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Original Article

TMPRSS6 rs855791 polymorphism and susceptibility to iron deficiency anaemia in non-dialysis chronic kidney disease patients in South Africa

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Abstract: Background: In genome-wide studies, there is a strong association between the TMPRSS6 allele A736V (rs855791) and significantly lower levels of serum iron, transferrin saturation, haemoglobin, and mean corpuscular volumes. The influence of this genetic variant on susceptibility to iron deficiency anaemia (IDA) in chronic kidney disease (CKD) patients is unknown. Methods: In this cross-sectional study, we measured the full blood count and TMPRSS6 T>C polymorphism in black adult participants (n=260) with CKD and healthy controls (n=146) at the Charlotte Maxeke Johannesburg Academic Hospital, South Africa. Results: The overall prevalence of anaemia in the CKD and control population was 46.9% and 19.6% respectively. Twenty-six per cent of CKD participants were iron deficient. The prevalence of rs855791 C homozygosity was similar among iron deficient and non-iron deficient anaemia groups (86.1% vs 84.2%, P=0.723). When the analysis was confined to subjects with or without functional iron deficiency anaemia, C homozygote (88.3% vs 84.4%, P=0.425) was similar for both groups. Conclusions: Our study suggests that homozygosity for TMPRSS6 rs855791 C genotype does not influence IDA in non-dialysis CKD patients in our population.

Keywords: Iron deficiency anaemia, CKD, TMPRSS6, rs855791

Introduction

Anaemia is present in the majority of patients with chronic kidney disease (CKD), occurring in 15.4% of patients with CKD [1, 2]. While the major cause of anaemia in CKD is a relative deficiency of erythropoietin, iron deficiency anaemia contributes to anaemia of CKD [1, 2]. Thus there is a need to identify genetic factors that may be associated with iron deficiency anaemia. Iron is essential for multiple biological functions in all tissues, but especially for synthesis of haemoglobin, as shown by anaemia that results from iron deficiency [3]. Over the past decade, heritable overtly pathological iron deficiencies have been accredited to mutations in several key genes that regulate iron homoeostasis [4] and thus the need to identify genetic factors that may be associated with iron deficiency anaemia.

The TMPRSS6 (transmembrane serine protease 6) gene encodes matriptase-2. Matripase-2 is an essential component of a pathway that detects iron deficiency, represses hepcidin transcription in the liver by cleaving membrane-bound hemojuvelin, and permits enhanced dietary iron absorption [5]. The TMPRSS6 SNP A736V (rs855791) has been found to be related to lower levels of serum iron, transferrin saturation, haemoglobin, and mean corpuscular volume in genome-wide association studies conducted in the general population [5]. Heritability estimates suggest that genetic factors contribute 20-30% of the variation in blood iron concentration [6]. This single nucleotide

polymorphism (SNP) rs855791 is located in the functional part of TMPRSS6 and causes a non-synonymous substitution that reduces the ability of the enzyme to inhibit hepcidin transcription [7]. Thus, TMPRSS6 A736V influences iron homoeostasis and erythropoiesis in normal subjects, although in another study conducted in patients with iron deficiency, based on presence or absence of anaemia, TMPRSS6 was found not to differ in women with iron deficiency with or without anaemia in the studied population [8, 9]. It remains uncertain whether the association is mediated by iron, or is through a direct effect of the variants on erythropoiesis.

Most studies conducted on the genetics of iron metabolism were conducted in persons of European ancestry. The few data available from the Asian population pointed to a potential role of ethnic variability in the heritability of specific measures of iron status [10, 11]. There are few studies that analysed the genetics of iron metabolism in African populations, where the burden of iron deficiency is immense [12]. Furthermore, it has been documented that the highest level of genetic diversity (both nuclear and mitochondrial) in the global human population is within the African population [13-15]. Genetic risk of disease in the African population would likely be more complex because of increased levels of possible interactions between the genetic diversity and numerous differential environmental factors that impact iron absorption [16].

The aim of this study was to investigate the role of the genetic variant rs855791 on susceptibility to iron deficiency anaemia in pre-dialysis CKD patients and to determine whether this variant influences iron status in CKD patients.

Materials and methods

Venous blood samples were collected from patients (n=265) attending the renal outpatient clinic of the Charlotte Maxeke Johannesburg Academic Hospital, South Africa and apparently healthy controls (n=141) which comprised of patients' relatives and hospital staff, from 1 May 2016 to 31 December 2016. Venous blood samples were collected, and standard methods were used for haematological measurements. Written informed consent was obtained from all participants, and the study protocol was approved by the Human Research Ethics Committee of the University of the Witwatersrand (M150929).

DNA extraction

Genomic DNA was extracted from whole blood using a Maxwell DNA purification kit (AS1010, Promega, WI, USA), as per the manufacturer's protocol. DNA concentrations were determined by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, DE, USA) and quality assessed with the A260/280 ratios. A 260/280 ratio of 1.8-2.0 was considered indicative of good quality DNA. Samples that had suboptimal DNA concentrations (<10 ng/ul) were re-extracted using a modified salting out method [17].

Genotyping

The region of the TMPRSS6 gene containing the rs855791 T>C polymorphism was amplified using the polymerase chain reaction (PCR). Primers were as described in Pei et al. (2014) [14, 18]: TMPRSS6F 5'-TAG AGA ACA GGG GCT CCA GG-3'; TMPRSS6R 5'-ATG TGG GCA GCA TCC TTT C-3'. The PCR reactions each contained 1x KAPA2G Robust HotStart Ready Mix (KAPA Biosystems, Massachusetts, USA), 0.125 μM of each of the forward and reverse primers and 50 ng extracted DNA. Thermocycling conditions were 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds, 65°C for 15 seconds, 72°C for 20 seconds and a final extension of 72°C for 1 minute. This resulted in the amplification of a single 249 bp fragment. The PCR products was digested with the restriction endonuclease Stu 1 (New England Biolabs, MA, USA). Genotype was determined by fragment size, under UV light in gel documentation system (Bio-Rad) and 10% of the samples were directly sequenced to confirm the genotyping results.

Statistical analysis

Normally distributed continuous variables were presented as means ± standard deviations, while non-normally distributed variables were presented as medians (interquartile ranges). Categorical variables were presented as numbers and percentages. The relationship between categorical variables and TMPRSS6 categories were tested using the Chi-square or Fisher's exact test. Analysis of variance (ANOVA)

Table 1. Comparison of demographic, biochemical and haematological characteristics of participants by TMPRSS6 gene

Parameter	CKD patients				Apparently healthy controls			
	TT (n=5)	TC (n=34)	CC (n=221)	*P*-value	TT (n=2)	TC (n=19)	CC (n=122)	*P*-value
Age (mean ± SD)	53.8±17.8	52.8±15.5	52.6±14.1	0.983[#]	37.5±3.5	43.6±16.2	40.3±11.9	0.528[#]
Gender n (%)								
Male	5 (100%)	11 (32.4)	121 (54.8)	0.005	0 (0.0)	7 (36.8)	53 (43.4)	0.415
Female	0 (100)	23 (67.7)	100 (45.3)		2 (100.0)	12 (63.2)	69 (56.6)	
Iron umol/Median (IQR)	10.3 (7.4-12.9)	10.8 (8.5-12.9)	9.9 (5-13.9)	0.814[$]	8.95 (7.7-10.2)	11.7 (8.7-19.6)	12.6 (10.9-14.5)	0.223[$]
TSAT % median (IQR)	18 (15-22)	19 (12-20)	16.5 (13-21)	0.479[$]	17 (13-21)	22 (19-28)	21 (17-23)	0.279[$]
Ferritin ng/ml median (IQR)	104 (58-198)	92 (70-100)	99 (44-140)	0.266[$]	69.5 (15-124)	66 (51-144)	77 (56-144)	0.776[$]
Haemoglobin g/l median (IQR)	12.5 (10.7-14)	14.3 (12.9-14.8)	12.5 (10.9-14.2)	0.310[$]	13.2 (12.6-13.8)	13.6 (12.3-15.1)	13.95 (13-15.5)	0.540[$]
Mean corpuscular volume fll (MCV) median (IQR)	88.1 (83.5-91.6)	92.9 (83.4-93.2)	88.2 (84.6-90.6)	0.856[$]	90.7 (88.2-93.2)	90 (86.8-90.9)	88.2 (84.2-91.6)	0.592[$]
Mean corpuscular haemoglobin pg/cell (MCH) median (IQR)	28.7 (26.9-30.1)	30.3 (26.8-31.8)	28.8 (27.6-30.1)	0.856[$]	32 (29.2-34.8)	29.6 (27.9-30.1)	29.7 (27.2-30.4)	0.525[$]
Mean corpuscular haemoglobin concentration (MCHC) g/dL median (IQR)	32.5 (31.4-33.4)	32.6 (32.2-33.2)	32.7 (31.4-33.7)	0.748[$]	34 (33.2-34.8)	32.5 (31.9-32.9)	32.6 (31.9-33.5)	0.229[$]
%Hypochromic red cells median (IQR)	7.9 (3.4-15.7)	10.5 (5.5-10.8)	9.9 (1.7-22.1)	0.940[$]	5.1 (3.0-7.2)	4.1 (2.1-11.9)	3.3 (1.8-5.6)	0.826[$]
Reticulocyte haemoglobin content (CHr) median (IQR)	27.9 (27.1-30.6)	29.8 (27.9-30.6)	27.8 (26.9-29.9)	0.412[$]	27.8 (26.8-28.7)	29.1 (27.9-31.6)	28.5 (27.2-30.6)	0.390[$]
GDF-15 median (IQR)	1012 (406.9-1458.3)	549.9 (303-598)	1170.3 (335.7-1636)	0.188[$]	978.95 (648.1-1309.8)	309.9 (159.9-1101)	438.7 (175.5-1183.4)	0.519[$]
Hepcidin	8.1 (4-35.8)	4.2 (3.9-5.1)	4.8 (4-15.2)	0.403[$]	3.2 (3.1-3.2)	2.9 (1.9-13.1)	2.9 (2.1-11.3)	0.960[$]

[#]*P*-value for analysis of variance (ANOVA). [$]*P*-value for Kruskal Wallis' test.

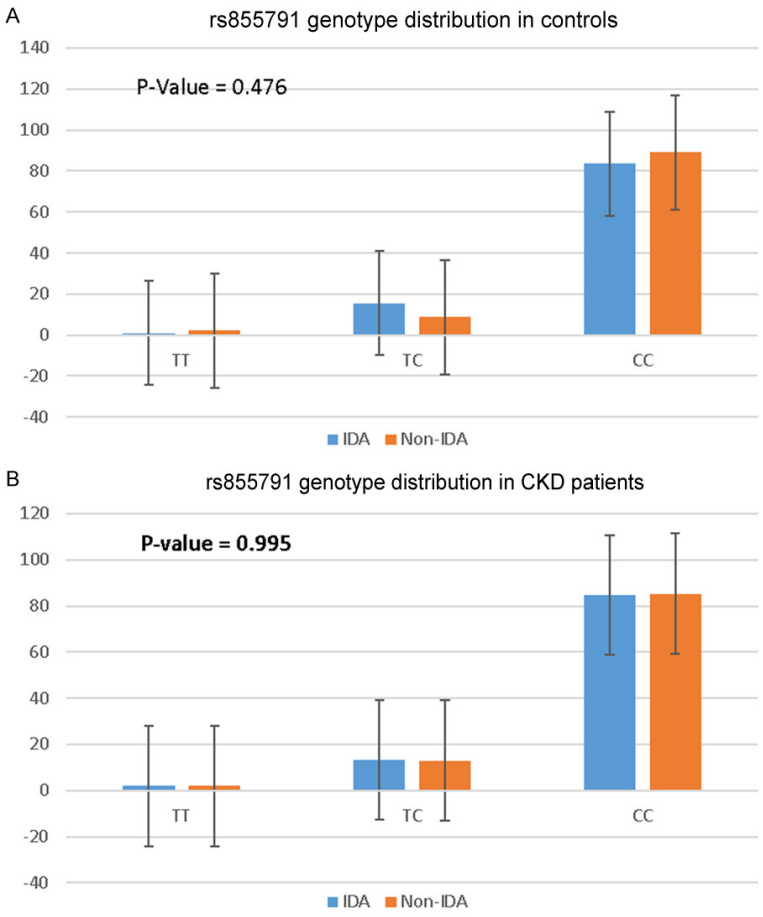

Figure 1. A. Prevalence of polymorphism by anaemic status among the controls. B. Prevalence of polymorphism by anaemic status among the CKD patients.

Table 2. TMPRSS6 rs855791 genotypes distribution between IDA and Non-IDA groups

Anaemia status	TMPRSS6 rs855791 (n, %)			*P*-Value
	TT	TC	CC	
IDA n (%)	4 (1.9)	24 (11.9)	173 (86.1)	0.73
Non-IDA n (%)	3 (1.5)	29 (14.4)	170 (84.2)	

or Kruskal Wallis' test was used to determine the association between continuous socio-demographic, biochemical and haematological characteristics and TMPRSS6 alleles. The relationship between TMPRSS6 polymorphisms and iron deficiency anaemia was evaluated using univariable and multivariable binary logistic regression controlling for potential confounders. Statistically significant level was set at *P*-value <0.05 (confidence interval of 95%). Stata version 14 (Stata Corp, USA) statistical software was used for analysis.

Results

Among the CKD groups, there was no statistically significant relationship between the prevalence of the TMPRSS6 alleles and most of the demographic characteristics (**Table 1**); only gender has a statistically significant relationship with TMPRSS6 alleles (*P*-value =0.005). Thus, while the majority (n=5/7) of TT alleles were found in males, only one-third (32.4%, n=11/53) of the TC alleles were found in males; (*P*-value =0.005) (**Table 1**).

Among the controls, there was no statistically significant difference in the proportion, mean or median variables of the demographic and clinical characteristics and among the TMPRSS6 alleles (**Table 1**).

From **Figure 1A**, in the control arm, there was no statistically significant difference in the prevalence of each category of TMPRSS6 rs855791 among the anaemic and non-anaemic groups (See also Table S1). Also, from **Figure 1B**, in the CKD arm, there was no statistically significant difference in the prevalence of each category of TMPRSS6 rs855791 among the anaemic and non-anaemic groups (See also Table S1).

Table 2 showed that on bivariate analysis, there was no statistically significant difference in the proportions of IDA Vs non-IDA among CC (86.1% Vs 84.2%), TC (11.9% Vs 14.4%), and TT (1.9% Vs 1.5%) categories of TMPRSS6 rs855791 gene. **Figure 2** also showed that ferritin and haemoglobin levels among the TMPRSS6 rs855791 categories was not statistically different.

Table S1 showed that on bivariate analysis, in the CKD group, there was no statistically significant difference (*P*-value =0.995) in the proportions of IDA Vs non-IDA among CC (59.7% Vs

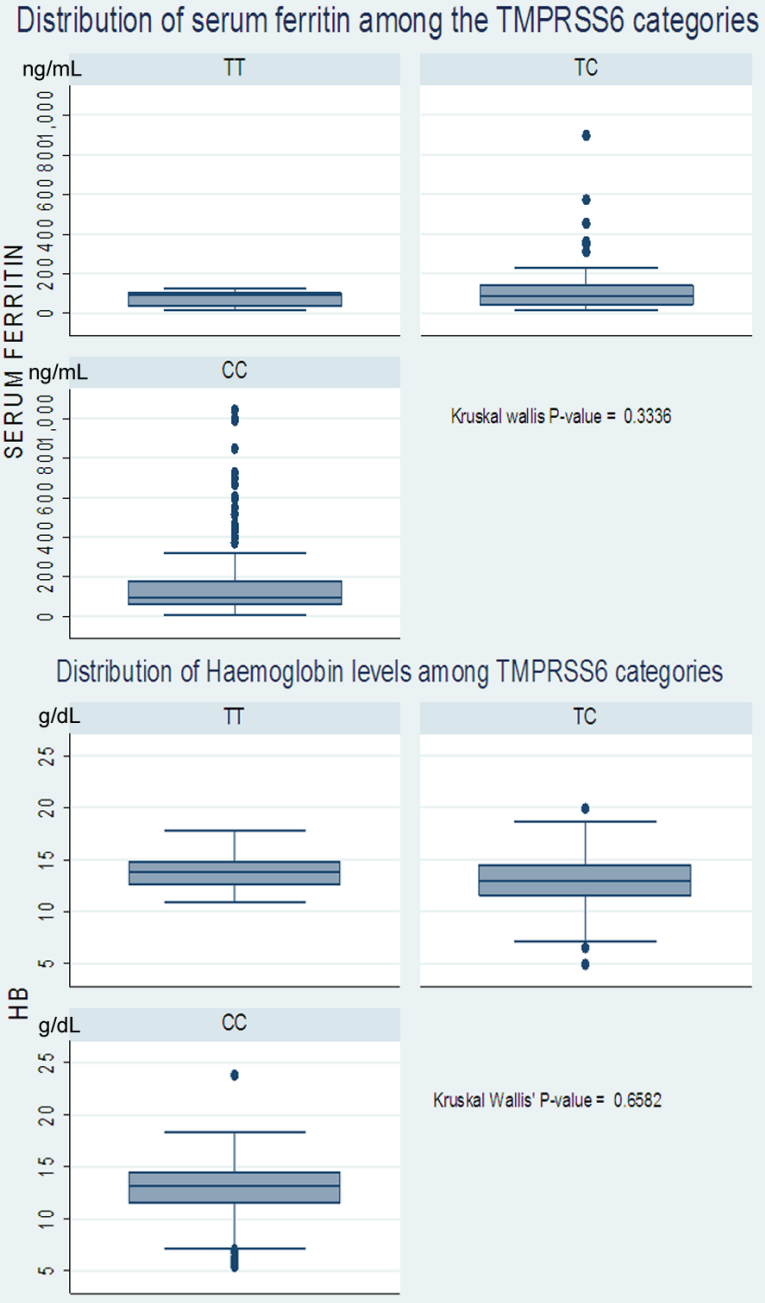

Figure 2. Showing distribution of haematological parameters among TMPRSS6 rs855791 categories.

40.3%), TC (58.8% Vs 41.2%), and TT (60.0% Vs 40.0%) categories of TMPRSS6 rs855791. Similarly, in the controls, there was no statistically significant difference in proportion of IDA and non-IDA among the categories of TMPRSS6 rs855791.

From the multivariable analysis (**Table 3**), there was a trend towards statistical significance for the relationship between TMPRSS6 rs855791 genotypes and iron deficiency anaemia. The TC genotype tends to be protective against iron deficiency anaemia as there was a 45% lesser odds of iron deficiency anaemia among participants who had TC genotypes as compared to participants who had CC genotypes (Adj OR: 0.55, 95% CI: 0.29-1.06, *P*-value =0.074). The CKD participants had about 3-fold higher odds of iron deficiency as compared to the controls (Adj OR: 3.2, 95% CI: 1.95-5.26, *P*-value <0.001). Similarly, participants who were 50 years or older had about 2-fold higher odds of iron deficiency anaemia as compared to participants younger than 50 years. (Adj OR: 1.81, 95% CI: 1.13-2.89, *P*-value <0.014).

Females also had about 2-fold higher odds of iron deficiency anaemia as compared to males. (Adj OR: 1.81, 95% CI: 1.13-2.89, *P*-value <0.014). Furthermore, there was a 54% lesser odds of iron deficiency anaemia among participants who had high reticulocyte haemoglobin (>28) or hepcidin (≥50) level (**Table 3**). The area under receiver operator characteristic (ROC) curve (AUC) of the regression model was about 77.0%. Thus, the model can discriminate iron deficiency anaemia 77% of times (see Figure S1).

Discussion

We report the first study focussing on the genetic aetiology of iron status in black non-dialysis CKD patients in South Africa. The prevalence of iron deficiency anaemia (IDA) among non-pregnant females in South Africa was 24.3% [19]. We found no association between the TMPRSS6 (rs855791) SNP, and parameters

Table 3. Multiple logistic regression of the relationship between TMPRSS6 rs855791 polymorphism and iron deficiency anaemia

Factor	Odds Ratio	95% CI	*P*-value	Adj Odds Ratio^	95% CI	*P*-value
TMPRSS6 rs855791						
CC	1.00	Ref	Ref	1.00	Ref	Ref
TT	1.31	0.29-5.95	0.726	1.83	0.39-8.48	0.442
TC	0.81	0.45-1.45	0.486	0.55	0.29-1.06	0.074
Study group						
Control	1.00	Ref	Ref	1.00	Ref	Ref
CKD	3.18	2.07-4.88	<0.001	3.20	1.95-5.26	<0.001
Age (years)						
<50	1.00	Ref	Ref	1.00	Ref	Ref
≥50	2.26	1.51-3.37	<0.001	1.81	1.13-2.89	0.014
Gender						
Male	1.00	Ref	Ref	1.00	Ref	Ref
Female	2.03	1.37-3.02	<0.001	2.12	1.33-3.38	0.002
Iron (umol/L)						
<7	1.00	Ref	Ref	1.00	Ref	Ref
≥7	0.23	0.13-0.43	<0.001	0.34	0.17-0.68	0.002
Reticulocyte haemoglobin pg/ml						
<28	1.00	Ref	Ref	1.00	Ref	Ref
≥28	0.34	0.23-0.51	<0.001	0.46	0.29-0.73	0.001
Hepcidin level ng/ml						
<50	1.00	Ref	Ref	1.00	Ref	Ref
≥50	0.76	0.48-1.20	0.240	0.46	0.26-0.80	0.006
MCHC g/dl	0.87	0.74-1.02	0.083	1.11	0.96-1.28	0.157
MCH pg/cell	0.85	0.77-0.93	<0.001	0.88	0.79-0.98	0.018

^Model corrected for age, gender, study group, serum iron level, reticulocyte haemoglobin and hepcidin level. Mean-Variance inflation factor =1.2. Hosmer-Lemeshow. *P*-value =0.2401.

of iron status including IDA in our CKD population, whereas previous researchers have found an association in European and Asian populations [20-22]. The exact mechanism through which TMPRSS6 is related to iron deficiency is still under investigation.

We found iron deficiency anaemia to be commoner (about 3-fold) among patients with CKD compared to controls; this finding is in agreement with previous studies [23-25]. There are several factors that may explain the higher prevalence of iron deficiency among CKD patients; compliance with oral supplements is difficult, as iron supplements need to be taken in between meals, and they cause gastrointestinal side effects, and intestinal absorption of oral iron may be impaired in CKD, and reduced iron absorption caused by use of phosphate binders.

Our study has also provided the first report of rs855791 allelic frequency in South African black non-dialysis CKD population. The C allele frequency in our IDA and control groups was 92.0% and 91.3% respectively. These values are much higher than the findings in the Taiwanese population (48.6% vs 50%), the European population (39-47%) and among other Asian population [18, 21, 26]. However, the C allele frequency has been reported to be 82-93% in an African population [18], which is similar to our findings. The high prevalence of the CC genotype may have an advantage of enhanced iron absorption during food shortages or may provide some protection against infective parasites like malaria which invade the red blood cells [27, 28]. These results further strengthen the hypothesis that genetic variations of the TMPRSS6 gene may contribute to variations in iron status depending on different racial and environmental factors, and more studies are needed in the African population to further ascertain the association between TMPRSS6 and iron deficiency anaemia.

In our study we found that TC genotypes tended to have a protective role against the development of iron deficiency anaemia, and TT did not have an association with the development of iron deficiency anaemia. This finding is at variance with the findings of Kumar et al. [25], [29] where the TT genotype had a pathological role, and the CC genotype was protective against iron deficiency anaemia. Gonçalves et al. [30] also found TT genotype to be significantly higher in the IDA group among Portuguese women. Differences in our findings could be explained by differences in the studied population as our study was conducted in patients with kidney disease while their studied population included pregnant women. In addition, environmental and racial factors could also contribute to these differences.

Polymorphisms of TMPRSS6 were found to be associated with a variety of iron parameters, including lower serum iron, haemoglobin, ferritin, mean corpuscular volume [20, 31-33]; our study is at variance with these findings. While Danquah et al's. [24, 28] findings were in agreement with our findings a possible explanation for differences in our findings could be explained by differences in sample size as other researchers had larger sample sizes. Other reasons could be abundant infections, and inflammatory processes affecting African CKD patients, which affect the validity of these markers on iron status. Nevertheless, more studies are needed to further explore these findings.

In this present study, increased levels of hepcidin were protective against iron deficiency anaemia; patients with hepcidin level >50 ng/ml are 56% less likely to develop iron deficiency anaemia. This finding supports the literature that lower levels of serum hepcidin are associated with iron deficiency anaemia [34, 35]. We found that serum hepcidin was not significantly associated with TMPRSS6 rs855791 in the fully adjusted models, and there was no decrease in strength of the association between this SNP and iron parameters; hence our data does not support an intermediate role for the association of rs855791 polymorphism with iron parameters in CKD patients. This finding is similar to the findings of Galesloot et al. [36]. Traglia et al. [37] found no association between rs855791 and hepcidin in an apparently healthy population. However, other studies were in disagreement with our findings [26, 38]. A possible explanation for a negative association of TMPRSS6 variants with iron deficiency anaemia and iron parameters may be explained by a likely omission of environmental and genetic factors that may alter hepcidin concentrations independent of iron regulation, and different methods used to measure hepcidin; we used mass spectrometry while others used enzyme-linked immunoassay (ELISA). Further research with larger sample size are needed to further explore this finding.

Conclusion

The present study has shown that the TMPRSS6 rs855791 CC genotype is frequent in a South African Black population (both CKD and controls). The TMPRSS6 rs855791 SNP previously reported to be associated with iron deficiency anaemia in the general population was not associated with iron deficiency anaemia in our CKD population. The SNP rs855791 was not associated with serum hepcidin in this study, and this further confirms that serum hepcidin, whether corrected for iron stores or not, is not the intermediate variable in the association of SNPs with iron parameters. Further studies are needed to elucidate the role of iron deficiency anaemia and hepcidin between the SNPs and iron parameters in CKD patients.

Acknowledgements

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Ethical approval for the study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg (reference number MD150929), and participants gave informed consent at the beginning of each data collection session throughout the study.

Disclosure of conflict of interest

None.

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Table S1. Association between anaemia and genotype distribution among participants with CKD and controls

IDA status	CKD				Controls			
	TMPRSS6 rs855791 (n, %)			*p*-value	TMPRSS6 rs855791 (n, %)			*p*-value
	TT	TC	CC		TT	TC	CC	
IDA group	3 (60.0)	20 (58.8)	132 (59.7)	0.995	1 (50.0)	4 (21.1)	41 (33.6)	0.476
Non-IDA group	2 (40.0)	14 (41.2)	89 (40.3)		1 (50.0)	15 (79.0)	81 (66.4)	

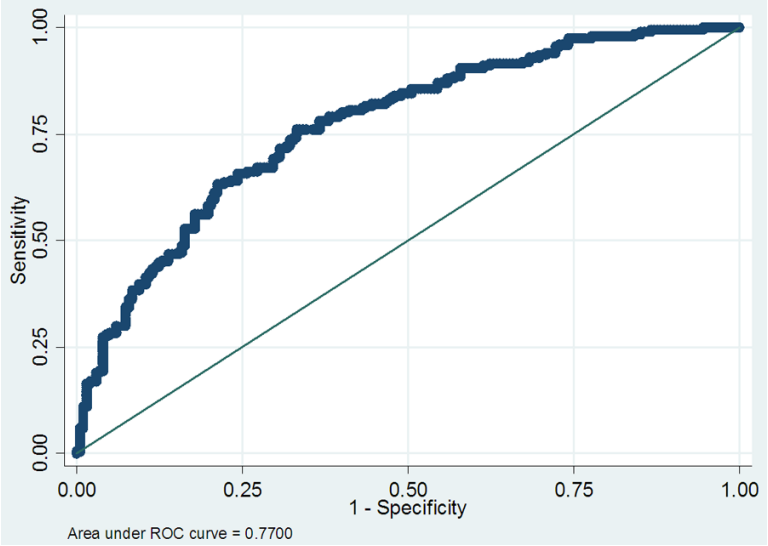

Figure S1. The area under Receiver operator characteristic (ROC) Curve of the regression model that predicts anaemia.