحليلية (الحالة ومجموعة ضابطة) لاستقصاء العلاقة بين تعدد الأشكال الجيني لانزيم البروتين سيرين 6 عابر الغشاء سي1795 تى الموجود في المنطقة 15الغير محمل بالصفات الجينة وخطر فقر الدم بسبب نقص الحديد .تم تعيين مجموعه من 200 شخص لهذه الدراسة ، 100 مريض يعانون من فقر الدم بسبب نقص الحديد و 100 متطوعين أصحاء كمجموعة ضابطة . تم جمع بيانات المرضى(العمر , النوع, تركيز خضاب الدم , متوسط حجم الكرية و معدل انتشار كريات الدم الحمراء) من الملفات الطبية للمرضى بواسطة الاستبيان، 3مل من الدم تم جمعها من جميع المشاركين في حاويات EDTA المضادة للتخثر. تم فصل الجزء المستهدف الحمض النووى بواسطة طريقة التمليح، ثم تحليل تعدد الشكل الجيني للبروتين السيرين 6 عابر الغشاء بتفاعل البلمرة المتسلسلة ,الاجزاء المتضخمة فرقت بواسطة 2% من الجل المصبوغ ببرومايد الإثيديوم وأظهرت النتائج بنظام توثيق الجل التى انتجت جزء واحد عند 249 تمثل سي متماثلة اللواقح (سي سي) وتم تحليلها بواسطة الحزمة الإحصائية للعلوم الإجتماعية الاصدارة 16. اختلافات ذات دلالة معنوية في مرضى ($P \leq 0.00$).تم استقصاء الجين(انزيم بروتين سيرين6 عابر الغشاء) بواسطة تفاعلات البلمرة المتسلسلة . النمط الوراثى تى تى من تعدد الاشكال الجينى لانزيم البروتينى سيرين 6 عابر الغشاء مرتفع بنسبة متكررة في مرضى فقر الدم بسبب نقص الحديد (71.4%) . اظهرت النتائج إنخفاض فى تركيز الخضاب فى الدم ومتوسط حجم الكرية الحمراء بينما إرتفع معدل توزيع الخلايا الحمراء معنويا($P \leq 0.00$) للمرضى فقر الدم بسبب نقص الحديد مقارنة مع الاصحاء، بينما الجين الطبيعى و الطفرة الجيني فى مرضى فقر الدم بسبب نقص الحديد لا توجد بها تفاعل مع فحوصات الدم الكامل. الخلاصة اوضحت الدراسة ارتباط ذو دلالة احصائية بين تعدد الاشكال الجينى لانزيم البروتين سيرين6 عابر الغشاء وخطر فقر الدم بسبب نقص الحديد بين مرضى السودانيين في ولاية الخرطوم. يجب اجراء مزيد من الدراسات باستخدام طرق اخرى مثل تعدد شكل طول جزء الحصر و تسلسل الحمض النووى.

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cDNA	Complementary DeoxyriboNucleic Acid
CI	Confidence Interval
DMT1	Divalent Metal Transporter1
HAMP	Hepcidin Anti-Microbial Peptide
Hb	Hemoglobin
i.e.	that is
IDA	Iron Deficiency Anemia
LDL	low Density lipoprotein
LIC	liver Iron Concentration
MCH	Mean Corpuscular Hemoglobin
MCV	Mean Corpuscular Volume
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
RBCs	Red Blood Cells
RNA	Ribonucleic Acid
SLC11A2	Solute Carrier Family 11 Member 2
sTFR	Soluble Trans-Ferrin Receptor
UTR	Un-Translated Region
IRE	Iron Responsive Element
RFLP	Restriction Fragment length Polymorphism

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1. INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Blood is formed from (water) plasma which carries certain cells and in which are dissolved many ions and molecules. The cellular elements of blood include erythrocytes (required for the transport of oxygen), leukocytes (defense against microbial attack) and platelets which regulating the balance between clotting and bleeding (Hoffbrand *et al* ,2006; Blann and Ahmed; 2014).

Anemia is a reduction in the haemoglobin concentration in the blood, in comparison with what would be found in a normal individual of the same age and gender (Bain and Gupta, 2003).

According to WHO iron deficiency anemia is the most common and widespread nutritional disorder in the world. As well as affecting a large number of children and women in non-industrialized countries, it is the only nutrient deficiency which is also significantly prevalent in virtually all industrialized nations (WHO, 2008). In Sudan IDA is common in Sudanese children, 58% of mothers gave history of anemia during pregnancy and 67% of poor nutrition indicating low income (Omer and Ahmed, 2015).

The causes of anemia center on three major pathophysiological categories are blood loss ,impaired red cell production and accelerated red cell destruction (hemolysis in excess of the ability of the marrow to replace these losses).Anemia may be a sign of an underlying disorder (Hoffbrand *et al* ,2006). The classification of anemia's are depend on this categories: accelerated erythrocyte destruction (hemolytic Inherited defects acquired disorders and hemolytic _hemoglobin disorders), blood loss (acute and

chronic) and impaired RBC production (aplastic ,iron deficiency ,sideroblastic anemia, anemia of chronic disease and megaloblastic) . The most frequent forms of anemia result from either blood loss or iron deficiency conditions (Turgen; 2012).

Homozygous inactivation of the TMPRSS6 gene leads to excessive HAMP production, impaired dietary iron absorption and microcytic anemia in mice and iron-refractory iron deficiency anemia (IRIDA) in humans (Nai A *et al*, 2012).

Iron refractory iron deficiency anaemia is a recently recognized recessive disorder that causes microcytic hypochromic anaemia. It is due to mutations of the trans-membrane protease serine 6 (TMPRSS6) gene, which encodes matriptase-2, a type II trans-membrane serine protease mainly expressed by hepatocytes (Silvestri *et al*, 2008).

1.2 literature review:

Iron deficiency anemia is the most advanced stage of iron deficiency. The iron-deficient erythropoiesis does not result in anemia until all iron has been mobilized from the marrow so that iron stores are absent, it's characterized by decreased or absent iron stores, low serum iron concentration, low transferrin saturation, and low blood hemoglobin concentration (Pasricha *et al* ,2013).

Mature erythrocyte is a round biconcave disc the diameter of erythrocyte in human range between 7 and 8 μm. Basic structural properties of various red cell components (haemoglobin, enzymes, and membrane) . Haemoglobin is responsible for transport of oxygen from lungs to the tissues and of carbon dioxide from tissues to the lungs (Hoffbrand *et al* ,2006). Haemoglobin (MW 64,500 daltons) is composed of haem (consisting of iron and protoporphyrin) and globin. The globin portion of the molecule consists of four (or two pairs) of polypeptide chains. One haem group is bound to each polypeptide chain (Kawthkar ,2013).

Iron is found in green vegetables and meats in ferric protein and heamprotein complexes respectively. Fortified breakfast cereals are also an important source of iron. The risk of iron deficiency is further increased by the habitual consumption of diets with insufficient amounts of bioavailable. Free iron is toxic and is, therefore, incorporated into heam or bound to protein within the body (Gargani, 2012). Heam consists of an iron atom at the center of a protoporphyrin ring. Transferrin transports up to two molecules of iron to tissues that have transferrin receptors, like bone marrow. Both ferritin and heamosiderin store iron in its ferric form. Ferritin is a water-soluble compound, consisting of protein and iron. Haemosiderin is

insoluble and consists of aggregates of ferritin that have partially lost their protein component (Gargani, 2012).

1.2.1 Epidemiology and causes of iron deficiency anemia

The prevalence of iron deficiency varies greatly according to host factors: age, gender, physiological, pathological, environmental, and socioeconomic conditions (WHO,2008) .

IDA is the most frequent acquired microcytic anemia, common in individuals with high iron requirements as it occurs in infancy, during the growth spurt in adolescents, in menstruating females and in pregnancy. Low dietary iron intake, mal-absorption and chronic blood losses are the commonest causes of microcytic anemia (Iolascon *et al* , 2008).

1.2.2 Other Microcytic Anemia:

Other microcytic anemia include the thalassaemic haemoglobinopathies which due to decreased Hb per cell because of the deficiency of one of the two types of globin chains that assemble to form the Hb tetramer (Hb A) of adult life. Hereditary sideroblastic anaemia, another example of microcytic red cells, is traditionally known to be result of a deficiency of haem synthesis and characterized by the morphological feature of ringed sideroblasts, i.e. erythroblasts with mitochondrial iron deposition visible at Perl's staining of bone marrow smears (Hentze *et al*, 2010).

1.2. 3 Pathophysiology of IDA:

A common pathophysiological feature of IDA is decreased haem formation. It is well known that haem is a powerful controller of different cell functions. Among others, haem regulates a kinase (Haem-regulated eIF

2alpha kinase or HRI), which is highly expressed in erythroid cells, that phosphorylates the alpha-subunit of the eukaryotic translational initiation factor 2 (eIF2alpha). When haem concentration declines, HRI blocks the kinase, thereby preventing further protein synthesis (Chen, 2007). globin is the main protein synthesized by erythroblasts: that is why translational control by haem ensures the needed balance between globin and haem synthesis. HRI has an essential protective role in iron deficiency, avoiding excessive protein synthesis and is responsible for hypochromia as an adaptive mechanism for erythrocyte survival (Zhang *et al*, 2009).

It has also been proposed that the erythrocyte size depends on the total iron present in the erythroid precursors and especially on the ability of red cell precursors to release iron through ferroportin B, a ferroportin isoform lacking the 5′ UTR IRE element at the RNA level, and thus unresponsive to iron (Zhang *et al*, 2011b). low hepcidin in IDA would allow Ferro- protein to export iron from red cell precursors leading to microcytosis, whereas high hepcidin in anemia of chronic disease (ACD), which is characterized by iron-restricted erythropoiesis, would cause iron retention in red cell precursors and lack of microcytosis (Keel and Abkowitz, 2009).

Mutations in the intestinal iron (Fe2+) transporter gene, SLC11A2 (DMT1) causing microcytic anemia and hepatic iron overload have been described (Beaumont *et al*, 2006). Recently, genetic defects in Trans-membrane serine protease 6 (TMPRSS6) resulting in iron refractory iron deficiency anemia (IRIDA) have been described (Finberg *et al*, 2008).

1.2.4 Diagnosis and management of iron deficiency anemia:

Powers and Buchanan, (2014) reported that the IDA diagnostic by CBC which show decreased in Hb level , low hematocrit and low RBCs

indices followed by peripheral blood film: RBCs are hypochromic microcytosis: pencil forms and target cells. Then iron profile in which Serum ferritin < 20, finally bone marrow: intermediate and late erythroblasts show micro-normoblastic maturation. Fe stain (Prussian blue) shows decreased iron in macrophages (Powers and Buchanan ,2014).

1.2. 5 Treating iron deficiency anemia:

It is important to underlying cause of iron deficiency. Oral iron supplementation used to normalizer haemoglobin levels. Also iron sucrose administered intravenously can be use if no response for oral iron therapy (Hatton *et al*, 2013).

The main reason for failure to respond to oral iron therapy is poor compliance. However, if the losses (for example, bleeding) exceed the amount of iron absorbed daily, the haemoglobin concentration will not rise as expected; this will also be the case in combined deficiency states. The presence of underlying inflammation or malignancy may also lead to a poor response to therapy. Finally, an incorrect diagnosis of iron deficiency anaemia should be considered in patients who fail to respond adequately to iron replacement therapy (Provan ,2003).

1.2.6 Prognosis:

The prognosis for iron deficiency itself is excellent, and the response to either oral or parenteral iron is similar. Pica may resolve and soreness and burning of the mouth abate. Hematologically, mild reticulocytosis begins within 35 days and then declines. The hemoglobin concentration begins to increase after the first week and has usually returned to normal within 6 weeks. Complete recovery from microcytosis may take up to 4 months. With oral iron dosage totaling 200 mg/day or less, the plasma ferritin remains <12

g/dl until the anemia is corrected and then gradually rises as storage iron is replaced over the next several months (Hoffman *et al* ,2000)

1.2.7 Iron-Refractory, Iron Deficiency Anemia (IDIRA):

It is a familial disorder characterized by iron deficiency anemia unresponsive to oral iron treatment but partially responsive to intravenous iron therapy. IRIDA patients harbor loss-of function mutations in TMPRSS6, a type II trans-membrane serine protease primarily expressed by the liver. Both humans and mice with TMPRSS6 mutations show inappropriately elevated levels of the iron regulatory hormone hepcidin. (Karin, 2009).

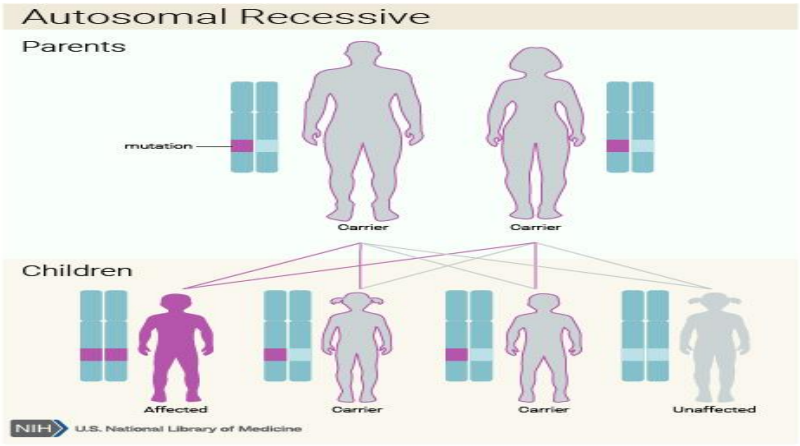

Figure (1.1): The mutation among offspring sons.(NIH,2008)

1.2.8 Discovery of IRIDA :

IRIDA was first described clinically in 1981 by Buchanan and Sheedhan. Finberg *et al*. (2008) noted that there were mutations in the trans-membrane protease serine 6 causes IRIDA, the mode of transmission being autosomal recessive. TMPRSS6 encodes matriptase-2 (MT-2), a trans-

membrane serine protease of the type-two trans-membrane serine protease (TTSP) family, which is mainly expressed in the liver (Chambers *et al*, 2009).

First shown by the discovery of a homozygous mutation of TMPRSS6 in mask mice having microcytic anemia. TMPRSS6 knockout mice have a similar phenotype: these mice develop anemia, lose trunk hairs and show decreased iron absorption because of high hepcidin levels, block ferroportin-mediated iron release to plasma. In cell models this serine protease cleaves the BMP co-receptor haemojuvelin, attenuating the BMP-mediated hepcidin activation (Silvestri *et al*, 2008).

The gene was cloned in mask mice, a product of N-ethyl-N-nitrosourea (ENU) mutagenesis, which had microcytic anaemia and a truncated matriptase-2 devoid of the catalytic domain (Du *et al*, 2008). Folgueras *et al*, (2008) reported that TMPRSS6 knockout mice have a similar phenotype. Matriptase-2 is the first hepcidin inhibitor with a documented in vivo effect in patients. Although epidemiological data are lacking, IRIDA is considered 'the most frequent' among the rare microcytic anaemias.

1.2.9 IDA among children

Some authors reported that diagnosis of IRIDA usually occurs in childhood. Whereas anaemia is mild-moderate and growth and development is normal, microcytosis and hypochromia are remarkable and disproportionate to the degree of anaemia (Finberg *et al*, 2008). transferrin saturation is extremely low, while ferritin may be normal or at least not as low as one would expect from transferrin saturation (Tanaka *et al*, 2010).

Melis *et al*. (2008) reported from the limited followup data, anaemia seems to be more severe in children than in adults. In the affected individuals, who belong to a large kindred originating from and living in a small Sardinian village.

Adult patients show mild anaemia, but persistently low transferrin saturation and remarkable microcytosis. Hepcidin levels are high/normal when measured in patient serum or urine, in any case inappropriate to iron deficiency, given that in the latter condition hepcidin expression is strongly suppressed. Patients from about 50 unrelated families of different ethnic origin have been described as being either homozygous or compound heterozygous for TMPRSS6 mutations, predicted to result in loss of function of the encoded protein (Camaschella and Poggiali, 2011).

1.2.10 Pathophysiology of IRIDA:

Missense mutations in TMPRSS6 gene are spread along the entire gene sequence, affecting not only the protease catalytic domain, but also other domains that could affect protein-protein interaction (Guillem *et al*, 2012).

Most mutations in vitro studies have shown that causal mutations have decreased activity and are unable to inhibit hepcidin promoter at the same rate of the wild type protein in a luciferase-based assay in cells transfected with haemojuvelin (Ramsay *et al*, 2009; De Falco *et al*, 2010). Hepcidin is a small peptide hormone produced by the liver that is detectable in serum and urine, is a central regulator of iron homeostasis (Nemeth and Ganz,2006).

There is no evidence of genetic heterogeneity of IRIDA. Only heterozygous TMPRSS6 mutations have been found in a few patients, although regulatory regions are not usually explored by sequencing (Finberg

et al, 2008). It is possible that single nucleotide polymorphisms (SNPs) or specific haplotypes play some role in the disease (Kloss-Brandstatter *et a*l, 2012), and that cases showing microcytosis without anaemia are due to mild TMPRSS6 mutations. TMPRSS6 haplo-insufficiency renders mice more susceptible to iron deficiency in conditions of iron restriction (Nai *et al*, 2010) or in the presence of increased requests, such as pregnancy (Finberg *et al*, 2010).

Common genetic variants (i.e. SNP) in the TMPRSS6 gene in several populations are associated with changes in the normal erythrocyte and iron parameters (Ganesh *et al*, 2009; Chambers *et al*, 2009).

Nai *et al*, (2011) noted that SNP rs855791 with a missense change in the serine protease domain may influence serum hepcidin levels and iron parameters in normal subjects. The ethnic background and the environment are also important: in Chinese the TMPRSS6 genetic variant rs855791 is associated with iron deficiency in the elderly, although it is unknown whether this variant is associated with increased hepcidin levels, That the coexistence of other polymorphisms that increase iron absorption or environmental factors/diet accounts for the observed difference (An *et al*, 2012).

Du *et al*, (2008) noted that deficient of TMPRSS6 activity upregulates hepcidin transcription and reduces the expression of ferroportin thereby resulting in retention of iron within the cells.

1.2. 11 Iron cycle:

The mechanisms of adaptation to iron deficiency are centered on the suppression of the hepatic hormone hepcidin and the tissue hypoxia that develops consequent to anemia (Zhang *et al*, 2011).The production of

erythropoietin (EPO) by the kidney increases in response to enhanced levels of hypoxia-inducible factor 2α (HIF-2α). As a consequence of the stimulation of erythropoietin, erythropoiesis is increased and hypochromic microcytic red cells are produced owing to the low availability of iron (Benyamin *et al*, 2009).

Senescent red cells are destroyed by macrophages, and their iron is recycled. The increase in erythropoiesis suppresses the production of hepcidin. In mice, this function is mediated by erythroferrone (ERFE), which is secreted by the erythroblasts21 to maintain adequate iron absorption and efficiency in erythropoiesis (Kautz *et al* ,2014). HIF-2α increases the expression of the duodenal divalent metal transporter 1 (DMT1) on the apical surface of enterocytes to increase the transfer of dietary iron from the lumen to enterocytes (Mastrogiannaki *et al*, 2013).

Hepcidin levels are depressed in response to a reduction in the physiologic signals that maintain its production (e.g., increases in levels of iron-bound transferrin and in the iron content of the liver) (Hentze *et al*, 2010), to the increased activity of the inhibitor transmembrane protease, serine 6 (TMPRSS6), to the reduction in levels of the activator bone morphogenetic protein 6 (BMP6), and to increased inhibition from erythropoietin-stimulated erythropoiesis (Camaschella ,2013). Ferroportin (FPN), which is no longer being degraded because of the low levels of hepcidin, exports the available iron across the enterocyte basal membrane and from macrophage stores to the circulation. Once stores are exhausted, levels of circulating iron decrease, even if absorption from the lumen is increased. Reduced levels of iron in the liver trigger increases in the synthesis of the iron carrier transferrin (referred to as apotransferrin when

not bound to iron), further decreasing levels of iron-bound transferrin, the ligand of the transferrin receptor. Consequently, the uptake of iron from transferrin receptors by all cells and organs (example, skeletal muscles and the heart) is reduced (Nemeth *et al*, 2004).

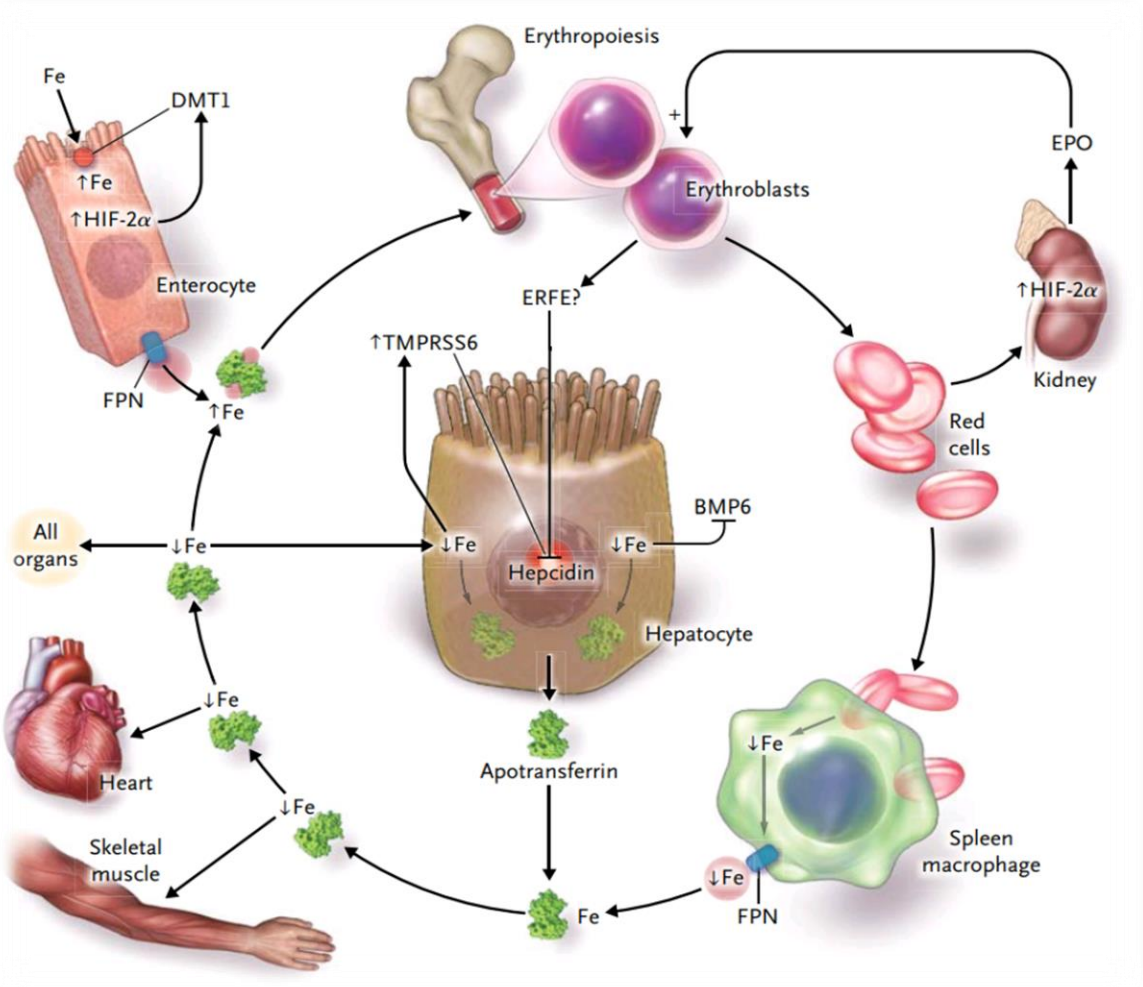

Figure1.2 Iron cycle (Camaschella,2015)

Table 1.1 Variation of iron parameters in the atypical microcytic anaemias compared with IRIDA and IDA (Camaschella C,2012).

Variants	**IRIDA**	**IDA**
Family history	Siblings	Sometimes
Anaemia at birth	No	No
Degree of anaemia	Moderate	Variable
MCV	Very low	Variable
MCH	Very low	Variable
Serum Iron	Very low	Low
Transferrin	High	high
Transferrin saturation	Very low	Low
Serum ferritin	Normal/ low	Low
sTFR	High	High
LIC	Low	Low
Serum hepcidin	Normal / high	Low

1.2.12 Trans-Membrane Serine Protease 6 (TMPRSS6) :

Matriptase-2 is a trans-membrane serine protease, TMPRSS6 is a member of type 2 trans-membrane serine proteases family mapped on 22q12-13 with unique structural features including a serine protease domain, a trans-membrane domain, a short cytoplasmic domain, and a stem region containing an LDL (Nai e*t al* ,2010) .

1.2.13 Function of TMPRSS6 gene:

TMPRSS6 gene synthesis protein (Matriptase 2), which plays an essential role in iron hemostasis that negatively regulates hepcidin expression by cleaving membrane-bound hemojuvelin. Matriptase-2 has a complex ectodomain, including a C-terminal serine protease domain and its activation requires an autocatalytic cleavage (Velasco et al, 2002).

Matriptase-2 can degrade in vitro extracellular matrix components such as fibronectin, fibrinogen, and type I collagen and to activate single-chain uPA although with low efficiency compared with matriptase.

Matriptase-2 shares the structural organization of TTSPs, including the short cytoplasmic domain, a type II transmembrane sequence, a stem region with 2 CUB (complement factor C1s/C1r, urchin embryonic growth factor, bone morphogenetic protein) domains and 3 LDLR (low-density lipoprotein receptor) tandem repeats, and the carboxy-terminal serine protease domain(Velasco *et al* 2002; Hooper *et al* , 2003)

1.2.14 Gene mutation:

Two nonsense mutations were identified by sequencing the TMPRSS6 gene: one heterozygous cDNA 1179T G substitution in exon 10 introducing a nonsense Y393X codon in the protein and a cDNA1795C T substitution in exon 15 introducing another protein nonsense codon R599X These mutations are both predicted to delete the serine protease domain from the encoded protein unless the mRNA harbouring premature translation termination codon is rapidly degraded through the nonsense-mediated RNA decay surveillance pathway (NCBI, 2008; Chang *et al*, 2007) .

Hepcidin is the core of iron metabolism and is tightly regulated by several mediators. Matriptase-2 is an important one and down regulates

hepcidin expression through cleaving membrane-bound hemojuvelin, which can enhance hepcidin transcription. Complete loss of function mutation of matriptase-2 leads to a rare disease, iron-refractory iron deficiency anemia (Hentze *et al*, 2010).

The important role of TMPRSS6 in erythropoiesis is highlighted also by Genome Wide Association Studies. Common TMPRSS6 genetic variants, as rs855791, associate with serum iron and transferrin saturation, hemoglobin (Hb) and erythrocyte (MCV and MCH) traits in different populations. TMPRSS6 haplo-insufficient mice have increased susceptibility to iron deficiency. Altogether these results suggest that TMPRSS6 gene dosage may modify erythropoiesis and influence HAMP expression (Nai A *et al*,;2012).

Some studies reported the mutations in TMPRSS6 have been identified in 14 IRIDA patients from other populations (Finberg *et al*, 2008; Guillem *et al*, 2008; Silvestri *et al*, 2009).

Some authors demonstrating that normal murine systemic iron homeostasis could not be achieved through activity of a single wild type Tmprss6 allele. These results raise the possibility that human heterozygous carriers of TMPRSS6 mutations may harbor subtle abnormalities of iron homeostasis that increase their risk for developing iron deficiency in response to certain physiological stresses, such as pregnancy, decreased dietary intake or inflammation. While the frequency of such pathogenic TMPRSS6 mutations is likely to be extremely low (Soranzo *et al* ,2010) .

1.2.15 Complete Blood Count (CBC):

CBC is a comparatively inexpensive but powerful diagnostic tool in a variety of hematological and non-hematological conditions (Agarwa, 2013).

The author noted that MCV is average volume of the red blood cell {MCV= (hematocrit/red cell count) ×100}.Can be directly measured by automated cell counters. Red cell distribution width (RDW) is a quantitative measure or numerical expression of anisocytosis .It is a co-efficient of variation of the distribution of individual RBC volume, as determined by automated blood cell counting instrument. The RDW value reflects size variability in red cell.

1.2.16 Principles of operation of automated haematology counter

All variables depend on counting and sizing of particles, whether red cells, white cells or platelets either by electrical impedance or by light scattering. Automated instruments have at least two channels. In one channel a diluent is added and red cells are counted and sized. In another channel a lytic agent is added, together with diluent, to reduce red cells to stroma, leaving the white cells intact for counting and also producing a solution in which Hb can be measured. Further channels are required for a differential WBC, which is often dependent on study of cells by a number of modalities, e.g. impedance technology with current of various frequencies, light scattering and light absorbance. A separate channel or an independent instrument may be required for a reticulocyte count. A new challenge to automated instruments is the production of accurate red cell indices, as well as total haemoglobin concentration, in patients who are infused with haemoglobin-based blood substitutes. This can be achieved with Bayer instruments, which measure size and haemoglobin concentration of individual red cells (Bain, 2006).

1.3 Rationale:

Iron deficiency anemia (IDA) has been widely explored and understood as a nutritional disorder but there is a limited understanding of iron deficiency anemia, which is due to genetic alterations in iron homeostasis (Soranzo *et al*, 2009).

Human patients have a new condition involved in the pathogenesis of hereditary microcytic anemia, the seldom data study late the discovery of inherited microcytic anemia in Sudan.

The individuals who have mutant gene (TMPRSS6 C/T polymorphism) have high risk to be affected by IDA.

1.4 Objective

1.4.1 General Objective:

-To detect the relation SNP of Trans-membrane Serine Protease 6 (TMPRSS6) C1795T gene polymorphism and it relation with the iron deficiency anemia in Khartoum State.

1. 4.2 Specific objectives

1) To determine the frequency of TMPRSS6 C/T genotypic variants in patients with IDA using Polymerase Chain Reaction (PCR).
2) To compare the TMPRSS6 genotype distribution of IDA patients with healthy control.
3) To investigate the association between TMPRSS6 (C/T) polymorphism and risk of IDA.
4) To investigate TMPRSS6 (C/T) polymorphism with hematological parameters (Hb, MCV and RDW) in anemic patients and healthy control.

CHAPTER TWO

MATERIALS AND METHODS

2. MATERIALS AND METHODS:

2.1 Study design:

The analytical case control study was conducted to investigate the association between TMPRSS6 gene polymorphism and risk of IDA in Khartoum state.

2.2 Study area and duration:

The study was carried out at Mohamed El-Amin Pediatric Hospital and Al-Zahra care center, Khartoum through the period(May to July 2018).

2.3 Study populations:

The analytical study population was two groups (anemic patients and normal control) samples, 100 individuals diagnosed with iron deficiency anemia and 100 healthy who served as control in Khartoum state.

2.3.1 Inclusion criteria

All patients known diagnosed with IDA.

2.3.2 Exclusion criteria:

Patients with known other inherited microcytic anemia.

2.4 Data collection

Patient's data (age, gender, Hb, MCV and RDW) were collected from patient's medical files. Laboratory investigations were performed for (Hb, MCV and RDW) for normal individuals and questionnaire was constructed for these data (appendix) some individual answer the questionnaire verbally and the other wrote the answer.

2.5 Sample collection

Blood samples (3ml) were collected from each patient upon in containers containing EDTA as an anticoagulant for analysis.

2.6 Ethical consideration:

This study was approved by collage of medical laboratory science, Sudan University of Science and Technology. A verbal informed consent was obtained from each participant before samples collection.

2.7 Methodology of PCR test

2.7. 1 DNA extraction

Nine hundred microliter of red cell lysis buffer was added to 300 µl of whole blood in 1.5 ml eppendorf tube. The mixture was centrifuged at 8000 rpm for 3 minutes and the supernatant was discarded. This step was repeated 3-4 times till RBCs lysis was complete and a white pellet of WBCs was obtained. To the cell pellet, 300µlof white cell lysis buffer and 10µl of proteinase K. were added, Mixed thoroughly, and incubated over night at 37˚c . At the end of incubation, 100µl of 6MNaCl was added, vortexed and centrifuged at 8000 rpm for 5 minutes to precipitate the proteins. To Precipitate of DNA, The supernatant was transferred into a new eppendorf tube containing 300µlof isopropanol. DNA was precipitated by inverting the eppendorf tube slowly. Further, the eppendorf tube were centrifuged at 8000rpm for 10 minutes to pellet down the DNA. Supernatant was discarded, 70% ethanol was added and mixed slowly to remove any excess salts, the tubes were centrifuged at 8000 rpm for 5 minutes to pellet down the DNA. Supernatant was discarded and DNA air-dried. After thorough drying, 50µl of TE buffer was added to dissolve the DNA,1%

agarose gel electrophoresis use to detect genomic DNA isolated from human blood samples (Sugana *et al*., 2014).

2.7.2 Quality Control:

The known samples were used as control samples for PCR machines.

Purchase and used standards from national systems of standards likes NIBSC.

2.7.3 Polymerase chain reaction

Allele specific PCR was used for molecular detection of TMPRSS6 (C/T) polymorphism. The sequences of the forward and reverse are shown in (Table 2.1).

Table 2.1 Primer Sequences.

Name of the gene	Primer sequences
TMPRSS6	Forward-5'-TAG AGA ACA GGG GCT CCA GG - 3'
	Reverse-5'-ATG TGG GCA GCA TCC TTT C - 3'

Table 2.2 PCR reaction mixture

PCR reaction mixture	Volume
Template DNA	2µ/L
Primer (F: 100Pmol/µL)	1µ/L
Primer (R: 100Pmol/µL)	1µ/L
Primer (I: 100Pmol/µL)	1µ/L
Distilled water	15µ/L
Total reaction volume	20µ/L

The amplification process consisted of initial denaturation of 94˚Cfor 3 min, 35 cycles each consist of 94˚C for 30sec,57˚C for 30 sec,72˚C for 30 secthen final extension at 72˚C for 5 min.

2.7.4 Agarose gel electrophoresis

PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and analyzed under UV light. 5µL of the PCR product were loaded on the gel along with 4µL of 100bpdeoxyribonucleic acid (DNA) ladder applied with each batch of patients samples.

2.7.5 Interpretation of results

The well TMPRSS6 gene show band at 249bp and mutant TMPRSS6 C/T gene show no band on agrose gel.

2.8 Statistical analysis

Data was analyzed by statistical package for social sciences (SPSS), version 16. Qualitative data was represented as frequency and Percentage. Quantitative data was presented as mean ± SD. Association between qualitative variables was tested using Pearson's Chi square ($\chi 2$) and in depended t tests for quantitative data. Binary logistic regression analysis was used to investigate the association between genotypes and risk of IDA.

CHAPTER THREE

RESULTS

3. RESULTS

3.1 Demographic data:

This analytical study (case and control) aimed to investigate the association between TMPRSS6 gene C/T polymorphism and risk of iron deficiency anemia. Two hundred Sudanese subjects were enrolled in this study, 100 patients with IDA and 100 healthy volunteers as control group, investigated by hematological analysis (Hb, MCV and RDW) and PCR. The mean age of patients were 20±16 years and of control was 21±17 years respectively. About 51.79% of anemic patients were male and 47.73% of anemic patients were female. Also 48.21% of control was male and 52.27% of control was female.

3.2. PCR:

TMPRSS6 gene investigate by PCR, produce single band at 249 bp represented the well gene (figure 3.1). The mutant (TT) genotype of TMPRSS6 C/T polymorphism was higher frequent in IDA patients (71.4%). The result showed significant difference in genotype distribution when compared to anemic patients with normal control groups (P≤ 0.00) (**Table 3.1**). The results showed no statistically significant association between each of TMPRSS6 (T/C) genotypes with gender and age **(Table 3.2 and 3.3)** respectively**.**

Comparison of value of CBC sub-parameter (Hb, MCV and RDW) in anemic patients and normal control with TMPRSS6 (TT and TC/CC genotypes) polymorphism showed statistically significant difference (P≤ 0.00) but when compare the mutant gene and well gene in anemic group

there were no statistically significant difference (**Table 3.4, 3.5 and 3.6**) respectively.

The study investigate the risk of TMPRSS6 gene and IDA by logistic regression test, which show no interaction between TMPRSS6 genotypes with age and gender in IDA patients, but there were interaction between TMPRSS6 genotypes and hematological parameters (Hb, MCV and RDW) (**Table 3.7**).

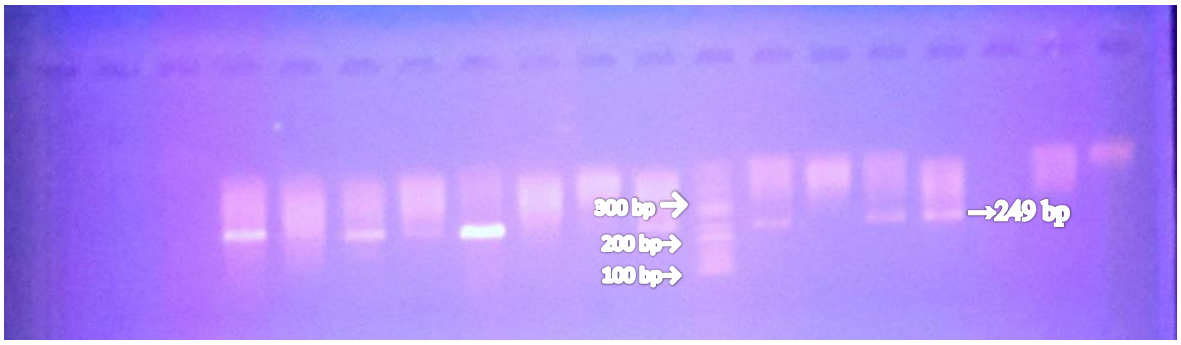

Figure 3.1 Amplified fragments of TMPRSS6.

Table3. 1. Genotype distribution in study groups.

Variable	TMPRSS6		total	P. value
	mutant gene	well gene		
Patient	68(77.4%)	32(28.6%)	100	≤ 0.00
Control	20(22.7%)	80(71.4%)	100	

Table3.2. Association between TMPRSS6 polymorphic genotypes and gender.

variable	TMPRSS6				p. value
	mutant gene		well gene		
	Anemic patient	Normal	Anemic patient	Normal	
Male	35.7%	8%	16.1%	40.2%	≤0.93
Female	31.8%	12.5%	15.9%	39.8%	

Table3. 3 comparison of mean age according to genotype

Sample	Mean of age (years)		p. value
	mutant gene	well gene	
Case	19±16	22±17	≤ 0.346
Control	20±17	21±16	

Table3.4 Comparison of mean Hb with case and control according to genotype

Variables	Mean of Hb g/dl		P. value
	mutant gene	well gene	
Case	8.93±1.4	9.22±1.8	≤ 0.40
Control	12.9±0.8	12.68±1.3	

Table3. 5 Comparison of MCV value with anemic patients and control according to genotype

Variables	Mean of MCV (fl)		P. value
	mutant gene	well gene	
anemic	66±5.5	68.4±5.7	≤ 0.08
Control	91.7±6.7	89.8±6.8	

Table3. 6 Comparison of RDW value with anemic patients and control according to genotype

Variables	Mean of RDW (%)		p. value
	mutant gene	well gene	
anemic	19.83±2.8	19.75±4	≤ 0.91
Control	12.58 ±1.8	13.07±2.1	

Table 3.7 Interaction between TMPRSS6 polymorphism with age, gender, HB, MCV and RDW in IDA patients.

Variables	95% C.l .		Odd ratio	P.value
	Lower	Upper		
Gender	0.437	1.713	0.865	0.055
Age	0.989	1.029	1.009	0.053
Hb	0.822	1.354	1.055	0.022
MCV	0.963	1.069	1.015	0.011
RDW	0.892	1.165	1.020	0.009

CHAPTER FOUR

Discussion, Conclusion, and Recommendations

4. Discussion, Conclusion, and Recommendations

4.1 Discussion

This study investigated the association of TMPRSS6 C1795T gene polymorphism with the risk of IDA.

TMPRSS6 gene investigated by PCR, which produce Single band at 249 bp represented well gene (C homozygous CC). The mutant (TT) genotype of TMPRSS6 C1795T polymorphism was higher frequent in IDA patients (71.4%).

There were statistically significant association between TMPRSS6 C/T polymorphism genotypes and risk of IDA ($P \leq 0.03$). Also reported means of Hb, MCV and RDW in patents and control (9g/dl, 12.7g/dl), (66 fl ,90 fl) and (19.8%, 12.9 %) respectively were significantly different ($P \leq 0.00$) The interpretation of lowing Hb concentration and MCV value and the increase of RDW is that TMPRSS6 polymorphisms is due to imbalance iron hemostasis (low heam decrease Hb synthesis) which lead to IRIDA. The present results are in agreement with Sung *et al* (2014) who reported (8.5 g/dl, 69 fl and 18%) values for Hb concentration, MCV and RDW respectively in IDA patients. An *et al (*2012) conducted study in Chinese population, reported that TMPRSS6 polymorphisms are significantly associated with decreased iron status which associated with lower hemoglobin levels and there were a common variants in TMPRSS6 as being a genetic risk factors for IDA ($P \leq 0.00$). Consistent with their associations to increased iron deficiency and anemia risk which agree with current result.

A study conducted on Italy population, observed that TMPRSS6 mutation leads to overproduction of hepcidin and defective iron absorption and utilization, which is a high risk factor for iron deficiency anemia (Melis *et al.*, 2008). TMPRSS6 homozygous mutation increases the risk of iron deficiency anemia by inappropriately elevated hepcidin expression in Tmprss6-/- results in chronically impaired uptake of dietary iron, reflected in decreased hepatic iron stores (Finberg *et al*, 2010). Significantly fewer C homozygotes in the IDA group compared to the healthy group have been reported by Sung *et al*. (2014), suggesting that homozygosis for TMPRSS6 C genotype has a protective role against IDA.

4.2 Conclusions

- There were statistically significant association between TMPRSS6 C1795T polymorphic genotypes and IDA risk. .
- There were interactions observed between TMPRSS6 C/T genotypes with means of Hb, MCV and RDW with IDA patients group when compere with normal individuals. But there were no statistically significant between well gene and mutant gene in IDA patients.

4.3 Recommendations

-Further studies should be conducted to identify the relationship between hepcidin level and iron profiles with TMPRSS6 C/T polymorphism.

-Future studies should be conducted by other techniques RFLP and DNA sequencing.

-future studies should be conducted by using TMPRSS6 gene mapping.

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Appendix

A. Reagents of PCR analysis:

Ethylenediamine –tetra-aceticacetate (EDTA)

One hundred from Diapotassium or disodium salt dissolve in 1 litre Water allow appropriate volumes to dry in bottle at 20˚C so as to give a concentration of 1.5± 0.25mg/ml of blood.

Red cells lysis buffer (RCLB)

TKM 1 Buffer low salt buffer (500 ml): 0.605 of tris HCL (10mM) pH 7.6 ,1.016 g of MgCL2 (10mM),0.372g of KCL (10mM),0.372g of EDTA (2mM) was dissolved in 500ml of distilled water, added 0.1ml of 100% triton –x to 9.9ml of distilled water.

White cells lysis buffer (WCLB)

TKM 2 Buffer / high salt buffer(100 ml) :0.121g of tris HCL (10mM) ph 7.6, o.o74g of KCL (10mM), 1.203g of MgCL2 (10mM), 0.074g EDTA (2mM), 0467g of Nacl (0.4M) was dissolved in 100 ml of distilled water.

6M Nacl

about 8.765g of Nacl was dissolved in 25ml of distille water.

TE Buffer

about 0.030g of tris HCL (10 mM) pH 8.0 and 0.009g of EDTA (1mM) was dissolved in 100ml of distilled water.

1XTBE buffer:

Ten ml added to 90 ml dH2O.

Preparation of Agarose Gel2% :

Two gram from a garose powder was dissolved in 50 ml from 1XTBE buffer and heated in microwave for 2 min (until agarose completely dissolved). then added 1.5 µL from ethidium bromide after cooling, mixed well and poured in to a casting tray that was taped up appropriately and was equipped with a sutable comb to from well in place. Any bubbles were removed and the gel allowed to set at room temperature. After solidification the comb was gently removed and spacer from the opened slides was removed.

Ethidium Bromide solution

Ten milligrams of ethidium bromide powder were dissolved into 500 µL deionized water ,and kept into brown bottle.

Primers (forward and reverse)

The stock solution were prepared by adding 250 µL from dH2O then were pelleted at 14.000 for 5min and placed at 4˚C over night . The working solutions were prepared by adding 10 µL from stock solutions and 90 µL from dH2O, mixed and placed at -20 ˚C

B / Questionnaire

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

Questionnaire

Association between TMPRSS6 gene and risk of iron difeciency anemia in Khartoum state.

العلاقة بين الجين العابر للاغشية وخطورة الإصابة بفقر الدم بسبب نقص الحديد

Name : .... ID number: .....

Address: .....

Age :..... years

Gender: male ☐ female ☐

Hb g/dl	MCV fl	RDW %

C/Figures

C1/ Sysmex for CBC

C2/PCR Machine

C3/ Electrophoresis machine

C4/ UV light machine