Statistical Analysis of Klotho Gene Polymorphisms and Their Association with Coronary Artery Disease Susceptibility in an Iraqi Population

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Abstract: Coronary artery disease (CAD) is one of the most prevalent cardiovascular disorders and a leading cause of morbidity and mortality worldwide. The present study aimed to investigate the association of Klotho gene polymorphisms (rs564481, rs751229348, and rs2501789490) with CAD susceptibility and to evaluate their relationship with sex and hypertension status. This observational case-control study included 60 CAD patients and 30 control subjects aged 30–70 years. Peripheral blood samples were collected from participants at the Specialized Center for Heart Diseases, Surgery, and Catheterization in Ramadi City, Anbar, Iraq, between September 2025 and November 2025. Genomic DNA was extracted, and Klotho gene polymorphisms were analyzed. The results showed that allele and genotype frequencies of rs564481 and rs2501789490 did not differ significantly between CAD patients and controls ($p > 0.05$). In contrast, rs751229348 demonstrated a significant association at the allele level, with the C allele showing a possible association with CAD susceptibility (OR = 1.89, $p = 0.036$). Genotype distributions of all investigated SNPs were consistent with Hardy–Weinberg equilibrium in both patients and controls ($p > 0.05$). Significant differences in genotype distribution according to sex were observed for rs564481 ($p = 0.005$), rs751229348 ($p = 0.005$), and rs2501789490 ($p = 0.001$). Furthermore, rs751229348 was significantly associated with blood pressure status ($p = 0.029$), whereas rs564481 and rs2501789490 showed no significant association with hypertension. In conclusion, the findings suggest that Klotho genetic variation, particularly rs751229348, may contribute modestly to CAD susceptibility and related cardiovascular traits in the studied population. However, these findings require confirmation through larger studies and functional investigations to clarify the biological role of Klotho polymorphisms in cardiovascular disease.

1 INTRODUCTION

Coronary artery disease (CAD) is a leading cause of global morbidity and mortality [1] characterized by chronic vascular inflammation. Due to the ageing population, the urgency of combating this disease has become increasingly critical [2]. Susceptibility to CAD is affected by multiple risk factors from environmental, metabolic and genetic areas [3]. With a good understanding of the molecular pathways involved in the disease, researchers have identified several predispositional genes for its development [4]. The Klotho gene (13q13.1) encodes a 140-kDa protein known as Klotho, consisting of 1,012 amino acids
and exhibiting substantial similarity with β-glucosidases [5]. The Klotho protein functions as a co-receptor for fibroblast growth factor 23 (FGF23) and is predominantly expressed in the kidneys and human vascular tissue. Recent studies have highlighted its essential function in regulating cardiovascular biology [6]. Klotho gene expression may be associated with cardiovascular disease [2], [7].

The Klotho protein has antioxidative and antiapoptotic characteristics by inhibiting the signalling pathways of insulin and insulin-like growth factor-1 (IGF-1) [8]. Extensive research on both animals and humans has demonstrated that Klotho mutant mice exhibit various traits akin to ageing, including diminished lifespan, hypoactivity, insulin resistance, atherosclerosis, vascular calcification, muscle and skin atrophy, osteoporosis, and pulmonary emphysema impacting multiple organ systems [9]. Although the mechanism of action remains incompletely elucidated, researchers have investigated many single-nucleotide polymorphisms (SNPs) in the human Klotho gene to elucidate the

association between Klotho SNPs and susceptibility to chronic illnesses [10], [11]. Genetic research on Klotho has predominantly concentrated on KL-VS polymorphisms located in exon 2. The polymorphisms F352V (rs9536314) and C370S (rs9527025) are linked to human longevity, cognitive function, neuroprotection, and the occurrence of concealed coronary artery disease (CAD). This study aimed to investigate the association between Klotho gene polymorphisms (rs564481, rs751229348, and rs2501789490) and susceptibility to CAD, as well as their relationship with sex and blood pressure status.

2 MATERIALS AND METHODS

2.1 Study Design

This observational case-control study included 60 patients and 30 controls aged 30 to 70 years diagnosed with coronary artery disease. Samples were collected from the Specialized Center for Heart Diseases, Surgery, and Catheterization in Ramadi City, Anbar, Iraq, from September 2025 to November 2025.

2.2 Inclusion and Exclusion Criteria

This study included patients with clinically confirmed coronary artery disease, diagnosed by a cardiologist based on electrocardiogram (ECG) findings, cardiac biomarkers, or coronary angiography. Individuals with tumors, respiratory diseases, congenital heart disease, cerebral infarction, chronic kidney disease, or autoimmune diseases were excluded from the study. Variables such as age, sex, BMI, smoking status, blood pressure, lipid profile, and family history were recorded for all participants and considered during statistical analysis.

2.3 Ethics Approval

This study was conducted in accordance with the ethical standards of the Declaration of Helsinki. The patient provided informed written and verbal consent prior to sample collection, which was preceded by review and approval of the study protocol and participant information by the local ethics committee at University of Anbar (Number 30231 on 11/9/2025).

2.4 Sample Collection

A sterile syringe was used to obtain 5 millilitres of venous blood. Aliquot 3 ml of this blood, transfer to a gel tube and let coagulate for 20 minutes at room temperature (25-30°C). The tubes were then centrifuged at 3000 RPM for 15 minutes to separate the serum which was kept in Eppendorf tubes at −20°C before used for immunohistochemical testing. The remaining 2 ml blood was placed into EDTA tubes and kept at −20°C for molecular analysis.

2.5 Genotyping of Klotho SNP

Genomic DNA was extracted from cryopreserved whole blood samples using a commercial extraction kit according to the manufacturer's protocol. Specific primers were designed to amplify regions containing the Klotho gene SNPs (rs564481, rs751229348, and rs2501789490), (Table 1). Primer specificity was evaluated in silico using the NCBI BLAST tool to ensure target-specific amplification and to avoid non-specific binding. Polymerase chain reaction (PCR) was performed under standard conditions, including an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 7 min. The PCR products were verified by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination to confirm the presence of specific amplicons of the expected size. After validation, the amplified PCR products were purified and sent for sequencing to Macrogen Inc. (South Korea) for SNP identification and confirmation.

2.6 Statistical Analysis

Statistical analysis was conducted with SPSS version 25 and Microsoft Excel 2010. Continuous variables were represented as mean ± standard deviation (SD), whilst categorical variables were displayed as counts and percentages Differences in allele and genotype frequencies among groups were assessed using the Chi-square test and Fisher's exact test was used when appropriate. The correlation between SNPs and CAD risk was assessed by calculating the odds ratio (OR) and 95% confidence interval (CI). The Hardy-Weinberg equilibrium (HWE) was evaluated for genotype distributions in each group utilizing the Chi-square test. A p-value less than 0.05 was deemed statistically significant.

3 RESULTS AND DISCUSSION

Table 2 shows that the distribution of Klotho SNP rs564481 genotypes differed significantly between males and females ($\chi^2 = 10.674$, $p = 0.005$), with CC and TT genotypes more frequent in males, while CT was more common in females. Similarly, Klotho SNP rs751229348 exhibited a significant association with sex ($\chi^2 = 10.780$, $p = 0.005$), where the CC genotype predominated in males and CA in females, while AA appeared at low frequencies in both sexes. A stronger association was observed for Klotho SNP rs2501789490 ($\chi^2 = 14.658$, $p = 0.001$), as CC was more frequent in males, whereas CA was more common in females, and AA was detected only in males.

Table 1: Sequences of primers used in the study.

Primer	Sequence (5′→3′ direction)	Product size	Reference
Klotho gene	F: TGTCTCAGTTTACCGACCTGAATGT	452	Design for present study
	R: ATTCATCGTTATCCAAAGCTTGACG		

Table 2: The relation between sex of samples study with SNPs variation among study groups.

SNPs	Categories	Sex		X^2	p-value
		Male	Female		
rs564481	CC	20	5	10.674	0.005
	CT	10	29		
	TT	20	6		
rs751229348	CC	36	15	10.780	0.005
	CA	13	23		
	AA	1	2		
rs2501789490	CC	43	24	14.658	0.001
	CA	4	16		
	AA	3	0		

Table 3: Relation between blood pressure status for samples study with SNPs variation among study groups.

SNPs	Categories	Blood pressure status		X^2	p-value
		Hypertension	Non-hypertension		
rs564481	CC	13	12	1.160	0.859
	CT	21	28		
	TT	7	9		
rs751229348	CC	24	27	5.570	0.029
	CA	15	21		
	AA	2	1		
rs2501789490	CC	31	36	2.763	0.060
	CA	7	13		
	AA	3	0		

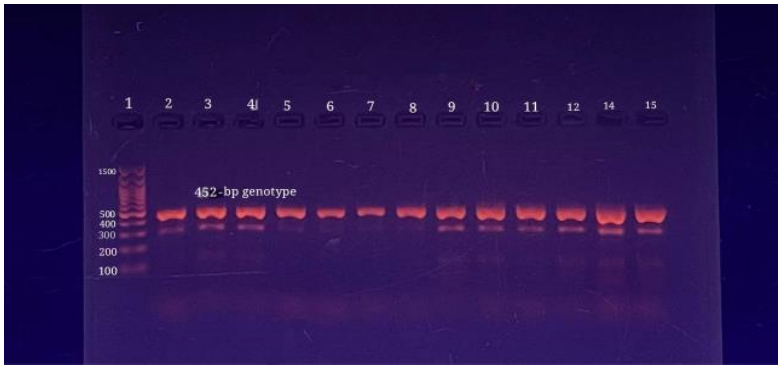

Figure 1: Agarose gel electrophoresis of PCR amplified Klotho gene fragments. Only one band was visualized at about 452 bp, indicating amplification of the target region. Lane M: DNA ladder (100-1500 bp); Lanes 1-15: amplified samples.

Table 4: Allele and genotype frequencies of Klotho SNPs (rs564481, rs751229348, rs2501789490) in controls and CAD patients.

SNPs	Alleles	All Subjects (n=90)	Controls Group (n=30)	CAD patients Group (n-60)	O.R	CI (95%)	p-value
rs564481	C	99 (55%)	36 (60%)	63 (52.5%)	0.74	0.37 - 1.44	0.214
	T	81 (45%)	24 (40%)	57 (47.5%)	1.36	0.69 - 2.68	0.215
rs751229348	C	138 (76.7%)	46 (76.7%)	92 (76.7%)	1.89	0.95 - 3.74	0.036
	A	42 (23.3%)	14 (23.3%)	28 (23.3%)	1.02	0.46 - 2.26	0.507
rs2501789490	C	154 (85.6%)	54 (90%)	100 (83.3%)	0.56	0.17 - 1.55	0.165
	A	26 (14.4%)	6 (10%)	20 (16.7%)	1.80	0.64 - 5.80	0.166

Genotype distribution varies depending on the categories of hypertension, SNPs variation relative to blood pressure status was assessed. The findings showed varying correlations of the analyzed SNPs. Table 3 demonstrates that there was no significant association between blood pressure status and Klotho SNP rs564481 genotypes ($\chi^2 = 1.160$, $p = 0.859$), with rates of CC, CT, and TT genotypes similar between the hypertension, and non-hypertension groups. Conversely, Klotho SNP rs751229348 was significantly associated with blood pressure status ($\chi^2 = 5.570$, $p = 0.029$), with the CC genotype being more frequent in hypertensives and the CA genotype being more common among non-hypertensives. The AA genotype was observed at low frequencies among groups. For Klotho SNP rs2501789490, no statistically significant association revealed ($\chi^2 = 2.763$, $p = 0.060$), but a trend toward variation was found: CC genotype used to be more than other group in hypertensive patients. PCR using conventional methods yielded amplification of the region targeted in Klotho. Agarose gel electrophoresis analysis of the obtained PCR products showed clear and specific bands at the expected fragment size (452 bp), confirming successful amplification and absence of non-specific products (Fig. 1).

Table 4 shows the distribution of the frequencies of the Klotho SNP rs564481, rs751229348, and rs2501789490 alleles between the control group and the cardiovascular patients. The results indicated no statistically significant differences in the frequencies of the Klotho SNP rs564481 and rs2501789490 alleles ($p > 0.05$), although there were slight differences between the two groups. In contrast, Klotho SNP rs751229348 showed a statistically significant association with the C allele (OR = 1.89, 95% CI = 0.95-3.74, $p = 0.036$), while the association with the A allele was not statistically significant. It should be noted that the C-allele frequencies reported in Table 4 appear numerically identical between controls and CAD patients (76.7% in both groups), which is not consistent with an OR of 1.89; this discrepancy should be re-verified against the raw genotyping counts. Furthermore, since the 95% CI for this association (0.95-3.74) narrowly includes 1.0, the result should be regarded as a borderline finding rather than a robust association.

Table 5 presents the genotype distribution of rs564481, rs751229348, and rs2501789490 among controls and CAD patients. No statistically significant differences were observed in genotype frequencies between the two groups for all studied SNPs ($p > 0.05$). Minor variations in genotype distribution were noted, with heterozygous genotypes (CT and CA) being more frequent in CAD patients compared to controls.

Table 6 presents the genotype distribution of Klotho SNP rs564481 and its conformity with Hardy-Weinberg equilibrium (HWE) among all subjects, controls, and CAD patients.

The observed genotype frequencies (CC, CT, and TT) were close to the expected values in all groups. The observed genotype frequencies agreed with the expected values in all groups, with no significant deviation from HWE ($p > 0.05$).

Table 7 presents the genotype distribution of Klotho SNP rs751229348 and its Hardy-Weinberg equilibrium (HWE) among all subjects, controls, and CAD patients. The observed genotype frequencies (CC, CA, and AA) were generally consistent with the expected values in all groups. No significant deviation from HWE was detected in all subjects ($p = 0.262$), controls ($p = 0.096$), or patients ($p = 0.847$), indicating that the genotype distribution of Klotho SNP rs751229348 follows the expected genetic equilibrium pattern.

Table 8 presents the genotype distribution of Klotho SNP rs2501789490 and its Hardy-Weinberg equilibrium (HWE) among all subjects, controls, and CAD patients. The observed genotype frequencies (CC, CA, and AA) were generally consistent with the expected values in all groups. No significant deviation from HWE was detected in all subjects ($p = 0.338$), controls ($p = 0.542$), or patients ($p = 0.215$), indicating that the genotype distribution of Klotho SNP rs2501789490 follows the expected genetic equilibrium pattern.

Table 5: Genotype form of (rs564481, rs751229348, rs2501789490) of all subjects, Control group and CAD patients' group.

SNPs	Genotype	All subjects (n=90)	C (Group) (n=30)	CAD patients (n=60)	O.R	CI (95%)	p-value
rs564481	CC	25 (27.8%)	11 (36.7%)	14 (23.3%)	0.53	0.18 - 1.54	0.216
	CT	49 (54.4%)	14 (46.7%)	35 (58.3%)	1.60	0.60 - 4.25	0.205
	TT	16 (17.8%)	5 (16.6%)	11 (18.4%)	1.12	0.31 - 4.58	0.547
rs751229348	CC	51 (56.7%)	16 (53.3%)	35 (58.3%)	1.230	0.46 - 3.24	0.410
	CA	36 (40%)	14 (46.7%)	22 (36.7%)	0.66	0.25 - 1.78	0.246
	AA	3 (3.3%)	0 (0%)	3 (5%)	1.530	0.12 - 82.85	0.593
rs2501789490	CC	67 (74.4%)	24 (80%)	43 (71.7%)	0.63	0.18 - 1.98	0.278
	CA	20 (22.2%)	6 (20%)	14 (23.3%	1.22	0.38 - 4.37	0.471
	AA	3 (3.4%)	0 (0%)	3 (5%)	1.0	0.29-1.0	0.291

Table 6: Number and percentage frequencies of (SNP rs564481) genotypes and their Hardy-Weinberg equilibrium (HWE) in Control group and CAD patients' group.

Groups	Genotype form						Allele frequency		p-value
	CC		CT		TT		C	T	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.			
All subjects	25	27	49	45	16	18	99	81	0.343
Controls	11	11	14	14	5	5	36	24	0.879
Patients	14	16	35	30	11	14	63	57	0.189

1 degree of freedom (d.f.) for Chi-squared distribution. Obs.: observed, Exp: expected.

Table 7: Number and percentage frequencies of (SNP rs751229348) genotypes and their Hardy-Weinberg equilibrium (HWE) in Control group and CAD patients' group.

Groups	Genotype form						Allele frequency		p-value
	CC		CA		AA		C	A	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.			
All subjects	51	53	36	32	3	5	138	42	0.262
Controls	16	18	14	10	0	2	46	14	0.0955
Patients	35	35	22	21	3	4	92	28	0.8474

1 degree of freedom (d.f.) for Chi-squared distribution. Obs.: observed, Exp: expected.

Table 8: Number and percentage frequencies of (SNP rs2501789490) genotypes and their Hardy-Weinberg equilibrium (HWE) in Control group and CAD patients' group.

Groups	Genotype form						Allele frequency		p-value
	CC		CA		AA		C	A	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.			
All subjects	67	66	20	22	3	2	154	26	0.338
Controls	24	24	6	5	0	1	54	6	0.542
Patients	43	41	14	17	3	2	100	20	0.215

1 degree of freedom (d.f.) for Chi-squared distribution. Obs.: observed, Exp: expected.

The present study investigated the association of Klotho gene polymorphisms (rs564481, rs751229348, and rs2501789490) with CAD, as well as their relationship with sex and blood pressure status. CAD remains one of the leading causes of morbidity and mortality worldwide, accounting for approximately one-third of global deaths [12]. In developing countries, including Iraq, there is growing concern regarding the increasing burden of CAD and its occurrence in relatively younger age groups [13]. Within this context, identifying potential genetic factors that may contribute to disease susceptibility is of particular importance. The results of the current study showed that, among the three investigated SNPs, only rs751229348 demonstrated a statistically significant association at the allelic level, whereas the remaining studied variants did not show significant differences in overall allele or genotype distribution between patients and controls.

These findings suggest that the investigated Klotho polymorphisms may have a limited or variant-specific association with CAD in the studied population, rather than a strong and consistent genetic effect. This observation agrees with previous reports indicating that the contribution of Klotho gene variants to cardiovascular disease may vary among populations due to differences in genetic background, environmental exposure, and gene-environment interactions [14]. The results of the current study revealed a strong association between sex, and all studied genetic variants, suggesting that genetic variation in the Klotho gene may be influenced by sex-linked biological factors [15]. The observed variation in genotype frequencies between males and females, particularly the higher frequency of certain homozygous genotypes in males and heterozygous forms in females, may suggest the presence of sex-related biological differences in the distribution of the studied SNPs. These differences are associated with genetic, physiological, or environmental factors that underlie the observed variation between males and females in susceptibility to cardiovascular disease. The precise mechanism behind this association is uncertain, specifically because hormonal parameters were not measured in the current investigation. Similar observations have been noted by earlier studies indicating that sex-related determinants can affect cardiovascular disease pattern and risk profile [7]. A Saudi Arabian study conducted by [16], on gender disparities in CAD revealed that the average age for men to present with CAD is 55 years, whereas for women it is 59 years. Men exhibited a higher propensity toward smoking. Smoking is a considerable risk factor, particularly for men; nevertheless, there is no gender disparity in the severity of CAD [17]. The results of the current study are consistent with a study [18], that found potential associations between the single nucleotide polymorphism (SNP) in the Klotho gene (rs9527025) and cardiovascular problems in patients with chronic kidney disease and end-stage renal failure, and did not show a statistically significant association between the rs9527025 genotype of the Klotho gene and chronic kidney disease [12] .The results of this study align with prior research emphasising the significance of Klotho gene variants in cardiovascular disorders. Various SNPs have been examined; however, research on KL-VS variations (including rs9536314 and rs9527025) has revealed their role in influencing cardiovascular risk and longevity [19]. Karslı et al. [2], indicated that KL-VS polymorphisms affect enzymatic activity and correlate with longevity [20]. Moreover, various studies indicate that specific variations of the Klotho gene may either heighten susceptibility to atherosclerosis or confer preventive effects, contingent upon the genetic background. Nevertheless, certain reports have indicated a lack of significant correlation between specific polymorphisms, such as C370S, and coronary artery disease, which partially aligns with the findings of the present study, implying that the influence of Klotho gene variants may differ based on the population and SNP type [21]. The association between the studied SNPs and blood pressure status was also examined in the current study. Among the investigated variants, only rs751229348 showed a statistically significant association with hypertension, whereas rs564481 and rs2501789490 did not show significant relationships. This finding may indicate a possible association between rs751229348 and blood pressure-related cardiovascular traits. Since the Klotho protein has been linked to vascular homeostasis, endothelial function, calcium metabolism, and oxidative stress regulation, such an observation may be biologically plausible [22]-[25]. However, it is important to emphasize that the present study was designed to assess association rather than causation, and therefore no direct mechanistic conclusion can be drawn from the current results.

The observed association for rs751229348 should also be interpreted in light of the corresponding odds ratio and confidence interval. Although this variant demonstrated statistical significance at the allelic level, the confidence interval was relatively broad and close to the null value, which may suggest that the strength of association is modest and potentially influenced by the sample size. Therefore, this result should be interpreted as a preliminary genetic association that requires confirmation in larger and independent study populations [26]. He current findings are generally consistent with the concept that CAD is a multifactorial disorder, resulting from the interaction of genetic susceptibility, physiological characteristics, and environmental risk factors [27].

Although the present results do not support a strong overall association between all studied Klotho variants and CAD, the significant finding observed for rs751229348 may indicate that some variants of this gene warrant further investigation in relation to cardiovascular traits [26]. This interpretation is also supported by previous studies demonstrating that Klotho deficiency or altered Klotho-related signaling may contribute to vascular dysfunction, endothelial impairment, and atherosclerotic changes, all of which are closely related to CAD pathophysiology [12], [27]. At the same time, the lack of significant differences between patients and controls for most

studied SNPs suggests that these variants, at least individually, may not serve as strong standalone genetic markers for CAD in the present population [28]. The relatively higher frequencies of some heterozygous genotypes among patients may reflect a minor or context-dependent genetic effect, which could become more relevant when combined with other genetic, clinical, or environmental factors [25], [27]. Accordingly, the present results should be interpreted within the broader framework of polygenic and multifactorial cardiovascular risk rather than as evidence of a single-gene effect.

The HWE analysis showed that genotype distributions for the studied SNPs were consistent with HWE expectations in both the patient and control groups. This finding supports the internal consistency of genotype distribution within the sampled population. However, HWE should be considered a supportive descriptive indicator rather than definitive evidence of data validity, as agreement with HWE alone does not fully exclude the possibility of genotyping error, sampling effects, or population stratification [28].

Despite the valuable findings of this study, some limitations should be acknowledged. The sample size was relatively limited, which may reduce the ability to detect weak genetic associations. In addition, the study focused on only three SNPs of the Klotho gene, and no functional or expression analysis was performed to determine their biological impact. Therefore, additional studies involving larger populations and functional genetic investigations are required.

4 CONCLUSIONS

The present study investigated the association between three Klotho gene polymorphisms (rs564481, rs751229348, and rs2501789490) and coronary artery disease susceptibility, with further evaluation according to sex and hypertension status. The findings demonstrated that allele and genotype distributions of rs564481 and rs2501789490 were not significantly different between CAD patients and control subjects, suggesting that these variants may have a limited contribution to CAD susceptibility in the studied population.

In contrast, rs751229348 showed a significant association at the allele level, particularly with the C allele (OR = 1.89, p = 0.036), indicating a possible role of this polymorphism in CAD-related genetic susceptibility. However, this association should be interpreted cautiously due to the moderate sample size and the need for confirmation in larger populations.

The results also revealed significant differences in the distribution of all investigated Klotho polymorphisms according to sex, suggesting that sex-related factors may influence the genetic distribution of Klotho variants and potentially contribute to differences in cardiovascular risk profiles. Furthermore, rs751229348 showed a significant association with blood pressure status, whereas rs564481 and rs2501789490 were not significantly associated with hypertension.

Hardy–Weinberg equilibrium analysis confirmed that the genotype distributions of all studied SNPs were consistent with expected genetic equilibrium, supporting the reliability of the obtained genotyping data. Overall, the findings indicate that Klotho gene polymorphisms may have a modest influence on CAD susceptibility and related cardiovascular risk factors, particularly rs751229348. Further studies with larger sample sizes, diverse populations, and functional investigations are required to clarify the biological role of Klotho genetic variations in cardiovascular disease development.

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