RESEARCH ARTICLE

# The Role of Klotho G395A Gene Polymorphism in Atherosclerotic Cardiovascular Disease and Mortality Risk Scores in Non-dialysis Chronic Kidney Disease

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## Abstract

**BACKGROUND:** Cardiovascular disease (CVD) is the leading cause of death in patients with chronic kidney disease (CKD). Klotho expression was reduced in patients with CKD, leading to vascular calcification, endothelial dysfunction, and atherosclerosis. We investigated the role of the klotho G395A gene polymorphism and plasma klotho level in the ten-year risk of atherosclerotic cardiovascular disease (ASCVD) and CVD mortality in CKD patients.

**METHODS:** We used the PCR-CTPP assay method to genotype klotho G395A single nucleotide polymorphism (SNP) in 72 non-dialysis CKD patients. The klotho level was determined using the enzyme-linked immunoassay (ELISA) method. Path analysis was used to determine the relationship between the klotho G395A SNP, plasma klotho level, ASCVD risk score, and CVD mortality risk score.

**RESULTS:** Our results showed that the GA genotype had lower plasma klotho levels than the GG genotype (path coefficient=-0.185, $p$=0.000). There was a significant negative correlation between plasma klotho level and the ASCVD risk score (r=-0.243, $p$=0.040), but no significant correlation was found between plasma klotho level and the CVD mortality risk score (r=-0.145, $p$=0.225). Path analysis showed that plasma klotho level had a significant negative direct effect on ASCVD risk score (path coefficient=-0.272, $p$=0.000) and an indirect effect on CVD mortality risk score (path coefficient=0.187, $p$=0.005).

**CONCLUSION:** Klotho G395A SNP might reduce lower plasma klotho levels, which increased ASCVD and CVD mortality risk scores in non-dialysis CKD patients. However, other risk factors such as age, CKD stages, hypertension, and smoking should be taken into consideration. Therefore, large-scale genetic association studies with adjusted variables could be conducted in various ethnic groups for a more robust result.

**KEYWORDS:** klotho, single nucleotide polymorphism, cardiovascular disease, chronic kidney disease

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## Introduction

Cardiovascular disease (CVD) is a significant cause of morbidity and mortality in patients with chronic kidney disease (CKD). The prevalence of coronary artery disease (CAD), atherosclerosis, microvascular disease, left ventricular hypertrophy, and myocardial fibrosis increases as glomerular filtration rate (GFR) decreases. (1) CVD is associated with the incidence of CKD (2) and contributing to 30-50% of all causes of death in patients with CKD worldwide (3). In addition to traditional risk factors such as diabetes and hypertension, the increased CVD mortality in patients with CKD is also impacted by an increase in oxidative stress and inflammation, which occurs concurrently with a decline in renal function.(4,5)

Therefore, continuous assessment of inflammation and oxidative stress is useful to predict CVD risk in the CKD patients.(6) Furthermore, genetic variants that induce defective expression of specific proteins, enzymes, and inflammatory mediators may increase the risk of vascular calcification and atherosclerotic CVD (ASCVD).(7) As vascular calcification is a strong predictor of CVD morbidity and mortality in the CKD population, defects in endogenous anti-calcification factors such as alterations in klotho levels may play an important role in determining the outcome of patients with CKD.(8)

Klotho gene is an aging-associated gene primarily expressed on the cell surface membranes of the proximal and distal kidney tubules.(9) Single nucleotide polymorphisms (SNPs) are the most common inherited variance identified in human genes. More than 10 SNPs have been identified in the human klotho gene, including the G395A SNP in the promoter region of the klotho gene.(9) G395A SNP is the most common variation of klotho gene in Asian populations (10) and is associated with CVD (11). This SNP may affect klotho expression, in which klotho deficiency has been linked to the increased risk of hypertension, CAD, and metabolic syndrome.(12)

The kidneys are the primary regulators that aid in maintaining plasma klotho levels under normal conditions. Patients with CKD had decreased klotho expression as failing to maintain klotho levels which subsequently can cause vascular calcification and increased phosphate levels.(13) Furthermore, vascular calcification in the subintima and media is associated with a higher risk of cardiovascular death. Declined klotho level has effects on arterial calcification, endothelial dysfunction, and the risk of atherosclerosis in people with CKD.(14) A previous study revealed that elevated circulating plasma klotho levels could reduce CVD risk, implying that kotho plays a preventive role in CVD.(12)

There have been many investigations focused on the role of klotho gene SNP in either CVD or CKD patients, but only a few studying both. Some studies also yield conflicting results, for example, G395A is correlated with CAD in Korean and Japanese (15), but not in European (16). On the other hand, carriers of the A allele of the G395A polymorphism were associated with increased systolic blood pressure levels in Koreans (12), but not in Japanese (17). Genetic polymorphisms could influence the expression of klotho levels that may play a role in mortality in CKD patients, according to the ethnic variation. To date, there has been no clear evidence to support the role of the klotho G395A gene polymorphism in ASCVD and CVD mortality risk in the Indonesian population, and further analysis is needed regarding the role of SNPs on klotho levels. Hence, in this study we aimed to determine the role of klotho G395A gene polymorphism and plasma klotho levels on the ten-year risk of ASCVD and CVD mortality in non-dialysis CKD patients.

## Methods

### Study Design

This was a cross-sectional study to determine the role of klotho G395A polymorphism and plasma klotho level on ASCVD and CVD mortality risk scores in CKD subjects. The research was a continuation of a previous study that looked at the relationship between polymorphisms in the ACE gene and ASCVD and CVD mortality scores.(18) Several CKD subjects have been added to the current study. A total of 72 Javanese non-dialysis CKD subjects from the kidney and hypertension outpatient clinic, Universitas Airlangga Hospital, Indonesia, were collected from June to September 2021. The study was approved by the Universitas Airlangga Hospital review board (local board reference number: 146/KEP/2021). All subjects gave their written informed consent prior to the commencement of the study. Non-dialysis CKD subjects aged 40-79 years with stable conditions were included in this study. Subjects with present or history of ASCVD, acute heart failure, uncontrolled arrhythmias, and those using angiotensin-converting enzyme (ACE) inhibitors were excluded.

**Data Collection**

Complete history and physical examination were performed for baseline characteristics of all subjects, including gender, age, ethnicity, body mass index (BMI), systolic/diastolic blood pressure, CKD stage, and history of risk factors and disease prior to the study, such as diabetes, smoking, hypertension, acute coronary syndrome (ACS), stroke, uncontrolled arrhythmia, and heart failure. Blood pressure was measured using an OMRON Professional Blood Pressure Monitor HBP-1120 (Omron Corporation, Kyoto, Japan). Before beginning blood pressure measurement, the subjects were asked to sit comfortably in a calm area for 5 minutes. The cuff was placed at the level of the heart, with the back and arms supported to avoid muscle contraction and isometric exercise-dependent rises in blood pressure.

Blood and urine samples were collected from all subjects at the time of recruitment. Five mL of blood was drawn by trained laboratory workers and put into a 5 mL venoject tube with EDTA anticoagulant. This included total cholesterol, high-density lipoprotein (HDL), serum creatinine, urine creatinine, and urine albumin level. The albumin-creatinine ratio (ACR) was measured using albumin and urine creatinine levels. The CKD-EPI Creatinine Equation (2009) was used to calculate the glomerular filtration rate (GFR).(19)

After that, we assessed the ASCVD risk score, which is the predicted ten-year risk of ASCVD, through the CKD patch equation (*https://ckdpcrisk.org/ckdpatchpce/*). This score consists of e-GFR, ACR, age, gender, race, systolic blood pressure, antihypertensive medication, HDL, total cholesterol, history of diabetes, and history of smoking. We also assessed the predicted ten-year risk of CVD mortality through the CKD patch score equation (*https://ckdpcrisk.org/ckdpatchscore/*), in which the measurement consists of e-GFR, ACR, age, gender, systolic blood pressure, total cholesterol, and smoking status. The CKD patch equation was based on the Chronic Kidney Disease Prognosis Consortium.(20)

**Peripheral Blood Mononuclear Cells (PBMC) Extraction and DNA Isolation**

Genotyping of the klotho gene SNP was performed on DNA extracted from PBMC. PBMCs were extracted from venous blood by the Ficoll-Paque® (Sigma-Aldrich, St. Louis, Missouri, USA) density gradient centrifugation method in 15/50 mL conical tubes.(21) Host DNA was isolated from PBMC using the QIAamp DNA Extraction kit (Cat. no. 51104; Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. PBMC extraction, DNA isolation, and SNP detection were performed in the laboratory of the Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya, Indonesia.

**Genotyping of Klotho Gene SNP**

Polymerase Chain Reaction (PCR) was carried out using Promega GoTaq® Green Master Mix (Cat. no. M7122, Promega, Madison, Wisconsin, USA) on DNA thermal Cycler machine: Applied Biosystem Veriti 96 Well (Thermo Fisher Scientific, Waltham, Massachusetts, USA). We used PCR with confronting 2-pair primers (PCR-CTPP) assay method to detect klotho G395A SNP (rs1207568). Primers used were: F1-5'GTTT CGTGGACGCTCAGGTTCATTCTC-3', and F2-5'GAGAAAAGGCGCCGACCAACTTTC-3' for forward primers, R1-5' GATCCCGCCCCCAAGTCGGGA-3' and R2-5'GTCCCTCTAGGATTTCGGCCAG-3' for reverse primers.(22) The amplification conditions were as follows: 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 30 seconds at 67°C, 45 seconds at 72°C, and a final extension of 5 minutes at 72°C. PCR products were then examined under the UV light using electrophoresis method with 2% agarose gel. The major homozygous (wild type) GG genotype was evidenced by the presence of two fragments (252 and 175 bp), the minor homozygous (SNP) A/A genotype was determined by the presence of two fragments (252 and 121 bp), while the heterozygous GA genotype showed three fragments (252, 175, and 121 bp), as shown in Figure 1.

**Plasma Klotho Levels Measurement**

Klotho levels were determined using the Enzyme-linked immunoassay (ELISA) method and the Human KL (klotho) ELISA Kit (Cat. no. E-EL-H5451, Elabscience, Houston,

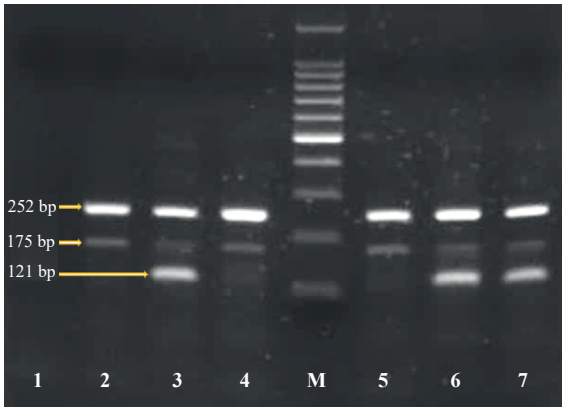


**Figure 1. PCR-CTPP products of klotho gene under 2% gel electrophoresis.** Lane M: marker; lane 1: negative control; lane 2, 4, 5: GG genotype; lane 3, 6, 7: GA genotype.

Texas, USA). Basic laboratory examinations except for plasma klotho levels were performed in private laboratories, while plasma klotho was measured in the laboratory of the Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya, Indonesia.

### Data Analysis

Statistical analysis was performed using IBM SPSS Statistics Software ver. 23 (IBM Corp, Armonk, NY, USA). Continuous data were presented as mean±standard deviation (SD), while dichotomous data were presented as frequency with percentage. A Shapiro-Wilk normality test was performed on numerical data to determine the normality of data distribution. For bivariate analysis, Spearman correlation tests were used to analyze the correlation between variables. Path analysis was then performed using Partial Least Squares-Structural Equation Modelling by Smart PLS 3.3.7, SmartPLS (GmbH Company, Oststeinbek, Germany) to determine the interrelationship between klotho G395 SNP, plasma klotho level, ASCVD risk score, and CVD mortality risk score. A *p*-value of <0.05 was considered as statistically significant.

## Results

### Subjects Characteristics

The baseline characteristics for 72 non-dialysis CKD subjetcs were shown in Table 1. The age range was 40-70 years, with mean of 57.33±6.93 years. Male and female subjects have same proportion. Most subjects had diabetes mellitus and hypertension less than ten years (55.6% and 75.0%, respectively). Most were non-smokers and had stage 3 CKD. From the physical examination, mean BMI and systolic blood pressure were above normal value (25.91±5.06 kg/m$^2$ and 142.89±22.61 mmHg, respectively). For laboratory values, serum creatinine, e-GFR, and ACR were abnormal (2.59±1.66 mg/dL, 32.78±15.89 mL/min/1.73m$^2$, and 584.23±908.68 mg/gram, respectively). Mean value of ASCVD and CVD mortality risk scores of the participants were also high.

### Correlation between Baseline Characteristics and Plasma Klotho Level

Table 2 showed the correlation between the baseline characteristics and plasma klotho level with ASCVD risk and CVD mortality risk scores. There was a significant positive correlation between age, systolic blood pressure, smoking history, and urine ACR with ASCVD risk and CVD mortality risk scores. There was a significant negative correlation between plasma klotho with ASCVD risk score (r=-0.243, *p*=0.040) and there was a strong positive correlation between ASCVD risk score and CVD mortality risk score (r=0.780, *p*=0.000) (Figure 2). There was a positive correlation between serum creatinine and CKD stage with CVD mortality risk score (r=0.436, *p*=0.000; r=0.371, *p*=0.001, respectively) and a negative correlation between e-GFR and CVD mortality risk score (r=-0.367, *p*=0.002).

**Table 1. Characteristics of non-dialysis CKD subjects.**

| Characteristics | Value (n=72) |
|---|---|
| Gender, n (%) | |
| Male | 36 (50.0) |
| Female | 36 (50.0) |
| Age (years), mean±SD | 57.33±6.93 |
| Diabetes, n (%) | |
| Non-diabetes | 19 (26.4) |
| Diabetes <10 years | 40 (55.6) |
| Diabetes 10-20 years | 8 (11.1) |
| Diabetes >20 years | 5 (6.9) |
| Hypertension, n (%) | |
| No hypertension | 10 (13.9) |
| Hypertension <10 years | 54 (75.0) |
| Hypertension 10-20 years | 6 (8.3) |
| Hypertension >20 years | 2 (2.8) |
| Smoking, n (%) | |
| Non smoker | 50 (69.4) |
| Current smoker | 4 (5.6) |
| Former smoker | 18 (25.0) |
| CKD stage, n (%) | |
| Stage 2 | 5 (6.9) |
| Stage 3 | 36 (50.0) |
| Stage 4 | 20 (27.8) |
| Stage 5 | 11 (15.3) |
| BMI (kg/m$^2$), mean±SD | 25.91±5.06 |
| Systolic blood pressure (mmHg), mean±SD | 142.89±22.61 |
| Diastolic blood pressure (mmHg), mean±SD | 81.01±11.48 |
| Total cholesterol (mg/dL), mean±SD | 182.13±50.54 |
| HDL (mg/dL), mean±SD | 39.94±12.18 |
| Serum creatinine (mg/dL), mean±SD | 2.59±1.66 |
| e-GFR (mL/min/1.73m$^2$), mean±SD | 32.78±15.89 |
| Urine ACR (mg/gram), mean±SD | 584.23±908.68 |
| Plasma klotho level (ng/mL), mean±SD | 1.03±2.07 |
| ASCVD risk score (%), mean±SD | 20.52±15.96 |
| CVD mortality risk score (%), mean±SD | 14.12±15.25 |

**Table 2. Correlation between variables in this study.**

| Variables | ASCVD Risk Score | | CVD Mortality Risk Score | |
|---|---|---|---|---|
| | r | *p* | r | *p* |
| Age | 0.435 | 0.000* | 0.484 | 0.000* |
| BMI | 0.057 | 0.634 | 0.044 | 0.711 |
| Systolic blood pressure | 0.273 | 0.020* | 0.443 | 0.000* |
| Diastolic blood pressure | -0.009 | 0.941 | 0.124 | 0.297 |
| Smoking history | 0.400 | 0.001* | 0.402 | 0.000* |
| HDL | -0.109 | 0.362 | -0.088 | 0.464 |
| Serum creatinine | 0.229 | 0.053 | 0.436 | 0.000* |
| CKD Stage | 0.146 | 0.221 | 0.371 | 0.001* |
| e-GFR | -0.163 | 0.172 | -0.367 | 0.002* |
| Urine ACR | 0.233 | 0.049* | 0.463 | 0.000* |
| Total cholesterol | 0.016 | 0.895 | 0.188 | 0.114 |
| Plasma klotho level | -0.243 | 0.040* | -0.145 | 0.225 |

*Significant if $p<0.05$.

**Frequency of Klotho G395A SNP**

Table 3 showed the allelic and genotypic frequencies of the klotho gene SNP in non-dialysis CKD patients. The GG genotype was found with greater frequency (72.2%) than the GA genotype (27.8%) in this population. In this study, no A/A genotypes were found in this population. Allelic frequency showed 86.11% and 13.89% for the G and A alleles, respectively, which indicates that the G allele was six times more common in Javanese population. We observed that allele frequencies in G395A polymorphism fulfill the Hardy-Weinberg equilibrium ($x^2$=1.87, $p$=0.392). The allele frequencies of G and allele A SNPs of the klotho G395A gene in this study were almost similar to the frequency of previously reported SNPs in the Asian population. According to dbSNP NCBI, reported frequencies in the Asian population were 84.2% for the G allele and 15.8% for the A allele, respectively.(23)

**Path Analysis Construction**

To assess the relationship between klotho gene SNP, plasma klotho level, ASCVD risk score, and CVD mortality risk score in non-dialysis CKD patients, a model was constructed using the SmartPLS software, as shown in Figure 3. The klotho gene SNP had a significantly negative direct effect on plasma klotho levels (path coefficient=-0.185, $p$=0.000). Plasma klotho itself had a significantly negative direct effect on ASCVD risk score (path coefficient=-0.272, $p$=0.000) and an indirect effect on CVD mortality risk score (path coefficient=-0.187, $p$=0.005), while ASCVD risk had a significantly positive direct effect on CVD mortality risk score (path coefficient=0.687, $p$=0.000) (Table 4). According to this model, the klotho SNP may result in a lower plasma klotho level, which increases ASCVD risk score and, ultimately, CVD mortality risk score. Nevertheless, other factors such as age, CKD stages, and smoking should also be taken as confounding factors, eventhough we have excluded subjects with severe infectious/inflammatory conditions and critically ill patients to reduce bias due to oxidative stress and acute inflammation and incorporated some of the factors in the CVD risk score.

## Discussion

This is the first study to our knowledge that has been conducted on genetic variations in the klotho gene in Javanese patients with CKD. In patients with non-dialysis CKD, the klotho GA genotype was associated with reduced plasma klotho levels. Additionally, we discovered that a decreased plasma klotho level was associated with an increased ASCVD risk score and CVD mortality risk score in bivariate and path analyses. This is in line with a previous study which observed that decreased serum klotho levels were independently linked with mortality and CVD incidence in patients with CKD.(24) This result is consistent with previous meta-analysis research that shows an inverse relationship between klotho levels and vascular calcification in individuals with CKD.(25)

The G395A SNP in the klotho gene is located in the promoter region and has been associated with an increased risk of developing multiple diseases, including CVD, which is one of the leading causes of worsening progression and

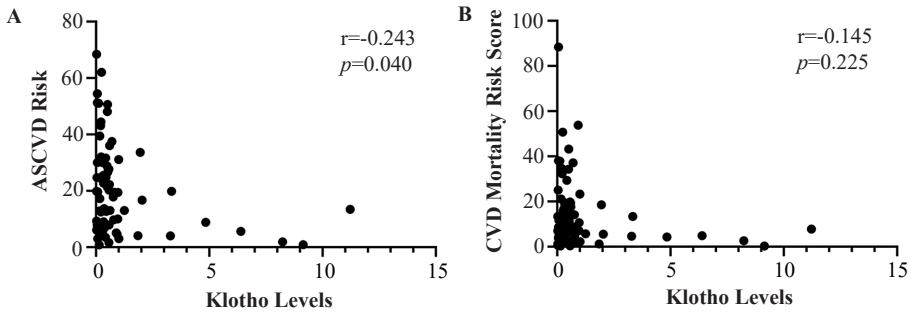


**Figure 2. Correlation between plasma klotho levels, ASCVD risk score, and CVD mortality risk score.** A: plasma klotho levels *vs.* ASCVD risk score; B: plasma klotho levels *vs.* CVD mortality risk score.

death in patients with CKD.(26) Klotho G395A SNP can influence the affinity of binding transcription factors, as the substitution of G → A in the promoter region will impair the amount of DNA-protein complex and may change the expression of the klotho gene, leading to lower plasma klotho level.(12)

This study found that plasma klotho levels could directly affect ten-year risk of ASCVD. This is because the decrease in plasma klotho levels will reduce phosphaturia, which further causes hyperphosphatemia. The increase in Ca x P product can increase the risk of vascular calcification.(27) In addition, a decrease in plasma klotho will decrease nitric oxide (NO) bioavailability, leading to endothelial dysfunction.(28) Decreased klotho levels are also associated with oxidative stress and chronic inflammation.(29) These three things will increase the risk of ASCVD and will ultimately contribute to CVD mortality. However, decreased klotho levels are not the only cause of ten-year risk of CVD mortality in CKD patients. Numerous pathogenic processes, including atherosclerosis, arterial stiffness, vascular calcification, congestive cardiomyopathy, capillary mismatch, and sudden cardiac death, have been implicated in this increased mortality risk.(30) Therefore, in this study, a decrease in plasma klotho levels did not have a direct effect on CVD mortality risk score, but an indirect effect instead, probably due to several other risk factors for CVD mortality in CKD patients.

ASCVD risk score was found to be associated with CVD mortality risk score in the current study. A patient with CKD has a higher mortality risk as their disease progresses, and CVD risk factors including inflammation and oxidative stress are significantly linked to mortality risk.(31) This finding is consistent with another cohort study, which found that mortality from CVD was sixfold higher than developing end-stage renal disease in non-dialysis CKD patients.(32)

Our findings demonstrated that the majority of patients in this population had the GG genotype (72.2%), with the G allele being six times more prevalent than the A allele. This finding was congruent with data from the dbSNP NCBI, which indicated that the G and A alleles had reported frequencies of 84.2% and 15.8% in the Asian population, respectively.(23) Another set of population genetics data from Ensembl indicated that the reported frequencies for the G and A alleles in the East Asian population were 83% and 17%, respectively. According to Ensembl data, the population with the highest frequency of the G allele is African (93%), whereas the population with the lowest frequency is American (73%).(33) Other research conducted in Asian countries confirmed the preponderance of the GG genotype. For instance, a study reported that

**Table 3. Allelic and genotypic frequency of klotho G395A SNP in non-dialysis CKD subjects.**

| Genotype | n | Frequency (%) |
|---|---|---|
| GG | 52 | 72.2 |
| GA | 20 | 27.8 |
| **Total** | **72** | **100** |
| **Allele** | **n** | **Frequency (%)** |
| G | 124 | 86.11 |
| A | 20 | 13.89 |
| **Total** | **144** | **100** |

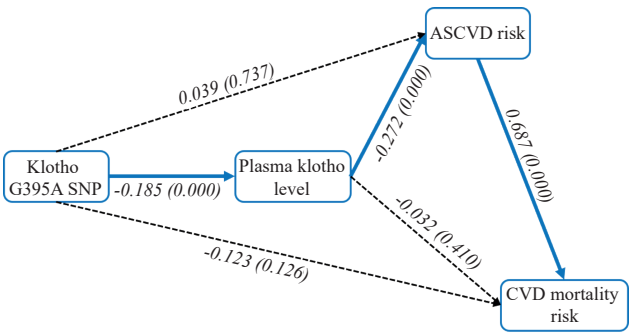


**Figure 3. Path analysis between klotho G/A SNP, plasma klotho level, ASCVD risk score, and CVD mortality risk score.**

**Table 4. Direct, indirect, and total effects of the path analysis.**

| Outcome | Direct Effect | | Indirect Effect | | Total Effect | |
|---|---|---|---|---|---|---|
| | Path Coefficient | *p*-value | Path Coefficient | *p*-value | Path Coefficient | *p*-value |
| CVD mortality risk | | | | | | |
| ASCVD risk → CVD mortality risk score | 0.687 | 0.000* | | | 0.687 | 0.000* |
| Plasma klotho level → CVD mortality risk score | -0.032 | 0.41 | -0.187 | 0.005* | -0.129 | 0.000* |
| Klotho G395A SNP → CVD mortality risk score | -0.123 | 0.126 | 0.068 | 0.389 | -0.056 | 0.569 |
| ASCVD risk | | | | | | |
| Plasma klotho level → ASCVD risk score | -0.272 | 0.000* | | | -0.272 | 0.000* |
| Klotho G395A SNP → ASCVD risk score | 0.039 | 0.737 | 0.05 | 0.017* | 0.09 | 0.435 |
| Plasma klotho level | | | | | | |
| Klotho G395A SNP → Plasma klotho level | -0.185 | 0.000* | | | -0.185 | 0.000* |

*Significant if $p<0.05$.

the majority of Korean respondents had the klotho G395A SNP with the GG genotype (63.2%), followed by the GA genotype (33%), and the AA genotype.(34) Another study discovered that the majority of Iranian participants with klotho G395A SNP were predominated by GG genotype (70%), followed by GA genotype (25%), and AA genotype (5%).(16) In Indonesia, a prior investigation on the klotho G395A SNP in the Medan population revealed that the majority of participants (52.2%) had the GG genotype, followed by the GA genotype (43.5%), and the AA genotype (4.3%).(10)

There was a link between the klotho G395A SNP and the occurrence of carotid artery calcification.(10) Conflicting finding was noted regarding the effect of G/A substitution on the expression of G395A in the promoter of the klotho gene. Nevertheless, the A allele had been reported to form fewer DNA-protein complexes compared to the G allele.(35) This indicated protein binding at the promoter may be attenuated by G/A substitution due to the decrease in klotho expression. The G395A polymorphism in the A allele was associated with coronary artery disease and ischemic stroke in patients with normal renal function, as well as in hemodialysis patients with vascular dysfunction.(36) Along with the substitution of the G395A polymorphism in the promoter region, it will disrupt the DNA-protein interaction, which may further weaken the function of klotho protein.(37) Nevertheless, there may be another factor apart from the klotho SNP at the G395A locus that influences the quantity of klotho protein, such as C1818T, a variant with no amino acid alteration situated on exon 4. Numerous investigations indicated that a silent mutation in an exon could result in the production of an alternative transcript with aberrant function or modify the degree of expression of the protein product.(17) Additionally, there is also C370S SNP in exon 2, which is one of the SNPs that make up the functional klotho variant KL-VS and can damage protein function by affecting its catalytic activity.(38)

## Conclusion

In conclusion, it may be inferred that the klotho GA genotype and a decreased plasma klotho level play a significant role on the ten-years risk of ASCVD and cardiovascular mortality in non-dialysis CKD. However, this study has some limitations. To begin, the sample size of this study was relatively small; as a result, our findings should be interpreted cautiously in light of the possibility of bias. Second, plasma klotho level, ASCVD risk, and mortality risk scores can still be influenced by other factors, such as aging, CKD stages, hypertension, smoking, and other factors. Third, this study did not examine inter-ethnic variation in the klotho gene polymorphism, despite the fact that SNP distribution and influence may vary between ethnic groups and populations. Thus, large-scale genetic association studies on the effect of the klotho SNP and plasma level on ASCVD risk and mortality risk scores in patients with CKD with adjusted variables could be conducted in various ethnic groups within the Indonesian population.

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## Authors Contribution

HS, BSP, and MT were involved in the conceptualization. CDKW prepared the methodology. SDS and HS collected the sample. CDKW and HS performed the laboratory analysis, while CDKW performed the formal analysis. CP and HS drafted the manuscript, while DSB and LA aided in reviewing and editing it. CDKW aided in the funding acquisition, while SDS, MT, and MYA provided the resources. And lastly, MT did supervision for the whole study.

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