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# Klotho gene polymorphism as a susceptibility factor for oxidative DNA damage in coronary artery disease

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**ABSTRACT**: The Klotho protein has been linked to the promotion of cardiovascular health maintenance through its capacity to enhance resistance against oxidative stress, thereby exerting protective effects. The potential involvement of Klotho gene polymorphisms in the regulation of human aging and age-related disorders, including coronary artery disease, chronic renal disease, osteoporosis, and stroke, is under investigation. The objective of our research was to investigate the association between the rs9527025 (Cys370Ser) polymorphism of the Klotho gene and coronary artery calcification, as well as the potential link between the rs9527025 polymorphism and oxidative DNA damage. The study involved a sample of 90 patients who had undergone coronary angiography. The genotyping of Cys370Ser in exon 2 of the Klotho gene was performed using the polymerase chain reaction. The evaluation of oxidative DNA damage was conducted using the alkaline comet test. The findings of the study revealed that there was no statistically significant association between the distribution of alleles in the Klotho SNPs and the occurrence of coronary artery disorders. In the meantime, the total comet score frequency was significantly associated with the rs9527025 polymorphism. The results of our study indicate that Klotho gene variants, particularly the C370S polymorphism (rs9527025), might play a role in influencing oxidative DNA damage in age-related diseases, such as coronary artery disease. In consequence, larger studies are required to confirm the association between Klotho deficiency and the progression of cardiovascular disease in order to elucidate risk factors for coronary artery disease and develop potential therapeutic strategies.

**KEYWORDS**: Cardiovascular disease; coronary artery disease; genetic polymorphism; Klotho; oxidative DNA damage.

## 1. INTRODUCTION

Coronary artery disease (CAD), a chronic vascular inflammation, stands as a prominent reason of global morbidity and mortality. As the population ages, the urgency to address this disease has grown exponentially [1]. Multiple factors, including environmental, metabolic, and genetic components, contribute to the susceptibility to CAD [2, 3]. Through a deeper understanding of the molecular mechanisms underlying the disease, researchers have successfully identified several genes that are susceptible to its occurrence.

The Klotho gene (13q13.1) encodes a 140-kDa protein called Klotho, comprising 1,012 amino acids and sharing significant homology with β-glucosidases [4]. The Klotho protein serves as a co-receptor for fibroblast growth factor 23 (FGF23) and is primarily expressed in the kidneys, as well as human vascular tissue [5, 6]. Recent investigations have emphasized its crucial role in regulating cardiovascular biology, suggesting that defects in klotho gene expression may be linked to cardiovascular disease [7-9].

The Klotho protein demonstrates antioxidative and antiapoptotic properties through its ability to hinder the signaling of insulin/insulin-like growth factor-1 (IGF-1) [10-12]. Numerous studies conducted on both animals and humans have provided evidence that Klotho mutant mice display a range of characteristics resembling aging, such as a reduced lifespan, hypoactivity, insulin resistance, atherosclerosis, vascular calcification, muscle and skin atrophy, osteoporosis, and pulmonary emphysema affecting multiple organ systems [4, 13].

Despite the mechanism of action not being completely understood, researchers have examined several single-nucleotide polymorphisms (SNPs) in the human Klotho gene to clarify the relationship between Klotho SNPs, and susceptibility to chronic diseases. The genetic studies on Klotho have primarily focused on KL-VS

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polymorphisms found in exon 2. These polymorphisms, named for the F352V (rs9536314) and C370S (rs9527025), have been associated with human longevity, cognition, neuroprotection, and the presence of hidden CAD [14].

In a previous study conducted by our team, we investigated the potential associations between Klotho C370S SNP (rs9527025) and cardiovascular complications in individuals diagnosed with chronic kidney disease and end-stage renal disease [15]. However, our findings did not show a significant correlation between the rs9527025 genotype of the Klotho gene and chronic kidney disease. In our present study, we aimed to reveal the relationship between the C370S genotype of the Klotho gene and the occurrence of coronary artery calcification. Furthermore, we conducted an analysis of DNA damage levels to assess the association between the rs9527025 polymorphism and oxidative DNA damage.

## 2. RESULTS

### 2.1. Clinical Characteristics of the Participants

Table 1 provides an overview of the main characteristics of 90 subjects, including their demographic and clinical data, as well as the relevant biochemical parameters associated with CAD risk. There were no statistically significant variations observed in the levels of serum glucose, triglyceride, LDL-cholesterol, HDL-cholesterol, and total cholesterol between patients diagnosed with CAD and the control group ($p > 0.05$). However, estimated glomerular filtration rates (eGFR) were found to be reduced in CAD patients ($p = 0.012$). Moreover, the CAD patients were categorized into subgroups based on the number of affected vessels: single-vessel disease (n = 33; 56.9%), two-vessel disease (n = 17; 29.3%), and three-vessel disease (n = 8; 13.8%).

**Table 1.** The main characteristics of the coronary artery disease patients and controls.

| Parameter | CAD patients (n=58) | Control (n=32) | *P* value |
|---|---|---|---|
| Gender (female/male) | 14/44 | 19/13 | 0.001 |
| Age (year) | 63.78±1.80 | 56.50±2.81 | 0.005 |
| Hypertension | 41 (70.7%) | 17 (29.3%) | 0.135 |
| DM2 | 27 (73.0%) | 20 (27.0%) | 0.192 |
| Current smoker | 33 (70.2%) | 14 (29.8%) | 0.291 |
| Total cholesterol (mg/dL) | 167.04±11.02 | 174.36±15.26 | 0.658 |
| LDL-cholesterol (mg/dL) | 95.56±5.45 | 101.36±11.83 | 0.663 |
| HDL-cholesterol (mg/dL) | 43.30±3.59 | 39.07±2.89 | 0.636 |
| Triglyceride (mg/dL) | 178.61±18.55 | 154.06±22.37 | 0.847 |
| Fasting glucose (mg/dL) | 118.43±6.72 | 118.63±10.64 | 0.433 |
| eGFR (mL/min/1.73m$^2$) | 62.51±5.04 | 89.04±7.36 | 0.012 |
| Total Comet Score (TCS) | 9.54±1.97 | 4.44±1.55 | 0.339 |

[a] CAD: coronary artery disease; DMT2: Diabetes mellitus type II; eGFR: estimated glomerular filtration rate.
[b] Adjusted for age and gender status.

### 2.2. Genotype Distribution of Klotho SNP rs9527025 and Its Associations with CAD Risk and Biochemical Profiles

Figure 1 displays the genotype frequencies and allele distributions of Klotho SNP rs9527025, which were consistent with the Hardy-Weinberg equilibrium (HWE) expectations. There was no statistically significant difference observed in the genotype distributions of the rs9527025 SNP between patients with CAD and healthy controls ($p > 0.05$). A multinomial logistic regression analysis was performed to investigate the relationship between allele distribution and the risk of CAD. The results of this analysis are presented in Table 2. The statistical analysis did not yield a significant association between the alleles of rs9527025 and the risk of CAD ($p = 0.450$). Moreover, no significant association was observed between the presence of single-vessel, two-vessel, or three-vessel disease and Klotho alleles ($p > 0.05$).

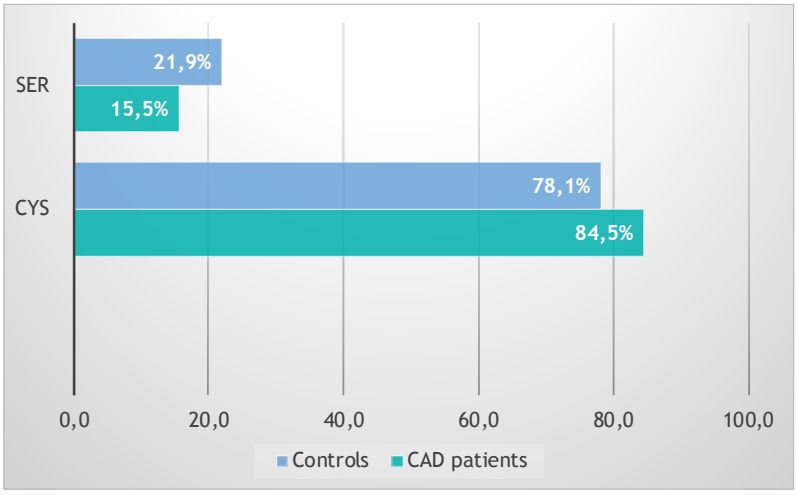


**Figure 1.** The allelic distribution of Klotho gene polymorphism in CAD patients and controls.

**Table 2.** Odds ratios and their 95% confidence intervals for alleles of Klotho genotypes for coronary artery disease.

| **rs9527025** | **CAD patients, *n* (% frequency)** | **Controls, *n* (% frequency)** | **OR (% 95 CI)** | ***P value*** * |
|---|---|---|---|---|
| Cys (Cys/Cys) | 49 (66.2) | 25 (33.8) | 1.524 (0.508-4.575) | 0.450 |
| Ser (Cys/Ser + Ser/Ser) | 9 (56.3) | 7 (43.8) | | |

The analysis of allele distributions of the SNP was conducted to examine the potential correlation between variants of the Klotho gene and clinical risk factors for CAD, as presented in Table 3.

**Table 3.** The distribution of baseline characteristics and the total comet score by Klotho alleles.

| **Parameter** | **Rs1800802** | | ***P* Value*** |
|---|---|---|---|
| | **Cys** | **Ser** | |
| Gender (female/male) | 27/47 | 6/10 | 0.939 |
| Hypertension | 47 (81.0%) | 11 (19.0%) | 0.740 |
| DM2 | 29 (78.4%) | 8 (21.6%) | 0.450 |
| LDL-cholesterol (mg/dL) | 99.26±6.07 | 93.63±10.36 | 0.622 |
| HDL-cholesterol (mg/dL) | 42.83±3.28 | 40.07±4.4 | 0.390 |
| Triglyceride (mg/dL) | 168.78±18.06 | 174.60±24.93 | 0.472 |
| Total cholesterol (mg/dL) | 168,52±11.67 | 171.27±12.99 | 0.902 |
| Fasting glucose (mg/dL) | 122.48±7.61 | 110.18±6.61 | 0.757 |
| eGFR (mL/min/1.73m2) | 70.18±5.96 | 73.00±7.39 | 0.846 |
| Total Comet Score (TCS) | 7.13±0.97 | 13.01±3.47 | 0.045 |

[a] DMT2: Diabetes mellitus type II; eGFR: estimated glomerular filtration rate.

### 2.3. Alkaline comet assay

The frequency of total comet score (TCS) in CAD patients (9.54 ± 1.97) was found to be higher than in the control group (4.44 ± 1.55); however, this difference was not statistically significant ($p > 0.05$). The TCS frequency showed a significant association with the rs9527025 polymorphism ($p = 0.045$, Figure 2). The presence of the rs9527025 missense mutation increases the prevalence of TCS. DNA damage was not shown to be significantly related to the subjects' age, gender, smoking habits, or biochemical profiles ($p > 0.05$).

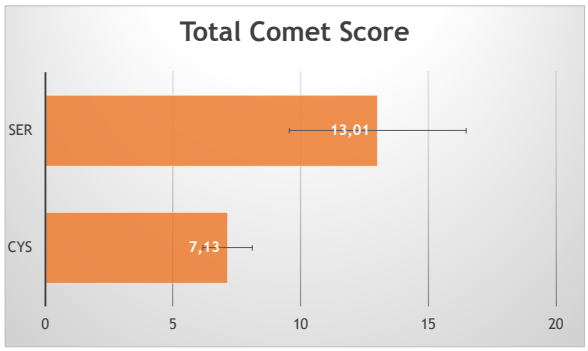


**Figure 2.** The total comet score (TCS) frequency of participants based on the allelic distribution of Klotho rs9527025 polymorphism

## 3. DISCUSSION

Cardiovascular disorders are complex and multifactorial diseases influenced by a combination of environmental and genetic factors, contributing to high rates of global morbidity and mortality [2, 3]. SNPs in the klotho gene have been linked to longevity and are also associated with various cardiovascular events, including arteriosclerotic diseases and coronary artery disease [14]. Furthermore, these genetic variations have been found to be associated with cardiovascular risk factors including elevated systolic blood pressure and reduced HDL cholesterol levels [10]. The KL-VS polymorphisms, specifically named for the F352V (rs9536314) and C370S (rs9527025) variations, have been prominently investigated in genetic studies related to the Klotho gene [14]. Arking et al. reported that the KL-VS allele of the Klotho gene is a functional variant that has an impact on longevity [16]. Moreover, they demonstrated that the introduction of serine at the position corresponding to Klotho amino acid 370 was not successful in restoring enzymatic activity. The heterozygosity of F352V and C370S in the coding region has been shown to provide protection against coronary arteriosclerotic heart disease [10]. They have shown that the KL-VS allele of the Klotho gene is associated with an increased risk of occult atherosclerosis in a high-risk sample composed of siblings of individuals with premature CAD. Majumdar et al. [9] found a positive association between KL-VS homozygosity of the Klotho gene and the occurrence of early-onset ischemic stroke, especially in individuals aged 40 years or younger. The multivariate analysis indicated that the A allele of the G395A SNP and the S allele of the C370S SNP were associated with a reduced risk of stroke-related death. In this study, we investigated the correlation between the C370S genotype of the Klotho gene and the presence of coronary artery calcification. According to our results, the allelic distribution of the C370S SNP was not significantly related to the presence of CAD. Consistent with our investigation, studies conducted in Massachusetts, USA, also found any significant association between the C370S SNP and cardiovascular diseases, such as premature acute CAD and vascular calcification, in both young and older patients [17]. In addition, we analyzed the association between the biochemical profiles of CAD patients and the C370S SNP. Despite several studies demonstrating a connection between reduced HDL, GFR levels and klotho variants, our investigation did not observe a similar association [10].

Atherosclerotic calcification, a process that occurs in the intimal layer, is closely associated with atherosclerosis. This process involves inflammation, the deposition of lipids, and cellular necrosis. Arterial stiffness is considered a significant independent predictor of mortality due to its associations with left ventricular hypertrophy, systolic hypertension, and reduced coronary perfusion [18]. In their study, Hu et al. [19] demonstrated that Klotho deficiency was linked to increased calcification severity, undetectable soluble Klotho levels, and elevated serum phosphorus levels. Conversely, Klotho overexpression was associated with reduced calcification, maintained Klotho levels, and normal renal function in a mouse model. Moreover, gene delivery of Klotho was observed to increase nitric oxide production, resulting in decreased blood pressure values and improved vascular endothelial function in rats with multiple risk factors for atherosclerosis [20]. According to the data, we categorized the CAD group into three subgroups based on the degree of significant stenosis in the main blood vessels: single-vessel disease, two-vessel disease, and three-vessel disease. However, no significant relationship was observed between the allele profiles of the C370S variant and the level of arterial stiffness in CAD patients.

Previous research has indicated that Klotho gene delivery has the potential to mitigate oxidative stress in mice. Additionally, Klotho protein has been shown to reduce apoptosis and senescence in vascular cells induced by hydrogen peroxide [21]. These findings suggest that Klotho may have antioxidative and

antiapoptotic properties, which could be beneficial in protecting against cellular damage and aging-related processes in the vascular system. In a recent study by Maekawa et al. [22], it was demonstrated that Klotho protein has the ability to inhibit the expression of adhesion molecules in the endothelium that are induced by tumor necrosis factor-a (TNF-a). This suppression of adhesion molecules subsequently leads to a reduction in monocyte adhesion to endothelial cells. These findings suggest that Klotho protein may have a protective role in preventing inflammation and vascular dysfunction, which are associated with the adhesion of immune cells to endothelial cells and the initiation of atherosclerosis. Indeed, the data presented in various studies indicate that Klotho plays a significant role in regulating oxidative stress mechanisms [20, 23]. Additionally, reduced Klotho expression is associated with the suppression of Wnt signaling, a pathway plays a significant role in diverse cellular processes, such as proliferation and differentiation, as well as oxidative stress. By regulating these processes, Klotho protein contributes to maintaining a healthy cardiovascular system and helps protect against conditions associated with oxidative stress and Wnt pathway dysregulation. Based on our analysis using the comet assay, we observed a significant association between the C370S polymorphism and oxidative DNA damage. Specifically, the TCS frequency was found to be significantly influenced by the presence of the C370S missense variant. Our findings indicate that individuals carrying the rs9527025 polymorphism tend to have a higher TCS frequency, suggesting a potential link between this genetic variant and increased oxidative DNA damage.

One of the limitations of this study is the absence of data on mRNA level analysis for Klotho, which could have provided valuable data to understand the impact of C370S variants on gene expression. Additionally, the study was conducted with a relatively small number of CAD patients, which may limit the generalizability of the results. Indeed, the careful classification of study groups based on the presence of CAD following coronary diagnostic angiography is a strength of this study. By ensuring that participants were accurately diagnosed with CAD, the researchers were able to establish a more reliable and relevant relationship between the C370S genotype and CAD. This approach helps to reduce potential confounding factors and enhances the validity of the study's findings.

## 4. CONCLUSION

Previous studies have linked Klotho polymorphisms to various cardiovascular events, such as arteriosclerotic diseases and coronary artery disease. However, we did not observe any correlation between the genotype distribution of the C370S SNP and the presence of CAD in our study. Indeed, the data from various studies strongly suggest that Klotho plays a significant role in regulating oxidative stress mechanisms. We found a significant association between the rs9527025 polymorphism (C370S) and oxidative DNA damage. The results of our study indicate that Klotho gene variants, particularly the C370S polymorphism (rs9527025), might play a role in influencing oxidative DNA damage in age-related diseases, such as CAD. Further validation of these findings through large-scale investigations could prove valuable for stratifying cardiovascular health risks.

## 5. MATERIALS AND METHODS

### 5.1. Sample Collection and Clinical Assessment

The study included 90 Caucasian participants who were recruited from the Cardiology Department of İstanbul Dr. Siyami Ersek Thoracic and Cardiovascular Surgery Training and Research Hospital in İstanbul, Turkey. The study enrolled patients who exhibited health complaints like atypical chest pain, chest distress, and angina, and were under suspicion of having CAD. Following a comprehensive medical inquiry and routine blood and urine tests, patients were referred to the Department of Cardiology to evaluate the potential presence of concurrent CAD. To evaluate arterial calcification, coronary angiography was conducted on all participants using the Judkins technique through the femoral artery. Out of the participants, 58 individuals were diagnosed as CAD patients, with at least one blood vessel showing luminal diameter narrowing exceeding 50.0%. The CAD group was categorized into three subgroups based on the extent of significant stenosis in the main blood vessels: 33 patients with single-vessel disease, 17 patients with two-vessel disease, and 8 patients with three-vessel disease. A control group (n = 32) was formed, consisting of subjects with normal coronary arteries and no cardiovascular disease. All study participants provided informed consent, and the study protocol was approved by both the local ethics committee of Marmara University and the Turkish Ministry of Health, Central Ethics Committee (ID: 09.2011.0020, 2011). The study excluded participants who had malignancies or a history of malignancies.

Demographic and clinical data, including relevant medical histories such as diabetes mellitus (DM) and hypertension, as well as family history, smoking habits, and alcohol consumption patterns, were collected from the medical records of the participants. Routine clinical methods were employed to measure various biochemical parameters linked to CAD, including serum glucose, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, and creatinine levels. Table 1 presents a summary of the demographic and biochemical profiles of the study participants.

### 5.2. Genotyping of Klotho SNP

Peripheral blood samples were obtained from the participants, and genomic DNA was extracted utilizing the Roche DNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). The genotyping of rs9527025 in exon 2 of the Klotho gene was conducted through the polymerase chain reaction (PCR) method, employing the CSL Gradient Thermal Cycler (Cleaver Scientific Ltd., Rugby, Warwickshire, UK). For the PCR reaction of the Klotho gene, a total volume of 30 µL was utilized, comprising 3 µL of genomic DNA, 200 µM of each dNTP, 2.5 mM MgCl2, 3 U of Taq polymerase, and forward and reverse primers at a concentration of 80 nM each. In this assay, three forward primers were employed: one primer was used to amplify the entire target region as a control, while the other two were specifically designed to detect the polymorphic region of interest. The sequences of four primers were presented in Table 4. The PCR reaction commenced with an initial incubation of 4 minutes at 94 °C, followed by a total of 30 cycles. Each cycle consisted of 30 seconds at 94 °C for denaturation, 60 seconds at 51 °C for annealing, and 60 seconds at 72 °C for extension. Finally, a final extension step was performed for 10 minutes at 72 °C to ensure the completion of the amplification process. The PCR products were visualized by running electrophoresis on a 1.5 % agarose gel that was subsequently stained with ethidium bromide.

**Table 4.** Sequences of primers used in the study.

| Primer name | Sequence |
|---|---|
| Forward primer 1 | 5′- CTGACTTTTTTGCTCTTT-3′ |
| Forward primer 2 | 5′ -TGACTTTTTTGCTCTTTG-3′ |
| Forward primer 3 | 5′-TGACTTTTTTGCTCTTTC-3′, |
| Reverse primer | 5′-GGCAGGAAATGAGAACTT-3′ |

a In each primer sequence, the polymorphic base is underlined.

### 5.6. Comet assay

Following the collection of approximately 5 mL of blood samples, peripheral lymphocytes were isolated from both CAD patients and healthy controls using Ficoll-Histopaque (Sigma-Aldrich, Taufkirchen, Germany) density gradient centrifugation. The viability of the cells was assessed using Trypan blue (Sigma-Aldrich, Taufkirchen, Germany) exclusion, with dye-excluding cells accounting for more than 90% of the total. The alkaline comet assay was performed following an adapted version of the methodology outlined by Singh et al. [24] to evaluate DNA damage levels. Approximately 50,000 prepared cells were combined with 1 % low melting point agarose (LMA) (Sigma-Aldrich, Taufkirchen, Germany) and then evenly distributed onto microscope slides coated with 0.7 % normal melting agarose. Following the solidification of the low melting point agarose (LMA), the slides were submerged in a lysing solution for a minimum of 1 hour at +4 °C. The lysing solution, which contained 10 mM Tris, 100 mM Na2EDTA, 2.5 M NaCl, pH 10, along with 10% DMSO and 1% Triton X-100, facilitated the preservation of the samples. To induce DNA unwinding and facilitate the detection of alkali-labile damage, the slides were immersed in an electrophoresis buffer with a composition of 0.3 M NaOH and 1 mM EDTA at pH 13 for a duration of 20 minutes. The DNA samples were subjected to electrophoresis at 300 mA and 15 V for a period of 30 minutes. Following electrophoresis, the cells were neutralized using 0.4 M Tris buffer at pH 7.5 and subsequently stained with 50 µL of ethidium bromide at a concentration of 20 µg/mL. The stained DNA images were analyzed using a fluorescent microscope equipped with a 400x objective lens (Olympus BX51 microscope, Tokyo, Japan). To evaluate DNA damage, a total of 100 cells were randomly chosen from each sample and visually examined to assess the appearance of comets. The degree of DNA migration in the cells was classified into five categories based on visual observation, as defined by Collins [25]. These categories ranged from class 0, indicating no DNA damage, to class 4, representing the maximum level of DNA damage.

The total comet score (TCS) was used as a parameter to assess and quantify the extent of DNA damage. The TCS was calculated using the following formula:

TCS= 0 (n)+1(n)+2(n)+3(n)+4(n), where "n" indicates the number of cells in each class.

### 5.7. Statistical Analyses

The statistical analyses were performed utilizing the Statistical Package for the Social Sciences (SPSS®) software, specifically version 21. The obtained data were presented as means ± standard deviation (SD), and a significance threshold of $p < 0.05$ was deemed to indicate statistical significance for all conducted analyses. To assess the Hardy-Weinberg equilibrium (HWE), the observed and expected genotype frequencies among the subjects were compared using the $\chi 2$ test. The study employed descriptive statistics and multivariate analysis to evaluate the relationships between clinical profiles and Klotho genotypes. The statistical analyses were appropriately controlled for any confounding variables, such as age and gender. The study employed multinomial logistic regression analysis to ascertain the odds ratio (OR) of the genotype in relation to the incidence of coronary artery disease (CAD).

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