




## ORIGINAL ARTICLE

# Association of a single nucleotide polymorphism combination pattern of the Klotho gene with non-cardiovascular death in patients with chronic kidney disease

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## ABSTRACT

**Background.** Chronic kidney disease (CKD) is associated with an elevated risk of all-cause mortality, with cardiovascular death being extensively investigated. However, non-cardiovascular mortality represents the biggest percentage, showing an evident increase in recent years. Klotho is a gene highly expressed in the kidney, with a clear influence on lifespan. Low levels of Klotho have been linked to CKD progression and adverse outcomes. Single nucleotide polymorphisms (SNPs) of the Klotho gene have been associated with several diseases, but studies investigating the association of Klotho SNPs with non-cardiovascular death in CKD populations are lacking.

**Methods.** The main aim of this study was to assess whether 11 Klotho SNPs were associated with non-cardiovascular death in a subpopulation of the National Observatory of Atherosclerosis in Nephrology (NEFRONA) study ( $n = 2185$  CKD patients).

**Results.** After 48 months of follow-up, 62 cardiovascular deaths and 108 non-cardiovascular deaths were recorded. We identified a high non-cardiovascular death risk combination of SNPs corresponding to individuals carrying the most frequent allele (G) at rs562020, the rare allele (C) at rs2283368 and homozygotes for the rare allele (G) at rs2320762 (rs562020 GG/AG + rs2283368 CC/CT + rs2320762 GG). Among the patients with the three SNPs genotyped ( $n = 1016$ ), 75 (7.4%) showed this combination. Furthermore, 95 (9.3%) patients showed a low-risk combination carrying all the opposite genotypes (rs562020 AA + rs2283368 TT + rs2320762 GT/TT). All the other combinations [ $n = 846$  (83.3%)] were considered as normal risk. Using competing risk regression analysis, we confirmed that the proposed combinations are independently associated with a higher {hazard ratio [HR] 3.28 [confidence interval (CI) 1.51–7.12]} and lower [HR  $6 \times 10^{-6}$  (95% CI  $3.3 \times 10^{-7}$ – $1.1 \times 10^{-5}$ )] risk of suffering a non-cardiovascular death in the CKD population of the NEFRONA cohort compared with patients with the normal-risk combination.

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**Conclusions.** Determination of three SNPs of the Klotho gene could help in the prediction of non-cardiovascular death in CKD.

**Keywords:** chronic kidney disease, Klotho, non-cardiovascular death, polymorphisms

## INTRODUCTION

Chronic kidney disease (CKD) is a worldwide health problem, the incidence and prevalence of which for 2017 were estimated at  $19.7 \times 10^6$  and  $697 \times 10^6$ , respectively [1]. It is predicted that CKD will become the fifth leading cause of years of life lost by 2040, showing a 100% increase compared with 2016 rates [2]. It has been widely documented that CKD patients show an increased risk of presenting with cardiovascular disease [3, 4], which could explain the high death rates [5, 6]. Consequently, most of the genetic studies aimed at identifying new single nucleotide polymorphisms (SNPs) associated with an increased risk of death in CKD patients are focused on cardiovascular risk [7–10]. Nevertheless, despite an evident increase in non-cardiovascular mortality in CKD patients [5, 11], no studies have focused on polymorphisms related to this type of death.

Klotho, a co-receptor of fibroblast growth factor 23 (FGF23), is a single-pass encoded protein that has 1012 amino acids and is subject to cleavage to produce soluble Klotho (sKlotho) [12]. In humans, this protein is expressed in a variety of tissues, showing particularly strong expression in kidney tubules [13]. Klotho gained relevance when it was described as an anti-ageing protein, as Klotho knockout mice showed a phenotype resembling human ageing, including shorter lifespan [14]. Furthermore, the main anti-ageing effects of Klotho were attributable to the protein produced in the kidney [15]. Currently there are many studies investigating Klotho effects on ageing in both humans and mice and many clinical trials involving the analysis of circulating sKlotho levels are being performed [16]. It has been demonstrated that the levels of both Klotho messenger RNA and protein, are decreased in CKD [17]. Furthermore, low levels of sKlotho have been linked to CKD progression [18–20] and adverse outcomes [19, 20]. However, some studies did not find a relationship between sKlotho levels and adverse outcomes in CKD patients [21, 22] after considering FGF23 levels. Indeed, Klotho effects do not have to be strictly limited to FGF23 signalling. Hu *et al.* showed that FGF23 was not indispensable for Klotho-induced phosphaturia [23] and that Klotho has the capacity to interfere with insulin, Wnt and transforming growth factor- $\beta$ 1 pathways in an FGF23-independent manner [24]. Altogether, it is clear that Klotho affects multiple age-related pathologies in FGF23-dependent and independent manners [25] and that premature death is present in the majority of patients with CKD, while the main causes are not restricted to greater cardiovascular disease incidence [4].

The Klotho gene (KL) is located on chromosome XIII and is composed of five exons that expand through >50 000 nucleotides [26]. According to the National Centre for Biotechnology Information, Klotho contains >11 000 SNPs [27]. Most of the genetic studies on Klotho have focused on the KL-VS polymorphisms located in exon 2 (name due to the F352V change of rs9536314 and C370S from rs9527025), which are related to human longevity [28], cognition [29], neuroprotection [30] and occult coronary artery disease [31]. The rs1207568 (G395A) SNP located at the promoter region has also been widely studied and has been associated with coronary artery disease [32], metabolic syndrome [33], kidney stones [34], mortality in dialysis [35] and immunoglobulin A nephropathy [36]. Other Klotho SNPs that emerged from genome-wide association studies have been

related to diabetic nephropathy [37], type 2 diabetes [38, 39], atheromatous progression [40], onset of stroke [41], chronic haemodialysis mortality [42] and the incidence of CKD [43].

Considering the abovementioned data, we decided to assess how 11 Klotho SNPs were related to all-cause death, cardiovascular death and non-cardiovascular death in the National Observatory of Atherosclerosis in Nephrology (NEFRONA) study [44].

## MATERIALS AND METHODS

### Study population

The NEFRONA study is a prospective, multicentre cohort study to analyse the morbidity and mortality of CKD patients in Spain [45]. From 2009 to 2012, we recruited patients between 18 and 75 years old with different degrees of CKD (950 Stage 3, 807 Stages 4 and 5 and 688 on dialysis) and 559 non-CKD volunteers (eGFR >60 mL/min/1.73 m<sup>2</sup>) and followed them for 4 years, collecting data every 6 months on fatal and non-fatal cardiovascular events (CVEs), non-cardiovascular death or initiation of renal replacement therapy. Physicians responsible for patient recruitment recorded the incidence of CVEs following the International Classification of Diseases, Ninth Revision, Clinical Modification. For out-of-hospital deaths, the cause of death was established upon interviewing family members. All included volunteers signed an informed consent provided by their respective local ethics committee that previously approved the study. Volunteers with a history of CVEs, significant carotid stenosis, active infections (human immunodeficiency virus, tuberculosis), pregnancy, life expectancy <12 months and having received any organ transplant or carotid artery surgery were not allowed to participate in the NEFRONA study.

For this study, controls and non-Caucasian volunteers were excluded. A flow diagram of the experiment design is shown in [Supplementary data, Figure S1](#).

### SNP selection and genotyping

DNA was extracted from blood samples of the NEFRONA study gathered in the REDinREN Biobank [46]. The 11 Klotho SNPs selected (rs9536254, rs567170, rs577912, rs580332, rs495392, rs2320762, rs562020, rs576404, rs385564, rs9536282 and rs2283368) were included in a previous study in which SNPs related to bone mineral disorders and vascular calcification were selected [43]. Moreover, SNPs should not have been previously related to cardiovascular or non-cardiovascular death. Genotyping was performed as previously described [40]. Hardy-Weinberg equilibrium (HWE) was performed by means of a chi-squared test and genotyping quality control was done by including Coriell Institute Biorepository replicates in each plate (samples NA07348, NA07349 and NA07351).

### Clinical data and biochemical variables

An itinerant team including a nurse and two technicians obtained anthropometric data, clinical data and blood samples. Family history of cardiovascular death and cardiovascular risk factors was obtained on recruitment day. Atherosclerosis assessment was performed at baseline as previously described

[47, 48] and plaques were defined according to the American Society of Echocardiography and the Mannheim Carotid Intima-Media Thickness (cIMT) Consensus as cIMT lumen protrusion  $\geq 1.5$  mm [49, 50].

### Statistical analysis

All statistical analyses were done using SPSS statistics software version 24.0.0.0 (IBM, Armonk, NY, USA). Quantitative variables are shown as average and standard deviation (SD), qualitative variables as *n* and percent and quantitative variables not following a normal distribution as median [quartile 1 (Q1)–quartile 3 (Q3)]. Comparisons between groups were done using Student's *t* test, chi-squared test and Mann–Whitney *U* test, respectively. For comparisons between more than two groups, we used an analysis of variance (ANOVA) and multiple comparisons between groups were performed with an honestly significant difference Tukey's test. Multivariable Fine and Gray competing risk regressions were performed for non-cardiovascular death, choosing as competing risk cardiovascular death and receiving a kidney transplant. Data are presented as hazard ratios (HRs) and 95% confidence intervals (CIs). *P*-values  $< 0.05$  were considered statistically significant.

The linkage disequilibrium of selected SNPs was calculated with CubeX [51].

## RESULTS

The analysis encompassed only patients with a Caucasian genetic background and CKD [2906 (96.73% of the NEFRONA study)] and subsequently only the ones with data on at least one of the Klotho SNPs analysed [2185 (72.7% of the NEFRONA study); [Supplementary data, Figure S1](#)]. During the 48 months of follow-up, we recorded 62 cardiovascular deaths (2.8%) and 108 non-cardiovascular deaths (4.9%), for 170 total deaths in our cohort (7.7%). [Table 1](#) shows that the variables related to both cardiovascular death and non-cardiovascular death were diabetes, advanced CKD stages and atheromatous plaque on the basal evaluation. Higher levels of intact parathyroid hormone, C-reactive protein and phosphate were also associated with both types of death; moreover, lower levels of albumin and serum calcium were related to both cardiovascular and non-cardiovascular death. Consequently, all these variables were associated with any death, which also showed an association with male sex and diastolic blood pressure. Pulse pressure, high-density lipoprotein cholesterol, glucose and pathological ankle–brachial index were associated with cardiovascular death, while hypertension, total cholesterol and low-density lipoprotein cholesterol were associated with non-cardiovascular death.

After proving that all the SNPs satisfied the HWE ([Supplementary data, Table S1](#)), we analysed the relationship of all genotypes with cardiovascular death, non-cardiovascular death and any death ([Supplementary data, Table S2](#)) and elaborated the models of dominance. [Supplementary data, Table S3](#) shows the allele frequencies in our cohort. As shown in [Table 2](#), under dominant assumption of the most frequent allele G of the rs562020 genotype, the carriers of the A allele in homozygosity showed a lower incidence of any death. When the dominant model for the less frequent C allele of rs2283368 was tested, we noted that TT carriers showed a lower incidence of any death and non-cardiovascular death and CT + TT carriers showed a higher incidence of both, as cardiovascular death was equal in both groups. It is obvious that the differences observed in any death under the dominant model of rs2283368 were

mainly due to the marked difference in non-cardiovascular death. Finally, the dominant model for the more frequent allele T of rs2320762 showed a greater incidence of non-cardiovascular death and of any death for the GG genotype.

Taking into account data from individual genetic models, we postulated a high non-cardiovascular death genotype combination. This combination corresponds to individuals carrying high-risk allele G of the rs562020 SNP, the high-risk allele C of the rs2283368 SNP and the high-risk genotype GG of the rs2320762 SNP (rs562020 GG/AG + rs2283368 CC/CT + rs2320762 GG); the three SNPs were not in linkage disequilibrium ([Supplementary data, Table S3](#)). In our population, 75 (7.3%) patients showed this genotype. Furthermore, we also postulated that carriers of the alternative genotypes would show fewer non-cardiovascular deaths (rs562020 AA + rs2283368 TT + rs2320762 GT/TT), with 95 (9.3%) patients showing this low-risk combination of genotypes. All the other combinations [846 (83.2%)] were considered as normal risk. It should be noted that all three SNPs could be correctly genotyped in 1016 patients (271 CKD3, 223 CKD 4–5 and 522 on dialysis), 46.4% of the initial study cohort. To test if our hypothesis was certain, the cohort was divided according to the three proposed risk combinations of genotypes and bivariate analysis was performed. As shown in [Table 3](#), the three groups only showed significant differences in non-cardiovascular death and any death incidence, and the only parameters that were close to reaching significance were the presence of basal atheroma plaques and glucose levels.

Finally, in order to determine the importance of the proposed genotypes in predicting non-cardiovascular death in our cohort, we performed a competing risk regression analysis adjusting for all the variables in the NEFRONA study that could contribute to non-cardiovascular death. As shown in [Table 4](#), after adjusting for all the possible confounding variables, the proposed combinations of genotypes still showed a significant association with non-cardiovascular death.

## DISCUSSION

This study identifies a combination of SNPs of three Klotho polymorphisms that are associated with a higher or lower risk of suffering non-cardiovascular death in a CKD population. Patients with CKD show an increased risk of death, and traditionally research has focused on the high rates of cardiovascular death presented by this population [3, 52]. However, CKD patients also show an increased risk of non-cardiovascular death [5, 11, 53], and less attention has been paid to this group of patients.

The main causes of non-cardiovascular death among CKD patients are infectious diseases and cancer. CKD patients show increased risk of infection in both early CKD stages [54] and in dialysis [55], and it has been recently shown that mice with reduced Klotho levels have lower survival rates after induced sepsis [56]. Unfortunately, the low numbers of deaths due to infection in the high- and low-risk groups (two and one, respectively) preclude further studies in this direction. Klotho also shows antitumor properties [57], and its levels are reduced in certain tumours, such as pancreatic [58] and breast [59], while high levels are considered indicate a more favorable prognosis in many cancer types, such as hepatocellular [60] and oesophageal [61] carcinomas. In our cohort, deaths attributable to cancer were three in the high-risk group and zero in the low-risk group, but again, the numbers are too low to perform further analyses. Despite this, we cannot discard the possible role of the described combination of genotypes in infections and/or cancer deaths in CKD patients.

Table 1. Epidemiological, clinical and biochemical parameters of cardiovascular, non-cardiovascular and any death of the cohort

| Variable  | Cardiovascular Death |                  |              | Non-cardiovascular death |                  |              | Any death        |                  |              |
|---|----------------------|------------------|--------------|--------------------------|------------------|--------------|------------------|------------------|--------------|
|   | Yes<br>(n = 62)      | No<br>(n = 2123) | P-value      | Yes<br>(n = 108)         | No<br>(n = 2077) | P-value      | Yes<br>(n = 170) | No<br>(n = 2015) | P-value      |
| Age (years)                                     | 63.02 (9.39)         | 58.21 (12.66)    | <b>0.000</b> | 64.78 (9.65)             | 58.01 (12.6)     | <b>0.000</b> | 64.1 (9.5)       | 57.86 (12.7)     | <b>0.000</b> |
| Sex (female), n (%)                             | 19 (30.6)            | 805 (37.9)       | 0.244        | 34 (31.5)                | 790 (38)         | 0.171        | 53 (31.2)        | 771 (38.3)       | 0.067        |
| Smoking (yes), n (%)                            | 38 (61.3)            | 1213 (57.1)      | 0.515        | 70 (64.8)                | 1181 (56.9)      | 0.103        | 108 (63.5)       | 1143 (56.7)      | 0.085        |
| Diabetes (yes), n (%)                           | 32 (51.6)            | 523 (24.6)       | <b>0.000</b> | 38 (35.2)                | 517 (24.9)       | <b>0.017</b> | 70 (41.2)        | 485 (24.1)       | <b>0.000</b> |
| Hypertension (yes), n (%)                       | 57 (91.9)            | 1933 (91.1)      | 0.810        | 91 (84.3)                | 1899 (91.4)      | <b>0.011</b> | 148 (87.1)       | 1842 (91.4)      | 0.056        |
| Dyslipidaemia (yes),<br>CKD stage, n (%)        | 39 (62.9)            | 1426 (67.2)      | 0.481        | 64 (59.3)                | 1401 (67.5)      | 0.077        | 103 (60.6)       | 1362 (67.6)      | 0.062        |
| Stage 3   | 16 (25.8)            | 851 (40.1)       | <b>0.001</b> | 26 (24.1)                | 841 (40.5)       | <b>0.001</b> | 42 (24.7)        | 825 (40.9)       | <b>0.000</b> |
| Stages 4 and 5                                  | 16 (25.8)            | 708 (33.3)       |              | 38 (35.2)                | 686 (33)         |              | 54 (31.8)        | 670 (33.3)       |              |
| Dialysis  | 30 (48.4)            | 564 (26.6)       |              | 44 (40.7)                | 550 (26.5)       |              | 74 (43.5)        | 520 (25.8)       |              |
| Body mass index (kg/m <sup>2</sup> )            | 29.01 (5.89)         | 28.25 (5.15)     | 0.261        | 28.21 (5)                | 28.28 (5.18)     | 0.884        | 28.5 (5.36)      | 28.26 (5.16)     | 0.561        |
| Systolic blood<br>pressure (mmHg)               | 147.9 (25.8)         | 142.9 (21.8)     | 0.074        | 142.5 (24.3)             | 143 (21.8)       | 0.791        | 144.5 (24.9)     | 142.9 (21.6)     | 0.429        |
| Diastolic blood<br>pressure (mmHg)              | 78.7 (13.7)          | 81.4 (11.6)      | 0.138        | 79.27 (11.5)             | 81.45(11.7)      | 0.06         | 79.08 (12.35)    | 81.53 (11.65)    | <b>0.009</b> |
| Pulse pressure (mmHg)                           | 69.1 (20.2)          | 61.5 (18.2)      | <b>0.001</b> | 63.23 (18.7)             | 61.63 (18.36)    | 0.377        | 65.41 (19.46)    | 61.39 (18.2)     | <b>0.006</b> |
| Haematocrit (%)                                 | 37.5 (5.5)           | 38.5 (5)         | 0.117        | 36.47 (5)                | 38.66 (5)        | <b>0.000</b> | 36.86 (5.22)     | 38.69 (5.02)     | <b>0.000</b> |
| Albumin (g/dL)                                  | 3.83 (0.46)          | 4.1 (0.46)       | <b>0.000</b> | 3.93 (0.5)               | 4.1 (0.46)       | <b>0.001</b> | 3.9 (0.49)       | 4.1 (0.45)       | <b>0.000</b> |
| iPTH (pg/mL), median (Q1-Q3)                    | 166 (105–264)        | 120 (68–220)     | <b>0.006</b> | 157 (76.6–263)           | 120 (68–220)     | <b>0.048</b> | 161.8 (86–263)   | 118 (68–217)     | <b>0.001</b> |
| C-reactive protein, median (Q1-Q3)              | 4 (1.4–8.1)          | 1.96 (0.94–4.32) | <b>0.002</b> | 4.6 (1.68–10.2)          | 1.92 (0.92–4.2)  | <b>0.000</b> | 4.24 (1.66–9.7)  | 1.9 (0.9–4.1)    | <b>0.000</b> |
| Total cholesterol (mg/dL)                       | 173.32 (51)          | 178.7 (38.9)     | 0.29         | 169.9 (39.7)             | 179.03 (39.2)    | <b>0.02</b>  | 171.15 (44)      | 179.2 (38.84)    | <b>0.011</b> |
| High-density lipoprotein<br>cholesterol (mg/dL) | 45.07 (15.34)        | 49.55 (15.39)    | <b>0.032</b> | 49.9 (17.67)             | 49.3 (15.28)     | 0.713        | 48.15 (17)       | 49.53 (15.2)     | 0.295        |
| Low-density lipoprotein<br>cholesterol (mg/dL)  | 98.13 (36.32)        | 102.14 (33.3)    | 0.381        | 93.25 (32.5)             | 102.47 (33.4)    | <b>0.01</b>  | 95.08 (34)       | 102.6 (33.1)     | <b>0.009</b> |
| Triglycerides (mg/dL)                           | 149.76 (79)          | 144.4 (81.2)     | 0.623        | 140.3 (81)               | 144.77 (81.21)   | 0.588        | 143.6 (80)       | 144.6 (81.2)     | 0.888        |
| Glucose (mg/dL)                                 | 122 (52.93)          | 107.2 (39.15)    | <b>0.035</b> | 104.2 (41.7)             | 107.8 (39)       | 0.358        | 110.6 (46.6)     | 107.4 (39)       | 0.385        |
| Calcium (mg/dL)                                 | 9.11 (0.58)          | 9.32 (0.59)      | <b>0.007</b> | 9.15 (07)                | 9.32 (0.58)      | <b>0.018</b> | 9.14 (0.66)      | 9.33 (0.58)      | <b>0.000</b> |
| Phosphate (mg/dL)                               | 4.62 (1.23)          | 4.03 (1.09)      | <b>0.000</b> | 4.19 (1.14)              | 4.04 (1.09)      | 0.167        | 4.35 (1.19)      | 4.02 (1.08)      | <b>0.000</b> |
| Sodium (mEq/L)                                  | 139.67 (3.26)        | 140.31 (3)       | 0.107        | 139.55 (4.2)             | 140.33 (2.99)    | 0.06         | 139.6 (3.87)     | 140.35 (2.98)    | <b>0.014</b> |
| Potassium (mEq/L)                               | 5.02 (0.73)          | 4.8 (0.6)        | <b>0.025</b> | 4.9 (0.7)                | 4.8 (0.59)       | 0.152        | 4.95 (0.71)      | 4.8 (0.59)       | <b>0.009</b> |
| Any plaque basal (yes), n (%)                   | 60 (96.8)            | 1489 (70.1)      | <b>0.000</b> | 98 (88.9)                | 1453 (70)        | <b>0.000</b> | 156 (91.8)       | 1393 (69.1)      | <b>0.000</b> |
| Pathology ankle-brachial<br>index (yes), n (%)  | 33 (53.2)            | 587 (27.9)       | <b>0.000</b> | 38 (36.2)                | 582 (28.2)       | 0.079        | 71 (42.5)        | 549 (27.5)       | <b>0.000</b> |

Values are expressed as average (SD) unless stated otherwise. Comparisons between groups performed with Student's t test for quantitative normally distributed variables, Mann-Whitney U test for non-normally distributed quantitative variables and Chi-squared test for categorical data. Statistically significant values are in bold. iPTH: intact parathyroid hormone.

Table 2. Proposed SNPs and models with effects on cardiovascular, non-cardiovascular and any death

| Type of death                   | rs562020_DOM G<br>(n = 2185) |               |              | rs2283368_DOM C<br>(n = 1017) |               |              | rs2320762_DOM T<br>(n = 2184) |                     |              | Whole cohort (n = 2185) |
|---------------------------------|------------------------------|---------------|--------------|-------------------------------|---------------|--------------|-------------------------------|---------------------|--------------|-------------------------|
|                                 | GG + AG<br>n = 1944          | AA<br>n = 241 | P-value      | CC + CT<br>n = 216            | TT<br>n = 801 | P-value      | GG<br>n = 318                 | GT + TT<br>n = 1866 | P-value      |                         |
| Cardiovascular death, n (%)     | 59 (3)                       | 3 (1.2)       | 0.114        | 7 (3.2)                       | 27 (3.4)      | 0.925        | 11 (3.5)                      | 51 (2.7)            | 0.471        | 62 (2.8)                |
| Non-cardiovascular death, n (%) | 101 (5.2)                    | 7 (2.9)       | 0.122        | 22 (10.2)                     | 33 (4.1)      | <b>0.000</b> | 26 (8.2)                      | 82 (4.4)            | <b>0.004</b> | 108 (4.9)               |
| All death, n (%)                | 160 (8.2)                    | 10 (4.1)      | <b>0.026</b> | 29 (13.4)                     | 60 (7.5)      | <b>0.006</b> | 37 (11.6)                     | 133 (7.1)           | <b>0.006</b> | 170 (7.8)               |

Comparisons between groups were performed with the chi-squared test. Statistically significant values are in bold.

The three polymorphisms identified in this work were previously analysed in a cohort of CKD patients on haemodialysis [42] and none of them was related to any type of death. Differences between our study and the study by Friedman et al. [42] could be attributed to the 11% non-Caucasian genetic

background in their work, the different composition of the cohort (the Friedman et al. study was exclusively haemodialysis patients) and/or the fact that they did not test combinations of different polymorphisms. Nevertheless, it is worth mentioning that in our population the rs577912 SNP that Friedman et al.

Table 3. Epidemiological, clinical, biochemical parameters and cause of death according to the three proposed genotypes

| Variable                                       | High-risk phenotype<br>(n = 75)                    | All others<br>(n = 846) | Low-risk phenotype<br>(n = 95)                  | P-value      |
|--|--|-------------------------|---|--------------|
|  | rs562020 GG/AG + rs2320762<br>GG + rs2283368 CC/CT |                         | rs562020 AA + rs2320762<br>GT/TT + rs2283368 TT |              |
| Age (years)                                    | 57.43 (12.32)                                      | 56.98 (13.28)           | 57.45 (11.91)                                   | 0.957        |
| Sex (female), n (%)                            | 30 (40)  | 325 (38.4)              | 41 (43.2)                                       | 0.656        |
| Smoking (yes), n (%)                           | 47 (62.7)  | 484 (57.2)              | 46 (48.4)                                       | 0.147        |
| Diabetes (yes), n (%)                          | 17 (22.7)  | 191 (22.6)              | 22 (23.2)                                       | 0.992        |
| Hypertension (yes), n (%)                      | 63 (84)  | 762 (90.1)              | 88 (92.6)                                       | 0.160        |
| Dyslipidaemia (yes), n (%)                     | 46 (61.3)  | 543 (64.2)              | 68 (71.6)                                       | 0.296        |
| Stage 3  | 20 (26.7)  | 233 (27.5)              | 18 (18.9)                                       | 0.429        |
| Stages 4 and 5                                 | 14 (18.7)  | 185 (21.9)              | 24 (25.3)                                       |              |
| Dialysis                                       | 41 (54.7)  | 428 (50.6)              | 53 (55.8)                                       |              |
| Months in dialysis                             | 26.49 (25.96)                                      | 27.59 (33.96)           | 28.02 (28.18)                                   | 0.961        |
| Type of dialysis (HD), n (%)                   | 28 (68.3)  | 276 (64.5)              | 33 (62.3)                                       | 0.830        |
| Body mass index (kg/m <sup>2</sup> )           | 27.45 (5.29)                                       | 27.77 (5.29)            | 27.95 (5.64)                                    | 0.751        |
| Systolic blood pressure (mmHg)                 | 140.4 (19.62)                                      | 142.3 (23.43)           | 140.05 (20.94)                                  | 0.719        |
| Diastolic blood pressure (mmHg)                | 82.07(10.25)                                       | 80.84 (12.75)           | 82.09 (12.09)                                   | 0.717        |
| Pulse pressure (mmHg)                          | 58.36 (15.7)                                       | 61.52 (19.1)            | 58 (17.76)                                      | 0.308        |
| Haematocrit (%)                                | 38.27 (4.58)                                       | 37.59 (5)               | 37.68 (3.9)                                     | 0.538        |
| Albumin (g/dL)                                 | 3.95 (0.5)   | 4.02 (0.44)             | 4.06 (0.4)                                      | 0.165        |
| iPTH (pg/mL)                                   | 238.97 (232.18)                                    | 211.71 (204.13)         | 211.5 (182.86)                                  | 0.590        |
| C-reactive protein                             | 4.53 (6.4)   | 5 (9.35)                | 3 (4.11)  | 0.214        |
| Total cholesterol (mg/dL)                      | 175.16 (44.94)                                     | 173.26 (39.83)          | 174.46 (39.49)                                  | 0.931        |
| High-density lipoprotein cholesterol (mg/dL)   | 49.19(18)  | 49.09 (16.1)            | 48.65 (12.93)                                   | 0.968        |
| Low-density lipoprotein cholesterol (mg/dL)    | 95.96 (40.5)                                       | 97.74 (33.6)            | 98.07 (33.47)                                   | 0.898        |
| Triglycerides (mg/dL)                          | 157.63 (107.56)                                    | 139.26 (73.77)          | 148 (71)  | 0.162        |
| Glucose (mg/dL)                                | 101.46 (32.65)                                     | 105.68 (41.52)          | 113.04 (49.18)                                  | 0.083        |
| Calcium (mg/dL)                                | 9.19 (0.75)  | 9.23 (0.62)             | 9.21 (0.57)                                     | 0.9          |
| Phosphate (mg/dL)                              | 4.2 (1.34)   | 4.34 (1.26)             | 4.47 (1.32)                                     | 0.250        |
| Sodium (mEq/L)                                 | 139.43 (3.27)                                      | 139.9 (3.29)            | 139.72 (2.92)                                   | 0.516        |
| Potassium (mEq/L)                              | 4.93 (0.65)  | 4.83 (0.65)             | 4.85 (0.62)                                     | 0.496        |
| Any plaque basal (yes), n (%)                  | 47 (62.7)  | 598 (70.7)              | 73 (76.8)                                       | 0.131        |
| Pathological ankle-brachial index (yes), n (%) | 19 (25.7)  | 256 (30.6)              | 36 (38.3)                                       | 0.184        |
| Cardiovascular death (yes), n (%)              | 3 (4)  | 30 (3.5)                | 1 (1.1)   | 0.417        |
| Non-cardiovascular death (yes), n (%)          | 10 (13.3)  | 43 (5.1)                | 2 (2.1)   | <b>0.003</b> |
| Any death (yes), n (%)                         | 13 (17.3)  | 73 (8.6)                | 3 (3.2)   | <b>0.005</b> |

Values are presented as average and SD unless stated otherwise. Comparisons between groups performed with ANOVA for quantitative variables and chi-squared test for categorical data. Statistically significant values are in bold. iPTH: intact parathyroid hormone.

Table 4. Coefficient of competing risk (n = 704)

| Variable                                   | HR (95% CI)  | P-value      |
|--|--|--------------|
| Age  | 1.046 (0.994–1.101)  | 0.09         |
| Sex (women)                                | 1.109 (0.474–2.598)  | 0.810        |
| Smoking (yes)                              | 1.544 (0.587–4.056)  | 0.380        |
| Diabetes (yes)                             | 1.209 (0.460–3.175)  | 0.700        |
| Hypertension (yes)                         | 0.709 (0.286–1.757)  | 0.460        |
| Dyslipidaemia (yes)                        | 0.495 (0.243–1.010)  | 0.054        |
| CKD Stages 4 and 5                         | 1.275 (0.365–4.453)  | 0.700        |
| Dialysis                                   | 2.22 (0.744–6.630)   | 0.150        |
| Diastolic blood pressure, mmHg             | 0.991 (0.960–1.023)  | 0.550        |
| Pulse pressure, mmHg                       | 0.990 (0.968–1.011)  | 0.350        |
| Haematocrit (%)                            | 0.925 (0.845–1.012)  | 0.085        |
| C-reactive protein                         | 1.015 (0.993–1.037)  | 0.150        |
| Total cholesterol, mg/dL                   | 1.009 (0.993–1.025)  | 0.270        |
| Low-density lipoprotein cholesterol, mg/dL | 0.993 (0.976–1.011)  | 0.440        |
| Any plaque basal                           | 2.574 (0.631–10.489)   | 0.190        |
| Sodium, mEq/L                              | 0.957 (0.833–1.100)  | 0.530        |
| Potassium, mEq/L                           | 1.410 (0.814–2.440)  | 0.220        |
| Klotho high-risk genotype                  | 3.286 (1.516–7.129)  | <b>0.003</b> |
| Klotho low-risk genotype                   | $6 \times 10^{-6}$ ( $3.3 \times 10^{-7}$ – $1.1 \times 10^{-5}$ ) | <b>0.000</b> |

Statistically significant values are in bold.

related to any death in their study is close to reaching a significant association in our study ( $P = 0.062$ ; [Supplementary data, Table S2](#)). The rs562020 SNP has been related to better cognitive function in people 92–100 years old and, in the same work, rs2283368 was significantly associated with cognitive decline >7 years [62]. In the Framingham cohort, the relationship between rs562020 and the presence of valvular or vascular calcification was analysed and no relationship was found [63], data that agree with our results showing no relationship between the polymorphism and cardiovascular death.

This work has certain limitations. The first limitation is that we did not analyse Klotho plasma levels, so we cannot correlate the proposed genotypes with protein levels. The second limitation is that we do not have a validation cohort to reassess the importance of the proposed combination of SNPs. Despite evident limitations, our work has certain strengths that we would like to highlight. First is that we focused on Klotho SNPs that have not been extensively studied, so we pave the way for further studies using the proposed genotypes in various diseases in which Klotho could be involved. Second is that the NEFRONA

study is very comprehensive, allowing us to correct for the effect of the proposed combination of genotypes by multiple possible confounding factors. Third is that our cohort is relatively big, with a prospective design and a relatively long follow-up time. Fourth is that we have considered a combination of three SNPs to check the relationship with all-cause death, an approach that could help to unravel synergistic effects of different polymorphisms that are missed by individual SNP analysis.

In summary, we have identified a combination of SNPs of the Klotho gene independently associated with higher and lower risks for non-cardiovascular death in CKD. Validation of these findings could be useful in the stratification of non-cardiovascular death risk in CKD patients.

## SUPPLEMENTARY DATA

Supplementary data are available at [ckjonline](http://ckjonline.com).

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## AUTHORS' CONTRIBUTIONS

V.J.M. and C.S. were involved in the study concept and design. Data acquisition was done by B.M. and data interpretation by V.J.M., C.S. and B.L.M. C.S. and V.J.M. were involved in the statistical analysis. Drafting of the manuscript was done by C.S. and V.J.M. All authors participated in critical revision of the manuscript for important intellectual content. Study supervision was done by V.J.M. The NEFRONA investigators collected baseline and prospective follow-up data for the cohort.

## CONFLICT OF INTEREST STATEMENT

None declared.

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