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Original Article

Exploring the Association of TMPRSS6 rs1421312 Variant with Hepcidin, sTfR1, and Iron Deficiency Anemia in Reproductive-Age Women

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ABSTRACT

Background: Iron deficiency anemia (IDA) continues to pose a significant global health problem affecting diverse demographic groups worldwide. A complex interplay between environmental and genetic factors affects the development of IDA. In this context, Variation in TMPRSS6 gene has been identified as a potential risk factor. This study examines the association between TMPRSS6 rs1421312 SNPs and key biomarkers of iron deficiency status. These included hepcidin, soluble Transferrin Receptor Type 1 (sTfR1), and the sTfR1-F Index, among non-pregnant women of reproductive age.

Methodology: To explore these associations, a cross-sectional design was employed. A total of 140 non-pregnant women, aged 17-50 years, were recruited based on low MCV and MCH. Participants were grouped into 60 IDA cases and 80 non-IDA cases based on a comprehensive analysis of their complete blood counts and serum iron studies. Additionally, DNA extraction and genotyping were carried out using allele-specific primers through PCR.

Result: The distribution of genotypes for TMPRSS6 rs1421312 in cases without IDA (non-IDA) was 9(11.25%) (CC), 67(83.75%) (TC), and 4(5%) (TT). Whereas in cases with IDA, it was 8(13.33%) (CC), 48(80%) (TC), and 4(6.66%) (TT). No significant difference was found between the groups (P = 0.840). Additionally, the results indicated that the variations in Hepcidin levels, sTfR1, and the sTfR1-Ferritin Index among the CC, TT, and TC genotypes were not statistically significant.

Conclusion: To the best of our knowledge, this represents the first such report from an Iraqi population. The current study concluded that no detectable association was found between the TMPRSS6 rs1421312 SNP and IDA. Additionally, no detectable associations were found with Hepcidin levels, sTfR1, or the sTfR1-Ferritin Index. Further studies are recommended to focus on the role of this SNP in IDA.

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Keywords: TMPRSS6 rs1421312, IDA, anemia, sTfR1, Hepcidin.

1 Introduction

Iron deficiency anemia (IDA) represents the most common form of anemia globally, and it remains a major public health issue [1]. According to the World Health Organization (WHO), IDA is a prevalent condition among women, children, and older adults worldwide. The prevalence is particularly high among pregnant and non-pregnant women and preschool children in Eastern Mediterranean countries, including those located in the Middle East [2]. The complex interaction between genetic and environmental conditions has a pivotal role in IDA development [3]. Despite the implementation of iron supplementation programs targeting vulnerable groups, including children and women of reproductive age, the prevalence of IDA remains high. Additionally, iron therapy does not treat all cases of this type of anemia [4,5].

Several rare genetic markers have been reported to have a significant effect on iron metabolism. In addition, genetic research has also revealed a number of common single-nucleotide polymorphisms (SNPs) that exert a lesser effect on iron metabolism by contributing to erythropoiesis [6]. Researchers have linked specific changes in genes (SNPs) controlling hepcidin, a key iron-regulating hormone, to low iron levels. The TMPRSS6 gene is the most common example [7]. However, studies in the Iraqi Kurdistan region investigating this connection are scarce.

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Genetic variants in the Transmembrane Serine Protease 6 (TMPRSS6) gene are considered risk factors for IDA. These genetic variants influence iron homeostasis by dysregulating hepcidin, the primary regulator of iron metabolism. The TMPRSS6 gene encodes the protein matriptase-2, which is a serine protease responsible for suppressing the transcription of the hepcidin gene. The TMPRSS6 gene produces matriptase-2, a serine protease that acts to inhibit hepcidin transcription. Consequently, TMPRSS6 variants could potentially lead to a rise in hepcidin levels, thereby affecting the IDA pathogenesis [8]. Hepcidin functions through ferroportin, a protein present predominantly in the cell membranes of enterocytes, macrophages, and hepatocytes, which mediates the export of iron from cells. Hepcidin triggers the internalization and breakdown of ferroportin [9,10]. As a result, high levels of hepcidin are linked to reduced dietary iron absorption from the intestines and also diminished release of stored iron, leading to lower iron concentrations in the bloodstream [11,12].

While the *TMPRSS6* gene plays a significant role, hepcidin synthesis is a complex process influenced by various factors beyond it, including Interleukin-6, bone morphogenetic proteins (BMPs), and other iron-regulating pathways. Specifically, bone morphogenetic protein 2 (BMP2) can stimulate hepcidin expression through its interaction with the BMP coreceptor, hemojuvelin [13].

Common genetic variants, including TMPRSS6 gene associated with IDA biomarkers, were previously investigated in various populations, such as South African and European populations [14], Saudi Arabia [3], and global populations [15]. Particularly, TMPRSS6 rs1421312 SNP has been found associated with serum Iron and transferrin saturations in African American and Italian populations; in addition to its commonness in African, European, Caucasian, Hispanic, and Asian populations [15]. Research on hepcidin has greatly contributed to a deeper understanding of iron metabolism, with many studies suggesting its potential as a clinical tool for diagnosing and managing iron-related disorders. [16].

Transferrin Receptor Type 1 (TfR1) is a membrane-bound protein crucial for the uptake of iron by cells. It binds to transferrin, the protein responsible for transporting iron, facilitating its entry into cells. In cases of iron deficiency anemia (IDA), TfR1 expression increases to meet the increased need for iron, as cells work to overcome the shortage [17]. The soluble form of TfR1 (sTfR1) is released into circulation and can serve as an indirect marker of body iron levels. In individuals with IDA, Elevated sTfR1 levels indicate the body's effort to boost iron absorption in response to depletion [18,19].

Many studies have investigated the link between hepcidin expression and different SNPs in the TMPRSS6 gene. For instance, a pilot study involving Egyptian children evaluated the effect of several TMPRSS6 SNPs on iron status, including their impact on hepcidin expression [7]. Although research specifically on the rs1421312 SNP is limited or nonexistent, these findings suggest a significant overall link between TMPRSS6 polymorphisms and hepcidin levels. It is true that other iron-related parameters, such as sTfR1 and the sTfR1-Ferritin Index, are based on the known function of the gene in iron metabolism.

The reason for selecting the TMPRSS6 rs1421312 SNP among all SNPs lies in the scarcity and conflicting nature of existing studies regarding its association with iron deficiency parameters. Moreover, by addressing this gap in the literature, our study aims to examine the association between TMPRSS6 rs1421312 SNPs and iron deficiency status among non-pregnant women of reproductive age in the Kalar district of Iraqi Kurdistan Region. We hypothesized that the rs1421312 variant is associated with altered hepcidin and iron indices. In general, this study aimed to assess the association between the TMPRSS6 gene single-nucleotide polymorphism and iron deficiency status, as well as hepcidin, soluble Transferrin Receptor Type 1 (sTfR1), and sTfR1-F Index in Iraq.

2 Materials and Methods

2.1 Study design

The present study utilized a cross-sectional design and obtained approval from the Research Ethics Committee of the University of Garmian (Approval No.: GRCEC114), consistent with the ethical principles of the Declaration of Helsinki. The sample consisted of 140 women aged 17 to 50 years who exhibited a low mean corpuscular volume (MCV) of less than 80 femtoliters and a low mean corpuscular hemoglobin (MCH) of less than 27 picograms. Individuals were excluded if they had evidence or a previous history of inflammatory disorders, *Helicobacter pylori* infection, active bleeding, occult blood, or other chronic medical conditions not specified in the inclusion criteria. Sociodemographic characteristics and medical histories were collected using a structured questionnaire, and informed consent was obtained from all participants prior to their participation. For analytical purposes, the participants were classified into two groups: 60 women diagnosed with iron deficiency anemia (IDA) and 80 women without iron deficiency anemia (non-IDA). Sample collection, biochemical analysis of iron study parameters, and complete blood count (CBC) analysis were previously performed by Al-Jaf et al. (2024) [20] to confirm IDA status.

2.2 Sample collection

The study was conducted between January 1st and May 15th, 2023, by collecting approximately 6 mL of venous blood samples from female patients attending private and public hospitals in the Kalar district of the Iraqi Kurdistan Region. Four milliliters of the sample were placed in collection tubes for an iron study using the COBAS C311 analyzer. The remaining blood samples were transferred into tubes containing ethylenediaminetetraacetic acid (EDTA) and analyzed for complete blood count (CBC) by using an automated method [20]. Hepcidin and soluble transferrin receptor type 1 (sTfR1) levels were estimated using a Human hepcidin, HEPC ELISA Kit and a Human sTfR1 Kit (China), an

automated ELISA washer (Biotech ELX50 Microplate Washer, USA), and a microplate reader (Biotech Microplate Reader, USA). The sTfR-Ferritin index was calculated as the ratio of sTfR concentration to the logarithm of the serum ferritin concentration. The calculation is as follows:

$$\text{sTfR-Ferritin Index} = \text{sTfR}/\log(\text{Ferritin})$$

2.3 DNA extraction

Genomic DNA was extracted from 200 µL of whole blood using the AddPrep Genomic DNA Extraction Kit, following the manufacturer's protocol. First, the blood sample was broken down by adding Lysis Solution and Proteinase K, then incubated at 56°C until the cells were fully lysed. Subsequently, the binding buffer was added to the lysate, and ethanol was introduced to facilitate the attachment of DNA to the spin column matrix. The mixture was then transferred onto the column and centrifuged, allowing the DNA to bind to the membrane. The column was washed sequentially with Wash Solution 1 and Wash Solution 2 to remove residual contaminants. After an additional centrifugation step to ensure complete removal of ethanol, the purified DNA was finally recovered by elution with the elution buffer. Finally, all DNA samples were stored at −20°°C until needed.

2.4 Genotyping

Polymerase chain reaction (PCR) was performed using a Taq DNA polymerase kit (Inviitrogen) according to the manufacturer's protocol. Each reaction contained 20 µl of master mix, prepared as follows:

Reagents	Volume
Add start master mix 2x	10 µl
F primers (5 pM)	1 µl
R primers (5 pM)	1 µl
Genomic DNA (Template)	5 µl
Nuclease-Free distilled Water	3 µl
Total Volume	20µl

PCR was performed in a thermal cycler (Applied Biosystems 2720, Foster City, CA, USA) using the following thermal profile: initial denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds; and a final extension at 72°C for 5 minutes.

Detection of the TMPRSS6 rs1421312 polymorphism utilized allele-specific primers as described by Al-Amer et al [3]. The primer sequences are listed below.

Forward1(Tallele): AGCCAGTGGCTTAGCCATTCCA
Reverse: GTGGTGGCAGCATCAGAGCAAAG
Forward2(Callele): AGCCAGTGGCTTAGCCATTCCG
Reverse: GTGGTGGCAGCATCAGAGCAAAG

The present study also utilized the primers described by Kocher et al. (1989) and Boakye et al. (1999) [21,22] for amplifying a 358 bp segment of the CytB gene from vertebrate mtDNA. This gene segment was used as a positive control to verify the efficacy of DNA extraction and the PCR reaction.

2.5 Differential Value and Statistical Analysis

WHO standards were adopted, defining iron deficiency as females with ferritin levels below 15 mg/L, transferrin saturation (TfSat) below 15%, and hemoglobin levels below 12 g/dL were considered to have iron deficiency [23]. Each completed questionnaire was assigned a unique identification number. The study's questions were encoded in preparations for data entry and analysis. The data was then entered into a Microsoft Excel spreadsheet to facilitate statistical analysis.

The current study's data were presented as mean values along with the standard error of mean (Mean ± S.E.M). Analysis was performed using both Microsoft Office Excel Spreadsheet 2021 and SPSS (Version 21.0) statistical software. Group mean differences were assessed using the independent two-sample t-test, also known as Student's t-test. Categorical variables were analyzed with the Chi-square test to identify associations. Statistical significance was defined as a *P-value* less than 0.05. Additionally, one-way analysis of variance (ANOVA) was used to compare means among groups defined by the TMPRSS6 rs1421312 SNP genotypes (CC, TT, and TC) for Hepcidin, soluble Transferrin Receptor type 1 (sTfR1), and the sTfR1-ferritin index.

3 Results

3.1 Detection of *TMPRSS6* (rs1421312) gene SNP

Figure 1 shows the results of allele-specific PCR for detecting the TMPRSS6 rs1421312 SNP, with PCR products separated using 1.5% agarose gel stained with ethidium bromide (Eth.Br.). The expected 135 bp amplicon size is visualized under UV light.

3.2 Distribution of *TMPRSS6* (rs1421312) gene SNP

The distribution of genotypes for TMPRSS6 rs1421312 in cases without iron deficiency anemia (non-IDA) was 9(11.25%) (CC), 67(83.75%) (TC), and 4(5%) (TT) whereas in cases with the iron deficiency anemia (IDA), it was 8(13.33%) (CC), 48(80%) (TC), and 4(6.66%) (TT) (Table 1). No statistically significant difference was observed between the groups (P = 0.840) (**Error! Reference source not found.** and

Figure 2).

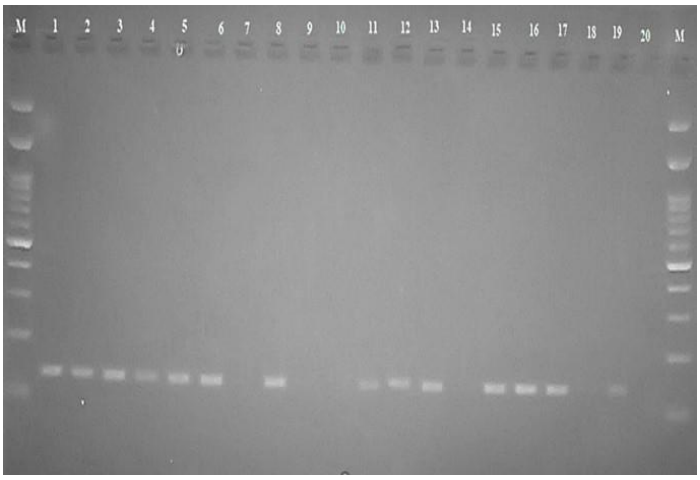


Figure 1: Agarose gel electrophoresis of Allele-specific PCR of *TMPRSS6* (rs1421312) PCR product size 135bp. The lanes included a 100-bp DNA ladder (Lane M), lanes 1-6,8,11-13,15-17, and 19, were positive for an expected amlicon size of 135 bp. Lane 7,9,10,14, and 18: were negative for the expected amplicon size of 135 bp, and Lane 20: had negative control.

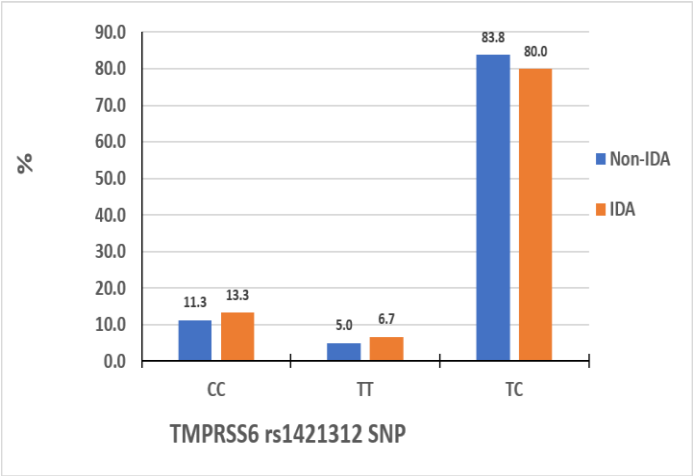


Figure 2: Distribution of TMPRSS6 (rs1421312) gene SNP in relation to serum iron status.

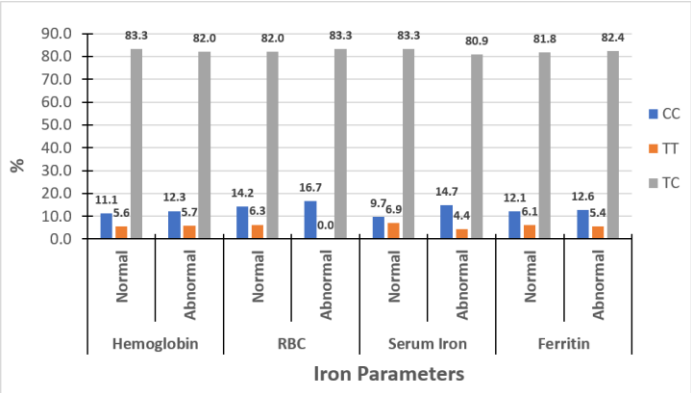
Table 1: Distribution of *TMPRSS6* (rs1421312) gene SNP in relation to serum iron status.

	N	CC	TT	TC	Chi-square	DF	P-Value
Non-IDA	80	9(11.25%)	4(5%)	67(83.75%)	0.348	2	0.840
IDA	60	8(13.33%)	4(6.66%)	48(80%)			
Total	140	17	8	115			

3.3 Distribution of *TMPRSS6* rs1421312 gene SNP in relation to some clinical parameters

Table **1)** and the results were as follows 13.57% of participants had normal hemoglobin, 91.42% had high RBCs, 51.42% had high iron, and 47.14% had high ferritin.

Table **1** and



The distribution of *TMPRSS6* rs1421312 was clinically evaluated, and it was analyzed for hemoglobin, RBC, serum iron, ferritin (

The data of the current study indicate that *TMPRSS6* rs1421312 was not significantly related to decreased hemoglobin (*P = 0.989*), decreased RBCs (P = *0.615*), decreased serum iron (*P* = 0.567), or decreased serum ferritin (*P* = 0.986) (

Figure **3**).

Table 1: Distribution of TMPRSS6 (rs1421312) gene SNP genotypes in relation to clinical parameters.

	N	CC	TT	TC	Chi-square	DF	P-Value
Hemoglobin							
Normal	18	2(11.11%)	1(5.55%)	15(83.33%)	0.023	2	0.989
Abnormal	122	15(12.29%)	7(5.73%)	100(81.96%)			
RBC							
Normal	128	15(14.15)	8(6.25)	105(82.03)	0.973	2	0.615
Abnormal	12	2(16.66%)	0(0.00%)	10(83.33%)			
Iron							
Normal	72	7(9.72)	5(6.94%)	60(83.33%)	1.133	2	0.567
Abnormal	68	10(14.70%)	3(4.41%)	55(80.88%)			
Ferritin							
Normal	66	8(12.12%)	4(6.06%)	54(81.81%)	0.028	2	0.986
Abnormal	74	9(12.61)	4(5.40%)	61(82.43%)			

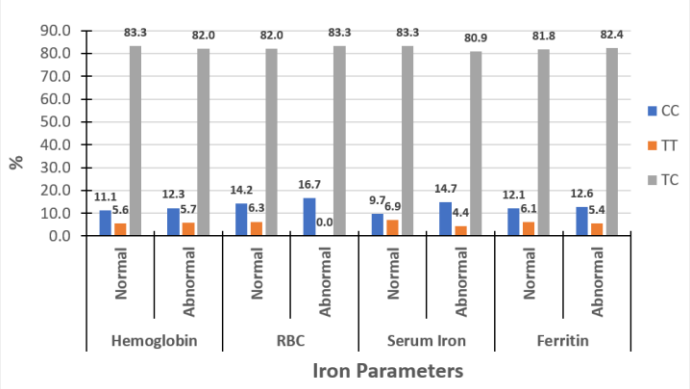


Figure 3: Distribution of TMPRSS6 (rs1421312) gene SNP genotypes in relation to clinical parameters.

Table **2**, **Error! Reference source not found.**b). Lastly, for the sTfR1-Ferritin Index, the mean values were 1.79 ± 0.30 for the CC genotype, 1.30 ± 0.34 for the TT genotype, and 2.20 ± 0.33

3.4 Association of Hepcidin, TfR1, and sTfR1-Ferritin Index with *TMPRSS6* (rs1421312) gene SNP in IDA

The ANOVA-test results for the association of Hepcidin, soluble Transferrin Receptor type 1 (sTfR1), and sTfR1-Ferritin Index with the TMPRSS6 rs1421312 SNP in iron deficiency anemia (IDA) showed no significant differences across the genotypes. For Hepcidin, the mean values for the CC, TT, and TC genotypes were 212.8 ± 37.30, 223.25 ± 59.85, and 267.0 ± 28.04, respectively, with F (2, 80) = 0.319, p = 0.728, indicating no significant effect (Table 3, **Error! Reference source not found.**a). Similarly, for sTfR1, the mean values for the CC, TT, and TC genotypes were 8.86 ± 0.93, 8.74 ± 1.45, and 10.30 ± 0.86, respectively, with F (2, 80) = 0.285, p = 0.753, showing no significant differences (

for the TC genotype, with F (2, 80) = 0.328, p = 0.722, confirming no significant differences between the genotypes (Table 3, **Error! Reference source not found.**c).

Table 2: Association of Hepcidin, TfR1, and sTfR1-Ferritin Index with *TMPRSS6* (rs1421312) gene SNP in IDA.

Parameter	TMPRSS6 rs1421312 SNP	Mean & Std. Error	df	F	*P-Value*
Hepcidin (pg/ml)	CC	212.8±37.30	2	0.319	0.728
	TT	223.25±59.85	80		
	TC	267.0 ±28.04	82		
sTfR1 (ug/ml)	CC	8.86±0.93	2	0.285	0.753
	TT	8.74±1.45	80		
	TC	10.30±0.86	82		
sTRf1-F Index	CC	1.79±0.30	2	0.328	0.722

	TT	1.30±0.34	80		
	TC	2.20±0.33	82		

4 Discussion

IDA is a prevalent health concern worldwide. Previously, iron deficiency was thought to arise from factors related to diet and environment. However, advancements in technology and increased knowledge of iron metabolism disorders have demonstrated that genetic factors significantly contribute to the development of iron deficiency [24,25]. The most prevalent SNPs linked to decreased iron status are in *TMPRSS6* gene, which codes for the MT-2 protein, which inhibits hepcidin formation [26,27].

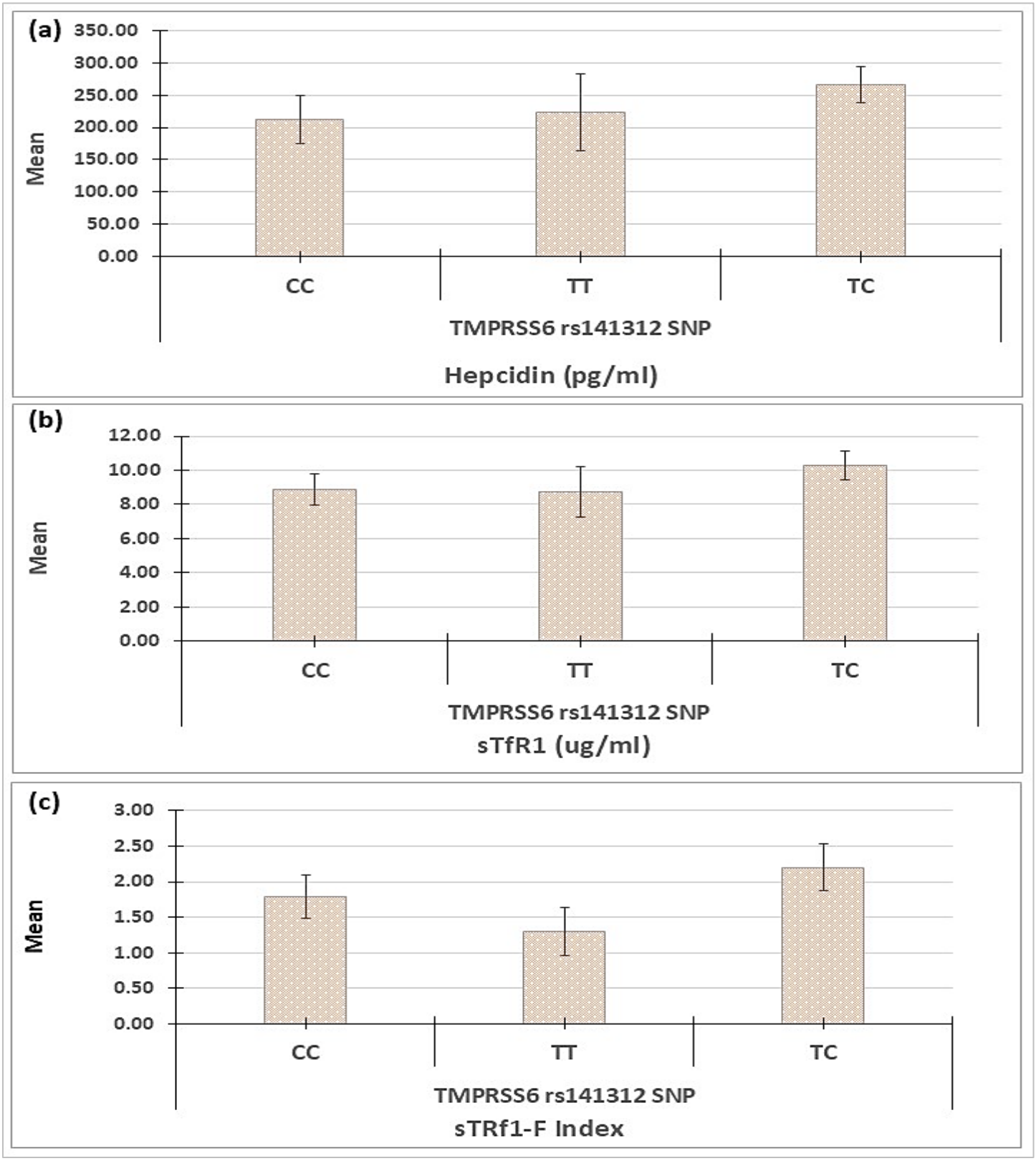


Figure 4: Association of iron-related biomarkers with the TMPRSS6 rs1421312 SNP in IDA patients. Serum levels of (a) Hepcidin, (b) Soluble transferrin receptor 1 (sTfR1), and (c) sTfR1-Ferritin Index are shown across genotypes (CC, CT, TT).

More than 50 *TMPRSS6* SNPs have been identified as being associated with iron levels status. The most extensively studied of these variations are rs855791, rs4820268, rs2235321, rs11704654, and rs2235324, all linked to reduced iron levels. Specifically, SNPs rs855791, rs4820268, and rs11704654 exert a considerable impact on measures of red blood cell function and iron levels, particularly among individuals of Asian descent [6, 28]. However, rs855791 and rs4820268 are much more commonly studied than rs1421312, which remains relatively under-researched with limited available data. The scarcity of information on rs1421312 highlights a gap in the literature, making it an intriguing target for further investigation.

To our knowledge, this is the first study to examine the relationship of TMPRSS6 rs1421312 SNPs with iron-deficient status in non-pregnant women of reproductive age in Kalar, Iraq. There is no previous research and conflicting results in the published work, and the study reveals a relationship between the TMPRSS6 rs1421312 mutation and markers of iron deficiency anemia (IDA) in this population.

The result of the study revealed a non-significant association observed in the distribution of *TMPRSS6* rs1421312 in cases without iron deficiency anemia (non-IDA) and those with IDA, as indicated by a p-value of 0.840.

Our study is in alignment with previous studies in female university students in Saudi Arabia, which showed no significant association between the TMPRSS6 rs1421312 SNP and iron deficiency anemia (IDA) [3]. However, our results do not agree with McLaren et al., who reported significant associations for rs2111833 and rs1421312 SNPs in mixed ethnic groups, particularly an inverse association for rs2111833 in Caucasians for serum iron levels [29]. In contrast, studies in African Americans found inconsistent associations of the rs1421312 risk allele with indicators of body iron status [14].

In this study, the TMPRSS6 rs1421312 SNP did not appear to significantly increase the risk of IDA within the population. The absence of a clear effect may reflect factors such as the relatively small sample size, unique population characteristics, or the influence of additional genetic and environmental variables. Future studies are warranted to explain these relationships [8,29].

This study also examined the association of the TMPRSS6 rs1421312 SNP with Hepcidin, soluble Transferrin Receptor 1 (sTfR1), and the sTfR1-Ferritin Index in individuals with IDA. Analysis of variance (ANOVA) results showed no significant differences across the genotypes for any of these markers, indicating that rs1421312 may have little effect on iron homeostasis (Table 3). In contrast, other TMPRSS6 variants, such as rs855791, have been reported to influence Hepcidin regulation and iron absorption, particularly through altered hepcidin expression [30]. The lack of a detectable effect for rs1421312 in our cohort could reflect differences in its functional role or genomic location.

In our study, rs1421312 showed no significant association with iron biomarkers such as hepcidin or sTfR1, suggesting that this SNP may play only a minor role in regulating iron metabolism compared with other TMPRSS6 polymorphisms [31]. It is possible that rs1421312 effect depends on interactions with other genetic or environmental factors not assessed in this study. It might be valuable to explore how factors such as lifestyle, diet, and underlying health conditions interact with genetic factors to impact IDA.

Conclusions

The present study did not find any significant association of the TMPRSS6 rs1421312 SNP with iron deficiency anemia (IDA) and its related hematological and biochemical parameters including hemoglobin, red blood cell count, serum iron, serum ferritin, hepcidin, sTfR1 and sTfR1-ferritin index in women of Iraqi Kurdistan. The relatively small sample size may have limited the statistical power to detect modest genetic effects. Thus, larger and more diverse population studies are recommended to better understand the potential role of the TMPRSS6 rs1421312 SNP in iron homeostasis and susceptibility to iron deficiency anemia.

Authors' contributions

Diyar Akbar Hasan Al-Jaf: Conceptualization, methodology, data collection, analysis, writing original draft. Ammar Lateef Hussein: supervision, review, editing. Sherko S. Niranji**:** Review and validation

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Conflict of interest

The authors declares that they have no conflicts of interests.

Data availability statement

The data are available upon request from the authors.

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