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Coding Variants of the *FMO3* Gene Are Associated with the Risk of Chronic Kidney Disease: A Case-Control Study

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Abstract

Background: Chronic kidney disease (CKD) is a global health concern involving roughly one-tenth of developed countries' populations. The flavin-containing dimethylaniline monooxygenase 3 (*FMO3*) gene encodes an enzyme that catalyzes trimethylamine N-oxide (TMAO), a toxin in CKD sufferers. This preliminary study aims to evaluate the association between coding region variations of *FMO3*, rs2266782G/A (E158K), rs2266780A/G (E308G), and rs1736557G/A (V257M), and the susceptibility to CKD.

Methods: A total of 356 participants were enrolled, including 157 patients diagnosed with CKD and 199 age-matched healthy individuals. Genotyping of *FMO3* gene variations was performed via PCR-RFLP and ARMS-PCR methods.

Results: Our findings revealed a significant association between rs2266780A/G and rs1736557G/A and CKD under different genetic models. Compared to the GGG haplotype of rs2266782/rs1736557/rs2266780, the GAG, GAA, AAG, and AAA haplotype combinations conferred an increased risk of CKD in our population. Interaction analysis revealed that some genotype combinations, including GA/AA/AA, AA/AA/AA, GA/AA/GA, and GG/AG/AA, dramatically increased CKD risk in the Iranian population. No correlation was found between *FMO3* polymorphisms and CKD stages.

Conclusions: These observations highlight the potential impact of coding variants of the *FMO3* gene on the onset of CKD. Further investigations into expanded populations and diverse races are needed to confirm our findings.

Keywords: Chronic kidney disease; *FMO3*, Genetic variant, Single-nucleotide polymorphism, Trimethylamine N-oxide.

Introduction

Chronic kidney disease (CKD) is a severe condition characterized by kidney function or structure abnormalities lasting more than three months and has serious health consequences (1, 2). Chronic kidney disease (CKD) is considered a global health issue and a crucial contributor to mortality, affecting virtually 10-16% of the population in advanced nations (3-5). It is estimated that CKD will develop into the fifth leading cause of death by 2040,

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revealing a twice-fold escalation in 2016 (6). This irreversible disorder manifests as renal damage, lessened glomerular filtration rates, high blood pressure, and hyperfiltration with or without proteinuria (7, 8). Besides, systemic complications correlated with metabolic or immunological unbalance could cause CKD.

Although the burden of CKD is nearly evident in developed countries, its complicated pathogenesis has yet to be utterly enlightened (9). The disease has a broad spectrum of etiology; thus, various factors play a part in its predisposition: infectious glomerulonephritides, renal vascular disease, genetic alteration, autoimmune diseases, and oxidative damage, to name a few (10). Furthermore, nephrotoxins, renal calculi, infection, environmental factors, and acute kidney diseases are growingly identified as critical warnings for kidney health (11). Based on previous findings, CKD is associated with an augmented risk of diabetes mellitus, hypertension, and cardiovascular disorders that can lead to premature death (6). Interestingly, a wide range of single-nucleotide polymorphisms (SNPs) has been identified that can simultaneously alter the risk of CKD and type 2 diabetes mellitus (T2DM) (12, 13). This provides evidence for the shared genetic background between such devastating conditions. On the other hand, Trimethylamine N-oxide (TMAO) and its precursors, 2-hydroxy-N,N,N-trimethylethanaminium (choline), and glycine betaine, are accumulated in CKD as uremic toxins and raise the risk of coronary diseases and mortality (14).

Flavin-containing monooxygenase 3 (FMO3) is a member of the drug-metabolizing enzymes and accelerates the NADPH-dependent cellular respiration of nitrogen-, sulfur-, and phosphorous-containing xenobiotics, such as therapeutic pharmaceuticals, dietetic ingredients, pesticides, and other foreign compound enzymes. Trimethylamine (TMA), TMAO, and choline are released from phosphatidylcholine, which is plentiful in lipid-rich nourishment (15). Phosphatidylcholine is degraded into choline by the intestine flora. A particular part of choline is converted to TMA and metabolized to produce oxidized TMAO by hepatic FMO3. Eventually, TMAO is excreted efficaciously by the normal kidney (16).

Some variations in the *FMO3* gene presumably modify the production of TMAO, which seems to be substantial for individuals suffering from CKD (17). Some rare genetic variants in the *FMO3* gene might diminish the catalytic activity of the FMO3, resulting in phenotypic attributes comprehended as characteristic trimethylaminuria or "fish-odor syndrome" (17). Some evidence has demonstrated that the mutant allele of rs2266782 (E158K) augments the catalytic activity of the *FMO3* by 35% more than the wild-type allele for both amine- and sulfide-containing substrates (18). It is documented that rs2266780 (E308G) and rs1736557 (V257M) polymorphisms diminish the enzymatic function of FMO3 *in vitro* and *in vivo* (19).

It has been established that genetic variations, and most importantly, SNPs, can impact the risk of chronic diseases (20-23). With this background, this preliminary investigation sought to study the correlation of *FMO3* rs2266780A/G, rs1736557G/A, and rs2266782G/A with CKD risk in a population from Iran. Discovering the contribution of genetic variations in the *FMO3* gene to CKD progression can help understand the pathophysiology of this chronic condition.

Materials and Methods

Ethical approval

The Local Ethics Committee of Zahedan University of Medical Sciences approved the study protocol (ethical code: IR.ZAUMS.REC.1400.047). The webpage for ethical approval is available at https://ethics.research.ac.ir/EthicsProposalViewEn.php?id=193164. Informed consent was taken from all subjects.

Population sample

We enrolled175 patients suffering from CKD who were admitted to the Clinic of

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Nephrology, Bu-Ali Hospital, Zahedan, from February 2021 to March 2022, and 199 irrelevant healthy subjects as the control group referred to the same healthcare center for periodic examinations. CKD has been characterized by the Kidney Disease Improving Global Outcomes (KDIGO) as a decline in glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for a minimum of three months, with or without a record of urinary protein excretion (24). According to eGFR, CKD sufferers were divided into five stages: Stage I (90 mL/min/1.73 m^2), Stage II (60–89 mL/min/1.73 m^2), Stage III (30–59 mL/min/1.73 m^2), Stage IV (15–29 mL/min/1.73 m^2), and Stage V (15 mL/min/1.73 m^2) (25). Expectant women and individuals who have been grafted before were excluded. Furthermore, patients who exhibited any manifestations of acute renal diseases, urinary tract infections, interstitial nephritis, diabetes mellitus, acute inflammatory response, neoplasms, polycystic kidneys, or other CKD-connected complications were excluded. The controls were chosen from healthy people without proteinuria or any kidney disease (CKD-EPI> 90 mL/min/1.73 m^2, estimated by the Chronic Kidney Disease in Epidemiology (CKD-EPI) experimentation equation (26). The whole blood sample was drained through phlebotomy and contained in sterilized vials for laboratory examinations. Likewise, 500 µL of whole blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes for isolation of genomic DNA.

Clinical and laboratory assessments

Clinical data such as age, gender, body mass index (BMI), level of albuminuria, and clinical stage by GFR were obtained from the individual's medical history. Serum creatinine and blood urea nitrogen (BUN) were measured via commercially accessible kits (Pars Azmoon®-Co, Tehran, Iran).

DNA extraction and Genotyping

Genomic DNA was separated from peripheral blood using the salting-out method (27). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele-specific amplification refractory mutation system (ARMS)-PCR were employed to determine *FMO3* gene variations. The sequences of the three SNPs were acquired from the *NCBI* databank, and specified primers were designed utilizing GeneRunner 3.05 (http://www.generunner.com/) and the *Primer1* database (accessible through http://primer1.soton.ac.uk/primer1.html/), which were synthesized by GenFanAvaran® Company (Iran, Tehran). Table 1 indicates the primer sequences used for the amplification of fragments. Polymorphic areas were amplified by combining 0.6 µL of genomic DNA (~90 ng/mL), 8 µL of Taq DNA Polymerase 2x Master Mix RED-$Mgcl_2$ 1.5 mM (AMPLIQON® Inc., Denmark), 0.7 µL of each primer (5 pmol) (GenFanAvaran®, Iran), and 5 µL sterile deionized H_2O. A 30-cycle PCR was conducted employing the following circumstances: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 35 sec, a specified annealing temperature (Table 2) for 30 sec, and an extension at 72 °C for 30 sec. Following *HinfI* for rs2266782 and *DraII* for rs2266780 digestions, the yields were disbanded by 2% agarose gel having a safe stain (SinaClon®, Iran). For genotyping the rs1736557 polymorphism, an ARMS-PCR method was applied.

Table 1. Sequences of the primers used for amplification of *FMO3* gene polymorphisms.

SNP	Primers	Sequence (5′ to 3′)	Annealing Temperature	Length of Amplicon (bp)	RE
rs2266782G/A (E158K)	F	GGGATGTTACCACTGAAAGG	62 ℃	A: 264 G: 65+199	*HinfI*
	R	CTTTAACAGAAGCGACCTTG			
rs2266780A/G (E308G	F	TACAGAGTCCTGAGGAAAGAGCC	59.5 ℃	A: 200 G: 100+100	*DraII*
	R	GACTCATCAAGGAAGGGGTAGGC			
rs1736557G/A (V257M)	R (G-allele)	CCATCTCTGACTGGTTGTGCG	61 ℃	Outer band (control): 300 Inner band (A or C): 120	-
	R (A-allele)	CCATCTCTGACTGGTTGTGCA			
	F (Outer)	CACCAGAATATCCACTACAAATG			
	F (Inner)	ACTAAAAGCCAGCAGGCATATCAC			

F: Forward; R: Reverse; bp: base pair; RE: restriction enzyme.

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Table 2. Demography of subjects in this study.

Parameter evaluated	CKD (n = 175 (mean±SD)	Controls (n = 199) (mean±SD)	*p*-value*
Age (Year)	49.91±14.71	47.28±17.60	0.052[a]
Gender			0.762
Female	94	110	
Male	81	89	
BMI (Kg/m^2)	26.82±3.97	27.75±5.36	0.143[a]
Albuminuria (%)		-	-
<30 mg/g	85 (48.6)	-	-
30-300 mg/g	62 (35.4)	-	-
>300 mg/g	28 (16.0)	-	-
Stage (Based on GFR)		-	-
I	43 (24.6)	-	-
II	35 (20.0)	-	-
III	62 (35.4)	-	-
IV	22 (12.6)	-	-
V	13 (7.4)	-	-
BUN (mg/dL)	33.26±18.13	12.73±4.58	**<0.001[a]**
Serum creatinine (mg/dL)	2.39±1.56	1.27±0.90	**<0.001[a]**

CKD: Chronic kidney disease; BMI: Body mass index; GFR: Glomerular filtration rate; BUN: Blood urea nitrogen.

Statistical analysis

The results were analyzed utilizing IBM SPSS® 22.0 statistics software (IBM® Company, New York, USA). Pearson's χ^2 test was employed to test the Hardy-Weinberg equilibrium (HWE) in the control group. The continuous and categorical variables were evaluated using the student's *t*-test and Fisher tests. Moreover, Fisher's exact test compared the allelic/genotypic frequencies between CKD cases and controls. To determine the connection between *FMO3* gene polymorphisms and CKD risk, we estimated odds ratios (ORs) with 95% confidence intervals (CIs). The SHEsis software platform was recruited to determine linkage disequilibrium (LD) among three investigated variants (28). The *p*-value <0.05 was deemed statistically meaningful.

Results

The control group comprised 94 females and 81 aged, gender, and BMI-adjusted males, with 110 females and 89 males in the CKD group. We observed no significant difference between the cases and controls concerning age, gender, and BMI. The clinical and demographic attributes of all subjects have been provided in Table 2. A noteworthy difference between the average creatinine levels in cases and controls indicated the proper selection of the controls.

Figure 1 demonstrates the gel photograph of the examined polymorphisms. Random re-genotyping of nearly 20% of the samples revealed a genotype correspondence pace of 99%.

Table 3 summarizes the allelic and genotypic frequencies of studied polymorphisms. Except for one SNP (rs2266780 A/G), this study observed no deviation from HWE in the controls (*p*-value for HWE >0.05). Our findings revealed no statistical correlation between the frequency of the rs2266782G/A variation and CKD incidence, neither in allelic nor genotypic models. Regarding rs2266780A/G, the A allele enhanced the risk of CKD under allelic, codominant homozygous, and recessive models of inheritance by 2.40, 5.09, and 10.99 folds, respectively. For rs1736557G/A, we found an enhanced risk of CKD under allelic (OR=4.27), codominant homozygous (OR=11.65), and recessive (OR=9.80) genetic patterns.

Table 4 shows the results of the haplotype analysis. Compared to the GGG haplotype of rs2266782/rs1736557/rs2266780, the GAG,

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GAA, AAG, and AAA haplotype combinations conferred an increased risk of CKD in our population. Based on the interaction analysis shown in Table 5, some genotype combinations, including GA/AA/AA, AA/AA/AA, GA/AA/GA, and GG/AG/AA, dramatically increased CKD risk in the Iranian population. We also assessed the correlation between genotypes of the studied SNPs and CKD stages and found no statistically significant association (p >0.05) (Data not shown).

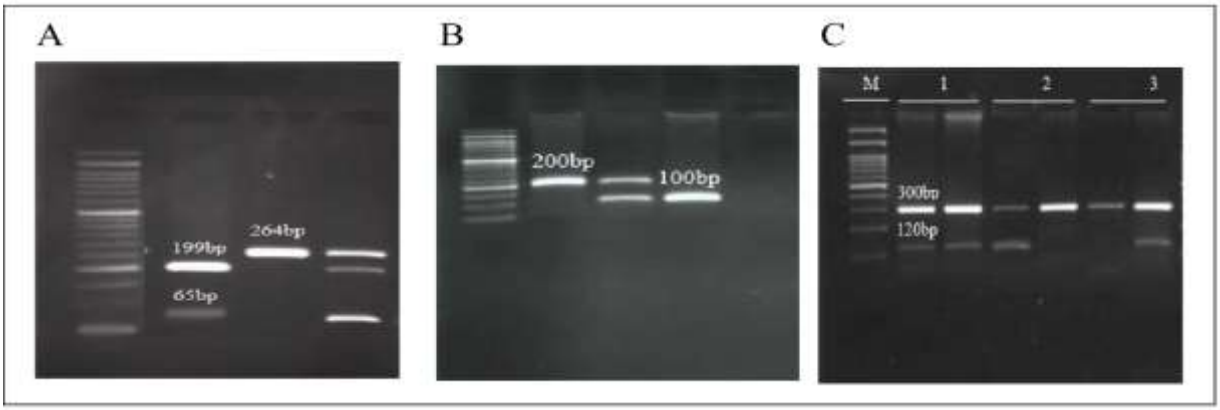

Fig. 1. The gel electerophoregram of genotyped *FMO3* variants. A: the different genotypes of rs2266782G/A, lane1: homozygote GG, lane2: heterozygote AG, lane3: homozygote AA; B: the observed genotypes of rs2266780A/G, lane1: homozygote AA, lane2: heterozygote AG, lane3: homozygote GG; C: the genotypes of rs1736557G/A, 1: heterozygote AG, 2: homozygote AA, 3: homozygote GG. M: DNA marker.

Table 3. Genotypic and allelic distribution of *FMO3* polymorphisms in patients with CKD and healthy attendees.

SNP	Type	CKD (%)	Control (%)	Model	*OR (95%CI)*	*p*-value*
rs2266782G/A	GG	58 (33.1)	65 (32.7)	Codominant1	0.93 (0.58-1.48)	0.660
	GA	78 (44.6)	94 (47.2)	Codominant2	1.09 (0.62-1.92)	0.900
	AA	39 (22.3)	40 (20.1)	Dominant	0.98 (0.63-1.51)	0.778
				Recessive	1.14 (0.69-1.87)	0.692
				Over-dominant	0.90 (0.60-1.35)	0.554
	G	194 (55.4)	224 (56.3)	Allelic	1 [reference]	-
	A	156 (44.6)	174 (43.7)	Allelic	1.03 (0.77-1.38)	0.869
rs2266780A/G	GG	34 (19.4)	35 (17.6)	Codominant1	0.33 (0.19-0.59)	**0.007**
	GA	47 (26.9)	145 (72.9)	Codominant2	5.09 (2.57-10.08)	**<0.001**
	AA	94 (53.7)	19 (9.5)	Dominant	0.88 (0.52-1.49)	0.407
				Recessive	10.99 (6.29-19.22)	**<0.001**
				Over-dominant	0.14 (0.09-0.22)	**<0.001**
	G	115(32.9)	215 (54.0)	Allelic	1 [reference]	-
	A	235 (67.1)	183 (46.0)	Allelic	2.40 (1.78-3.23)	**<0.001**
rs1736557G/A	GG	41 (23.4)	102 (51.3)	Codominant1	1.43 (0.86-2.40)	0.483
	GA	45 (25.7)	78 (39.2)	Codominant2	11.65 (6.31-21.53)	**<0.001**
	AA	89 (50.9)	19 (9.5)	Dominant	3.44 (2.20-5.37)	**<0.001**
				Recessive	9.80 (5.61-17.13)	**<0.001**
				Over-dominant	0.54 (0.34-0.84)	**0.001**
	G	127 (36.3)	282 (70.9)	Allelic	1 [reference]	-
	A	223 (63.7)	116 (29.1)	Allelic	4.27 (3.14-5.80)	**<0.001**

CI: confidence interval; OR: odds ratio; CKD: Chronic kidney disease. *P*-value* adjusted for BMI. Codominant 1 and Codominant 2 represent the heterozygous and homozygous codominant models, respectively.

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Table 4. Haplotype analysis of *FMO3* polymorphisms on CKD risk.

rs2266782G/A	rs1736557G/A	rs2266780A/G	CKD (%)	Control (%)	*OR (95%CI)*	*p*-value
G	G	G	11 (6.4)	41 (20.6)	1 [reference]	
G	G	A	20 (11.5)	39 (19.6)	1.91 (0.81-4.50)	0.137
A	G	G	13 (7.5)	34 (17.1)	1.42 (0.57-3.58)	0.453
A	G	A	20 (11.5)	27 (13.6)	2.76 (1.43-6.67)	0.022
G	A	G	18 (10.4)	19 (9.5)	3.53 (1.40-8.92)	**0.007**
G	A	A	47 (26.9)	13 (6.5)	13.47 (5.45-33.33)	**<0.001**
A	A	G	15 (8.6)	14 (7.1)	3.99 (1.49-10.71)	**0.005**
A	A	A	30 (17.2)	12 (6.0)	9.32 (3.62-23.95)	**<0.001**

OR: Odds ratio; CI: Confidence interval; CKD: Chronic kidney disease. *P <0.017* was considered statistically significant.

Table 5. Interaction analysis of *FMO3* polymorphisms on CKD risk.

rs2266782G/A	rs2266780A/G	rs1736557G/A	CKD (%)	Control (%)	*OR (95%CI)*	*p*-value
GG	AG	GG	3 (1.7)	34 (17.1)	1 [reference]	
GA	AG	GG	4 (2.3)	32 (16.1)	1.42 (0.30-6.83)	0.665
GA	AG	GA	5 (2.9)	25 (12.6)	2.27 (0.49-10.38)	0.286
GG	AG	GA	1 (0.6)	17 (8.5)	0.67 (0.06-6.90)	0.734
AA	AG	GG	6 (3.4)	16 (8.0)	4.25 (0.94-19.20)	**0.049**
GA	GG	GA	5 (2.9)	12 (6.0)	4.72 (0.98-22.82)	**0.042**
GA	AA	GG	12 (6.9)	10 (5.0)	13.60 (3.19-57.88)	**<0.001**
AA	AG	GA	2 (1.1)	9 (4.5)	2.52 (0.36-17.42)	0.342
GA	AG	AA	11 (6.3)	7 (3.5)	17.81 (3.92-80.92)	**<0.001**
AA	GG	GA	1 (0.6)	6 (3.0)	1.89 (0.17-21.32)	0.606
GG	GG	GA	2 (1.1)	5 (2.5)	4.53 (0.60-34.19)	0.121
AA	GG	GG	3 (1.7)	3 (1.5)	11.33 (1.55-82.79)	**0.006**
GA	GG	GG	5 (2.9)	3 (1.5)	18.88 (2.95-120.77)	**<0.001**
GG	AG	AA	11 (6.3)	3 (1.5)	41.55 (7.30-236.46)	**<0.001**
GG	GG	AA	5 (2.9)	3 (1.5)	18.89 (2.95-120.78)	**<0.001**
AA	AA	GA	5 (2.9)	2 (1.0)	28.33 (3.76-213.70)	**<0.001**
AA	AG	AA	4 (2.3)	2 (1.0)	22.67 (2.87-179.19)	**<0.001**
GA	AA	GA	13 (7.4)	2 (1.0)	73.67 (11.02-492.50)	**<0.001**
GA	GG	AA	5 (2.9)	2 (1.0)	28.33 (3.76-213.70)	**<0.001**
GG	AA	GG	2 (1.1)	2 (1.0)	11.33 (1.15-111.70)	**0.016**
AA	AA	AA	10 (5.7)	1 (0.5)	113.33 (10.59-212.98)	**<0.001**
AA	AA	GG	3 (1.7)	1 (0.5)	34.00 (2.65-436.56)	**<0.001**
GG	GG	GG	3 (1.7)	1 (0.5)	34.00 (2.65-436.56)	**<0.001**
GA	AA	AA	18 (10.3)	1 (0.5)	204.00 (19.76-2105.55)	**<0.001**

OR: Odds ratio; CI: Confidence interval; CKD: Chronic kidney disease. *P <0.05* was considered statistically significant.

Discussion

In the present investigation, for the first time, we reported that the *FMO3* coding polymorphisms, rs2266780A/G, and rs1736557G/A, but not rs2266782G/A, were correlated with CKD incidence. Briefly, the A allele of rs2266780A/G and rs1736557G/A enhanced CKD risk in our population. Statistically, rs2266780A/G was associated with CKD risk in all genotypic models except for the dominant (AA+AG vs. GG) pattern. Finally, the A allele of rs1736557G/A in AA vs. GG, GA+AA vs. GG, and AA vs. GG+GA modes markedly enhanced CKD risk. Also, different haplotype combinations conferred an increased risk of CKD. Our findings revealed that interactions between different genotypes might dramatically change the risk of CKD in our population. Deviations from HWE for rs2266780A/G in the studied groups can be explained by non-random mating or, more importantly, the small sample size.

The most common variant in the *FOM3* gene is the rs2266780, which is at a high allelic frequency in all populations. For

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instance, its commonness is approximately 45% among Africans, 40–45% among Europeans, and 30% among Asians (29). In another report, the rs2266782 polymorphism in a haplotype combination with c.560T> C variation decreased the enzyme function and resulted in severe trimethylaminuria (29). The rs1736557 variant diminished the enzyme's catalytic activity and is more common in Asians than Europeans and Africans (29). Generally, individuals who are homozygote recessive or combined heterozygote for genetic variation impairing the creation or function of FMO3 could diminish metabolizing trimethylamine to its N-oxide (30). Findings showed that the A allele of rs2266782G/A, the G allele of rs2266780A/G, and the G allele of rs1736557G/A are related to the high TMAO concentration in CKD patients. Furthermore, based upon an average follow-up of 3.3 years, most participants who carried the A allele at locus rs2266782 had shown reduced eGFR and higher mortality risk (15). In 2021, Haoran Wei et al. monitored 955 individuals with chronic heart failure and reported that participants with the GG genotype at the locus of rs2266782 had a higher level of TMAO (31). Another group of researchers has examined the relationship between *FMO3* gene variations and the incidence of T2DM in a sample of the Chinese population. Their findings failed to support a correlation between rs2266782 polymorphism and T2DM risk (32).

Human beings possess five functioning forms of FMO: FMO1, FMO2, FMO3, FMO4, and FMO5 (33). *FMO1*, *2*, *3*, and *4* are clustered on chromosome 1q24.3, and *FMO5* is located on chromosome 1q21.1 (33). *FMO3* is mainly expressed in the liver, which, in association with *FMO5*, consists of the abundant isoforms of FMO (34). The FMO requires FAD as a prosthetic group, NADPH as a co-factor, and molecular oxygen as a co-substrate for catalysis. The general part of FMO3 is related to catalyzing the oxidative metabolism of a broad spectrum of chemical substances, such as therapeutic pharmaceuticals and dietary-derived combinations (19). Hepatic oxidation of TMA to TMAO depends on FMO3. The amount of circulating TMAO has been recognized as a prognostic biomarker of morbidity in CKD sufferers due to kidney fibrosis and malfunction (35). The pathophysiological influence of TMAO has been approved in *FMO3*-overexpressed rats. These mice showed enhanced gluconeogenesis and lipogenesis due to defects in Kruppel-like factor 15 and PPARα pathways (36). The evidence reveals that the *FMO3* gene presumably functions in physiology and has been opted for by natural selection over time (37). The FMO3 has a wide range of substrates, from very small (as an illustration, TMA) to moderately sized (e.g., ketoconazole). While it is hypothesized that FMO3 is a medicine-metabolizing factor, the main activity of FMO3 seems to be the bioconversion of endogenous substrates, called TMA, or probably endogenous regulators of cardiovascular function (15). Moreover, FMO3 may activate some unrevealed cell signaling intermediaries playing a role in cholesterol equilibrium, resulting in a raised risk of cardiovascular disease and morbidity (38).

Our investigation had a few limitations. First, the sample size was relatively small. Second, we did not assess the expression level of the *FMO3* gene in participants. Cautiously interpreting the study findings, we believe the current investigation has presented a new insight into the clinical relationship of *FMO3* variants. Altogether, our findings suggest that rs2266780A/G and rs1736557G/A, but not rs2266782G/A, are associated with CKD risk in our population. Additionally, coding variants of *FMO3* in interaction with each other positively impact CKD vulnerability. These observations highlight the possible effect of coding variants of *FMO3* on CKD development. More extensive replicated examinations of various races are required to elucidate the link between these functional variants and the clinical outcome of the disease.

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Conflict of interest

The authors claim that they do not have any conflicts of interest.

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