Klotho as a biomarker of subclinical atherosclerosis in patients with moderate to severe chronic kidney disease

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Chronic kidney disease (CKD) has been associated with a higher risk of cardiovascular disease (CVD). CKD patients present a decrease in the levels of the protein Klotho that accompanies the decrease in kidney function. This protein has been related to protective effects against CVD. However, it is unclear whether circulating Klotho, and its expression in peripheral blood cells (PBCs) are also associated with subclinical atherosclerosis in CKD. The present study aimed to study the relationship between Klotho and subclinical atherosclerosis in a population of patients with moderate to severe CKD. We determined the serum levels and gene expression in PBCs levels of Klotho and three inflammatory cytokines in 103 patients with CKD and investigated their relationship with two surrogate markers of subclinical atherosclerosis: ankle-brachial index (ABI) and carotid intima-media thickness (CIMT).

Patients with subclinical atherosclerosis presented lower serum and PBCs expression levels of Klotho. Both variables were associated with the presence of subclinical atherosclerosis, being directly related with ABI and inversely with CIMT (P < 0.0001 for both). Multiple regression analysis demonstrated that both variables were significant determinants for ABI (adjusted R² = 0.511, P < 0.0001) and CIMT (adjusted R² = 0.445, P < 0.0001), independently of traditional and emergent cardiovascular risk factors. Moreover, both constituted protective factors against subclinical atherosclerosis [OR: 0.993 (P = 0.002) and 0.231 (P = 0.025), respectively]. Receiver operating characteristic analysis pointed to the utility of serum Klotho (area under the curve [AUC]: 0.817, 95% CI: 0.736–0.898, P < 0.001) and its gene expression in PBCs (AUC: 0.742, 95% CI: 0.647–0.836, P < 0.001) to distinguish subclinical atherosclerosis. The reductions in serum and PBCs expression levels of Klotho in CKD patients are independently associated with the presence of subclinical atherosclerosis. Further research exploring whether therapeutic approaches to maintain or elevate Klotho could reduce the impact of CVD in CKD patients is warranted.

Abbreviations

CKD Chronic kidney disease
CVD Cardiovascular disease
PBCs Peripheral blood cells
TNFα Tumor necrosis factor α
IL Interleukin
sCVD Subclinical atherosclerotic cardiovascular disease

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Patients with chronic kidney disease (CKD) present high rates of morbidity and mortality, mainly of cardiovascular (CV) origin. In fact, CKD patients are more likely to die from cardiovascular disease (CVD) than to develop end-stage renal failure. Different pathological mechanisms account for this higher risk of mortality including atherosclerosis, arterial stiffness, vascular calcification, congestive cardiomyopathy, capillary/myocyte mismatch in the heart, and sudden cardiac death.

Although the involvement of accelerated atherosclerosis in the CV mortality observed in CKD patients has recently been questioned, many clinical trials demonstrate that treatments aiming to reduce lipid burden are effective in reducing CV events, mainly in the early stages of CKD. Additionally, several studies have reported an increase in the incidence and severity of coronary heart disease as the GFR decreases and the prevalence of the disease since the largest proportion of systemic Klotho is generated by the kidneys and its levels are reduced in all stages of CKD. Mortality being associated with markers of vascular dysfunction and with the incidence of atherosclerosis.

Deficiency triggers endothelial dysfunction and vascular calcification. Moreover, several clinical studies also suggest that low serum levels of Klotho are associated with the prevalence and severity of CVD, all-cause mortality, and with the incidence of atherosclerosis, arterial stiffness, vascular calcification, congestive cardiomyopathy, capillary/myocyte mismatch in the heart, and sudden cardiac death.

Importantly, experimental models point to the existence of Klotho protective effects upon vascular system that include the maintaining of endothelial wall homeostasis and the promotion of vascular health, whereas Klotho deficiency triggers endothelial dysfunction and vascular calcification. Moreover, several clinical studies also show that low serum levels of Klotho are associated with the prevalence and severity of CVD, all-cause mortality, and with the incidence of atherosclerosis.

Methods
Patients. A cross-sectional single-center study was conducted recruiting patients from the Nephrology Service of the University Hospital Nuestra Señora de Candelaria (Santa Cruz de Tenerife, Spain). From January-December 2006, 387 patients were initially evaluated and 103 were finally enrolled in the study. Inclusion criteria included CKD patients in stages 3–4 (estimated glomerular filtration rate (eGFR) 15–60 ml/min/1.73 m² according to the Modification of Diet in Renal Disease Study–4 (MDRD–4 equation), who were older than 18 years of age, and who did not have history of known atherosclerotic cardiovascular disease. Exclusion criteria included history of heart failure; chronic inflammatory, immunologic, or tumor disease; positive serology to hepatitis B, hepatitis C, or HIV; acute inflammatory or infectious intercurrent episodes in the previous month; institutionalization; receipt of immunotherapy or immunosuppressive treatment; and inability or unwillingness to provide informed consent. Patients with ABI values ≥ 1.3 were also excluded. The study protocol was approved by the Institutional Ethics Committee of the University Hospital Nuestra Señora de Candelaria and complied with ethical standards of the Declaration of Helsinki. Written informed consent was obtained from all participants.
Biochemical parameters measurements and vascular assessments. All samples were drawn in the morning after 8 h of fasting. After centrifugation, serum samples were aliquoted and immediately frozen at −80 °C. Routine biochemical parameters were measured using standard methods. Concentrations of serum Klotho protein were measured by a solid phase sandwich ELISA using the human soluble α-Klotho assay kit (Immuno-Biological Laboratories, Takasaki, Japan) according to manufacturer’s instructions. The assay sensitivity was 6.15 pg/mL and the intra and inter-assay coefficients of variation were 2.7–3.5% and 2.9–11.4%, respectively. The serum levels of the inflammatory cytokines TNFa, IL6, and IL10 were measured by ELISA methods (Quantikine®, R&D Systems, Abingdon, UK). Minimum detectable concentrations were 0.10 pg/mL, 0.70 pg/mL, and 0.09 pg/mL, respectively. Intra- and inter-assay coefficients of variability were <10.8%. Serum Klotho, TNFa, IL6, and IL10 levels were expressed as pg/mL. High-sensitivity serum C-reactive protein (hsCRP) was measured by a high-sensitivity particle enhanced immunoturbidimetric fully automated assay (Roche Diagnostics GmbH, Mannheim, Germany) in a Cobas 6000 analyzer from the same manufacturer with a sensitivity of 0.3 mg/L and intra- and inter-assay coefficients of variation of 1.6% and 8.4%, respectively. hsCRP levels were expressed as mg/L.

The assessment of subclinical atherosclerotic disease was made by measurement of ABI and CIMT. The ABI was calculated using a portable pulse detector (Ultrasonic Mini Doppler ES-100VX; Hayashi Denki Co., Ltd., Kawasaki, Japan) with an 8 MHz probe. Measurements of CIMT was performed by a unique reader in a blinded fashion by ultrasonography of the carotid arteries with a high-resolution ultrasound (Philips ATL 5000 HDI, Royal Philips Electronics, Amsterdam, The Netherlands) equipped with a 6–13 MHz linear array transducer. We defined patients having subclinical atherosclerosis as those having ABI < 0.9 and/or CIMT ≥0.9 mm, according to the Guidelines for the management of arterial hypertension released by the Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC).25,26

Gene expression analysis. For analysis of gene expression in PBCs, 2.5 ml samples of whole-blood were collected in PAXgene blood RNA tubes (BD, Franklin Lakes, NJ) at the same time as serum samples. Total RNA was isolated from these tubes using a PAXgene blood RNA kit (Qiagen, Valencia, CA) according to manufacturer’s specifications and quantified using a Thermo Scientific NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, MA, USA). cDNA was obtained using a High-Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Foster City, CA, USA) for further analysis. Transcripts of Klotho gene (KL), TNF, IL6, IL10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as constitutive gene, were measured by real-time TaqMan quantitative PCR (qRT-PCR) with TaqMan Fast Universal PCR master mix (Thermo Fisher Scientific) in a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). TaqMan gene expression assays for each transcript were: Hs00183100_m1 [KL], Hs00174128_ml [TNF], Hs00985639_ml [IL6], Hs00961622_m1 [IL10], and Hs99999905_m1 [GAPDH]. The level of target mRNA was estimated by relative quantification using the comparative method (2−ΔΔCt) by normalizing to GAPDH expression. mRNA levels were expressed as arbitrary units (a.u.). Quantification of each cDNA sample was tested in triplicate. A corresponding non-transcriptase reverse transcriptase reaction was included as a control for DNA contamination.

Statistical analysis. Continuous variables were checked for the normal distribution assumption using the Kolmogorov–Smirnov statistic. Non-normally distributed variables were expressed as the median (interquartile range) and normally distributed variables were expressed as the mean ± SD. Categorical data were expressed as number and percent frequency. Differences between groups were analyzed using Chi-square test, Student’s t-test or the Mann–Whitney U-test as appropriate. The Spearman rank correlation was used to determine the correlations between two variables. Backward stepwise multiple regression analysis was performed to determine the independent association between patient clinical parameters as potential predictor variables (age, sex, body mass index (BMI), hypertension (HT), diabetes mellitus (DM), smoking, dyslipidemia, serum uric acid, eGFR, urinary albumin excretion (UAEx), phosphorus, hsCRP, serum and PBCs expression levels of TNFa, IL6, and IL10, and Klotho) and ABI and CIMT values as dependent variables. Tolerance and variance inflation factor were analyzed in order to exclude collinearity. A multiple logistic regression was performed to assess independent predictors of the presence of subclinical atherosclerosis. For this purpose, we adopted three models: in model 1, we introduced age, HT, smoking, DM, dyslipidemia, macroalbuminuria and eGFR. In model 2, we additionally included the serum and blood expression levels of IL6 and of the ratio TNFa/IL10. Finally, in model 3 we adjusted the analysis for the serum and PBCs expression levels of Klotho. Area under the curve (AUC) from receiver operating characteristic (ROC) analysis was performed to examine the ability of serum and PBCs expression levels of Klotho in distinguishing between patients with and without subclinical atherosclerosis and to identify the optimal cut-off values. A value of P < 0.05 was considered to be statistically significant. All analyses were performed using SPSS software version 25 (IBM Corp. Armonk, NY, USA).

Results

Characteristics of the patients and biochemical parameters. The characteristics of the study population including demographic and laboratory data are shown in Table 1. A total of 103 patients with CKD (52 males; mean age 67.3 ± 7.9 years) with a median eGFR of 38.63 (35.5–44) ml/min/1.73m2 were included. The median and interquartile range was 618.3 (492.9–785.4) pg/mL for serum Klotho concentrations and 1.9 (1.6–2.6) a.u. for Klotho mRNA levels in PBCs.

Forty-four of patients (42.7%) presented subclinical atherosclerosis: 41 patients had ABI <0.9 and 10 had CIMT ≥0.9 mm. We compared the differences between this group of patients to those without subclinical atherosclerosis. The prevalence of patients with DM (52.3 vs. 30.5%; P < 0.05), macroalbuminuria (79.5 vs. 40.7;
P < 0.001), and smokers (50 vs. 23.7%; P < 0.01) was higher in this group. No differences were observed in age, sex, BMI, HT, or dyslipidemia. Regarding laboratory data, CKD patients with subclinical atherosclerosis presented higher levels of glucose, UAE, and IL6, and reduced serum concentrations of Klotho as well as lower mRNA expression levels in PBCs. No differences were observed for the rest of inflammatory parameters according to the presence of subclinical atherosclerosis, including serum levels and expression in PBCs of TNFα and IL10, and the expression of IL6 in PBCs. Similarly, there were no differences in Klotho levels, neither soluble or PBCs expression, in subjects according to the presence of HT or dyslipidemia. Serum Klotho concentrations were lower in patients with DM and in smokers. Macroalbuminuric patients presented lower levels of both serum and PBCs expression of Klotho.

The characteristics of subjects stratified by tertiles of soluble and PBCs gene expression levels of Klotho are shown in Table 2. CKD patients with higher Klotho (tertiles 3), presented significantly higher ABI values (P < 0.001 for both Klotho determinations) and reduced CIMT (P < 0.001 for serum levels and P < 0.01 for KL expression in PBCs), which resulted in a lower prevalence of subclinical atherosclerosis (P < 0.001 for both determinations of Klotho). The prevalence of smokers and macroalbuminuric patients were lower in the higher tertiles.

### Table 1. Clinical characteristics. Biochemical assessments and gene expression analysis according to the presence of subclinical cardiovascular disease. BP blood pressure, ABI ankle-brachial index, CIMT carotid intima-media thickness, eGFR estimated glomerular filtrate rate, UAE urinary albumin excretion, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, hs-CRP high sensitivity C-reactive protein, TNFα tumor necrosis factor, IL interleukin KL Klotho gene.

| Characteristics                          | No subclinical atherosclerosis | Subclinical atherosclerosis | P value |
|------------------------------------------|-------------------------------|----------------------------|---------|
| N                                        | 59                            | 44                         |         |
| Age (years)                              | 67 ± 7.8                      | 67.8 ± 8.02                | 0.808   |
| Male (%)                                 | 30 (50.8)                     | 22 (50)                    | 0.619   |
| Systolic BP (mm Hg)                      | 140 (138–152)                 | 140 (134–148)              | 0.398   |
| Diastolic BP (mm Hg)                     | 88 (82–90)                    | 88 (82–90)                 | 0.864   |
| Body mass index (kg/m²)                  | 29 (28–31)                    | 29 (25.3–31)               | 0.941   |
| ABI                                      | 1.05 (0.97–1.10)              | 0.87 (0.83–0.89)           | < 0.001 |
| CIMT (mm)                                | 0.73 ± 0.066                  | 0.80 ± 0.096               | < 0.001 |
| Comorbidities                            |                               |                            |         |
| Diabetes mellitus (%)                    | 18 (30.5)                     | 23 (52.3)                  | < 0.05  |
| Hypertension (%)                         | 53 (89.8)                     | 42 (85.5)                  | 0.291   |
| Current smokers (%)                      | 14 (23.7)                     | 22 (50)                    | < 0.01  |
| Dyslipidemia (%)                         | 44 (74.6)                     | 33 (75)                    | 0.961   |
| Macroalbuminuria (%)                     | 24 (40.7)                     | 35 (79.5)                  | < 0.001 |
| Laboratory data                          |                               |                            |         |
| Hemoglobin (g/dl)                        | 11.8 (11.2–12.1)              | 11.9 (11.5–12.4)           | 0.203   |
| Creatinine (mg/dl)                       | 1.9 (1.38–2.8)                | 1.87 (1.54–2.39)           | 0.602   |
| eGFR (ml/min/1.73 m2)                    | 38.63 (36.1–47.8)             | 38.6 (34.4–42.4)           | 0.277   |
| UAE (mg/g)                               | 214 (132–600)                 | 979 (399–1277)             | < 0.001 |
| Albumin (g/dL)                           | 3.9 (3.5–4)                   | 3.74 (3.5–3.95)            | 0.347   |
| Total cholesterol (mg/dL)                | 179 (149–193)                 | 161 (153–201)              | 0.875   |
| HDL-C (mg/dL)                            | 42 (37–46)                    | 42 (36–46)                 | 0.625   |
| LDL-C (mg/dL)                            | 92 (80–109)                   | 103 (80.3–115.8)           | 0.259   |
| Triglycerides (mg/dL)                    | 157 ± 39.6                    | 143.1 ± 41.9               | 0.084   |
| Uric acid (mg/dL)                        | 6.63 ± 1.59                   | 6.61 ± 1.3                 | 0.942   |
| Glucose (mg/dL)                          | 79 (59–106)                   | 108 (83.3–143.8)           | < 0.05  |
| Calcium (mg/dL)                          | 9.2 (8.9–9.8)                 | 9.3 (8.9–9.8)              | 0.896   |
| Phosphorus (mg/dL)                       | 4.1 (3.8–5)                   | 4.2 (3.9–4.8)              | 0.928   |
| Klotho (pg/mL)                           | 744.92 (591.05–841.2)         | 523.96 (361–635.6)         | < 0.001 |
| hs-CRP (mg/L)                            | 5.29 (3.3–7)                  | 5.34 (3.43–7.3)            | 0.936   |
| TNFa (pg/mL)                             | 15.6 (12–17.9)                | 14.9 (11.9–17.6)           | 0.844   |
| IL6 (pg/mL)                              | 3.2 (2.4–4.9)                 | 7.1 (3.9–9.4)              | < 0.001 |
| IL10 (pg/mL)                             | 36.8 (24–50.3)                | 35 (29.4–42.4)             | 0.844   |
| KL mRNA (a.u.)                           | 2.2 (1.8–3)                   | 1.7 (1.5–2.1)              | < 0.001 |
| TNF mRNA (a.u.)                          | 1.9 (1.6–2.7)                 | 1.8 (1.29–2.3)             | 0.107   |
| IL6 mRNA (a.u.)                          | 1.8 (1.5–2.2)                 | 1.8 (1.5–2.3)              | 0.970   |
| IL10 mRNA (a.u.)                         | 1.25 (1.11–1.37)              | 1.27 (1.06–1.38)           | 0.896   |
|   | Tertile 1 < 559 pg/mL | Tertile 2 559–730 pg/mL | Tertile 3 > 730 pg/mL | P value for trend |
|---|----------------------|-------------------------|-----------------------|-------------------|
| **Characteristics** | | | | |
| N | 34 | 35 | 34 | - |
| Age (years) | 69.9 ± 6.8 | 64.9 ± 7.5 | 67.2 ± 8.6 | 0.031 |
| Male (%) | 16 (47) | 18 (51.4) | 15 (44.1) | 0.635 |
| Systolic BP (mm Hg) | 140 (134–148) | 140 (138–152) | 140 (135.8–148.5) | 0.356 |
| Diastolic BP (mm Hg) | 90 (82–90) | 88 (82–90) | 85.5 (74–90.5) | 0.32 |
| Body mass index (kg/m²) | 29 (26–31) | 28 (25–31) | 29.5 (28–31.3) | 0.292 |
| ABI | 0.89 (0.84–0.94) | 0.92 (0.87–1) | 1.06 (0.99–1.13) | < 0.001 |
| CIMT (mm) | 0.816 ± 0.092 | 0.758 ± 0.077 | 0.71 ± 0.065 | < 0.001 |
| **Comorbidities** | | | | |
| sCVD (%) | 25 (73.5) | 17 (48.6) | 2 (0.06) | < 0.001 |
| Diabetes mellitus (%) | 16 (47.1) | 15 (42.9) | 10 (29.4) | 0.371 |
| Hypertension (%) | 32 (94.1) | 34 (97.1) | 29 (85.3) | 0.218 |
| Current smokers (%) | 16 (47.1) | 14 (40.4) | 6 (17.6) | < 0.05 |
| Dyslipidaemia (%) | 25 (73.5) | 25 (71.4) | 27 (77.1) | 0.235 |
| Macroalbuminuria (%) | 25 (73.5) | 22 (62.9) | 12 (35.3) | < 0.01 |
| **Laboratory data** | | | | |
| Hemoglobin (g/dl) | 11.8 (11.3–12.1) | 12 (11.4–12.4) | 11.8 (11.3–12.3) | 0.703 |
| Creatinine (mg/dl) | 1.87 (1.49–2.61) | 1.8 (1.4–2.8) | 1.9 (1.48–2.68) | 0.542 |
| eGFR (ml/min/1.73 m²) | 36.7 (34.1–39.5) | 39.4 (35.4–43.1) | 38.62 (36.5–51.92) | 0.096 |
| UAE (mg/g) | 1007 (238–1210) | 359 (190–1240) | 180 (117.3–534.5) | < 0.001 |
| T-cholesterol (mg/dL) | 181 (155–201) | 156 (142–193) | 179 (152–190) | 0.359 |
| HDL-C (mg/dL) | 42 (38–46) | 42 (36–45) | 41.5 (34.5–46) | 0.507 |
| Triglycerides (mg/dL) | 148.6 ± 35.9 | 143.3 ± 39.2 | 161.7 ± 46.2 | 0.267 |
| Uric acid (mg/dl) | 7.1 ± 7.2 | 6.21 ± 1.49 | 6.56 ± 1.51 | < 0.05 |
| Glucose (mg/dL) | 103.5 (85.2–145) | 99 (79–140) | 83.5 (75–120) | 0.064 |
| Calcium (mg/dL) | 9.2 (8.9–9.7) | 9.3 (9–10) | 9.34 (9.8–9.8) | 0.562 |
| Phosphorus (mg/dL) | 4.3 (3.8–4.9) | 4.2 (3.8–4.9) | 4.1 (3.8–5) | 0.974 |
| hs-CRP (mg/L) | 6 (3.3–7.5) | 4.87 (3.21–6.8) | 6.23 (3.4–7.26) | 0.283 |
| TNFα (pg/mL) | 15.9 (10.9–17.3) | 15.6 (12.8–18.4) | 15.1 (11.8–18.3) | 0.764 |
| IL6 (pg/mL) | 7.1 (3.9–8.9) | 4.4 (2.5–9) | 3.1 (2.28–3.9) | < 0.001 |
| IL10 (pg/mL) | 35 (29.1–40.7) | 35.6 (25–48.3) | 38.4 (23.1–55.4) | 0.644 |
| IL6 mRNA (a.u.) | 1.9 (1.5–2.63) | 1.8 (1.4–2.3) | 1.95 (1.6–2.9) | 0.115 |
| IL10 mRNA (a.u.) | 1.9 (1.6–2.83) | 1.9 (1.5–2.3) | 1.75 (1.5–2.12) | 0.525 |
| IL10 mRNA (a.u.) | 1.29 (1.14–1.39) | 1.27 (1.06–1.39) | 1.235 (1.1–1.34) | 0.284 |
| **Characteristics** | | | | |
| N | 32 | 36 | 35 | - |
| Age (years) | 67.6 ± 7.8 | 69.5 ± 7.3 | 64.9 ± 8 | 0.064 |
| Male (%) | 14 (43.8) | 18 (50) | 20 (57.5) | 0.427 |
| Systolic BP (mm Hg) | 140 (134–148) | 140 (138–152) | 140 (136–152) | 0.451 |
| Diastolic BP (mm Hg) | 87 (82–91.5) | 89 (82–90) | 88 (82–90) | 0.912 |
| Body mass index (kg/m²) | 28.5 (26–31) | 29.5 (26.3–31) | 29.5 (26–31) | 0.894 |
| ABI | 0.89 (0.86–0.99) | 0.905 (0.873–0.967) | 1.06 (0.97–1.14) | < 0.001 |
| CIMT (mm) | 0.799 ± 0.094 | 0.777 ± 0.079 | 0.72 ± 0.073 | < 0.01 |
| **Comorbidities** | | | | |
| sCVD (%) | 20 (62.5) | 18 (50) | 6 (18.8) | < 0.001 |
| Diabetes mellitus (%) | 12 (37.5) | 16 (44.4) | 13 (37.1) | 0.319 |
| Hypertension (%) | 29 (90.6) | 34 (94.4) | 32 (91.4) | 0.223 |
| Current smokers (%) | 14 (43.8) | 18 (50) | 4 (11.4) | < 0.05 |
| Dyslipidaemia (%) | 26 (81.3) | 25 (69.4) | 26 (74.3) | 0.314 |
| Macroalbuminuria (%) | 26 (81.3) | 20 (55.6) | 13 (37.1) | < 0.01 |
| **Laboratory data** | | | | |
| Hemoglobin (g/dl) | 11.8 (11.6–12.5) | 11.8 (11.4–12.2) | 11.8 (11.2–12.1) | 0.557 |
| Creatinine (mg/dl) | 2.1 (1.5–2.6) | 1.88 (1.42–2.65) | 1.64 (1.4–2.8) | 0.742 |

Continued
*P* higher tertiles of serum (*P* was also reduced in subjects in the higher tertiles of serum Klotho (*Klotho* (*Klotho*) correlated with ABI (*r* = 0.556, < 0.0001). *P* correlated (r = 0.346, 0.0001). *P* levels (r = − 0.177, < 0.0001). *P* eGFR (r = 0.333, 0.0001) and inversely correlated with UAE (r = − 0.455, < 0.0001). We found a trend for an inverse correlation between age and serum Klotho levels (*r* = − 0.177, *P* = 0.073). Serum Klotho levels were directly and significantly correlated with eGFR (*r* = 0.219, *P* = 0.026) and inversely correlated with UAE (*r* = − 0.455, *P* < 0.0001), glucose (*r* = − 0.266, *P* = 0.007), and serum levels of IL6 (*r* = − 0.515, *P* < 0.0001). PBCs mRNA KL levels were also lower in higher tertiles of PBCs mRNA levels of TNFα (*r* = 0.199, *P* = 0.044; *r* = 0.227, *P* = 0.021, respectively), and inversely correlated with UAE (*r* = − 0.387, *P* < 0.0001), serum IL6 levels (*r* = 0.455, *P* < 0.0001).

Regarding subclinical atherosclerosis markers, both serum and PBCs mRNA Klotho levels were positively correlated with ABI (*r* = 0.556, *P* < 0.0001) and positively related with CIMT (*r* = − 0.541, *P* < 0.0001 and *r* = − 0.437, *P* < 0.0001, respectively). Among inflammatory markers, only serum IL6 levels presented significant associations with subclinical atherosclerosis, being inversely related with ABI (*r* = − 0.568, *P* < 0.0001) and positively related with CIMT (*r* = 0.558, *P* < 0.0001). PBCs expression of TNF only correlated with ABI (*r* = 0.244, *P* = 0.013) and with UAE (*r* = 0.29, *P* = 0.003).

To test the independent association between the levels of soluble and PBCs expression of Klotho and the two markers of atherosclerosis, backward stepwise multiple regression analysis was performed with ABI and CIMT as dependent variables. The results showed that serum and blood mRNA Klotho levels, together with serum IL6, were positively related and significantly associated with the values of ABI (adjusted R² = 0.537, *P* < 0.0001) and CIMT (adjusted R² = 0.37, *P* < 0.0001) (Table 3). Collinearity was assessed by examining tolerance and the variance inflation factor (VIF) for each variable in both regression analysis. Tolerance and VIF values were higher than 0.60 and lower than 1.5 for all variables in any of the analysis. Therefore, collinearity was excluded.

The multivariate logistic regression modelling results, using the presence/absence of subclinical atherosclerosis as the dependent variable, are presented in Table 4. Traditional risk factors for CVD (age, HT, smoking, DM, and dyslipidemia) were entered as covariates (model 1), with additional models in which markers of renal function (eGFR and macroalbuminuria) (model 2), and inflammatory cytokines (model 3) were added. Results of these analyses showed that both serum and PBCs mRNA levels of Klotho were covariates associated with subclinical atherosclerosis, indicating that the levels of both variables are protective factors for the presence of

### Table 2. Clinical characteristics and general biochemical assessments stratified by tertiles of serum Klotho (pg/mL) (A) or PBCs KL mRNA (a.u.) (B) levels. BP blood pressure, ABI ankle-brachial index, CIMT carotid intima-media thickness, sCVD subclinical cardiovascular disease, eGFR estimated glomerular filtrate rate, UAE urinary albumin excretion, T-cholesterol total cholesterol, HDL-C high-density lipoprotein cholesterol, hs-CRP high sensitivity C-reactive protein, TNFα tumor necrosis factor, IL interleukin.

| B                   | Tertile 1 <1.7 a.u | Tertile 2 1.7–2.3 a.u | Tertile 3 >2.3 a.u | P value for trend |
|---------------------|-------------------|----------------------|-------------------|------------------|
| eGFR (ml/min/1.73 m²) | 36.7 (34.3–41)    | 38.6 (35–39.6)       | 42.3 (38–54)      | < 0.001          |
| UAE (mg/g)          | 800 (323–1185)    | 395 (180.5–1200)     | 214 (120–450)     | < 0.01           |
| Albumin (g/dL)      | 3.7 (3.5–3.9)     | 3.8 (3.6–4)          | 3.9 (3.4–4)       | 0.212            |
| T-cholesterol (mg/dL) | 179 (155–197)     | 165 (146.5–184)      | 180 (155–196)     | 0.518            |
| HDL-C (mg/dL)       | 41.5 (35–42)      | 42 (39–46)           | 42 (37–46)        | 0.177            |
| Triglycerides (mg/dL) | 150.4 ± 30.6      | 151 (120.3–184.3)    | 145 (124–180)     | 0.88             |
| Uric acid (mg/dL)   | 6.5 ± 1.4         | 6.74 ± 1.6           | 6.57 ± 1.39       | 0.778            |
| Glucose (mg/dL)     | 92.5 (79.5–127)   | 96 (81–140.8)        | 87 (77–138)       | 0.613            |
| Calcium (mg/dL)     | 9.1 (8.8–9.6)     | 9.2 (9–9.8)          | 9.4 (9–9.8)       | 0.293            |
| Phosphorus (mg/dL)  | 4.3 (3.9–5)       | 4.2 (3.9–4.9)        | 7 (6.6–7.4)       | 0.244            |
| hs-CRP (mg/L)       | 5.75 (3.92–7.32)  | 5.12 (3–7.3)         | 5.3 (3.2–7.1)     | 0.9              |
| TNFa (pg/mL)        | 13.1 (10.9–17.7)  | 15.2 (12.7–16.3)     | 16.4 (12–19.7)    | 0.119            |
| IL6 (pg/mL)         | 6.4 (3.6–9.3)     | 4.8 (3.43–7.78)      | 2.9 (2.4–3.9)     | < 0.001          |
| IL10 (pg/mL)        | 30.9 (23.2–42.3)  | 38.2 (33–46.4)       | 36.1 (24.7–54)    | 0.115            |
| TNFα mRNA (a.u.)    | 1.8 (1.2–2.6)     | 1.8 (1.5–2.1)        | 2.1 (1.8–3)       | < 0.05           |
| IL6 mRNA (a.u.)     | 1.95 (1.5–2.78)   | 1.8 (1.6–2.2)        | 1.8 (1.5–2.2)     | 0.339            |
| IL10 mRNA (a.u.)    | 1.26 (1.2–1.4)    | 1.3 (1.1–1.4)        | 1.25 (1.14–1.39)  | 0.802            |
this condition in CKD patients in stage 3–4 (Table 4 and Fig. 1). The results were similar when analyzing these variables separately.

ROC curve analysis. The ROC curve analysis was used to determine the ability of the serum and PBCs gene expression levels of Klotho to distinguish between subjects according to the presence of subclinical atherosclerosis (Fig. 2). AUC for serum and PBCs expression levels of Klotho were 0.817 (95% CI: 0.736–0.898, P < 0.001) and 0.742 (95% CI: 0.647–0.836, P < 0.001), respectively. The optimal cut-off values for subclinical atherosclerosis were 553.04 pg/ml (specificity 56.8% and sensitivity 88.1%) for serum Klotho, and 2.05 a.u. (specificity 75% and sensitivity 62.7%) for mRNA KL expression in PBCs.

Discussion
In this study, we show that CKD patients with subclinical atherosclerosis present reduced levels of serum and mRNA expression in PBCs of Klotho as compared with CKD patients without this clinical status. In addition, both Klotho variables are significantly associated with two markers of subclinical atherosclerosis, ABI and CIMT, independently of cardiovascular risk factors and inflammatory parameters. Specifically, both determinations directly correlated with ABI, whereas presented an inverse association with CIMT. Moreover, in multivariate analyses, both Klotho variables were found to be independent determinants of ABI and CIMT, and independent predictors of subclinical atherosclerosis. Whether reduction in serum and PBCs gene expression levels of Klotho directly promote or favor the progression of atherosclerosis in CKD is an intriguing question that requires further study.

Atherosclerosis is a major complication in CKD patients that dramatically increase CVD morbidity and mortality. In this study, we employed two widely used methods for assessing the presence of subclinical atherosclerosis: ABI and CIMT. ABI is a quick, noninvasive way to check peripheral artery disease, being considered as an indirect indicator of general atherosclerosis and an independent predictor of cardiovascular and all-cause mortality in CKD and hemodialysis (HD) patients. CIMT, an ultrasound based quantitative parameter widely used as a direct marker of atherosclerotic disease. We have chosen CIMT in this study to determine subclinical atherosclerotic disease due to its independent association with increased cardiovascular risk and impaired renal function, being able to predict ischemic events and long-term mortality in patients with different stages of CKD, and in predialysis and HD patients. Our results pointed to Klotho as an independent determinant of ABI and CIMT, even after adjusting for age, gender, blood pressure, smoking, DM, dyslipidemia, and other factors. Additionally, both Klotho variables in our study were significant predictors of subclinical atherosclerosis

### Table 3. Multiple backward stepwise regression analysis for ABI and CIMT as dependent variables. ABI ankle-brachial index, CIMT carotid intima-media thickness, PBCs peripheral blood cells, IL interleukin, KL Klotho gene.

| | Adjusted R² | β | Standard error | t | P |
|---|---|---|---|---|---|
| ABI | 0.537 | | | | |
| Current smokers | −0.206 | 0.019 | −2.375 | 0.0075 | |
| Diabetes mellitus | −0.163 | 0.017 | −2.342 | 0.0212 | |
| Serum IL6 (pg/mL) | −0.288 | 0.003 | −3.454 | 0.00083 | |
| Serum Klotho (pg/mL) | 0.236 | 0.000052 | 2.841 | 0.0055 | |
| PBCs KL mRNA (a.u.) | 0.238 | 0.013 | 2.963 | 0.0037 | |

### Table 4. Multivariate logistic regression analysis for the presence of subclinical atherosclerosis with serum Klotho and KL PBCs mRNA levels as independent variables. OR values are expressed per unit variation. Model 1 was adjusted by age, hypertension, current smokers, diabetes mellitus, hyperlipidemia. Model 2 was Model 1 adjusted by macroalbuminuria and eGFR. Model 3 was Model 2 adjusted by serum levels of IL6 and TNFα/IL10 and PBCs mRNA levels of IL6 and TNF/IL10. eGFR estimated glomerular filtrate rate, IL interleukin, TNFα tumor necrosis factor.

| | Unadjusted Model 1 | Model 1 | Unadjusted Model 2 | Model 2 | Unadjusted Model 3 | Model 3 |
|---|---|---|---|---|---|---|
| Serum Klotho (pg/mL) | 0.993 (0.99–0.996) | <0.001 | 0.992 (0.988–0.996) | <0.001 | 0.993 (0.989–0.998) | 0.003 |
| PBCs KL mRNA (a.u.) | 0.259 (0.12–0.707) | 0.006 | 0.24 (0.086–0.673) | 0.007 | 0.218 (0.069–0.694) | 0.01 |

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in a full adjusted model that included important cofounders such as age, HT, smoking, eGFR, DM, dyslipidemia, macroalbuminuria, and the serum and PBCs expression levels of the cytokines IL6, TNFα, and IL10.

Previous clinical studies have suggested that Klotho might play a role in the pathogenesis of atherosclerotic disease. Genetic variants of Klotho have been associated with high CIMT in hypertensive patients32 and with the risk of early-onset occult coronary artery disease35. Low soluble Klotho levels have been related to CVD in cross-sectional clinical studies carried out in elderly subjects (> 65 years old)22, in subjects with preserved renal function subjected to angiography20, and in diabetic patients21. Similar results have been observed in patients in HD, where diminished levels of circulating Klotho were related to cardiovascular events and mortality34. However, studies about the relationship between Klotho and markers of subclinical atherosclerosis in CKD patients are scarce and none of them have considered the expression of Klotho in PBCs. A recent cross-sectional study suggested that decreased soluble Klotho levels in 114 CKD patients were associated with signs of arterial stiffness, determined by increased brachial-ankle pulse wave velocity (baPWV) (adjusted OR = 0.60, 95% CI = 0.39–0.98, P = 0.0075)23. Similarly, a prospective study carried out in 63 patients with CKD showed that patients with serum soluble Klotho levels in the lower quartile (< 309 pg/mL) had significantly higher cardiovascular and all-cause mortality rates (hazard ratio = 4.17, 95% CI = 1.29–13.48, P = 0.018)35.

Experimental studies point to the existence of a role of Klotho in the maintenance of the vascular health. Klotho-deficient mice show increased vascular endothelial permeability, impaired endothelial-dependent vasodilatation, reduced excretion of nitric oxide metabolites, and impaired angiogenesis and vasculogenesis3–7,16,17,36,37. These dysfunctions were recovered after parabiosis with wild-type mice16. In that study, serum Klotho stimulated endothelium-derived NO production, which ameliorated endothelial dysfunction and prevented vascular remodeling16. This protective effect on vascular cells was also observed in vitro, where Klotho was able to protect endothelial and vascular smooth muscle cells from inflammation and oxidation, critical factors in the progression of atherosclerosis38–41. Klotho attenuated cellular apoptosis and senescence in human umbilical vein endothelial cells (HUVECS) via mitogen-activated kinase and extracellular signal-regulated kinase pathways39. In rat aortic smooth muscle cells, Klotho gene transfer downregulated Nox2 protein expression and intracellular superoxide production, and attenuated angiotensin II-induced superoxide production, reducing oxidative damage and apoptosis41. Finally, Klotho may have a role in the modulation of endothelial inflammation by reducing TNFα-induced expression of adhesion molecules and NF-κB activation39 and by inhibiting retinoic-acid-inducible gene-1 induced expression of IL6 and IL8 in HUVECS40.

Results of our study are in line with these previous findings and extends the association between Klotho and CVD to the reduced expression of Klotho in PBCs. Macrophages, monocytes, lymphocytes, and other PBCs participate in the immune response with a central role in the development of the inflammatory response associated with the atherogenic process. This role ranges from a contribution to the low-grade systemic inflammation that accompanies cardiovascular disease (secretion of pro- or anti-inflammatory factors into the systemic circulation) to the resolution of the local response of the vascular wall (environmental signal transduction, uptake of LDL or oxLDL particles, engulfment of dead cells, secretion of inflammatory cytokines or pro-resolving molecules, etc.)42,43. Klotho has been previously detected in PBCs and marked reductions in Klotho expression in PBCs have been related with aging and with the development of pathologies with an inflammatory component including atherosclerosis44,45. Although there are few studies that delve into the mechanisms of action, the up-regulation

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**Table: Multivariate odds-ratio (95% CI) for subclinical atherosclerosis**

| Factor                        | OR (95% CI)   | P-value |
|-------------------------------|---------------|---------|
| Age (years)                   | 0.930 (0.851-1.017) | 0.110   |
| Hypertension                  | 1.569 (0.146-16.877) | 0.710   |
| Current smokers               | 1.721 (0.472-6.281) | 0.411   |
| Diabetes mellitus             | 2.129 (0.635-7.133) | 0.221   |
| Hyperlipidaemia               | 1.330 (0.354-5.002) | 0.673   |
| Macroalbuminuria              | 2.332 (0.670-8.123) | 0.184   |
| eGFR (mL/min/1.73m²)          | 1.062 (0.976-1.156) | 0.162   |
| IL6 (pg/mL)                   | 1.135 (0.917-1.404) | 0.244   |
| Ratio TNFα/IL10 (pg/mL)       | 1.517 (0.133-17.279) | 0.737   |
| IL6 mRNA (a.u.)               | 0.596 (0.234-1.514) | 0.276   |
| Ratio TNF/IL10 mRNA (a.u.)    | 0.723 (0.289-1.810) | 0.489   |
| Serum Klotho (pg/mL)          | 0.993 (0.988-0.998) | 0.003   |
| PBCs Klotho mRNA (a.u.)       | 0.260 (0.076-0.892) | 0.032   |

**Figure 1.** Multivariate odds ratio for subclinical atherosclerosis displayed as the odds ratio (OR) with 95% confidence intervals (CIs).
of Klotho in these cells could be beneficial in antagonizing the progression of atherosclerotic lesions through anti-inflammatory effects. In this sense, the expression of Klotho in these cells has been related with the attenuation of lipopolysaccharide (LPS)-induced acute inflammation in macrophages and with the inactivation of NF-kB signaling and the promotion of M2 polarization in these cells, which infiltrates atheromatous plaques and develop a defensive and repair response to vascular damage. Moreover, it has also been related with the suppression of the stress response of the Golgi apparatus and endoplasmic reticulum, the reduction of the levels of oxidant radicals and pro-inflammatory cytokines, as well as with an increase in the production of anti-inflammatory cytokines and the preservation of the immune function in senescent monocytes. All these mechanisms play key roles in the inflammatory response exerted by PBCs in the atherosclerotic process and, therefore, make Klotho expression in these cells an interesting target in such scenario. The presence of Klotho in these cells could be considered, albeit indirectly, as a possible actor involved in the preservation of vascular function.

Although our study provides novel information about the relationship between Klotho and subclinical atherosclerosis in CKD patients, we acknowledge several limitations. First, serum concentrations of vitamin D, fibroblast growth factor-23, and parathyroid hormone factors related to Klotho and calcium phosphate metabolism, with potential impact on atherosclerosis, were not measured in our study, and therefore a possible influence on the relationship between Klotho and CVD cannot be completely ruled out. Moreover, protein expression levels of Klotho in PBCs were not assessed in these patients and mRNA variations in Kl expression may not reflect substantial modifications in protein levels. Second, the sample size was relatively small, which does not allow generalization of the results. Thirdly, although we accounted for confounding of traditional and CKD-related cardiovascular risk factors, a potential for uncontrolled or residual confounding that could affect our results could be plausible. Finally, given the cross-sectional design of the study, we can only demonstrate associations without definitive inferences on their direction or causality. Our observations only show a relationship with an already developed clinical scenario and they do not provide information about the independent implication of Klotho levels variations in the development and progression of the atherosclerotic lesion.

Nevertheless, our study presents some strengths that deserve to be highlighted: it was a population-based sample of adults; the presence and severity of subclinical atherosclerosis were carefully analyzed by two different markers of atherosclerosis; and data included conventional and CKD-related cardiovascular risk factors, including the serum levels and PBCs expression of inflammatory parameters.

Figure 2. ROC curves of serum and mRNA PBCs levels of Klotho for distinguishing subclinical atherosclerosis.
Conclusions

In conclusion, in the present study we determined the association between serum concentrations and PBCs Klotho expression levels with markers of subclinical atherosclerosis in CKD patients, while simultaneously explored the possible confounding effects caused by CKD-MBD and inflammation-related risk factors. Further experimental and clinical studies are warranted to confirm our findings, to explore the effects and relationships of Klotho on the cardiovascular system, to evaluate the role of Klotho as a potential novel biomarker of CVD, and to assess the effect of therapeutic strategies directed to increase Klotho levels on atherosclerotic disease in CKD.

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Author contributions
J.F.N.-G. designed the study; J.D.C., C.F., E.M.N., N.P.G., and A.G.L. carried out the experiments; C.M.F, J.D.C., and J.F.N.G. analyzed the data and drafted and revised the paper. All authors approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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