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Association of Transmembrane Serin Protease 6 Gene (TMPRSS6-gene) Polymorphism with Iron and Ferritin Status in Iraqi Adults Patients with Iron Deficiency Anemia

A thesis

Submitted to the Council of College of Medicine, University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master in Science Clinical Biochemistry

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بسم الله الرحمن الرحيم

﴿ وَمَا تَوْفِيقِي إِلَّا بِاللَّهِ ﴾

صَدَقَ اللَّهُ الْعَلِيُّ الْعَظِيمُ

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Supervisor certification

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We, the examination committee, certify that we have read thesis entitled " **Association of Transmembrane Serin Protease 6 Gene (TMPRSS6-gene) Polymorphism with Iron and Ferritin Status in Iraqi Adults Patients with Iron Deficiency Anemia**

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Dedication

This work is dedicated to:

My Mother…My mother's love is all I have to complete my life Iam here thanks to her, may God have mercy on her...

To… My lovely sons Sajad and Zain who have suffered a lot, and I love you all are very much...

To… Every knowledge student…

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List of Abbreviations

Abbreviations	Details
BMI	Body mass index
BMP6	morphogenetic protein 6
Cdna	Complementary DeoxyriboNucleic Acid
CHF	chronic heart failure
CI	Confidence Interval
CKD	chronic kidney disease
CPD	chronic pulmonary disease
DcytB	duodenal cytochrome B
DMT1	divalent metal transporter 1
DNA	Deoxyribonucleic Acid
EDTA	Ethylene di-amine tetra acetic acid
ELISA	Enzyme linked immune sorbent assay
ERFE	Erythroferrone
ESA	erythropoiesis-stimulating agents
FPN	Ferroportin
GI	Gastrointestinal
GWAS	Genome Wide association study
HAMP	Hepcidin antimicrobial peptide
Hb	Hemoglobin
HO	haem oxidase
HWE	Hardy-Weinberg equilibrium
hypoxia-inducible	HIF-2α factor

IBD	inflammatory bowel disease
IDA	iron deficiency anaemia
IRIDA	iron-refractory iron deficiency anemia
LDLR	low-density lipoprotein receptor
MCH	Mean Corpuscular Hemoglobin
MCV	Mean Corpuscular Volume
mRNA	Messenger Ribonucleic Acid
NSAIDs	Nonsteroidal anti-inflammatory drugs
PNH	paroxysmal nocturnal hemoglobinuria
PPI	proton-pump inhibitors
QoL	quality of life
RBCs	Red Blood Cells
RFLP	Restriction Fragment length Polymorphism
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SPSS	Software Package for Social Science
Taq Polymerase	Thermus aquaticus polymerase
TBE buffer	Tris borate EDTA buffer
TSAT	transferrin saturations
UPA	Usability Professional Association
WHO	World Health Organization

Summary

Iron deficiency anemia (IDA) is the highest nutritional deficiency worldwide. It is a multifactorial disease, with a higher morbidity rate.

TMPRSS6 polymorphisms importantly rs855791 and AQP1 rs10244884 are found to play an essential role in iron homeostasis in the human body play an essential role in iron homeostasis in the human body.

The rs855791 (T > C) polymorphism is highly associated with iron levels, and multiple blood parameters, leading to IDA and the same as in the AQP1rs10244884.

These analytical study aimed to investigate the association between TMPRSS,AQP1 and risk of iron deficiency anemia. One hundred subjects were recruited for this study.

One hundred subjects were recruited for this study 50, patients affected with iron deficiency anemia and 50 healthy volunteers as a control group. Patients data (age sex, Hb, MCV, MCH, MCHC, iron, ferritin, ascorbic acid (was collected from patient's medical files by questionnaire. Five ml of blood were got from every subject by vein 2ml was set into EDTA tube and mixed carefully, the blood is stored in an EDTA tube at (-20°C) and used for DNA extraction. The rest of the 3ml drawn gradually into dispensable tubes containing isolating gel genomic DNA was extracted by salting out method and the TMPRSS6C/T polymorphism and AQP1were analyzed using polymerase chain reaction. Amplified fragments separated on 2% agarose gel stained with ethidium Bromide and demonstrated by gel documentation system, which produce single band at 249 bp represented C homozygous (CC) in caseTMPRSS6 and produce single band at 192bp represented C homozygous about AQP1 .

The data analyzed by computer program SPSS version 7 TMPRSS 6-gene investigated by PCR. The TT genotype of TMPRSS 6 C/T polymorphism was higher frequent in IDA patients.

But the well gene and mutant gene within the IDA patients had no interaction with the CBC parameters) Hb, MCV, MCHC, MCH, RBC.

AQP1 gene investigated by PCR, the TT genotype of AQP1 polymorphism was higher frequent in IDA patients.

But the well gene and mutant gene within the IDA patients had no interaction with the CBC parameters) MCV, RBC, MCHC, MCH.except HB.

It is significant with mutant gene, and we find there are no relationship between Age, Sex and BMI with IDA, and the result show relationship between Ascorbic acid and SNPS.

In conclusion, there were statistically significant association between TMPRSS 6 C/T polymorphism, AQP1 and risk of IDA among Iraqi patients in Baghdad state.

Chapter One

Introduction and Literature Review

1. Introduction

1.1 Iron Defiency Anemia

1.1.1 Background

Anemia is the most common hematologic disorder, iron deficiency being the leading cause worldwide [1]. Often, anemia is the presenting sign of a more serious underlying condition that, if left untreated, can generate consequent morbidity[2].

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). Many factors can cause IDA among women including dietary deficiency or gastrointestinal disturbances as well as multiple pregnancies due to low iron stores and insufficient socio-economic requirements [3].

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 [4].

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 [5].

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 [6].

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 [7].

1.1.2 Definition

The WHO has recognised iron deficiency anaemia (IDA) as the most common nutritional deficiency in the world, with 30% of the population being affected with this condition [8]. While IDA is more prevalent in children and women, adult men are also susceptible depending on their socioeconomic status and health conditions [9].

Although the most common causes of IDA are gastrointestinal (GI) bleeding and menstruation in women, decreased dietary iron intake and absorption are also culpable causes [10].

Iron is required for various cellular functions, including but not limited to enzymatic processes, DNA synthesis, oxygen transport and mitochondrial energy generation [11-12] such as, the symptoms of IDA can vary over a wide range. Shortness of breath, fatigue, palpitations, tachycardia and angina can result from reduced blood oxygen levels. This resultant hypoxemia can subsequently cause a compensatory decrease in intestinal blood flow, leading to motility disorder, malabsorption, nausea, weight loss and Central hypoxia can cause headaches, vertigo and lethargy as well as cognitive impairment with several studies showing an improvement in cognitive functions once anaemia has normalized [13-14].

It is well known that IDA significantly affects quality of life (QoL) [15]. With recent evidence demonstrating that treating IDA improves QoL, regardless of the underlying cause for anaemia [16-17].

1.1.3 Pathophysiology

Iron is an essential element and is controlled and iron recycling [18].

Dietary iron can be found in two forms:

Haem and non-haem iron. Haem iron is easily absorbable and arises from haemoglobin (Hb) and myoglobin in the form of animal meat, poultry and fish. Non-haem iron is mostly found in plant food but is not as easily absorbable. Compounds such as phytate, oxalate, polyphenols and tannin, which are found in plants, diminish the uptake of non-haem iron, as do some drugs, such as proton pump inhibitors [19-20]. Ascorbic acid, citrate and gastric acid, conversely, facilitate iron absorption [21].

In a healthy diet, approximately 5–15mg of elemental iron and 1–5mg of haem iron are ingested daily although only 1–2mg is ultimately absorbed into the intestine, predominantly in the duodenum and proximal jejunum [22].

Show in the (**Fig. 1.1**) for details on the iron absorption .

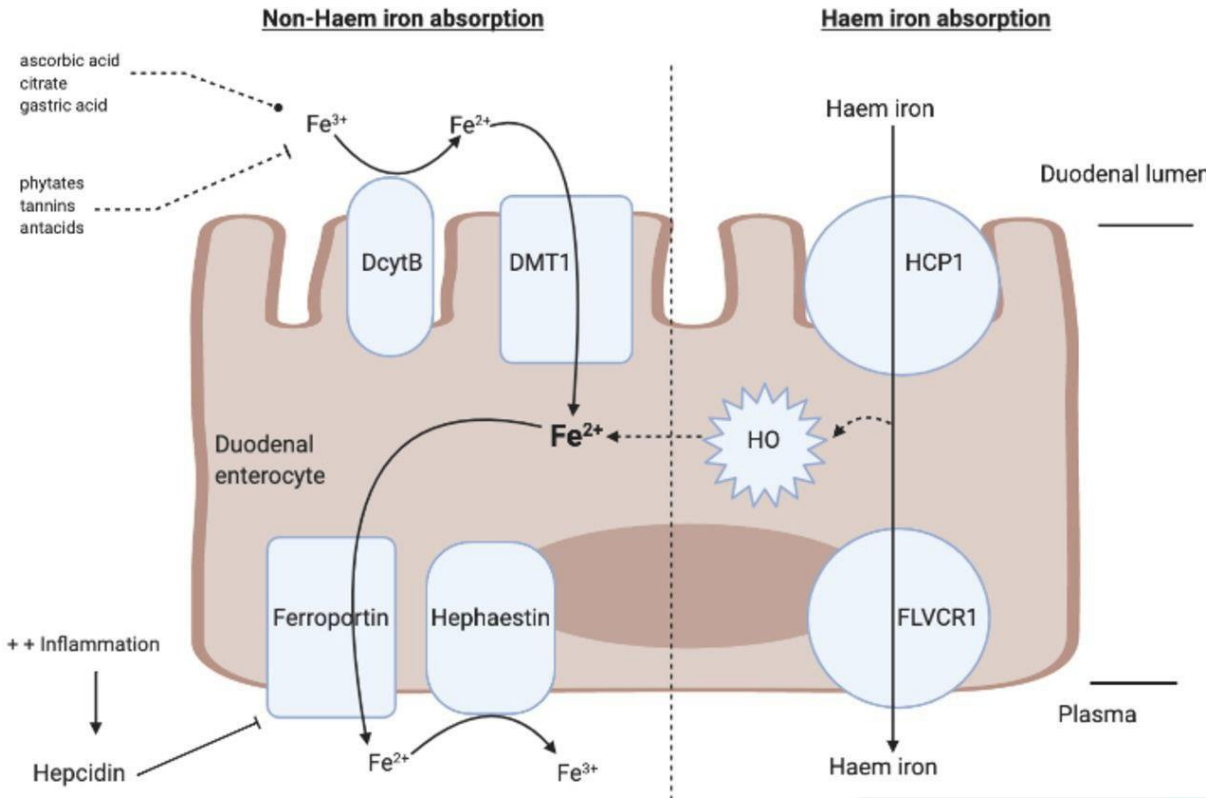


Figure [1.1]: Iron absorption pathways.[23]

1.1.4. Assessment and diagnosis

The WHO defines anaemia as blood Hb level below 130g/L in men and 120g/L in women, In isolated iron deficiency, serum ferritin (the storage molecule for iron) should be less than 30ug/L[24]. However, ferritin is an acute phase protein and can be increased in the presence of inflammation [25]. Thus, if there is evidence of concomitant inflammation, such as elevated C reactive protein, ferritin less than 100ug/L is indicative of IDA. Serum iron and transferrin saturations (TSAT) will be reduced with TSAT less than 20% required for the diagnosis of IDA[26].

1.1.5 Epidemiology

Anaemia affects one-third of the world population with IDA being the top cause[27]. IDA is highly prevalent in preschool children (<5 years), women in the reproductive age and pregnant women with prevalence rates, reaching up to 41.7%, 32.8% and 40.1%, respectively (2016 Global Health Observatory data)[28].

Reliance on a vegan diet, malabsorption syndromes and heavy menstrual bleeding are also high-risk categories in high-income countries, with around two-third of women with heavy menstrual bleeding having ID/IDA [29-30].

IDA is more difficult to treat in the elderly population and only represents around 30% of anaemia cases, as other types of anaemia may exist [31].

Frequent blood donation is also a poorly recognized cause of IDA. In one study of 2425 individuals, 16.4% and 48.7% of frequent male donors showed the absence of iron stores and iron-restricted erythropoiesis, respectively, with the corresponding proportions of 27.1 and 66.1% for females[32]. Although formal estimates on the prevalence of genetic forms of IDA are lacking, IRIDA is thought to represent less than 1% of the cases of IDA seen in medical practice[29]. The reported prevalence of ID/IDA in chronic inflammatory conditions varies greatly amongst different studies, depending on the thresholds of iron parameters used to define ID.ID affects anywhere between 37% and 61% of patients with chronic heart

failure [33-34] and between 24% and 85% of patients with chronic kidney disease[35-36], higher rates being associated with advanced disease. It is also documented in 13-90% of patients with inflammatory bowel disease depending on disease activity and severity, and whether measured in the outpatient or inpatient setting [35]. ID and IDA have also been reported in 42.6% and 33% of cancer patients, respectively, and associated with advanced disease, close proximity to cancer therapy and poor performance status in patients with solid tumours [37-38].

In the surgical setting, preoperative anaemia is present in around one-third of patients undergoing major surgery with over two-third of cases having IDA. ID is also observed in over half of surgical patients without anaemi, the prevalence of postoperative anaemia can reach up to 90% [39].

1.1.6 Etiology

There are multiple physiologic, environmental, pathologic and genetic causes of iron deficiency (ID) that lead to IDA (**Fig. 1.2**). More importantly, etiologies may vary considerably or tend to coexisting different patient populations (children, women and elderly), geographies (developing and developed countries) and specific clinical conditions [40-41].

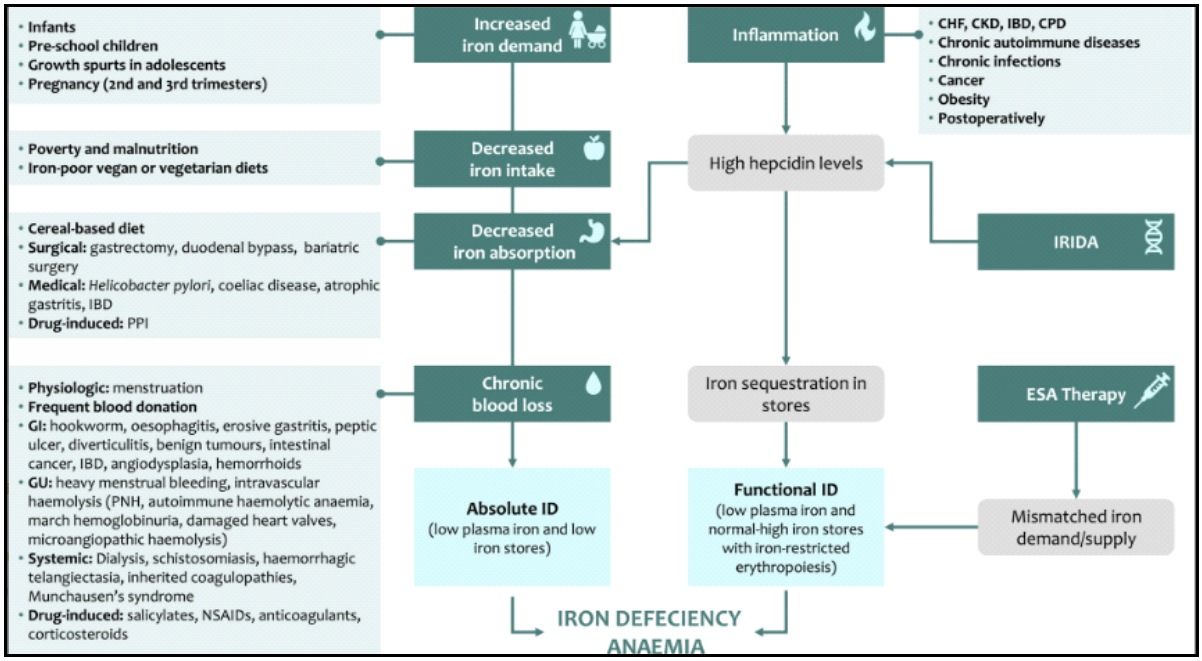


Figure [1.2]: Various etiologies of iron deficiency anemia. CHF, chronic heart failure; CKD, chronic kidney disease; CPD, chronic pulmonary disease; ESA, erythropoiesis-stimulating agents; IBD, inflammatory bowel disease; ID, iron deficiency; IRIDA, iron-refractory iron deficiency anemia; NSAIDs, Nonsteroidal anti-inflammatory drugs; PNH, paroxysmal nocturnal hemoglobinuria; PPI, proton-pump inhibitors [42].

1.2 Absolute iron deficiency anaemia

Absolute ID' refers to the reduction of total body iron stores (mostly in macrophages and hepatocytes) which may or may not progress in severity leading to IDA [29].

Absolute ID may occur in instances of increased demand, decreased intake, decreased or malabsorption, or chronic blood loss. Increased demand is usually physiologic and commonly noted in infants, preschool children, growth spurts in adolescents and pregnancy (mostly second and third trimesters)[43] Decreased iron intake can be a direct consequence of poverty and malnutrition as the case with many children and pregnant women in developing countries or attributed to iron-poor vegan or vegetarian diets.[29-43] D ecreased absorption is recognized in certain dietary practices, with several inhibitors of iron absorption

recognized such as calcium, phytates (present in cereals) and tannins (present in tea and coffee) [11].

It is also attributed to surgical procedures including gastrectomy, duodenal by pass and bariatric surgery (especially Roux-en-Y gastric bypass) which increase stomach pH and decrease conversion to ferrous iron. Certain medical conditions are also known to be associated with decreased iron absorption such as infection with Helicobacter pylori (competition for iron, increased pH and reduction of vitamin C), coeliac disease (gluten-induced enteropathy), atrophic gastritis (increased pH) and inflammatory bowel disease [[44-]. It should be noted that such conditions of decreased iron absorption, in most instances, render patients refractory to oral iron therapy [45].

The use of proton-pump inhibitors may also contribute to decreased iron absorption [46].

1.2.1 Women and pregnancy

In women with heavy menstrual bleeding, IDA is associated with reduced quality of life and general well-being, and severe anaemia may lead to hospitalization[47-48]. More importantly, it results in many women entering pregnancy with anaemia. In pregnant women, IDA is associated with an increased risk of preterm labour, low neonatal weight and perinatal complications [47].

Severe IDA is also associated with increased newborn and maternal mortality, due to lower tolerance to excessive blood loss during delivery and increased risk of infections[48-49]. Infants born to anaemic mothers are more likely to have IDA themselves [50].

ID also carries a negative impact on the mother–child relationship and the child's cognitive development, an effect measurable for up to 10 years despite iron repletion [51].

1.2.2 Clinical Implications

IDA usually develops slowly from the progression of ID. The full range of symptoms and signs associated with IDA at presentation and follow-up are reviewed elsewhere [52], whilst certain clinical consequences are described in this section and [**Fig. 1.3**]. It should be noted, however, that due to nonspecificity of symptoms and the co-occurrence of IDA with multiple morbidities, confounding effects of underlying diseases on the observed association between IDA and outcomes cannot be fully dismissed. IDA is associated with decreased cognitive performance and delayed motor and cognitive development in children, decreased physical performance and quality of life in adults, especially women in the reproductive age group, and cognitive decline in the elderly [53-54]. Although these symptoms remain nonspecific, they can be attributed to low delivery of oxygen to body tissues in IDA. They may also occur as a direct effect of ID [55-56], probably due to reduced iron levels in muscle or brain tissue, and impact on energy production, myoglobin synthesis and brain development. Additional effects of ID attributed to the impact of low iron levels on DNA replication and cell cycle (oral lesions, hair loss, nail abnormalities), immune response (increased susceptibility to infections), myelogenesis and neurotransmission (restless leg syndrome) and inhibition of cytochrome P450 production (altered drug metabolism) are reviewed elsewhere[57].

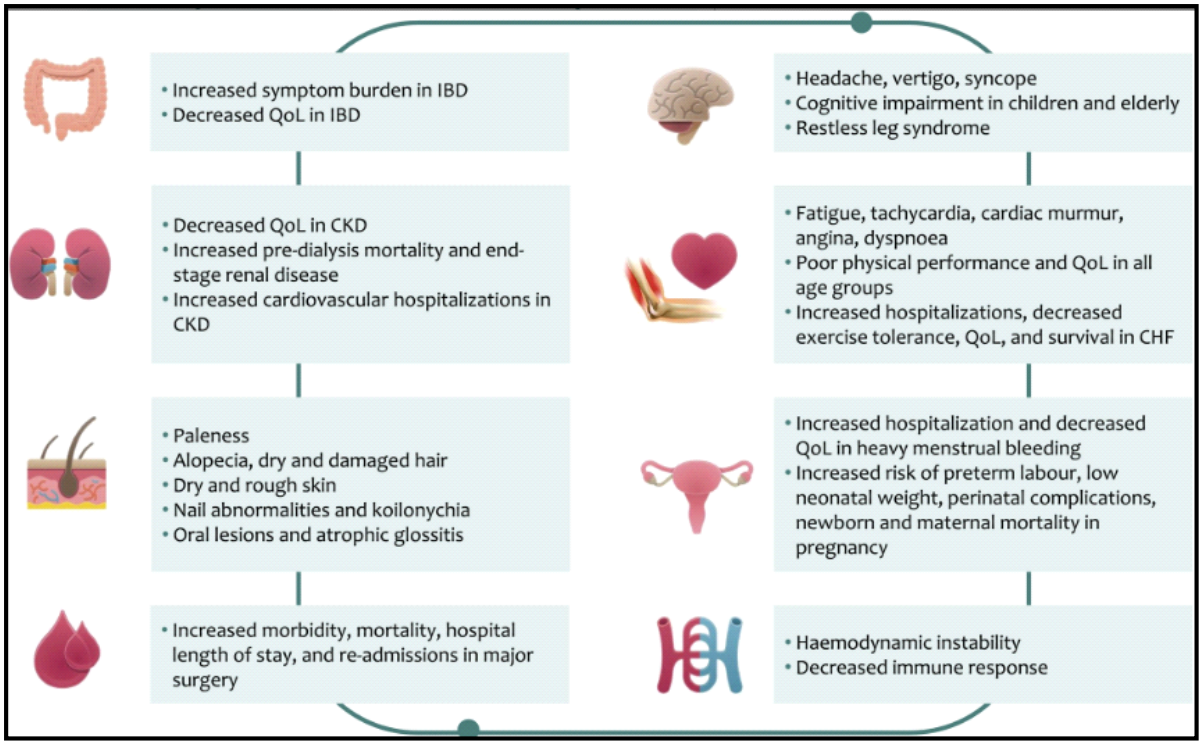


Figure [1.3]: Clinical implications of iron deficiency anemia. CHF, chronic heart failure; CKD, chronic kidney disease; IBD, inflammatory bowel disease; QoL, quality of life [41].

1.3 The Parameters

1.3.1 Iron

One of the important trace essential minerals, iron, controls the differentiation and development of living cells, as well as Iron may interact and carry oxygen to various areas of the body via transfer of electrons across cells, which has an influence on genome synthesis, as well as contribute in a variety of metabolic process that are necessary to life [59].

The liver is a major storage organ for iron. Approximately one third of the body's total iron is deposited in hepatocytes, sinusoidal mesenchymal cells, and reticuloendothelial cells[60]. Liver plays an important role in iron metabolism. It is the major production site of the iron regulatory peptides: ferritin, transferrin, and hepcidin. Liver derangements, therefore, have a direct effect on iron regulation[61].

1.3.2 Ferritin

Ferritin is highly symmetrical and persistent iron-containing protein that was crystallized, named, and discovered in 1937. Because it contains a huge cavity that really can store a lot of iron, it was named the major iron storage protein [62].

Ferritin's capacity to bind iron ions and promote mineralization among those ions by coupling its ferroxidase action with the chemical characteristics of the cavity environment is one of its most essential features, The mineral core, which may contain reach to 4000 Fe atoms in mineral form, is protected and kept in solution by the protein covering [63].

Ferritin would be almost abundant, and it has a wide range of functions. It's mostly present in the cytoplasm, but it's also find in the nucleus, mammalian mitochondria, plant plastids, insect endoplasmic reticulum (ER), and circulation plasma. Ferritins have such a ferroxidase action that uses the same chemicals as the harmful Fenton reaction, thus they operate as antioxidants and control the availability of ferrous iron within cells. Over the last few decades, periodic reviews on ferritin have appeared, each focusing on a different aspect of this molecule [64].

1.3.3 Ascorbic acid

Ascorbic acid functions primarily as a cofactor for microsomal monooxygenases (hydroxylases) and oxidases. In most animals, ascorbic acid is synthesized from glucose in the liver or kidney. In some animals, however, a deficiency of gluconolactone oxidase, a last step in ascorbic acid synthesis, results in the need for a dietary source. The enzymes for ascorbic acid production in the cold-blooded vertebrates (fishes, amphibians, and reptiles) are located in the kidneys [65].

Present-day birds, whose ancestors appeared about the same time as the mammals, have a kidney-liver transition. The older order of present-day birds,

such as the ducks, pigeons, and hawks, synthesize ascorbic acid in their kidneys, whereas in the more recent order they produce ascorbic acid both in their kidneys and livers (e.g., of the perching and song birds). Mammals produce ascorbic acid in the liver. Of the mammals that do not produce ascorbic acid (e.g., primates and guinea pigs), so-called pseudogenes for L-gulonolactone oxidase exist. The 164-nucleotide sequence of exon X of this gene contains nucleotide substitutions throughout its sequence with a single nucleotide deletion, a typical example of a pseudogene [66].

Ascorbic acid (Vitamin C) has been shown to play a role in iron metabolism in the body. Vitamin C can increase the absorption of non-heme iron, which is the type of iron found in plant-based foods. By improving the absorption of nonheme iron, Vitamin C may help to prevent iron-deficiency anemia. Additionally, Vitamin C can reduce oxidative stress in the body, which can help to maintain healthy iron levels. Therefore, adequate intake of Vitamin C is important for maintaining healthy iron levels and preventing anemia [67].

Vitamin C has several mechanisms that contribute to its role in iron metabolism**:**

- Iron absorption: Vitamin C enhances the absorption of non-heme iron, the type of iron found in plant-based foods, by reducing it to its more absorbable ferrous form (Fe^{2+}) and preventing its reoxidation to its less absorbable ferric form (Fe^{3+}).
- Antioxidant: Vitamin C acts as an antioxidant, neutralizing reactive oxygen species that can interfere with iron metabolism. This helps to maintain healthy iron levels in the body.
- Enzyme activation: Vitamin C is required for the activation of some enzymes involved in iron metabolism, such as ferroxidase, which is involved in the release of iron from cells.

- Regulation of iron transport: Vitamin C has been shown to regulate the expression of iron transport proteins, such as transferrin, and to modulate the uptake of iron by cells.
- Vitamin C plays an important role in iron metabolism, and adequate intake of Vitamin C is important for maintaining healthy iron levels and preventing iron-deficiency anemia [68].

1.4 Iron-Refractory Iron Deficiency Anemia

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 [69].

1.4.1 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 transmembrane 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 [70].

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 ferroportinmediated iron release to plasma. In cell models this serine protease cleaves the BMP co-receptor haemojuvelin, attenuating the BMP-mediated hepcidin activation [71].

The gene was cloned in mask mice, a product of N-ethyl-Nnitrosourea (ENU) mutagenesis, which had microcytic anaemia and a truncated matriptase-2 devoid of the catalytic domain [72].

1.4.2 Pathophysiology

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 [73].

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 [74].

Hepcidin is a small peptide hormone produced by the liver that is detectable in serum and urine, is a central regulator of iron homeostasis [75].8*

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 [76].

It is possible that single nucleotide polymorphisms (SNPs) or specific haplotypes play some role in the disease [77]. 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 [78] or in the presence of increased requests, such as pregnancy [79].

Common genetic variants (i.e. SNP) in the TMPRSS6 gene in several populations are associated with changes in the normal erythrocyte and iron parameters [80].

1.4.3 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 [81].

The production of 11 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 [82].

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 [82].

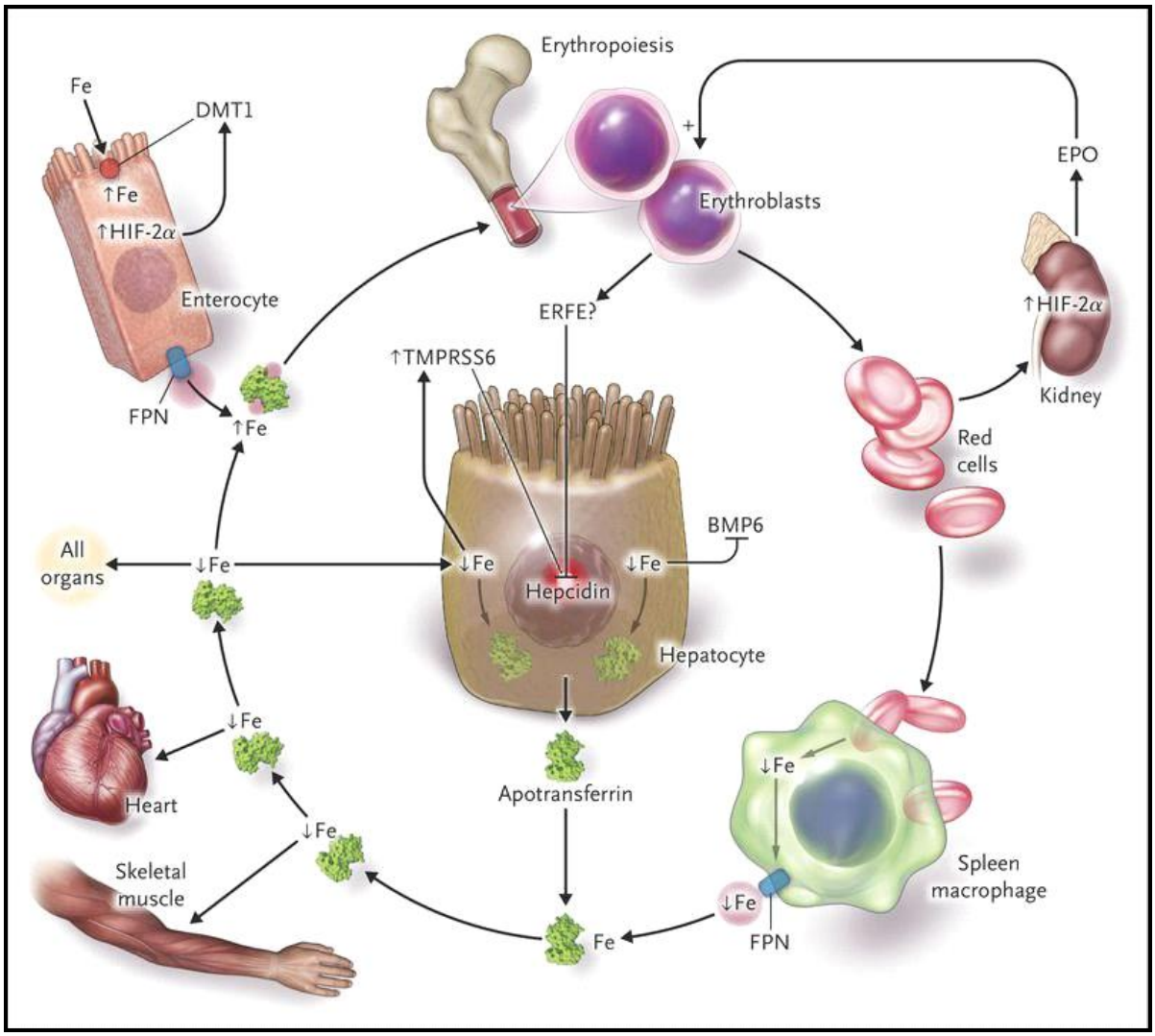
HIF-2α increases the expression of the duodenal divalent metal transporter 1 (DMT1) on the apical surface of enterocytes to increase the transfer o f dietary iron from the lumen to enterocytes [83].

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) [84].

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 [85].

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) [86].



[Figure. 1.4]: 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 [27].

1.4.4 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 [88].

1.4.5 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 [89].

Matriptase-2 can degrade in vitro extracellular matrix components such as fibronectin, fibrinogen, and type I collagen and to activate singlechain 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 [90].

1.4.6 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 [91].

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 [92].

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 [93].

Some studies reported the mutations in TMPRSS6 have been identified in IRIDA patients from other populations [94].

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 [95].

Aims of this study

1- Study the genetic variations of trans-membrane serine protease-6 (TMPRSS6) SNPs rs855791 and rs10244884 and its relationship with the levels of iron and ferritin and study the correlations with age, and body max index (BMI).

2- Study the levels of ascorbic acid and suggested the correlation with these SNPs.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and kits

The chemicals, laboratory kits and reagents used in this research are showed in the [**Table 2.1**].

Table [2.1]: Chemicals

No.	Chemicals	Company	Origin
2-	Agarose	CONDA	Spain
7-	DNA ladder 1000bp	Cyntol	Russian
8-	DNA loading dye	Promega	USA
4-	Ethidium- Bromide Solution	Bio- basic	Canada
10-	Firritn ELISA Kit	Solar biotech	USA
6-	GO Taq® G2 Green Master Mix	Promega	USA
9-	Iron	Abbott	USA
1-	Primers	Macrogen	Korea
5-	Restriction enzyme	Biolabs	England
3-	TBE (tris-borate EDTA)	INTRON Biotechnology	Korea
11-	Tris Borate EDTA (TBE)	Thomas baker	India
12-	Vit-cELISA Kit	Solar biotech	USA

2.1.2 Instruments and Equipments

The [**Table 2.2**] below showing the instruments and equipments used in this study.

Table [2.2]: Instruments and equipments

No.	Apparatuses	Company	Origin
6-	Auto hemoanalyzer	Abbott	USA
13-	Centrifuge	Hettich	Germany
1-	Conventional PCR	analytik jena	Germany
11-	Deep freeze	GFL	Germany
3-	Electrophoresis	Bioneer	South Korea
4-	ELISA Washer	Paramedical	Italy
7-	Incubator	Binder	Germany
5-	Microliter plate reader for ELISA test (Paramedical, Italy)	Paramedica	Italy
2-	Microlitre centrifuge	Hermle Labortechnik	Germany
9-	Micro-pipettes 10-100 micro liter	Nexty	Japan
8-	Micro-pipettes 200, 300, 400, 600, 900,1000 micro liter	Slamed	Germany
12-	Refrigerator and freeze	LG	South Korea
10-	Vortex	Heidolph	Germany

2.1.3 Subjects

This study was designed as a case-control study conducted on 100 subjects those divided into two groups: the first one includes patients with Iron defecience anemia, and the second one includes individuals as healthy a control group.

The present study was completed in Al-Imam Ali general hospital in Baghdad. The collection of samples was directed during the period from 12th of December 2022 until 1st of April 2023. Questionnaires were created to collect

data from the control and patients groups. Also all subjects were selected in random state.

2.1.4 Patients Group

This group was consisted of 50 patients with Iron Defeciency (11 male + 39 female). The patients' ages between (20-50) years. All patients were diagnosed by physicians and according to especially criteria.

2.1.5 Control Group

The control group was consisted of 50 individuals (12 male + 38 female), they were taken from out people and relative. This group's age between (20-50) years old.

2.1.6 Inclusion criteria

1. Persons with IDA.
2. Age from 20 to 50 years.
3. Pregnancy

2.1.7 Exclusion criteria

Patients with known other inherited microcytic anemia.

2.1.8 Samples Collection

Venous blood sample were pulled from control and patients by utilizing the disposable syringe as a part of the sitting position and in Random status. Five ml of blood were got from every subject by vein, 2 ml was set into EDTA tube and mixed carefully, the blood is stored in an EDTA tube at (-20)°C and used for DNA extraction. The rest of the 3 ml drawn gradually into dispensable tubes containing isolating gel. while the rest of the blood in gel - containing tubes was let to clot at room temperature for 30 minutes and after that centrifuged at 3000 xg for 10 min, then partitioned into little aliquots and kept in (-20)ºC until analysis for estimation of Iron, Ferritin, Ascorbic acid.

2.1.9 Study design

Volunteers were under through anthropometric measurements at the beginning, which include height, and weight measurements. Serums of each sample were running under both ELISA technology to get the levels of Ferritin and Ascorbic acid, as well as genetic analysis technology such as PCR and RFLP-technique for detect the target gene (SNP gene) and also used molecular absorption technology to analyze serum Iron by the use Abbott c4000.

2.1.10 Measurement of Body Mass Index (BMI)

The BMI is defined as the body weight divided the square of the height of the body in meters and is expressed worldwide in kg/m^2 units, resulting from kilograms of mass and meters of height [96].

The BMI number and classifications are listed below according WHO.

Severely underweight - BMI less than 18.5 kg / m^2

Table 2-3 the range of BMI in adult is classified according to the following

Weight standard	BMI
Under weight	Below 18.5
Healthy weight	18.5 – 24.9
Overweight	25.0 – 29.9
Obese	30.0 and higher

(Also referred to as severe, extreme, or massive obesity) [97].

2.2 Biochemical study

2.2.1 Determination of Serum Human Ferritin:

Concentration Ferritin concentration is measured by enzyme linked immunosorbent assay kit (ELISA). [Biosoruce Technology Laboratory] [98].

2.2.2 Assay Principle

This ELISA kit uses sandwich–ELISA as the method for the accurate quantitative detection of human Ferritin. The plate has been pre-coated with human FE antibody. FE present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human FE.

Antibody is added and binds to FE in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated FE antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step.

Substrate solution is then added and color develops in proportion to the amount of human FE. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.2.3 Reagents Preparation:

All reagents provided are stored at 2-8°C.

Wash buffer

A volume of 20 ml of concentrated wash buffer was diluted into 480ml of distilled water to yield 500 ml of washing buffer.

Standard:

Reconstituted the 120µl of the standard (480ng/ml) with 120µl of standard diluent to generate a 240ng/ml standard stock solution.

The lid was tightened and the standard was let to stand for 15 minutes and turned it upside down for several times. Then serial dilutions were made as needed. The recommended concentrations were as follows: 240, 120, 60, 30, 15 and 0 ng/ml.

2.2.4 Assay procedure

1- All reagents were brought to room temperature before use.

2- Fifty microliter of the each standard was added to the standard well.

3- A volume of 40 µl of sample and then 10 µl of ferritin antibody were add to testing sample wells.

4- A volume of 50 µl of streptavidin -HRP reagent was dispensed into each well. Covered with a sealer and incubated for 60 minutes at 37°C.

5- The wash process was repeated for five times by filling each well with wash buffer (approximately 350 µl).

6- The liquid was removed at each step was essential to good performance then remaining wash solution was removed by aspirating and the plate was invert and blot it against clean paper towels.

7- A 50µl volume of substrate solution A and 50µl volume of substrate solution B were add to each well.

8- The microplate wells covered with a new sealer then incubated for 10 minutes at 37°C.

9- The reaction was stopped by adding 50 µl of stop solution to each well. The color was changed from blue to yellow color.

10- The absorbance was read the optical density at 450 nm using microtiter plate reader within10minutes.

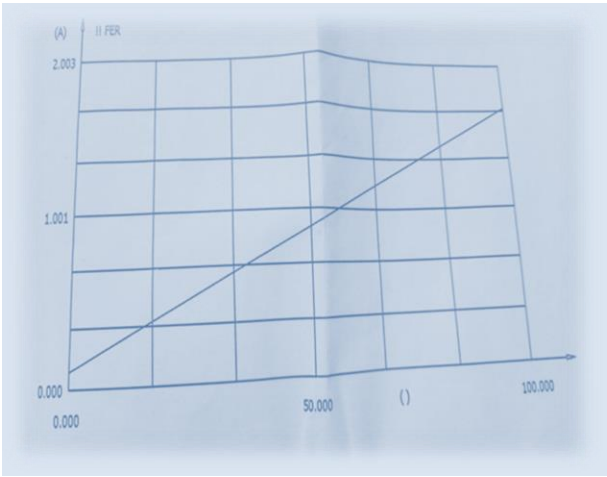


Figure [2.1]: Standard curve of ferritin

2.2.5 Determination of Iron

2.2.5.1 Principles of procedure

PRINCIPLES OF PROCEDURE

At a pH of 4.8, iron is released from transferrin to which it is bound, and then quantitatively reduced to a ferrous state. The iron forms with Ferene-S*, a stable colored complex of which the color intensity is proportional to the amount of iron in the sample. Particular reaction conditions and a specific masking agent almost entirely eliminate the interference from copper.

* Ferene-S = 3-(2-pyridyl)-5,6-bis-[2-(5-furylsulfonic acid)]-1,2,4-triazine

Methodology: Ferene

REAGENTS

Reagent Kit

MULTIGENT Iron is supplied as a liquid ready-to-use, two‑component kit which contains:

6K95-30

3 x 88 mL

3 x 11 mL

Estimated tests per kit: 918*

6K95-41

10 x 88 mL

10 x 11 mL

Estimated tests per kit: 3,060*

*Calculation is based on the minimum reagent fill volume per kit.

Reactive Ingredients Concentration

Acetate buffer pH 4.8 1.4 mol/L

Guanidine hydrochloride 4.5 mol/L

Thiourea 65 mmol/L

Ferene-S ≥ 20 mmol/L

Ascorbic acid ≥ 0.5 mol/L

Nonreactive Ingredients: contains buffer and contains buffer and Stabilizer

2.2.6 Determination of Ascorbic acid (Biosource technology laboratory)

2.2.6.1 Principle

Vit.C ELISA kit applies the competitive enzyme immunoassay technique utilizing a polyclonal anti-Vit.C antibody and an Vit.C-HRP conjugate. The assay sample and buffer are incubated together with Vit.C-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450nm in a microplate reader.

The intensity of the color is inversely proportional to the Vit.C concentration since Vit.C from samples and VC-HRP conjugate compete for the anti-Vit.C antibody binding site. Since the number of sites is limited, as more sites are occupied by Vit.C from the sample, fewer sites are left to bind Vit.C-HRP conjugate.

A standard curve is plotted Vit.C ELISA kit applies the competitive enzyme immunoassay technique utilizing a polyclonal anti-Vit.C antibody and an Vit.C-HRP conjugate. The assay sample and buffer are incubated together with Vit.C-HRP conjugate in pre-coated plate for one hour.

After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex.

Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450nm in a microplate reader. The intensity of the color is inversely proportional to the Vit.C concentration since Vit.C from samples and Vit.C-HRP conjugate compete for the anti-Vit.C antibody binding site.

Since the number of sites is limited, as more sites are occupied by Vit.C from the sample, fewer sites are left to bind Vit.C-HRP conjugate. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The Vit.C concentration in each sample is interpolated from this standard curve. Relating the intensity of the color (O.D.) to the concentration of standards[100].

2.2.6.2 Reagents and Preparation

1- Bring all kit components and samples to room temperature(20-25°C) before use.
2- Samples - Please predict the concentration before assaying. If concentrations are unknown or not within the detection range, a preliminary experiment is recommended to determine the optimal dilution. PBS (pH 7.0-7.2) or 0.9% physiological saline can be used as dilution buffer.
3- Wash Solution - Dilute 10 mL of Wash Solution concentrate (100×) with 990 mL of deionized or distilled water to prepare 1000 mL of Wash Solution (1×). If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. The 1× wash solution is stable for 2 weeks at 2-8°C.
4- Do not dilute the other components which are ready- to-use.

2.2.6.3. Assay Procedure

Please read Reagents Preparation before starting assay procedure. It is recommended that all Standards and Samples be assayed in duplicate. It is strongly recommended to do a preliminary experiment before measuring all samples.

1- Secure the desired numbers of coated wells in the holder then add 100 µL of Standards (Shake the bottle of each standard gently by hand and Pipette up and down the solution of standard for 3times before adding) or Samples to the appropriate well. Add 100µL of PBS (pH 7.0-7.2) in the blank control well.

2- Dispense 10µL of Balance Solution into 100µL samples only, mix well. (NOTE: This step was required when the sample was cell culture supernatants, body fluid and tissue homogenate; if the sample was serum or plasma, then this step should be skipped.

3- Add 50µL of Conjugate to each well (NOT blank control well). Mix well. Mixing well in this step is important. Cover and incubate the plate for 1 hour at 37°C.

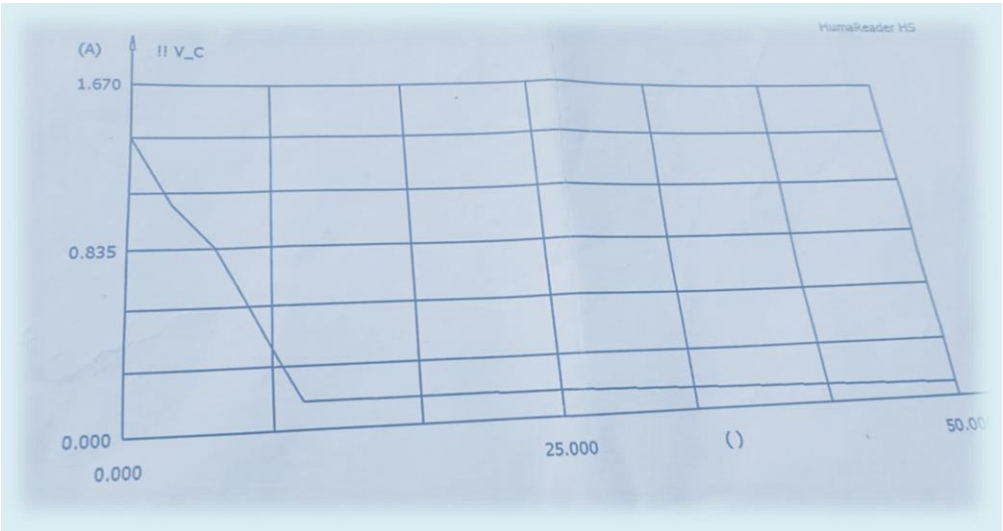


Figure [2.2]: Standard curve of Vit.C

2.3 Methodology of PCR test

2.3.1 DNA extraction

RBC Lysis

1. An anticoagulant-treat collecting tube was used to collect fresh human blood.
2. A total of 300 µl of fresh blood was added to a 1.5 milliliter micro-centrifuge tube to fill it halfway (not provided). If the sample is bigger than 300 µl, fill a sterilized 15 milliliters centrifuge tube with it (up to 1 ml).
3. Left the sample combination to remain at 25°C for ten minutes.
4. It was centrifuged at 3000-xg for five minutes, and all the supernatants are removed.
5. Then 100 µl of RBC Lysis Buffer was added to the pellet and the cells were resuspended by pipetting.

Cell Lysis

1. Then 200 µl of FABG Buffer was added to the sample mixture, and mixed well by vortexing.
2. Permitted the sample combination to rest at room temperature for ten minutes, or until it became clear. During incubation, the test tube inverted every 3min.
3. In a 70°C water bath, preheated the needed Elution Buffer (for Step 5 DNA Elution).

DNA Binding

1. The samples vortexed for 10 seconds after added 200µl of ethanol (96-100%). If a precipitate has developed, the sample was pipetted to mix it.
2. The FABG Column Installed to the Collection Tube. Carefully the sample was transferred to the FABG Column. For one minute, centrifuge at 18,000 x g. the FABG Column replaced in a new Collection tube after discarding the old one.

Column Washing

1. The FABG Column was filled with 400 µl of W1 buffer and centrifuged for 30 seconds at 18,000 x g. The FABG Column returned to the collection tube after discarding the flow-through.
2. The FABG Column was filled with 600µl of wash buffer and centrifuge d for 30 seconds at 18,000 xg. The FABG Column returned to the collection tube after discarding the flow-through.
3. Dried the column tube by centrifuging for a further 3 minutes at 18,000 x g.

Elution

1. The FABG Column was transported to a 1.5mL micro-centrifuge tube after drying.
2. 100µl of Pre-heated Elution. Buffer or TE added to the membrane center. Of FABG Column.
3. The FAGB Column placed in an incubator at 37°C for 10 minutes.
4. The DNA was eluted by centrifugation at maximum speed for 1 minute at 18,000x g.
5. The DNA was kept at 4 °C or -20 °C.

2.3.2 DNA concentration and purity

The concentration and purity of the isolated DNA were analyzed by a Nano drop spectrophotometer. DNA concentration measurements were recorded as µg/ml, while DNA purity was taken from the ratio obtained from the A260/280 absorbance formula. The 260/280 and 260/230 ratios were calculated to determine both the quantity and quality of the DNA. Whenever the 260/280 ratio was less than 1.7 and/or the 260/230 ratio was less than 1.7, the sample was re-extracted [101].

Table [2.4]: The set of primers used

Gene	Type primer	Sequence
TMPRSS6 rs855791 (V736A)	Forward Reverse	F: 5'-TAG AGA ACA GGG GCT CCA GG-3 R: 5'-ATG TGG GCA GCA T
Aquaporin 1 gene rs10244884	Forward Reverse	F: 5'- ATAGGTGCCACCCATGCTCC - : 5'- GCCTCTGCTCTGCTGACTCG-3' 3'

2.3.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was the most effective way and standard method to separate, identify, purify DNA fragments (DNA fragments of varied sizes rangged from 100 bp to 25 kb) and it is simple, rapid to perform. The location of DNA within the gel can be determined directly used stained. Bands containing as little as 1-10 mg of DNA can be detected by direct examination of the gel in ultraviolet light. Electrophoresis was the movement of a charged molecules, chiefly proteins and nucleic acids under the influence of an electric field, Loading dyes used in gel electrophoresis serve three major purposes:

1- They add density to the sample, allowing it to sink into the gel.
2- The dyes provide color and simplify the loading process.
3- The dyes move at standard rates through the gel, allowing for the estimation of the distance that DNA fragments have migrated. The rate of migration of a DNA molecule through a gel is determined by the following:

- size of DNA molecule.
- agarose concentration.
- DNA conformation.
- voltage applied.
- presence or use of staining.
- type of agarose and electrophoresis buffer.

2.3.4 Gel Electrophoresis for Analyze DNA Quality

1. Preparation of Tris-borate-EDTA buffer (TBE): A weight of 27g of Tris base with 14 g boric acid, and 1.86 g of EDTA (pH 8) dissolved in 500 ml distilled water.
2. The gel was prepared at a concentration of 1% for DNA extraction (2% for PCR production) by dissolving 0.3 of agarose in a 30 ml buffer solution TBE 0.5X and then heated the mixture for 1 min in the microwave.
3. The homogeneous solution of agarose was left until its temperature reaches 55°C, then 3µl of ethidiom bromid (10 mg/µl) was added to it and mixed with the mixture by turning the beaker.
4. The homogeneous mixture of agarose was poured into the gel tray and left to polymerize for 30 minutes.
5. After hardening, the agarose was transferred to an electrophoresis device and immersed in a TBE running buffer at a concentration of 0.5X. 6. Ten µl of DNA extraction (five µl of PCR production) was combined with 2 µl of loading-dye and carefully loaded by a mechanical pipette into the wells of the gel.
6. The electrophoresis was set to 100 volts and 70 A for 20 minutes for DNA extraction (50 minutes for PCR production) and the devise was carried out.
7. After completing the electrophoresis, the gel was imaged and the image was analyzed in order to determine the molecular weights of the DNA segments.
8. The gel was then photographed and analyzed using the CS analyzer® software to determine the extracted DNA molecular weight.
9. The gel was exposed to the UV light and the DNA bands was visible.

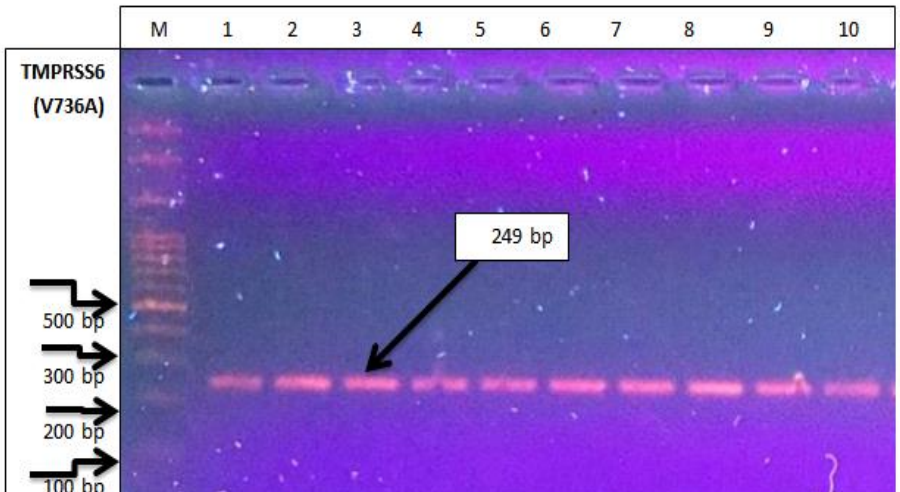
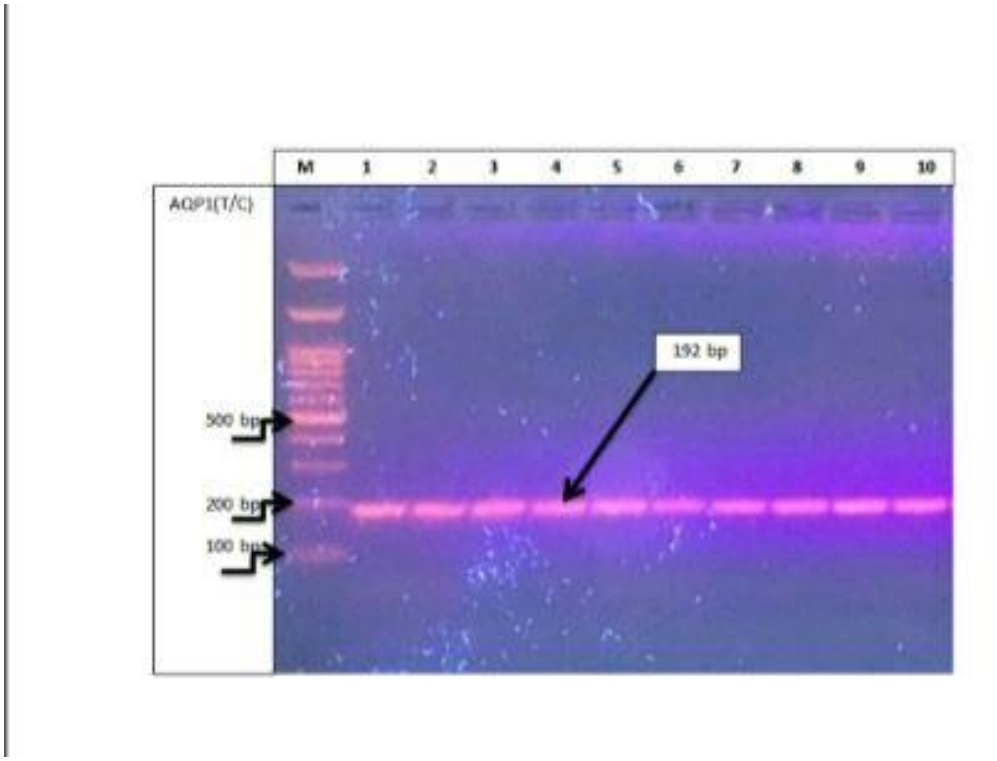


Figure [2.3]:A- Electrophoresis for Genomic DNA (TMPRSS6). Extracted genomic DNA agarose gel electrophoresis, the electrophoresis was conducted on 2% agarose, 100 volts for 20 min.

B- - Electrophoresis for Genomic DNA (AQP1). Extracted genomic DNA agarose gel electrophoresis, the electrophoresis was conducted on 2% agarose, 100 volts for 20 min



B- - Electrophoresis for Genomic DNA (AQP1). Extracted genomic DNA agarose gel electrophoresis, the electrophoresis was conducted on 2% agarose, 100 volts for 20 min.

2.3.5 Restriction enzyme add

10 µl of PCR product was taken and added to the PCR tube, 2.5 µl of the restriction enzymes and 2.5 µl of CutSmart® Buffer, and 10 µl of nuclease free water added to the same PCR tube to complete the total volume of the mixture,

25µl. The mixture was incubated at a temperature of 37°C for two hours in incubator, and after two hours the activity of the restriction enzyme was proven at a temperature of 65°C for 20 minutes.

2.3.6 The Polymerase Chain Reaction (PCR) steps

1- Initialization step

The reaction was heated to about 94-96°C (this study used 95.0°C). It required for DNA polymerases activation.

2- Denaturation step

This step was included heat the reaction to about 94-98°C (this study used 95.0 °C) for melted the DNA template by distraction of hydrogen bonds. The single stranded DNA molecules were yielded in this step.

3- Annealing step

The temperature of reaction was lowered to 50-65 °C (this study used 60.0°C). Allowed for the primers to annealed with single stranded of DNA template. The Taq polymerase attached to the primer- template hybrid and begined the formation of DNA.

4- Extension / Elongation step

The optimum activity of Taq polymerase occurs at 72-80 °C and (this study used 72.0 °C) was used with this enzyme.

DNA polymerase synthesizes a new DNA strand that complementary to the DNA template strand by adding dNTPs in 5 to 3 direction. The extension time depends on both the length of the DNA target and the type of DNA polymerase.

2.3.7 PCR Components of Amplification

The PCR G0 Taq® G2 Green Master- Mix Kit was used for amplification of ARG1gene. The PCR G0 Taq® G2 Green Master - Mix Kit was a premixed ready-to- use solution containing G0 Taq® G2 DNA Polymerase, MgCl2, dNTPs, and reaction buffers at optimal concentrations for efficient. Amplification of a wide range of DNA templates by PCR. G0 Taq® G2 Green Master Mix contains two dye (blue and yellow) that allow monitoring of progress during electrophoresis. The reactions assembled with G0 Taq® G2 Green Master Mix have sufficient density for direct loading onto agarose gels. G0 Taq® G2 DNA Polymerase exhibits 5 ' 3 ' exonuclease activity.

2.3.8 Thermocycler Program of Amplification

The PCR Thermocycler program that gave the results of amplification of TM PRSS6andAQP1 gene is shown in [**Table 2.4**]. Thermocycler program for

Table [2.5]: PCR Amplification of TMPRSS6 and AQP1 gene

Name of cycle	Temp °C	Time	No. of cycle
Initial denaturation	95	5 min	1 cycle
Denaturation	95	1min	35 cycle
Annealing	60	1min	35 cycle
Extension	72	1min	35 cycle
Final extension	72	5min	1 cycle

2.3.9 Agarose Gel Electrophoresis for PCR products

After execution PCR, 5 µL of each sample were loaded on 2 % agarose gel and stained with Ethidiume Bromide. The electrophoresis was performed at (55V for 45 minutes) to evaluate the PCR product.

2.3.10 Optimization of PCR Conditions

Started the optimization of PCR reaction for rs855791 genotyping and AQP1 with the thermo- cycling condition listed in the [**Table 2.5**].

Table [2.6]: Optimized reaction mixture for PCR

Composition	Concentration	Volume
Master mix	2.5 X	8
Mgcl2	25 MM	0.5
Forward primer	10 PM	1
Revers primer	10-20 ng/µl	1
DNA sample		2
Nucleases free water		7.5
Total volume		20

2.3.11 PCR-RFLP

Restrictive Digestive Digestion for the TMPRSS6 rs855791 Gene and AQP1 rs10244884 PCR-RFLP.

PCR-RFLP was a two-part method as indicated by its name. The first part consists of a traditional PCR with primers surrounded the possibly mutated area. The PCR product was then subjected to restriction enzymes which have the ability to cut DNA at specific sequences, also called restriction sites, thus creating DNA fragments. The restriction enzymes can either be used as positive or negative markers meaning that if the DNA was mutated the enzyme can either gain a restriction site, creating more fragments and of different sizes than with normal DNA, or lose a site that normally exists in un mutated DNA.

The latter would then result in less fragments and of different sizes than with normal DNA. After restriction, the fragments were analyzed by gel electrophoresis, which creates visible bands, and by assessing; how far the bands have travelled through the gel in comparison to a DNA ladder with known band sizes the restriction fragments' sizes can be estimated. The number of fragments combined with their sizes allows the original sequence to be identified as mutated [102].

The following ingredients were combined to produce the restriction reaction:

1- An amount of (5 µl) of PCR product.
2- The restriction enzyme (Taq 1) 0.25 µl.
3- Restriction buffer 1.5 µl (each restriction enzyme has its own restriction buffer, which is given by the manufacturer).
4- Bovine serum albumin (BSA) 0.15 g/L added 0.1 µl.
5- Using molecular grade water, the reaction mixture was brought to an end at 15 µl add 20 µl of mineral oil.
6- The reaction mixture was incubated for 24 hours at 65°C in a water bath.
7- The RFLP reaction product was resolved on 2% Agarose gel electrophoresis as described in [**Fig. 2.4**] as figure of RFLP.

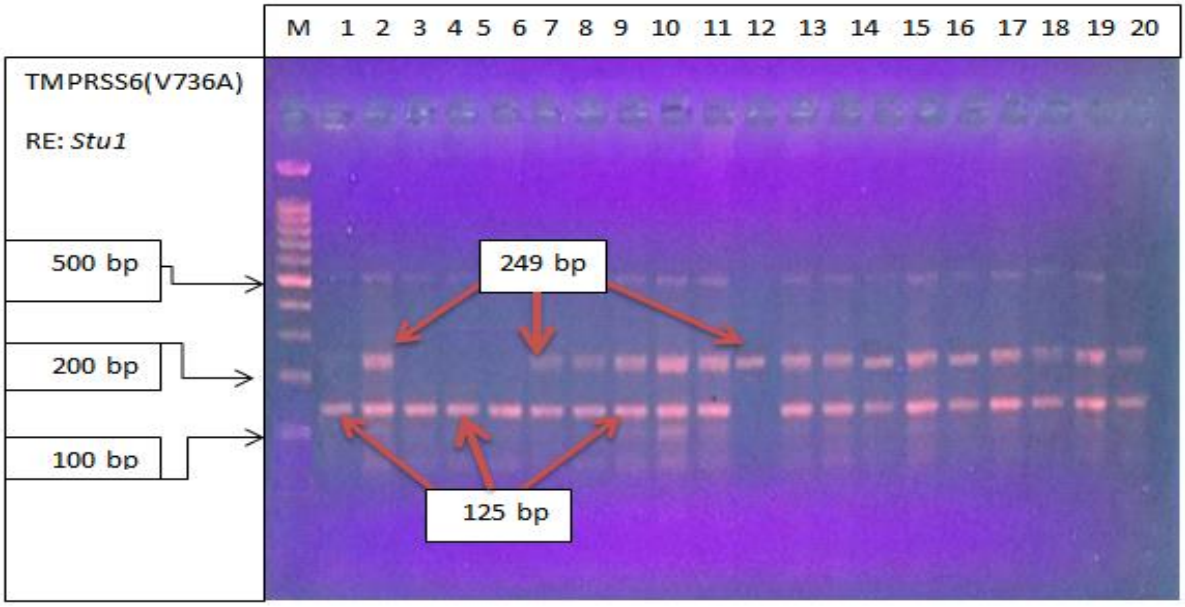
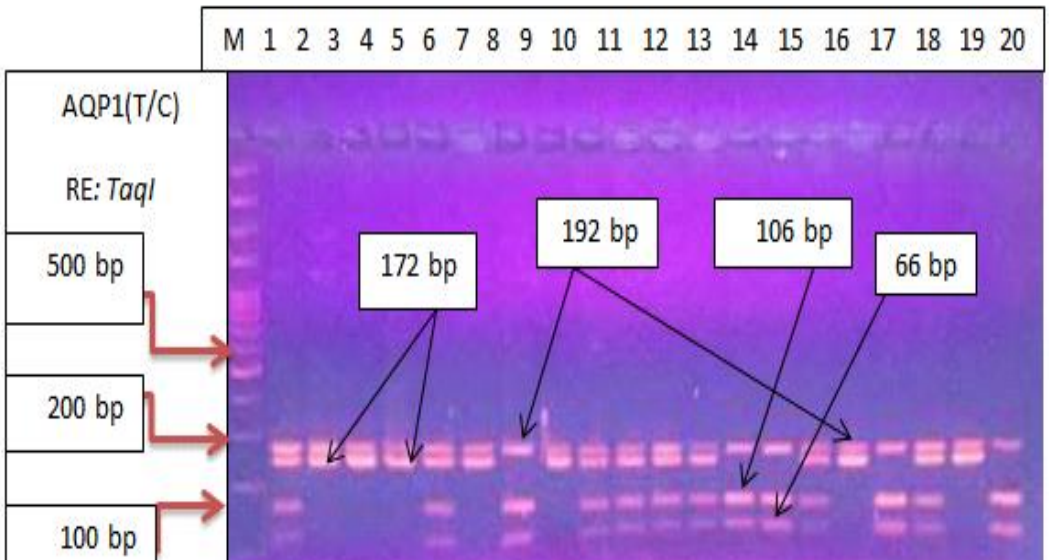


Figure [2.4]:A- Genotyping of TMPRSS6 by RFLP. PCR products were digested by stu1. Single band 249 bp represented homozygous C and single band at 125 bp represented homozygous T. Two bands represented heterozygous TC.



Figure(2-4): (b) Genotyping of AQP1 by RFLP. PCR products were digested by stu1. Single band 192 bp represented homozygous C and single band at 172 bp represented homozygous T. Two bands represented heterozygous TC.

2.4 Statistical analysis

The statistical analysis of this prospective study performed with the statistical package for social sciences (SPSS) 20.0 and Graphpad prism Version 7. Numerical data were tested for normality testing using Shapiro-Wilk test found that the data were abnormally distributed. The data described as mean and standard deviation and independent sample t test used for comparison between two groups. Categorical data were described as count and percentage. Chi-square test or Fisher exact test used to estimate the association between variables. Odds ratio and its 95% [103].

Chapter Three

Results and Discussion

3. Results and discussion

This study was designed to investigate the role of TMPRSS6 gene rs855791 mutation in adult patients affected with IDA in Iraq.

A total of 100 subjects were enrolled in the study. Genotyping was performed using PCR-RFLP technique. The PCR product (249 bp) and (192pb) were digested with stu1 restriction enzyme to detect T > C polymorphism [**Fig. 3.1**]. The genotype frequencies of patients and control subjects were performed in Hardy-Weinberg equilibrium for both SNPs.

To the best of our knowledge, most of the genetic parameters in this study have been done for the first time, therefore, our current study is considered to be the first trial in regard to the genetic relationship between iron deficiency anemia and genetic mutation not only in Iraq, but also around the world.

3.1 General characteristic of the study group

3.1.1. Age

In the present study, the age of subjects showed a non-significant difference ($p>0.05$) between the control group and iron deficiency anemia patients group as demonstrated in **Table 3.**

Iron deficiency anemia is a global disease that may affect people of any age due to variable causes, although the elderly are also at risk as the prevalence of anemia increase with age, but the evidence is scarce [104].

This age matching helps to eliminate differences in parameters' results that may originate due to the significant variation in age.

Table (3.1): Descriptive analysis of age in the study groups

		Study groups		P value
		Control	Patients	
Age		31.02±8.42	33.76±6.37	0.709^{NS}
Age groups	≥30 years	42	48	0.665^{NS}
		42.0%	48.0%	
	31-40 years	38	34	
		38.0%	34.0%	
	<40 years	20	18	
		20.0%	18.0%	

NS: none statistical significance (p>0.05).

3.1.2. Sex

Regarding gender, our results also showed a non-significant difference in gender between the IDA patients and the controls (p>0.05).

However, women of reproductive age are at higher risk of anemia compared to men due to the increased requirement for iron, resulting from regular blood loss during menstruation [105], as well their specific dietary habits, namely their lower intake of meat compared to men [106]. Therefore, it is indicated that the main risk factors for anemia development in women are an improperly balanced diet and high blood loss during menstruation [107]. On this account, providing sufficient amounts of iron in everyday meals and improving the bioavailability of non-heme iron is a matter of great importance in this population group [108].

Table (3.2): Descriptive frequency of sex in the study groups

		Study groups	
		Control	Patients
Sex	Female	38	39
		76.0%	78.0%
	Male	12	11
		24.0%	22.0%
P value		0.832^{NS}	

NS: none statistical significance (p>0.05).

3.1.3 Body Mass Index (BMI)

In this study, patients with a normal body mass index were selected. Thus, results in **Table 3.3**, showed a non-significant difference ($p>0.05$) in BMI between patients with IDA and control group.

Table (3.3): Descriptive analysis of body mass index in the study groups

	Study groups	
	Control	**Patients**
BMI	23.52	22.72
	3.06	3.04
p value	0.265^{NS}	

3.1.4 Baseline and clinical characteristics of participants

Hematological and biochemical results in the current study revealed that there was highly significant differences ($p<0.001$) in mean values of Hb, RBC, MCV, MCH, MCHC, iron, ferritin and vitamin C values between the patients and the controls.

Table (3.4): Descriptive analysis of hematological and biochemical parameters in study groups

		Study groups		p value
		Control	**Patients**	
Hb g/Dl	Mean	13.89	11.11	<0.001**
	SD	1.26	0.73	
RBC 10*12/L	Mean	4.66	4.15	<0.001**
	SD	0.69	0.44	
MCV fl	Mean	81.36	72.39	<0.001**
	SD	5.45	7.31	
MCH pg	Mean	30.73	23.07	<0.001**
	SD	6.86	2.36	
MCHC g/L	Mean	33.07	29.39	<0.001**
	SD	3.00	2.61	
Iron mg/L	Mean	97.06	50.50	<0.001**
	SD	26.61	29.76	
Ferritin mg/L	Mean	43.78	19.48	<0.001**
	SD	10.80	4.14	
Vit C mg/dL	Mean	6.90	4.84	<0.001**
	SD	1.73	1.85	

****: high statistical significance.**

In this study, patients with IDA had significantly lower hematological and biochemical parameters levels than control group. This result agree with several studies; one of these studies by (Johnson Wimbley and Graham, 2011) reported that CBC is evaluated to find out the cause of anaemia. IDA severity depends on hemoglobin and RBCs levels. MCV, MCH and MCHC levels are also used for diagnosis of Iron deficiency anemia [109].

The present results were 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 [110].

This study also showed that RBC count and Hb are important factors in the diagnosis of IDA as reported globally by (Nagababu et al., 2008). Interestingly, we observed that majority of the RBC parameters were significantly reduced in IDA cases vs controls. Similarly, reduced values of RBC's parameters were observed in IDA patients by [111].

Ascorbic acid effectively increases the absorption of ferrous ions (Fe^{3+}) and ferric ions (Fe^{2+}) [112]. It results from the reducing properties of vitamin C which make the iron soluble in a wide range of pH levels and allows iron to be absorbed through iron transporters in the small intestine [113]. Vitamin C intakes ranging from 100 to 200 mg/day will maintain blood concentration at adequate to saturating status (50–75 μmol/L) [114].

As ascorbic acid is a potent enhancer of non-heme iron absorption [115], some intervention studies administering products rich in vitamin C in conjunction with iron sources showed improved iron status [116].

3.1.5 Association of TMPRSS6 rs855791 (V736A) with IDA

SNP rs855791

The rs855791 (V736A) genotype frequencies were in HWE for both cases and controls. Assessed the association of rs855791 with the pathogenicity of IDA in co-dominant, additive, dominant, and recessive models. The results

found that rs855791 (V736A) is significantly associated with IDA as observed in codominant model (P < 0.05, OR: 1.4 and 95% CI: 1.08-2.8). Similarly, significant association was found in Additive model (P < 0.05, OR: 1.7 and 95% CI: 1.1- 3.01). However, non-significant P-values were obtained in Dominant and Recessive models (P > 0.05) [**Table 3.5**].

Among the four studied genetic model's, significant P-values (0.038, and 0.041 respectively) were observed in co-dominance and additive models, confirming that rs855791 is significantly associated with IDA. We did not find significant correlation between blood and Fe parameters with TMPRSS6 rs855791 genotypes and we find highly significant correlation between blood and Ascorbic acid with TMPRSS6 rs855791 genotypes.

Table (3.5): Different genetic models for TMPRSS6 rs855791 (T > C) association with IDA

rs855791	Study groups		P value	Odds ratio (CI95%)
	Control	Patients		
Codominant				
CC Homozygous	12 (24.0)	6 (12.0)	0.038*	1.4 (1.08-2.8)
CT Heterozygous	22 (44.0)	21 (42.0)		
TT Homozygous	16 (32.0)	23 (46.0)		
Additive				
C allele	46 (46.0)	33 (33.0)	0.041*	1.7 (1.1-3.01)
T allele	54 (54.0)	67 (67.0)		
Dominant				
CC	12	6	0.192	0.4 (0.14-1.3)
TC+TT	38	44		
Recessive				
TT	16	23	0.218	1.8 (0.8-4.1)
TC+CC	34	27		

Type II transmembrane serine protease 6 (matriptase 2) encoded from the transmembrane serine protease 6 gene (*TMPRSS6*) mapped on 22q12.3 in hepatocytes. *TMPRSS6* plays a role in iron homeostasis by regulating the hepcidin hormone. The negative regulation of hepcidin transcription by *TMPRSS6* results in promoting intestinal iron absorption to maintain iron homeostasis [117].

One common SNP variation, termed rs855791, has been described to involve iron status and red blood cell (RBC) indices, including MCV and mean corpuscular haemoglobin (MCH). The SNP rs855791 is a common variant of *TMPRSS6*playing a role in the determination of the various levels of RBC indices and iron parameters in the population [118]. The SNP locates in exon 17 of *TMPRSS6* as T>C substitution in nucleotide position 2207, inducing missense change from valine (V) to alanine (A) as GTC>GCC; V736A. The three SNP genotypes include homozygous C allele (C/C), T allele (T/T), and heterozygous T/C. Several studies of allele frequency and prevalence of SNP variation were described. The C allele frequency was highly distributed in the Caucasian population, similar to the Rwandan population in Sub-Saharan Africa [119]. On the other hand, different allele frequencies were reported in the Asian population, where T alleles, including T/T and T/C genotypes, presented at higher frequencies than those of the C/C genotype presented in the Taiwanese, Japanese, and Indian populations [118,119].

In a study previously undertaken of female students from the northern region of Saudi Arabia, the rs855791 SNP in *TMPRSS6* was found to be significantly associated with poor iron status [120].

Al-Amer et al. [120] reported that rs855791 was significantly associated with low serum iron and serum ferritin but showed no association with low Hb, red blood cell (RBC), platelets and white blood cell (WBC).

Meanwhile, a study of Turkish IDA patients found that mutations in *TMPRSS6* rs855791, rs4820268, and rs2413450 are associated with increased RBC and TIBC in IDA patients [121].

3.1.6 Association of Aquaporin1 rs10244884 with IDA

The rs10244884 genotype frequencies were performed in HWE for both cases and controls.

The association of rs10244884 was assessed with the pathogenicity of IDA in co-dominant, additive, dominant, and recessive models.

The results demonstrated that rs10244884 is significantly associated with IDA as observed in codominant model ($P < 0.05$, OR: 2.1and 95% CI: 1.2-6.3).

Similarly, significant association was found in Additive model ($P < 0.05$, OR: 1.3 and 95% CI: 1.1- 01.72). However, non-significant P-values were obtained in Dominant and Recessive models ($P > 0.05$).

Among the four studied genetic model's, significant P-values (0.012, and 0.045 respectively) were observed in co-dominance and additive models, confirming that rs10244884 is significantly associated with IDA. We did not find significant correlation between blood and Fe parameters with AQP1rs10244884 genotypes and we find high significant correlation between blood and Ascorbic acid with AQP1rs 10244884 genotypes.

Table (3.6): Different genetic models for AQP1rs10244884 (T > C) association with IDA

	Study groups		P value	Odds ratio (CI95%)
rs10244884	**Control**	**Patients**		
Codominant				
CC Homozygous	8 (16.0)	17 (34.0)	0.012*	2.1 (1.2-6.3)
TC Heterozygous	28 (56.0)	23 (46.0)		
TT Homozygous	14 (28.0)	10 (20.0)		
Additive				
C allele	44 (44.0)	57 (57.0)	0.045*	1.3 (1.01-1.72)
T allele	56 (56.0)	43 (43.0)		
Dominant				
TT	14 (28.0)	10 (20.0)	0.483^{NS}	0.642(0.24-1.5)
TC+CC	36 (72.0)	40 (80.0)		
Recessive				
CC	8 (16.0)	17 (34.0)	0.063 ^{NS}	2.7 (1-7)
TC+TT	42 (84.0)	33 (66.0)		

SNP rs855791 could be a causative variant, operating through altered protease activity or substrate binding, and **rs10244884** was found to be in the linkage disequilibrium with rs855791, causing the **rs10244884** to exhibit similar signals [121]. This association could be explained by changes in protease function and hepcidin-mediated iron homeostasis management.

In human GWAS, common genetic variations of *TMPRSS6* have been linked to erythrocyte and iron parameters [122,123]. The SNP rs855791 has recently been identified as the top-hit in GWAS linked to changes in SI, TS, erythrocyte MCV, Hb levels, and glycated Hb levels [124]. SNP of rs855791 results in a non-synonymous substitution at the protease's catalytic and active site, which is strongly associated with iron status and erythrocyte characteristics, thus leading to IDA and/or IRIDA [120]. T allele variations of the rs855791 in the *TMRSS6* gene have been linked to an increased risk of ID and iron-deficient anaemia (IDA) [125]. In a Taiwanese case–control study, homozygotes for the SNP rs855791 CC had a lower prevalence of IDA than people with the CT or

TT variant [118]. Meanwhile, variants (TT) in rs855791 are also related to lower TS and serum ferritin (SF), higher hepcidin, and higher ratios of hepcidin to iron indices in European and Saudi Arabia populations [120,125].

In contrast, a study of reproductive-age Pakistani women found that the rs855791 T allele is linked to the incidence of IDA [126]. A similar finding of the risk allele among Pakistani women was reported in a recent study by Buerkli et al.[127]. In a study of iron absorption in Taiwanese women utilising stable iron isotopes, the T allele was linked to a higher risk of developing iron deficiency, with serum iron and transferrin saturation being lower in the TT version of rs855791 [128]. This revealed that women with the *TMPRSS6* rs855791 (2321 C > T) polymorphism have impaired iron homeostasis, which affects oral iron absorption and may increase the risk of IDA. The reason why rs855791 has a big impact on contributing to the risk of IDA and exhibits in most of the IRIDA cases [126] is that rs855791 is a non-synonymous variant in which it causes an alanine to valine amino acid change at a position of 736 nearby catalytic and binding sites of MT-2 in the *TMPRSS6* sequence [129].

Pei *et al* [130] further confirmed this role of rs855791C>T, reporting that it protects women against iron-deficiency anemia. The results of our study demonstrate that the heterozygous (TC) genotype confers protection against iron-deficiency anemia, in agreement with previous studies [131]. The findings of our study contradict those of Kumar *et al.*[132] and Macdougall *et al.*[133], who reported that CC genotype is protective in IDA, whereas TT genotype is pathological. Later studies by Gonçalves *et al.*[134] reported that patients with IDA were less likely to have CC genotypes. Variations in the study may be attributable to a difference in the study population and ethnicity. These data demonstrated that TMPRSS6 (rs855791C>T) genotypes serve as effective predictors of IDA. Therefore, patients with IDA should be tested for TMPRSS6 (rs855791C>T) genotype. This can help guide baseline investigations and treatment decisions [135].

Genotyping was performed using PCR–RFLP technique. The PCR product (249 bp) and (192bp) were digested with stu1 restriction enzyme to detect T > C polymorphism (**Fig. 3.1**). The genotype frequencies of patients and control subjects were in Hardy–Weinberg equilibrium for both SNPs.

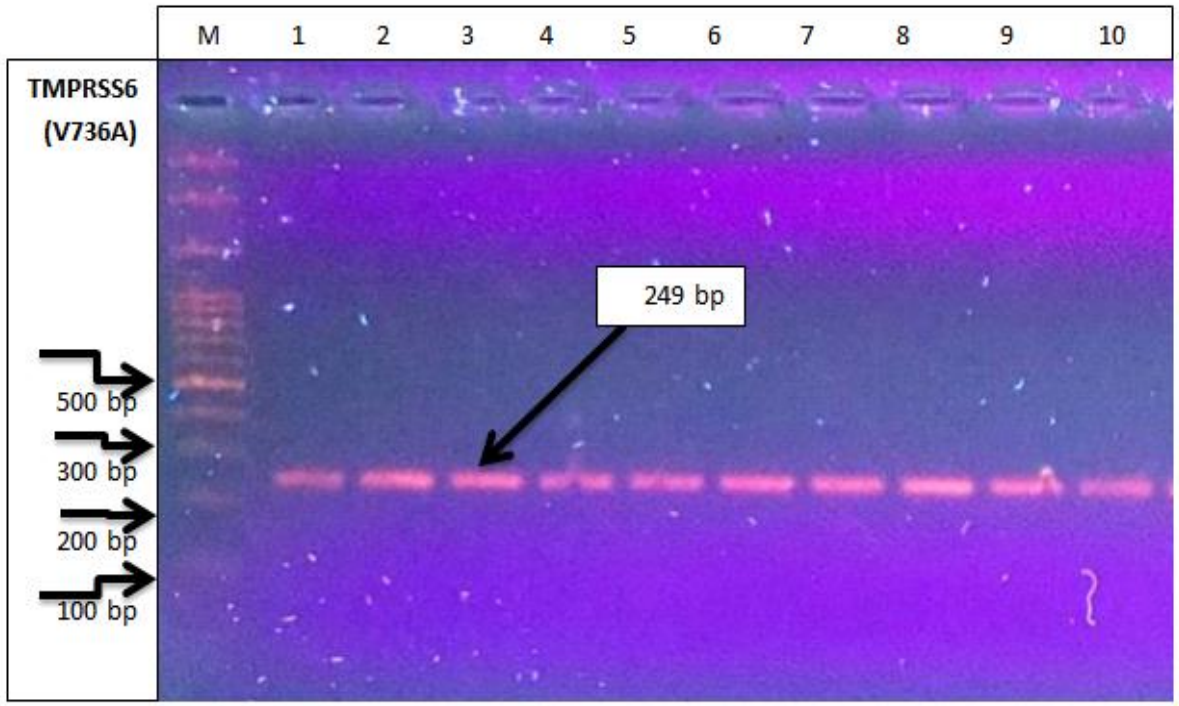


Figure. (3.1): (a) Genotyping of TMPRSS6 by PCR (PCR product = 249 bp).

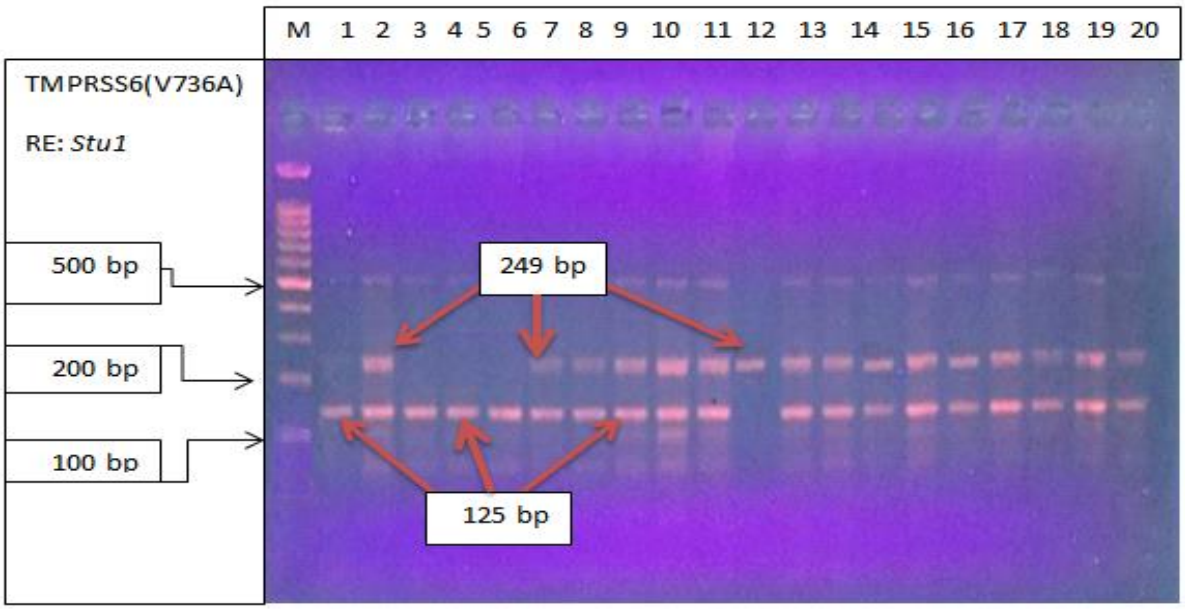


Figure. (3.1): (b) Genotyping of TMPRSS6 by RFLP. PCR products were digested by stu1. Single band 249 bp represented homozygous C and single band at 125 bp represented homozygous T. Two bands represented heterozygous TC.

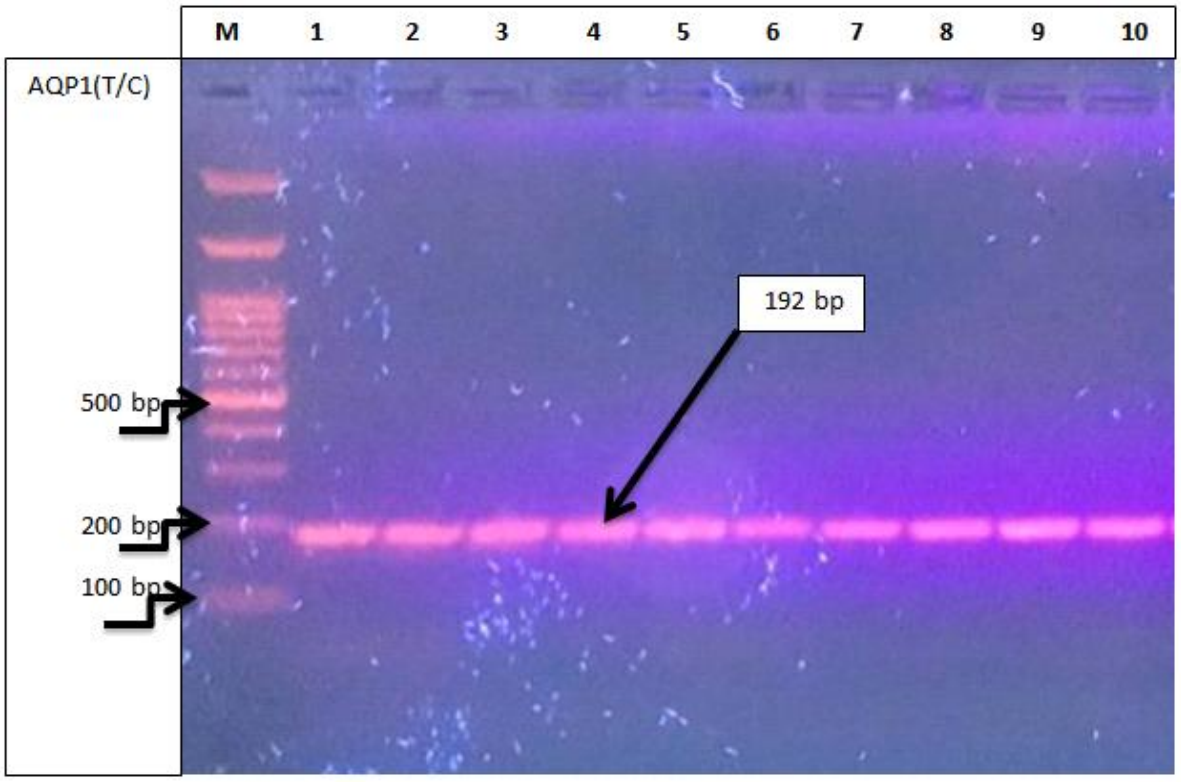


Figure. (3.1): (c) Genotyping of AQ1 by PCR (PCR product = 129 bp).

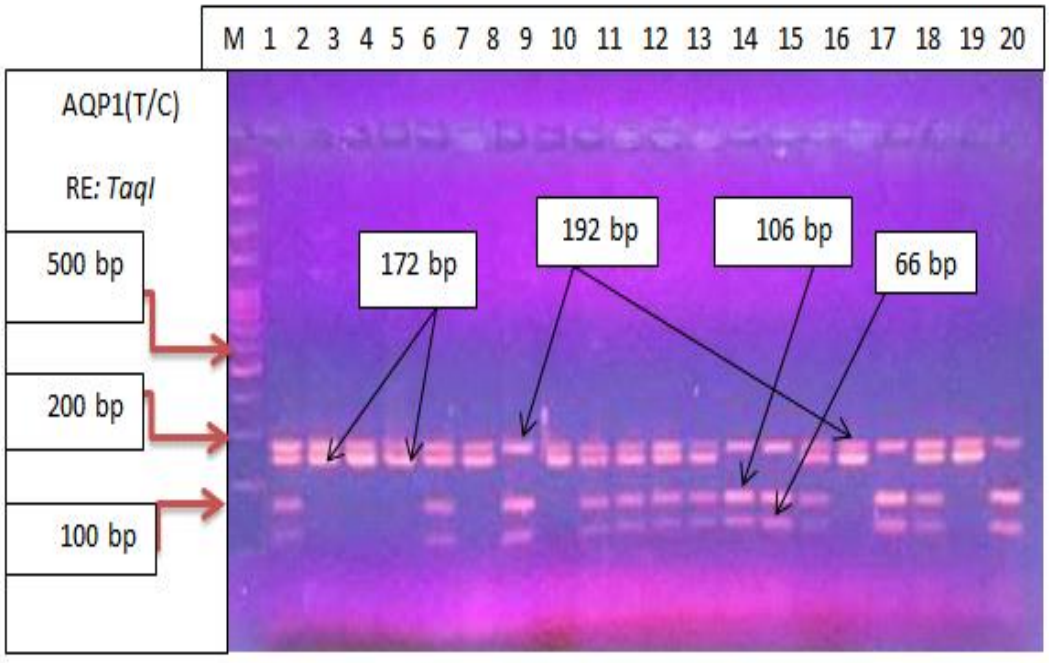


Figure. (3.1): (d) Genotyping of AQP1 by RFLP. PCR products were digested by stu1. Single band 192 bp represented homozygous C and single band at 172 bp represented homozygous T. Two bands represented heterozygous TC.

3.1.7 Association Analysis Between Hematological Traits and AQP1 rs10244884 Polymorphism

The results in table 3-7 showed that there was a significant association between Hb level and mutant C allele (10.11) compared with T allele (11.12) similarly CC genotype have lower Hb level (10.26), CT genotype (10.88) and TT genotype (11.4).

Serum iron level was significantly associated with mutation of AQP1. Mutant C allele have 47.23 compared with T allele (54.84). CC and CT genotypes have similar iron values compared with TT genotype with elevated iron level.

Vit C have lower values in CC and CT genotypes compared with TT genotype with significant difference.

Table (3.7): The Results of Association Analysis Between Hematological Traits and AQP1 rs10244884 Polymorphism

	rs10244884				
	Genotype			Allele	
	CC	CT	TT	C	T
Hb	10.26±0.60	10.88±0.86	11.40±0.35	10.11±0.74	11.12±0.72
p value	0.008*			0.015*	
RBC	4.08±0.42	4.12±0.45	4.35±0.42	4.10±0.43	4.23±0.45
p value	0.075 NS			0.147 NS	
MCV	72.45±7.56	73.13±6.77	70.59±8.10	72.72±7.23	71.95±7.48
p value	0.435 NS			0.603 NS	
MCH	22.73±1.69	23.44±2.97	22.79±1.61	23.02±2.31	23.14±2.45
p value	0.347 NS			0.800 NS	
MCHC	28.36±1.49	30.36±2.59	28.90±3.39	29.17±2.23	29.68±3.05
p value	0.002*			0.357 NS	
Iron	48.76±23.90	44.96±14.81	66.20±52.57	47.23±20.69	54.84±38.50
p value	0.025*			0.045 *	
VitC	4.89±1.91	4.45±1.70	5.67±1.88	4.71±1.83	5.02±1.88
p value	0.047*			0.417 NS	
Ferritin	19.88±3.91	19.74±3.83	18.20±5.08	19.82±3.86	19.02±4.49
p value	0.302 NS			0.351 NS	

3.1.8 Association Analysis Between Hematological Traits and TMPRSS6 rs855791 Polymorphism

The results in **table 3.8** showed that there were significant reduction in serum iron level with CC and CT genotypes in comparison with TT genotype (p=0.037). Similarly, C allele have lower serum level compared with T allele (p=0.05).

Vit C also have reported as lower levels in CC genotype and TC genotype compared with TT genotype (p<0.001) C allele have significantly lower level compared with T allele (p=0.007).

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≤ 0.00). Consistent with their associations to increased iron deficiency and anemia risk which agree with current result [126].

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 [143]. 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 [136]. Significantly fewer C homozygotes in the IDA group compared to the healthy group have been reported by Sung et al. (2014) [116], suggesting that homozygosis for TMPRSS6 C genotype has a protective role against IDA.

Table (3.8): The Results of Association Analysis Between Hematological Traits and TMPRSS6 rs855791Polymorphism

	rs855791				
	Genotype			Allele	
	CC	CT	TT	C	T
Hb	11.25±0.67	11.22±0.79	10.98±0.67	11.23±0.74	11.05±0.72
p value	0.235 NS			0.255 NS	
RBC	4.15±0.16	4.15±0.51	4.16±0.43	4.15±0.42	4.16±0.45
p value	0.998 NS			0.968 NS	
MCV	68.82±9.26	72.09±6.30	73.60±7.44	70.90±7.58	73.12±7.11
p value	0.123 NS			0.164 NS	
MCH	22.48±2.06	23.00±2.27	23.29±2.53	22.81±2.20	23.20±2.44
p value	0.563 NS			0.446 NS	
MCHC	28.97±1.93	28.99±1.79	29.87±3.28	28.98±1.83	29.59±2.91
p value	0.246 NS			0.275 NS	
Iron	46.17±12.88	42.81±14.02	58.65±40.01	44.03±13.62	53.69±34.74
p value	0.037*			0.050*	
VitC	3.83±2.41	4.31±1.57	5.60±1.66	4.14±1.91	5.19±1.73
p value	<0.001**			0.007*	
Ferritin	18.33±4.74	20.19±4.08	19.13±4.01	19.52±4.38	19.46±4.05
p value	0.291 NS			0.953 NS	

These studies demonstrated that *TMPRSS6* gene polymorphisms were associated with elevated hepcidin levels in end-stage renal failure [137] and chronic kidney disease patients with IDA [118]. Mutations in the *TMPRSS6* gene were also reported in patients with IRIDA, a rare autosomal recessive disorder in which the hepcidin levels were shown to be elevated. [138] Therefore, it is critical to understand the metabolic mechanism of the normal *TMPRSS6* gene and how the polymorphisms in the *TMPRSS6* gene cause IDA. Differences in the frequency and the trends of linkage disequilibrium of risk alleles may account for the limited replication of association results among global

populations. As a result, there is a need to study the population-specific genetic variations that may influence iron status [139].

Type II transmembrane serine protease 6 (matriptase 2) encoded from the transmembrane serine protease 6 gene (*TMPRSS6*) mapped on 22q12.3 in hepatocytes. *TMPRSS6* plays a role in iron homeostasis by regulating the hepcidin hormone. The negative regulation of hepcidin transcription by *TMPRSS6* results in promoting intestinal iron absorption to maintain iron homeostasis [113,140].

However, multiple *TMPRSS6* SNPs have been identified across all studies, profoundly associated with IRIDA, IDA, low iron, and blood indices. SNPs that have been identified were classified as synonymous, missense, intron, 5'-UTR and intergenic variants. The most frequently reported *TMPRSS6* SNP were rs855791 and rs10244884, linked to biomarkers of poor iron status and low blood indices [137].

Chapter Four

Conclusions and Recommendations

4. Conclusions and Recommendations

4.1 Conclusions

1-There were statistically significant association between TMPRSS6 polymorphic genotypes AQP1 and IDA risk.

2-TMPRSS6 and its polymorphism can also lead to IRIDA.

3-This study will help the health care workers, and researchers to further work on the extrinsic and intrinsic factors of IDA in Iraqi adults patients.

4.2 Recommendations

1. Further studies should be conducted to identify the relationship between hepcidin level and iron profiles with TMPRSS6 C/T polymorphism.
2. Future studies should be conducted by other techniques RFLP and DNA sequencing.
3. Future studies should be conducted by using TMPRSS6 and AQP1 gene mapping.

References

REFERENCES

1- Abdelrhman, A. H., Abdelgadir, E. A., & Khalid, K. M.. Molecular Study of Hepcidin HAMP (-582A/G) Gene Polymorphisms and Measurement of Serum Hepcidin Level among Sudanese Patients with Anemia of Chronic Kidney Disease. International Journal of Clinical Skills, (2020), 14(2), 318-323.

2- ACHEBE, Maureen M.; GAFTER-GVILI, Anat. How I treat anemia in pregnancy: iron, cobalamin, and folate. Blood, The Journal of the American Society of Hematology, 2017, 129.8: 940-949.

3- AL-AMER, Osama, et al. Study the association of transmembrane serine protease 6 gene polymorphisms with iron deficiency status in Saudi Arabia. Gene, 2020, 751: 144767.

4- ALLAIN, Charles C., et al. Enzymatic determination of total serum cholesterol. Clinical chemistry, 1974, 20.4: 470-475.

5- ALSHEIKH, Mona. Prevalence and risk factors of iron-deficiency anemia in Saudi female medical students. Saudi Journal for Health Sciences, 2018, 7.3.

6- AN, Peng, et al. TMPRSS6, but not TF, TFR2 or BMP2 variants are associated with increased risk of iron-deficiency anemia. Human molecular genetics, 2012, 21.9: 2124-2131.

7- ANDRO, M., et al. Anaemia and cognitive performances in the elderly: a systematic review. European Journal of Neurology, 2013, 20.9: 1234-1240.

8- ANDRO, M., et al. Anaemia and cognitive performances in the elderly: a systematic review. European Journal of Neurology, 2013, 20.9: 1234-1240.

9- Arosio, Paolo, Leonardo Elia, et al. Ferritin, cellular iron storage and regulation. IUBMB life. 2017; 69(6): 414-422.

10- ATKINSON, Mark A.; MACLAREN, Noel K. The pathogenesis of insulin-dependent diabetes mellitus. New England journal of medicine, 1994, 331.21: 1428-1436.

11- AUERBACH, Michael; ADAMSON, John W. How we diagnose and treat iron deficiency anemia. American journal of hematology, 2016, 91.1: 31-38.

12- MILLER, Clare E.; BAIN, Barbara J. The utility of blood and bone marrow films and trephine biopsy sections in the diagnosis of parasitic infections. Mediterranean Journal of Hematology and Infectious Diseases, 2015, 7.1.

13- BATAR, Bahadir, et al. The role of TMPRSS6 gene variants in iron-related hematological parameters in Turkish patients with iron deficiency anemia. Gene, 2018, 673: 201-205.

14- BATAR, Bahadir, et al. The role of TMPRSS6 gene variants in iron-related hematological parameters in Turkish patients with iron deficiency anemia. Gene, 2018, 673: 201-205.

15- Bathla S, Arora S. Prevalence and approaches to manage iron deficiency anemia (IDA). 2021:1–14.

16- Beck, K.; Conlon, C.A.; Kruger, R.; Coad, J.; Stonehouse, W. Gold kiwifruit consumed with an iron-fortified breakfast cereal meal improves iron status in women with low iron stores: A 16-week randomised controlled trial. *Br. J. Nutr.* **2011**, *105*, 101–109.

17- BENYAMIN, Beben, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. Nature genetics, 2009, 41.11: 1173-1175.

18- Biosource technology laboratory. Human Ferritin ELISA Kit user manual. USA; 2022.

19- Biosource technology laboratory. Human Vit. C ELISA Kit user manual. USA; 2022.

20- BORATYN, Grzegorz M., et al. Magic-BLAST, an accurate RNA-seq aligner for long and short reads. BMC bioinformatics, 2019, 20.1: 1-19.

21- BRISSOT, Pierre, et al. Rare anemias due to genetic iron metabolism defects. Mutation Research/Reviews in Mutation Research, 2018, 777: 52-63.

22- BRUNER, Ann B., et al. Randomised study of cognitive effects of iron supplementation in non-anaemic iron-deficient adolescent girls. The Lancet, 1996, 348.9033: 992-996.

23- BUERKLI, Simone, et al. The TMPRSS6 variant (SNP rs855791) affects iron metabolism and oral iron absorption–a stable iron isotope study in Taiwanese women. haematologica, 2021, 106.11: 2897.

24- Burton JK, Yates LC, Whyte L, *et al*. New horizons in iron deficiency anaemia in older adults. 2020; 49: 309–18.

25- Cable RG, Glynn SA, Kiss JE et al. Iron deficiency in blood donors: analysis of enrollment data from the REDS-II Donor Iron Status Evaluation (RISE) study. Transfusion 2011; 51: 511–22.

26- Camaschella C, Nai A, Silvestri L. Iron metabolism and iron disorders revisited in the hepcidin era. *Haematologica.* 2020; 105(2): 260-272.

27- CAMASCHELLA, Clara. Iron and hepcidin: a story of recycling and balance. Hematology 2013, the American Society of Hematology Education Program Book, 2013, 2013.1: 1-8.

28- Camaschella C. Iron deficiency. Blood 2019; 133: 30–9.

29- Camaschella C. Iron-deficiency anemia. New England journal of medicine. 2015; 372(19):1832–43.

30- CAMASCHELLA, Clara. Iron and hepcidin: a story of recycling and balance. Hematology 2013, the American Society of Hematology Education Program Book, 2013, 2013.1: 1-8.

31- CAPPELLINI, Maria Domenica, et al. Iron deficiency across chronic inflammatory conditions: International expert opinion on definition, diagnosis, and management. American journal of hematology, 2017, 92.10: 1068-1078.

32- ÇEKIÇ, Cem, et al. The effect of intravenous iron treatment on quality of life in inflammatory bowel disease patients with nonanemic iron deficiency. Gastroenterology Research and Practice, 2015, 2015.

33- CHAMBERS, John C., et al. Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. Nature genetics, 2009, 41.11: 1170-1172.

34- Chaparro CM, Suchdev PS (2019) Anemia epidemiology, pathophysiology, and etiology in low- and middle-income countries. Ann N Y Acad Sci 1450:15–31.

35- COLOMER, Julia, et al. Anaemia during pregnancy as a risk factor for infant iron deficiency: report from the Valencia Infant Anaemia Cohort (VIAC) study. Paediatric and perinatal epidemiology, 1990, 4.2: 196-204.

36- Congdon EL, Westerlund A, Algarin CR et al. Iron deficiency in infancy is associated with altered neural correlates of recognition memory at 10 years. J Pediatr 2012; 160: 1027–33.

37- Cooke AG, McCavit TL, Buchanan GR, Powers JM. Iron deficiency anemia in adolescents who present with heavy menstrual bleeding. J Pediatr Adolesc Gynecol 2017; 30: 247–50.

38- Crielaard BJ, Lammers T, Rivella S. Targeting iron metabolism in drug discovery and delivery. *Nat Rev Drug Discov* 2017; 16: 400–23.

39- Danquah, I.; Gahutu, J.B.; Zeile, I.; Musemakweri, A.; Mockenhaupt, F.P. Anaemia, iron deficiency and a common polymorphism of iron-regulation, *TMPRSS6* rs855791, in Rwandan children. *Trop. Med. Int. Health* **2014**, *19*, 117–122.

40- De Falco L, Silvestri L, Kannengiesser C et al. Functional and clinical impact of novel TMPRSS6 variants in iron-refractory iron-deficiency anemia patients and genotype-phenotype studies. Hum Mutat 2014; 35: 1321–9.

41- De Silva AD, Mylonaki M, Rampton DS. Oral iron therapy in inflammatory bowel disease: usage, tolerance, and efficacy. 2003; 9: 316–20.

42- Disler PB, Lynch SR, Charlton RW, *et al*. The effect of tea on iron absorption. *Gut* 1975; 16: 193–200.

43- DOPSAJ, Violeta, et al. Associations of common variants in HFE and TMPRSS6 genes with hepcidin-25 and iron status parameters in patients with end-stage renal disease. Disease Markers, 2019, 2019.

44- DOPSAJ, Violeta, et al. Associations of common variants in HFE and TMPRSS6 genes with hepcidin-25 and iron status parameters in patients with end-stage renal disease. Disease Markers, 2019, 2019.

45- DU, Xin, et al. The serine protease TMPRSS6 is required to sense iron deficiency. Science, 2008, 320.5879: 1088-1092.

46- ELMAHDY, Magda, et al. TMPRSS6 gene polymorphism and serum hepcidin in iron deficiency anemia. The Egyptian Journal of Hospital Medicine, 2018, 73.7: 7090-7103.

47- Enns C.A., Jue S., Zhang A.S. The ectodomain of matriptase-2 plays an important nonproteolytic role in suppressing hepcidin expression in mice. Blood. 2020; 136: 989–1001.

48- FINBERG, Karin E., et al. Down-regulation of Bmp/Smad signaling by Tmprss6 is required for maintenance of systemic iron homeostasis. Blood, The Journal of the American Society of Hematology, 2010, 115.18: 3817-3826.

49- Folgueras, A.R., de Lara, F.M., Pendas, A.M., Garabaya, C., Rodriguez, F., Astudillo, A., Bernal, T., Cabanillas, R., Lopez-Otin, C. and Velasco, G. (2008). *Membrane-bound serine protease matriptase-2 (Tmprss6) is an essential regulator of iron homeostasis.* Blood, **112**: 2539–2545.

50- Fraser IS, Mansour D, Breymann C, Hoffman C, Mezzacasa A, Petraglia F. Prevalence of heavy menstrual bleeding and experiences of affected

women in a European patient survey. Int J Gynaecol Obstet 2015; 128: 196–200.

51- Friedman L S. Chapter 16: Liver, Biliary Tract, & Pancreas Disorders. Papadakis. 2015; 6.

52- Garcia-López S, Bocos JM, Gisbert JP, *et al*. High-Dose Intravenous treatment in iron deficiency anaemia in inflammatory bowel disease: early efficacy and impact on quality of life. *Blood Transfus* 2016; 14: 199–205.

53- GHO. Global Health Observatory data repository 2016. Available at.

54- GICHOHI-WAINAINA, Wanjiku N., et al. Common variants and haplotypes in the TF, TNF-α, and TMPRSS6 genes are associated with iron status in a female black South African population. The Journal of nutrition, 2015, 145.5: 945-953.

55- Gisbert JP, Bermejo F, Pajares R, *et al*. Oral and intravenous iron treatment in inflammatory bowel disease: hematological response and quality of life improvement. *Inflamm Bowel Dis* 2009; 15: 1485–91.

56- Gonçalves L, Nobre Jesus G, Afonso C, Vieira A, Maia R, Correia L, *et al*. The role of TMPRSS6 gene variants in different types of iron deficiency anaemia-from the rare severe hereditary IRIDA to the common mild acquired IDA. Reunião Científica do Anemia Working Group Portugal 28-29 novembro 2014.

57- GUILLEM, Flavia, et al. Inactive matriptase-2 mutants found in IRIDA patients still repress hepcidin in a transfection assay despite having lost their serine protease activity. Human mutation, 2012, 33.9: 1388-1396.

58- De Silva AD, Mylonaki M, Rampton DS. Oral iron therapy in inflammatory bowel disease: usage, tolerance, and efficacy. 2003; 9: 316–20.

59- Hallberg L, Rossander L, Skånberg AB. Phytates and the inhibitory effect of bran on iron absorption in man. *Am J Clin Nutr* 1987; 45: 988–96.

60- Heidelbaugh JJ. Proton pump inhibitors and risk of vitamin and mineral deficiency: evidence and clinical implications. Ther Adv Drug Saf 2013; 4: 125–33.

61- HENTZE, Matthias W., et al. Two to tango: regulation of Mammalian iron metabolism. Cell, 2010, 142.1: 24-38.

62- HENTZE, Matthias W., et al. Two to tango: regulation of Mammalian iron metabolism. Cell, 2010, 142.1: 24-38.

63- Hershko C, Camaschella C. How I treat unexplained refractory iron deficiency anemia. Blood 2014; 123: 326–33.

64- Hoffbrand, A.V. Moss, P.A. and Pettit, J.E.(2006) *Essential Haematology*. 5th ed. Oxford: black well, pp:40-43.

65- HOOPER, John D., et al. Mouse matriptase-2: identification, characterization and comparative mRNA expression analysis with mouse hepsin in adult and embryonic tissues. Biochemical Journal, 2003, 373.3: 689-702.

66- JALLOW, Momodou W., et al. A recall-by-genotype study on polymorphisms in the TMPRSS6 gene and oral iron absorption: a study protocol. F1000Research, 2021, 8: 701-701.

67- JALLOW, Momodou W., et al. 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, 2021, 5.3: nzab014.

68- JANKOWSKA, Ewa A., et al. Iron deficiency: an ominous sign in patients with systolic chronic heart failure. European heart journal, 2010, 31.15: 1872-1880.

69- Jimenez KM, Gasche C. Management of iron deficiency anaemia in inflammatory bowel disease. 2019; 142: 30–6.

70- Jingfang L, et al. Iron metabolism and type 2 diabetes mellitus: A meta-analysis and systematic review. Journal of diabetes investigation. 2020; 11(4): 946-955.

71- Johnson-Wimbley TD, Graham DY. Diagnosis and management of iron deficiency anemia in the 21st century. Therap Adv Gastroenterol. 2011 May;4(3):177-84.

72- Jung L H, et al. Effect of excess iron on oxidative stress and gluconeogenesis through hepcidin during mitochondrial dysfunction. The Journal of nutritional biochemistry.2015; 26(12): 1414-1423.

73- Karin, E. F. (2009), *Iron-refractory iron deficiency anemia*. Seminars in Hematology, **46**: 378–386.

74- Kassebaum NJ, Jasrasaria R, Naghavi M et al. A systematic analysis of global anemia burden from 1990 to 2010. Blood 2014; 123: 615–24.

75- Kautz, L., Jung, G., Valore, E.V., Rivella, S., Nemeth, E. and Ganz, T. (2014). Identification of erythroferrone as an erythroid regulator of iron metabolism. Nature Genetic, 46: 678-84.

76- KINYOKI, Damaris, et al. Anemia prevalence in women of reproductive age in low-and middle-income countries between 2000 and 2018. Nature medicine, 2021, 27.10: 1761-1782.

77- Krijt, J.; Frýdlová, J.; Gurieva, I.; Přikryl, P.; Báječný, M.; Steinbicker, A.U.; Vokurka, M.; Truksa, J. Matriptase-2 and hemojuvelin in hepcidin regulation: In vivo immunoblot studies in *Mask* Mice. *Int. J. Mol. Sci.* **2021**, *22*, 2650.

78- Kulnigg-Dabsch S, Resch M, Oberhuber G, Klinglmueller F, Gasche A, Gasche C. Iron deficiency workup reveals high incidence of autoimmune gastritis with parietal cell antibody as reliable screening test. Semin Hematol 2018; 55: 256–61.

79- Kumar A, Brookes MJ. Iron therapy in inflammatory bowel disease. *Nutrients* 2020; 12: 3478.

80- Kumar PK, Srivastava RK, ShirinJ, Singh S, Ani K. Effect of TMPRSS6 gene polymorphism on morphometry of placenta and foetal outcome. IJSRR 2018; **7**:2116–2134.

81- LONE, Nasira Munawar, et al. Role of TMPRSS6 rs855791 (T> C) polymorphism in reproductive age women with iron deficiency anemia from Lahore, Pakistan. Saudi journal of biological sciences, 2021, 28.1: 748-753.

82- Lopez A, Cacoub P, Macdougall IC, *et al*. Iron deficiency anaemia. 2016; 387: 907–16.

83- Lynch S, Pfeiffer CM, Georgieff MK, et al. Nutrition. *The Journal of nutrition.* 2018;148(suppl_1):1001S-1067S.

84- MACDOUGALL, Iain C., et al. FIND-CKD: a randomized trial of intravenous ferric carboxymaltose versus oral iron in patients with chronic kidney disease and iron deficiency anaemia. Nephrology Dialysis Transplantation, 2014, 29.11: 2075-2084.

85- Madhikarmi NL, Murthy KRS. Antioxidant enzymes and oxidative stress in the erythrocytes of iron deficiency anemic patients supplemented with vitamins. Iran Biomed J. 2014; 18(2):82.

86- MASTROGIANNAKI, Maria; MATAK, Pavle; PEYSSONNAUX, Carole. The gut in iron homeostasis: role of HIF-2 under normal and pathological conditions. Blood, The Journal of the American Society of Hematology, 2013, 122.6: 885-892.

87- CAMASCHELLA, Clara. Iron and hepcidin: a story of recycling and balance. Hematology 2013, the American Society of Hematology Education Program Book, 2013, 2013.1: 1-8.

88- MELIS, Maria Antonietta, et al. A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. haematologica, 2008, 93.10: 1473-1479.

89- Milman, N.T. A review of nutrients and compounds, which promote or inhibit intestinal iron absorption: Making a platform for dietary measures that can reduce iron uptake in patients with genetic haemochromatosis. *J. Nutr. Metab.* **2020**, *2020*, 7373498.

90- Mirza FG, Abdul-Kadir R, Breymann C, Fraser IS, Taher A. Impact and management of iron deficiency and iron deficiency anemia in women's health. Expert Rev Hematol 2018; 11: 727–36.

91- Mitsuyoshi H, Yasui K, Yamaguchi K, et al. Pathogenic role of iron deposition in reticuloendothelial cells during the development of chronic hepatitis C. Int J Hepatol. 2013; 2013:686420.

92- Mohd Atan, F.N.E., Wan Mohd Saman, W.A., Kamsani, Y.S. *et al.* TMPRSS6 gene polymorphisms associated with iron deficiency anaemia among global population. *Egypt J Med Hum Genet* **23**, 147 (2022).

93- Monsen ER, Hallberg L, Layrisse M, *et al*. Estimation of available dietary iron. *Am J Clin Nutr* 1978; 31: 134–41.

94- Munoz M, Acheson AG, Auerbach M et al. International consensus statement on the peri-operative management of anaemia and iron deficiency. Anaesthesia 2017; 72: 233–47.

95- Munoz M, Laso-Morales MJ, Gomez-Ramirez S, Cadellas M, **Nunez-Matas MJ, Garcia-Erce JA. Pre-operative haemoglobin** levels and iron status in a large multicentre cohort of patients undergoing major elective surgery. Anaesthesia 2017; 72: 826–34.

96- Musallam KM, Taher AT. Iron deficiency beyond erythropoiesis: should we be concerned? Curr Med Res Opin 2018; 34: 81–93.

97- NAGABABU, Enika, et al. Iron-deficiency anaemia enhances red blood cell oxidative stress. Free radical research, 2008, 42.9: 824-829.

98- NAI, Antonella, et al. Deletion of TMPRSS6 attenuates the phenotype in a mouse model of β-thalassemia. Blood, The Journal of the American Society of Hematology, 2012, 119.21: 5021-5029.

99- NAI, Antonella, et al. Deletion of TMPRSS6 attenuates the phenotype in a mouse model of β-thalassemia. Blood, The Journal of the American Society of Hematology, 2012, 119.21: 5021-5029.

100- NAI, Antonella, et al. Increased susceptibility to iron deficiency of Tmprss 6-haploinsufficient mice. Blood, The Journal of the American Society of Hematology, 2010, 116.5: 851-852.

101- NALADO, Aishatu Muhammad, et al. 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, 2019, 10.1: 1.

102- National Center for Biotechnology Information (NCBI). (2008). Entrez-Gene. http://www.ncbi.nlm.nih.gov/sites/entrez/dbgene. Accessed May 1, 2008.

103- Nemeth, E. and Ganz, T. (2006). Regulation of iron metabolism by hepcidin. Annual Review Nutrition. 26: 323-342.

104- NEMETH, Elizabeta, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. science, 2004, 306.5704: 2090-2093.

105- Pandey S, Kunder S, Singh J, Bajaj N, Dwivedi S, Baghel VS, Pandey D, Pandey SK. Effect of TMPRSS6 rs855791 (T>C) polymorphism on pathophysiology of iron-deficiency anemia. Menoufia Med J 2022; 35: 1314-7.

106- PEI, Sung-Nan, et al. TMPRSS6 rs855791 polymorphism influences the susceptibility to iron deficiency anemia in women at reproductive age. International journal of medical sciences, 2014, 11.6: 614.

107- Peyrin-Biroulet L, Williet N, Cacoub P. Guidelines on the diagnosis and treatment of iron deficiency across indications: a systematic review. Am J Clin Nutr 2015; 102: 1585–94.

108- Prentice, A.M.; Mendoza, Y.A.; Pereira, D.; Cerami, C.; Wegmuller, R.; Constable, A.; Spieldenner, J. Dietary strategies for improving iron status: Balancing safety and efficacy. *Nutr. Rev.* **2017**, *75*, 49–60.

109- PEI, Sung-Nan, et al. TMPRSS6 rs855791 polymorphism influences the susceptibility to iron deficiency anemia in women at reproductive age. International journal of medical sciences, 2014, 11.6: 614.

110- Rosenfeld, D.L.; Tomiyama, A.J. Gender differences in meat consumption and openness to vegetarianism. *Appetite* **2021**, *166*, 105475.

111- Rowe, S.; Carr, A.C. Global vitamin C status and prevalence of deficiency: A cause for concern? *Nutrients* **2020**, *12*, 2008.

112- Sabatier, M.; Rytz, A.; Husny, J.; Dubascoux, S.; Nicolas, M.; Dave, A.; Singh, H.; Bodis, M.; Glahn, R.P. Impact of Ascorbic Acid on the In Vitro Iron Bioavailability of a Casein-Based Iron Fortificant. Nutrients 2020, 12, 2776.

113- Sanchez-Sabate, R.; Sabaté, J. Consumer attitudes towards environmental concerns of meat consumption: A systematic review. *Int. J. Environ. Res. Public Health* **2019**, *16*, 1220.

114- SEIKI, Toshio, et al. Association of genetic polymorphisms with erythrocyte traits: Verification of SNPs reported in a previous GWAS in a Japanese population. Gene, 2018, 642: 172-177.

115- Sekhar, D.L.; Murray-Kolb, L.E.; Kunselman, A.R.; Weisman, C.S.; Paul, I.M. Differences in risk factors for anemia between adolescent and adult women. *J. Womens Health* **2016**, *25*, 505–513.

116- Shah Y, Patel D, Khan N. Iron deficiency anemia in IBD: an overlooked comorbidity. *Expert Rev Gastroenterol Hepatol* 2021; 15: 771–81.

117- Shokrgozar N, Golafshan HA. Molecular perspective of iron uptake, related diseases, and treatments. 2019; 54: 10–16.

118- SILVESTRI, Laura, et al. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell metabolism, 2008, 8.6: 502-511.

119- SORANZO, Nicole, et al. Common variants at 10 genomic loci influence hemoglobin A1C levels via glycemic and nonglycemic pathways. Diabetes, 2010, 59.12: 3229-3239.

120- Sourabh S, Bhatia P, Jain R. Favourable improvement in haematological parameters in response to oral iron and vitamin C combination in children with iron refractory iron deficiency anemia (IRIDA) phenotype. Blood Cells Mol Dis. 2019; 75:26–9.

121- Stauder R, Valent P, Theurl I. Anemia at older age: etiologies, clinical implications, and management. Blood 2018; 131: 505–14.

122- Steiber A, Leon JB, Secker D et al. Multicenter study of the validity and reliability of subjective global assessment in the hemodialysis population. J Ren Nutr 2007; 17: 336–42.

123- Stein J, Dignass AU. Management of iron deficiency anemia in inflammatory bowel disease - a practical approach. *Ann Gastroenterol* 2013; 26: 104–13.

124- PEI, Sung-Nan, et al. TMPRSS6 rs855791 polymorphism influences the susceptibility to iron deficiency anemia in women at reproductive age. International journal of medical sciences, 2014, 11.6: 614.

125- TANAKA, Toshiko, et al. A genome-wide association analysis of serum iron concentrations. Blood, The Journal of the American Society of Hematology, 2010, 115.1: 94-96.

126- Tey T T, Yiu R, Leow W Q. Hepatitis B-Associated Symptomatic Iron Overload, with Complete Resolution after Nucleoside Analogue Treatment. Case Reports in Gastrointestinal Medicine. 2021.

127- TIETZ N.W Text book of clinical chemistry, 6th Ed C.A. Burtis, ER. Ashwood W.B. Saunders (2018): 1698-1704.

128- Tt Brownlie, Utermohlen V, Hinton PS, Giordano C, Haas JD. Marginal iron deficiency without anemia impairs aerobic adaptation among previously untrained women. Am J Clin Nutr 2002; 75: 734–42.

129- TURGEON, Mary Louise. Clinical hematology: theory and procedures. Lippincott Williams & Wilkins, 2005.

130- Urbaszek K, Drabińska N, Szaflarska-Popławska A et al (2021) Tmprss6 rs855791 polymorphism status in children with celiac disease and anemia. Nutrients.

131- VELASCO, Gloria, et al. Matriptase-2, a membrane-bound mosaic serine proteinase predominantly expressed in human liver and showing degrading activity against extracellular matrix proteins. Journal of Biological Chemistry, 2002, 277.40: 37637-37646.

132- Weerth D, Schrenck VJEjoci. Gastrin/cholecystokinin type B receptors in the kidney: molecular, pharmacological, functional characterization, and localization. 1998; 28(7):592-601.

133- World Health Organisation. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. Vitamin and Mineral Nutrition Information System 2011 Bathla S, Arora S. Prevalence and approaches to manage iron deficiency anemia (IDA). Crit Rev Food Sci Nutr 2021: 1–14.

134- WORLD HEALTH ORGANIZATION, et al. Global anaemia reduction efforts among women of reproductive age: impact, achievement of targets and the way forward for optimizing efforts. 2020.

135- WU, Alan HB. Tietz clinical guide to laboratory tests-E-book. Elsevier Health Sciences, 2006.

136- YEO, Tee Joo, et al. Iron deficiency in a multi-ethnic Asian population with and without heart failure: prevalence, clinical correlates, functional

significance and prognosis. European journal of heart failure, 2014, 16.10: 1125-1132.

137- Zaid Abdulrazzaq Ibrahim. Evaluate Iron Parameters Change and Gene Expression of Hepcidin and Ferritin in Pre- and Post-Menopausal Iraqi Women with Type 2 Diabetes Mellitus. A Thesis Submitted to the council of the faculty of medicine/ Al-Qadisiah university. 2022.

138- ZHANG, An-Sheng, et al. Suppression of hepatic hepcidin expression in response to acute iron deprivation is associated with an increase of matriptase-2 protein. Blood, The Journal of the American Society of Hematology, 2011, 117.5: 1687-1699.

الخلاصة

فقر الدم الناجم عن نقص الحديد هو اعلى نقص غذائي في العالم انه مرض متعدد العوامل مع معدل إمراضية عالية أن تعدد الأشكال الجيني TMPRSS6rs855791يلعب دورا أساسيا في تنظيم الحديد في جسم الإنسان ويلعب دورا أساسيا في توزان الحديد في الجسم، يرتبط تعدد الأشكال الجيني TMPRSS6 بشكل كبير بمستويات الحديد ومعايير الدم المتعددة.

هدف الدراسة التحليلية للتحقق من العلاقة بين تعدد الأشكال الجيني لأنزيم البروتين سيرين TMPRSS6 وخطر الإصابة بفقر الدم الناجم عن نقص الحديد وخطر فقر الدم بسبب نقص الحديد تم تعيين مجموعة من 100 شخص لهذه الدراسة 50 مريض يعانون من فقر الدم بسبب نقص الحديد و50 متطوعين أصحاء تم جمع البيانات للمرضى (العمر، النوع، تركيز خضاب الدم، متوسط حجم الكريه، متوسط حجم خضاب الدم، متوسط حجم تركيز خضاب الدم، الحديد، الفيريتين، حامض الاسكوربك اسد، الطول والجنس) من الملفات الطبية للمرضى بواسطه الاستبيان 5مل من الدم تم تجميعها من جميع المشتركين ووضع 2مل في تيوبات EDTA المضادة للتخثر وخزنه تحت درجة (-20) سيليزيه ويستعمل فيما بعد لاستخلاص DNA والباقي 3مل نضعه في جل تيوب للفحوصات الكيميائية تم استخراج الحمض النووي من الدم ثم تحليل تعدد الأشكال الجينيTMPRSS6and AQP1 بتفاعل البلمره لأجزاء المتضخمة فرقت بواسطة 2% من الجل المصبوغ ببرمابيد الاثيديوم وأظهرت النتائج بنظام الهلام الكهربائي التي أنتجت جزء واحد عند 249 تمثل سي متماثله (سي سي) وتم تحليلها بواسطة الحزمة الإحصائية الإصدارة 7 اختلافات ذات دلاله معنويه في مرضى ($P \leq 0.00$) تم كشف الجين انزيم البروتين سيرين 6 عابر الغشاء بواسطة تفاعلات البلمره المتسلسلة النمط الوراثي تي تي من تعدد الأشكال الجيني لانزيم البروتيني سيرسين 6 عابر الغشاء مرتفع بنسبه كبيره في مرضى فقرالدم بسبب نقص الحديد اظهرت النتائج انخفاض في, -HB-MCV MCHC- MCH وانخفاض في تركيز الحديد والفيريتين والاسكوربك اسد مقارنة مع الأشخاص الطبيعيين. لكن بالنسبة للجين الهجين والجين السائد بالنسبة لمرضى نقص الحديد لا يوجد أي تفاعل بين الجينات المذكورة والباراميترات للصورة الكاملة للدم كذلك بالنسبة للجين AQP1.

النمط الوراثي تي تي أيضاً موجود بنسبة كبيرة في مرضى فقر الدم بسبب نقص الحديد كذلك HB, MCHC, MCH, MCV, iron, ferritin and ascorbic acid ظهرت النتائج جدا منخفضة مقارنه بالأصحاء لكن الجين السائد والجين الهجين للمرضى المصابين بفقر الدم بسبب نقص الحديد لا يتفاعل مع البارميترات للصورة الكاملة للدم ماعدا خضاب الدم.

كذلك وجدنا أن لا يوجد اي علاقه بين الطول والعمر والجنس في مرض فقر الدم بسبب نقص الحديد أيضاً توجد علاقه بين الاسكوربك اسد والتغايير الجيني الخلاصة أوضحت الدراسة ارتباط ذو دلالة إحصائية بين تعدد الأشكال الجيني لأنزيم TMPRSS6 وAQP1 وخطر فقر الدم بسبب نقص الحديد بين مرضى العراقيين في مدينه بغداد يجب إجراء مزيد من الدراسات باستخدام طرق اخرى مثل تعدد شكل طول المتجزء وتسلسل الحمض النووي.

بسم الله الرحمن الرحيم

﴿ وَمَا تَوْفِيقِي إِلَّا بِاللَّهِ ﴾

صَدَقَ اللَّهُ الْعَلِيُّ الْعَظِيمُ

[سورة هود: آية (88)]

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية الطب / قسم الكيمياء الحياتية

دراسة

تعدد الأشكال الجيني لأنزيم البروتيني سيرين 6 عابر الغشاء مع نقص الحديد والفيريتين في مرضى العراق الكبار مع فقر الدم بسبب نقص الحديد

رسالة
مقدمة الى عمادة كلية الطب – جامعة بابل
كجزء من متطلبات نيل درجة الماجستير في العلوم /الكيمياء الحياتية السريرية

من قبل
سوسن هاشم حوشي حسن
بكالوريوس تقني تحليلات مرضية

باشراف

الأستاذ الدكتور
مؤيد الغزالي

الأستاذ المساعد الدكتور
حسين ناجي

1444هـ **العراق – بغداد** **2023م**