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Full Title of the Article： The association of the serum Klotho with the severity and mortality among adults with Cardiovascular-Kidney-Metabolic Syndrome

Running Title： Association of serum Klotho with the severity and mortality in CKM syndrome

Authors

Jiao Tang [1#], Zhehao Xu [2#], Li Ren[1], Jiahua Xu[1], Xin chen [1], Yian Jin[2], Ruiyun Liang [3], Huanji Zhang[1*],

[1] Department of Cardiovascular Medicine, The Eighth Affiliated Hospital of Sun Yat-sen University, China

[2] Department of General Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, China

[3] Department of Respiratory Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, China

[#] Co-first authors

[*]Corresponding author

Corresponding Author： Huanji Zhang, zhanghj65@mail.sysu.edu.cn

Highlights:

- This study is the first to reveal the relationship between serum Klotho levels and the severity and mortality of CKM syndrome.
- Serum Klotho is significantly associated with the severity of CKM syndrome
- Serum Klotho was U-shaped related to all-cause mortality in patients with CKM syndrome.
- Serum Klotho was L-shaped associated with cardiovascular mortality in patients with CKM syndrome stage 0-3 and linearly associated with cardiovascular mortality in patients with CKM syndrome stage 4.
- Low serum Klotho level is an independent risk factor for the progression of CKM syndrome as well as all-cause and cardiovascular mortality.

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Abstract

Background: Cardiovascular-kidney-metabolic (CKM) syndrome is characterized as a systemic disease resulting from the pathophysiological interplay among metabolic risk factors, chronic kidney disease (CKD), and cardiovascular disease (CVD). The Klotho protein may serve as a novel biomarker for age-related diseases, including hypertension, heart failure, diabetes, and cardiovascular mortality. However, the utility of serum Klotho levels as an indicator of severity and mortality risk in CKM syndrome remains uncertain.↵

Methods: This study involved 9,871 participants from the National Health and Nutrition Examination Survey (NHANES) conducted between 2007 and 2016. Serum Klotho levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit. CKM syndrome is characterized by the coexistence of subclinical or clinical cardiovascular disease, chronic kidney disease (CKD), and metabolic disorders. The optimal cutoff value was established through the maximum Youden's index. Multivariate weighted regression models were employed to calculate the odds ratio (OR) and hazard ratio (HR), along with the 95% confidence interval (95% CI), to evaluate the association between serum Klotho levels and the severity of CKM syndrome, as well as all-cause and cardiovascular mortality. Additionally, the receiver operating characteristic curve (ROC) and restricted cubic spline (RCS) curves were utilized to assess predictive efficacy and explore nonlinear relationships.↵

Results: Serum Klotho levels were significantly associated with the severity of CKM syndrome (OR=0.90; 95% CI, 0.86-0.93, $P < 0.001$). The relationship between serum Klotho levels and all-cause mortality was U-shaped, while the relationship with cardiovascular mortality was L-shaped. Specifically, low serum Klotho levels were associated with an increase in all-cause mortality by 21% and cardiovascular mortality by 76% among patients with CKM syndrome. Furthermore, serum Klotho levels demonstrated excellent predictive efficacy for both the severity and mortality associated with CKM syndrome.↵

Conclusions: This study is the first to elucidate the relationship between serum Klotho levels and the severity and mortality associated with CKM syndrome. This study indicates that low serum Klotho levels serve as reliable indicators of both the severity of CKM syndrome and the associated risk of mortality.↵

Keywords: Cardiovascular-Kidney-Metabolic syndrome, Metabolic Syndrome, Klothos, NHANES

Introduction

The intricate relationship has been highlighted by numerous observational studies and epidemiological data among metabolic syndrome (MS), chronic kidney disease (CKD), and cardiovascular disease (CVD)[1-4]. This triad frequently co-occurs in CVD patients, who often present with CKD and type 2 diabetes mellitus (T2DM). Furthermore, individuals with CKD and/or T2DM demonstrate worsened cardiovascular prognoses[4]. As understanding of the relevant pathophysiological mechanisms deepens, the connections among these conditions become increasingly clear. The American Heart Association emphasized the significant interactions by releasing a scientific statement in October 2023[5].This statement delineates a systemic condition marked by intricate relationships among a spectrum of metabolic risk factors, CVD and CKD. These interrelated components contribute significantly to the overall health status of individuals affected by the syndrome. The syndrome is stratified into four progressive stages, which ranges from stage 0 to 4, serving to illustrate the potential for worsening multiorgan dysfunction over time. With the advancement of stages, there is a correlated escalation in the likelihood of encountering unfavorable cardiovascular outcomes. This classification system is crucial for understanding the severity of the syndrome and for guiding appropriate clinical interventions[5, 6].

Klotho is a critical factor in mammalian aging and encodes a protein that exists in multiple isoforms, each possessing distinct functions[7]. Currently, three unique Klotho isoforms have been recognized: the complete transmembrane form, the shortened soluble form, and the released variant[8]. The intact form of Klotho serves a vital function as a co-receptor for fibroblast growth factor 23 (FGF23). This interaction is essential for maintaining the balance of phosphate levels in the body[8]. The circulating soluble and secreted forms of Klotho, collectively referred to as α-Klotho, are thought to exert endocrine or paracrine effects on various organ systems, including the kidneys, bones, brain, cardiovascular system, lungs, and vascular endothelium[9-13]. A decline in Klotho levels is generally associated with the aging process[7]. Recent retrospective studies suggest that Klotho could emerge as a new biomarker for several age-related diseases. These conditions encompass a spectrum of health issues, including heart failure, hypertension, diabetes, and cardiovascular mortality[14]. The identification of Klotho as a biomarker could facilitate early detection and intervention for these ailments,

ultimately contributing to better management and improved outcomes for affected individuals.

Given the rising incidence of CKM syndrome and its significant impact on CVD progression, assessing the predictive value of serum Klotho levels is of paramount importance. This assessment is vital for predicting the severity of the condition and the associated mortality risk in patients with CKM syndrome, thereby facilitating more effective risk stratification and timely interventions.

Methods

The study used data from the National Health and Nutrition Examination Survey (NHANES) spanning five cycles from 2007 to 2016. NHANES allows for a thorough analysis of various health indicators and nutritional trends within this demographic, providing valuable insights into the prevalence of different health conditions and the dietary patterns that may influence them[15, 16]. The board's endorsement also serves as a guarantee that the research methods are scientifically sound and that the study's objectives align with ethical principles, further reinforcing the credibility and integrity of the research endeavor[17].

Study Population

The analysis excluded 37,197 participants who lacked sufficient information on Klotho, 2,641 participants under the age of 20, 547 individuals with missing values for CKM indicators, and 709 individuals with missing covariate and mortality data. Consequently, the final sample comprised 9,871 participants with complete data (Fig. S1).

Klotho concentrations assessment

This investigation focused on quantifying Klotho protein levels in serum samples, which were meticulously stored at minus 80 degrees Celsius at the Centers of Disease Control and Prevention. Following proper sample preservation, the specimens were transported on dry ice to Laboratories at the University of Washington in Seattle, for a comprehensive analysis conducted between 2019 and 2020[7]. The Klotho levels were ascertained utilizing an ELISA kit procured from IBL International in Japan. Data obtained from the ELISA assays were systematically entered into the Oracle management system, ensuring a high level of data integrity. Subsequent verification of the data was performed by a designated regional supervisor, further validating the precision and reliability of our findings[8, 18]. Each serum sample was subjected to duplicate ELISA measurements to ensure the robustness of our results. The mean values of these measurements were calculated in strict accordance with the manufacturer's instructions.

Any discrepancies noted between the two measurements that exceeded 10% warranted a reanalysis of the sample in question. The laboratory procedures, including quality assurance and monitoring for Klotho concentration analysis, adhered to the NHANES guidelines[19].

CKM Syndrome assessment

CKM syndrome is defined by the simultaneous occurrence of subclinical or clinical CVD, CKD and MS, with specific definitions outlined in Table S2[6, 20, 21]. Clinical CVD refers to the presence of overt, diagnosable heart and vascular diseases, which can be confirmed through medical history, physical examination, or diagnostic tests. Clinical CVD encompasses a background of myocardial infarction, stroke, chronic heart failure, or coronary heart disease[22]. Subclinical CVD, identified by a 10-year CVD risk of $\geqslant 20\%$ or the presence of high-risk CKD, on the other hand, refers to early signs of heart and blood vessel disease that are not apparent through symptoms or routine physical examination (Table S1). It can only be detected through specific tests or measurements. To assess eGFR, this study utilized 2021 Chronic Kidney Disease Epidemiology Collaboration creatinine equations, which do not account for race or ethnicity (Fig. S2). The 10-year CVD risk was estimated through a simplified CKM risk algorithm that incorporated factors such as age, gender, cholesterol levels, diabetes status, blood pressure, kidney function, and the use of antihypertensive medications and statins (as detailed in Table S1). Metabolic disorders include overweight/obesity, abdominal obesity, prediabetes, diabetes, hypertension, dyslipidemia, and metabolic syndrome[23, 24]. aa

To account for the varying clinical severity of CKM syndrome, participants were classified into four distinct CKM stages[25]. Stage 0 indicated the absence of abnormalities, while stage 1 was characterized by obesity or prediabetes alone. Stage 2 included individuals with at least one additional metabolic disorder or CKD. Stage 3 was characterized by the existence of subclinical CVD alongside metabolic disorders or CKD. Finally, stage 4 represented clinical CVD in conjunction with metabolic disorders or CKD. The specific criteria for each stage are detailed in Table S2 [26].

Assessments of Other Covariates

The study investigated various covariates including race, age, gender, BMI, education level, PIR, smoking and alcohol consumption to evaluate their potential impact on obscuring the relationship between serum Klotho levels and symptoms of CKM syndrome. In this study, we categorized educational achievement into three distinct levels: those who did not complete primary education (below 9th grade), those with some high school education (9th to 11th grade), and those who pursued education beyond high school (post-secondary education). Alcohol intake was divided into two categories: those who do not consume alcohol and those who do.

Meanwhile, smoking habits were classified as individuals who have never smoked and those who are current smokers. Within the scope of this study, hypertension was defined by SBP measurements averaging 140 mmHg or greater, and DBP readings averaging 90 mmHg or greater. This definition was applied alongside either a confirmed medical diagnosis or documented use of antihypertensive medications. For diagnosis of diabetes mellitus, this study considered glucose levels of 7.0 mmol/L or above, levels of glycohemoglobin > 6.5%, the presence of antidiabetic medications or insulin use, or a diagnosis recorded by a healthcare provider. These parameters align with standard clinical practices for identifying these conditions [27].

Statistical Analysis

Conducting this study in alignment with the analytical protocols of the 2007-2016 NHANES, we utilized strata, primary sampling units, and sample weights to adeptly navigate intricacies of the survey's design. Continuous variables describing the population were reported as means (± SE) or as percentages. Subjects were stratified into two categories according to their Klotho concentrations, using the group with superior Klotho levels as the comparative reference. This categorization was established through a rigorous ROC analysis to ensure its clinical significance.

This study utilized multiple linear regression analyses, adjusted for various covariates, to investigate the connection between serum Klotho and CKM syndrome stages ranging from 0 to 4. Additionally, adjusted for various covariates, multivariable Cox proportional hazards models were employed to ascertain the correlation between Klotho and the risks of all--cause and CVD mortality in participants with CKM syndrome at stages 0-3 and stage 4. We investigated the correlation between Klotho levels and the risk of all-cause and CVD mortality using restricted cubic splines to delineate the dose-response association across the CKM syndrome stages ranging from 0 to 4. Additionally, we compared the dose-response relationship between stages 0-3 and stage 4.

To enhance the robustness of the findings, this study conducted supplementary analyses, including subgroup analyses, to address demographic differences between individuals with varying Klotho levels and those with CKM syndrome. This study, utilizing ROC curve analysis , evaluated the predictive power of Klotho levels for CKM syndrome stages 0 to 4 with all-cause and CVD mortality. The values of AUC were determined to signify the predictive accuracy of these models. Data analysis was conducted utilizing R software, specifically version 4.2.3.

Results

Characteristics of participation

The basic features of participants, categorized according to the CKM syndrome group, are detailed in Table 1. This study included 18,373 participants with the average age of 57.47 years old (±10.87). Within this group, 50.58% (n=4,993) were women, and 16.64% (n=1,643) had diabetes. Importantly, notable differences were found among the CKM groups regarding sex, age, BMI, race, alcohol consumption, smoking status, educational attainment, diabetes prevalence, hypertension, physical activity, and the distribution of the PIR.

Correlation of Klotho and CKM syndrome

The findings indicated that for each SD increase in Klotho levels, there was a significantly reduced likelihood of presenting with CKM syndrome, reflected by an OR of 0.90 ($^{95\%\ CI}$, 0.86-0.93, $P < 0.001$). Moreover, the optimal cutoff of Klotho was determined through a rigorous ROC analysis aimed at maximizing the Youden index, establishing a threshold at 722 (Table S3). When using the higher Klotho group as a reference, the lower Klotho group demonstrated a significantly elevated odds ratio for CKM syndrome, reporting the OR of 1.20 (95% CI, 1.11-1.30, $P < 0.001$). This reveals that those with Klotho levels falling below the threshold of 722 are more likely to develop cardiorenal metabolic syndrome, underscoring the probability of using Klotho as a biomarker for evaluating risk.

Regression analysis of Klotho and all-cause as well as cardiovascular mortality

Table 3 delineates this study's findings on the relationship between Klotho and rates of mortality attributable to all causes and cardiovascular diseases. This table demonstrate the correlation between Klotho concentrations and likelihood of all-cause and CVD mortality across stages 0-3 and stage 4 of CKM syndrome. The study demonstrates a significant inverse correlation between increased Klotho levels and the risk of all-cause mortality (HR= 0.94; 95% CI, 0.89-1.00, $P = 0.044$) and CVD mortality (^{HR} = 0.65; $^{95\%\ CI}$, 0.57-0.75, $P < 0.001$). Designating the group with elevated Klotho levels as the reference, individuals with decreased Klotho levels displayed a heightened risk for all-cause mortality (^{HR}= 1.21; $^{95\%\ CI}$, 1.08-1.36, $P < 0.001$) and mortality due to cardiovascular causes (^{HR} = 1.76; $^{95\%\ CI}$, 1.41-2.19, $P < 0.001$). These findings highlight the protective function of Klotho against mortality risks,

particularly concerning cardiovascular health, and imply that lower Klotho levels may act as predictive markers for adverse outcomes in CKM syndrome.

Nonlinear relationships of Klotho with all-cause and CVD mortality

Figure 1 illustrates the results of the RCS analysis, which investigates the nonlinear connection between the levels of Klotho and the risks associated with all-cause and cardiovascular mortality among individuals with CKM syndrome stages 0-3 and stage 4. Following adjustments for all relevant covariates in the comprehensive analytical model 3, the findings revealed that Klotho levels demonstrated a nonlinear relationship with both all-cause mortality and CVD mortality across the CKM syndrome stages (P nonlinear < 0.05). Notably, a U-shaped relationship between serum Klotho levels and the risk of all-cause mortality has been identified, suggesting that both extremely high and low Klotho levels are associated with an increased likelihood of all-cause mortality. Conversely, patients at CKM syndrome stages 0-3 exhibited an L-shaped correlation with cardiovascular mortality, whereas those at stage 4 demonstrated a linear correlation. Within this framework, elevated Klotho levels were correlated with a lower likelihood of cardiovascular mortality.

The predictive performance of the Klotho for CKM syndrome, all-cause mortality, and cardiovascular mortality

Figure 2 and Table S4 present the ROC curves from the analysis. The fully adjusted model for CKM syndrome yielded an AUC of 0.748, with a 95% CI spanning 0.733 to 0.763. This AUC reflects a sensitivity of 0.663, suggesting that the model correctly identifies approximately 66.3% of those with CKM syndrome, while a specificity of 0.710 indicates that about 71.0% of individuals without the syndrome are also accurately recognized by the model. In the analysis of all-cause mortality, the AUC was determined to be 0.769, accompanied by a 95% CI of 0.755 to 0.783. This AUC indicates a model sensitivity of 0.679 and a specificity of 0.733. For cardiovascular mortality, the AUC was found to be 0.741, with a 95% CI from 0.715 to 0.767, corresponding to a sensitivity of 0.722 and a specificity of 0.667.

Stratification connection of Klotho for all-cause and CVD mortality

Figures S5-6 present a detailed stratified analysis that examines the correlation between Klotho levels and the risk of mortality from all causes and cardiovascular disease (CVD), with subgroups based on gender, smoking status, alcohol intake, hypertension, and diabetes. The

results from this analysis did not reveal any significant interactions between Klotho levels and the subgroups that were investigated. This suggests that the association between Klotho levels and mortality risk might be consistent across the various subgroups studied (*P* for interaction > 0.05).

Discussion

This research pioneers an investigation into the correlation between Klotho levels and the severity and mortality associated with CKM syndrome. Involving 9,871 adults, the research demonstrated the significant association between serum Klotho and the severity for CKM syndrome. This study reveals that decreased Klotho levels are independently associated with an increased risk of CKM syndrome. Furthermore, the results suggest that lower Klotho levels may elevate all-cause mortality by 21% and CVD mortality by 76% in individuals diagnosed with CKM syndrome.

Klotho, originally discovered by Kuro-o and colleagues in 1997, plays a crucial role in modulating various age-related phenotypes in mice[28]. Mice with impaired expression of the Klotho gene exhibit a syndrome resembling accelerated aging, characterized by a shortened lifespan, sterility, atherosclerosis, osteoporosis, skin atrophy, and emphysema[28]. Conversely, studies have indicated that when Klotho is overexpressed in mice, there is a corresponding extension of their lifespan, highlighting the gene's potential impact on longevity[29]. The Klotho gene gives rise to a transmembrane protein, predominantly found in the kidney. This protein comprises a sizable extracellular domain, a transmembrane region, and a brief intracellular segment, playing pivotal roles in renal function and mineral metabolism[8, 30]. The transmembrane Klotho interacts with the fibroblast growth factor receptor (FGFR) to form a complex that enhances its binding affinity for FGF23, thereby regulating phosphate excretion and the production of active vitamin D in the kidney[8, 19]. Additionally, the transmembrane Klotho is cleaved by membrane-bound proteases, such as α- and β-secretase, resulting in the release of the entire extracellular domain, which circulates as soluble Klotho. This soluble form exerts a wide range of effects on tissue and cellular functions, even in the absence of Klotho expression[8, 30].

CKM syndrome is a systemic condition marked by intricate pathophysiological mechanisms that encompass a range 4 metabolic risk factors, CKD and CVD[5]. Previous studies have demonstrated that Klotho influences numerous metabolic pathways that are crucial for both the pathogenesis and prevention of CVD[31]. These pathways include the inhibition of lipid peroxidation and inflammation[32], the prevention of cardiac fibrosis development[33], the protection against endothelial damage and vascular calcification, and the reduction of vascular

stiffness[13]. Importantly, expression levels of Klotho mRNA have been found to be markedly reduced in the kidneys of individuals with CKD[34]. In the early stages of CKD, specifically at stages ⩽2, there is a notable decrease in both serum and urinary Klotho levels. This initial decline is then followed by a subsequent increase in serum levels of FGF23 [35]. The extent of the reduction in urinary Klotho is positively correlated with the severity of eGFR decline in CKD patients[35, 36]. Elevated circulating FGF23 has been independently associated with adverse renal and cardiovascular outcomes[35, 37, 38]. The interplay between Klotho and FGF23 is integral to the pathogenesis of CKD and CVD[35, 39]. Accordingly, it becomes imperative to explore the intricate interplay between Klotho levels and the associated risk of progression and mortality within CKM syndrome.

Furthermore, this study employed RCS curve analysis to reveal a U-shaped link between Klotho levels and all-cause mortality, which reveals that both excessively low and high Klotho levels are correlate with an unfavorable prognosis. This finding is consistent with a prior prospective study that identified a similar U-shaped correlation in patients with rheumatoid arthritis (RA), underscoring the necessity of maintaining serum Klotho within an optimal range to potentially mitigate early mortality[40]. Additionally, the RCS curves indicated an L-shaped association between Klotho and cardiovascular mortality experiencing CKM syndrome stages 0-3, while a linear association was observed for CKM syndrome stage 4. This implies that reduced Klotho levels significantly elevate risk of CVD mortality. Therefore, serum Klotho could act as a vital biomarker for forecasting disease progression and mortality in individuals with CKM syndrome. Elevating serum Klotho levels could help reduce cardiovascular mortality in individuals with stages 0-3, while both increasing serum Klotho and maintaining it within a specific range may represent a novel therapeutic strategy for the stage 4 population.

Addressed

This study is the first to investigate the relationship between serum Klotho levels and the severity and mortality of CKM syndrome. It underscores the potential of serum Klotho as a biomarker for predicting both the severity and mortality associated with CKM syndrome. Notably, among individuals with CKM syndrome stage 4, serum Klotho levels demonstrate a linear correlation with cardiovascular mortality, suggesting its viability as a therapeutic target. ↵

This study has several limitations. Firstly, retrospective studies inherently cannot eliminate the possibility of bias, and cross-sectional designs do not allow for causal inferences. Secondly, the study's focus on European populations limits the generalizability of the findings to other demographic groups. Consequently, future large-scale prospective studies are essential to explore the longitudinal association between Klotho levels and the severity and mortality risk of CKM syndrome.↵

Conclusions

This study underscores the significant correlation between serum Klotho levels and the severity of CKM syndrome. Low serum Klotho levels serve as a reliable indicator of both CKM syndrome severity and associated mortality risk. Furthermore, serum Klotho levels may be utilized as a novel biomarker for staging CKM syndrome and guiding various treatment strategies. Elevating serum Klotho levels could represent a promising therapeutic approach for individuals with stage 4 CKM syndrome.

Declarations

Author contribution statement: TJ and ZH planned and designed the study, analyzed and interpreted the data, and wrote the manuscript. RL, CX, JH, and YA analyzed the data and created the tables presented in the manuscript. HJ and RY, as corresponding authors, provided important advice in the study design, supervised and coordinated the study conduct process, and revised the manuscript and tables. All authors have contributed to the manuscript and approved the submitted version.

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Data Availability Statement: The data associated with this study have been deposited in the NHANES. NHANES data and survey methodology are publicly available and accessible on the website (https//www.cdc.gov/nchs/nhanes/index.htm). Further inquiries can be directed to the corresponding author.

Declaration of interest statement: The authors declare no conflicts of interest. On behalf of all the authors, the corresponding author states that there are no conflicts of interest.

Additional information: No additional information is available for this study.

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FIGURE LEGEND

Figure 1 Nonlinear associations Klotho and all-cause mortality, cardiovascular mortality among CKM stage 0-3 and stage 4 populations

A: The relationship between Klotho and all-cause mortality among CKM stage 0-4 populations (fully adjusted RCS Model). B: The relationship between Klotho and cardiovascular mortality among CKM stage 0-4 populations (fully adjusted RCS Model). C: The relationship between Klotho and all-cause mortality among CKM stage 0-3 populations (fully adjusted RCS Model). D: The relationship between Klotho and cardiovascular mortality among CKM stage 0-3 populations (fully adjusted RCS Model). E: The relationship between Klotho and all-cause mortality among CKM stage 4 populations (fully adjusted RCS Model). F: The relationship between Klotho and cardiovascular mortality among CKM stage 4 populations (fully adjusted RCS Model). Adjustments in the model accounted for the following variables: age, sex, education level, ethnicity, marital status, family PIR, smoking status, drinking status, BMI, diabetes, and hypertension.

Figure 2 ROC curve analysis of Klotho with age, sex, race, and BMI in predicting CKM stages 0 to 4 and all-cause or cardiovascular mortality. The AUC for CKM syndrome and all-cause and cardiovascular mortalities were 0.748, 0.769, and 0.741, respectively.

Table 1 Baseline characteristics of individuals classified by stages of CKM

Characteristic	Cardiovascular- Kidney- Metabolic Syndrome					*P*-value
	0, N = 846	1, N = 1,545	2, N = 4,500	3, N = 1,954	4, N = 1,026	
Age	52.91 ± 10.035	53.65 ± 9.978	58.98 ± 10.452	55.29 ± 10.813	64.56 ± 9.690	<0.001
BMI	22.04 ± 1.992	29.36 ± 5.221	29.83 ± 6.164	31.27 ± 6.716	31.13 ± 6.941	<0.001
PIR	2.96 ± 1.738	2.91 ± 1.658	2.73 ± 1.655	2.45 ± 1.615	2.21 ± 1.523	<0.001
Gender (%)						<0.001
Male	426 (50.35%)	676 (43.75%)	2,750 (61.11%)	421 (21.55%)	605 (58.97%)	
Female	420 (49.65%)	869 (56.25%)	1,750 (38.89%)	1,533 (78.45%)	421 (41.03%)	
Race (%)						<0.001
Non-Hispanic White	76 (8.98%)	242 (15.66%)	708 (15.73%)	353 (18.07%)	112 (10.92%)	
Non-Hispanic Black	67 (7.92%)	174 (11.26%)	484 (10.76%)	265 (13.56%)	77 (7.50%)	
Mexican	431 (50.95%)	707 (45.76%)	1,962 (43.60%)	880 (45.04%)	536 (52.24%)	
Other Hispanic	139 (16.43%)	324 (20.97%)	958 (21.29%)	292 (14.94%)	232 (22.61%)	
Others	133 (15.72%)	98 (6.34%)	388 (8.62%)	164 (8.39%)	69 (6.73%)	
Education (%)						<0.001
Less Than 9th Grade	188 (22.22%)	383 (24.79%)	1,276 (28.36%)	617 (31.58%)	370 (36.06%)	
9-11th Grade	353 (41.73%)	720 (46.60%)	2,199 (48.87%)	999 (51.13%)	511 (49.81%)	
High school grades and more	305 (36.05%)	442 (28.61%)	1,025 (22.78%)	338 (17.30%)	145 (14.13%)	
Marital (%)						0.117
Married/Living with a partner	574 (67.85%)	994 (64.34%)	2,935 (65.22%)	1,250 (63.97%)	639 (62.28%)	
Widowed/Divorced/Never married	272 (32.15%)	551 (35.66%)	1,565 (34.78%)	704 (36.03%)	387 (37.72%)	
Diabetes (%)						<0.001

No	846 (100.00%)	1,545 (100.00%)	3,665 (81.44%)	1,501 (76.82%)	671 (65.40%)	
Yes	0 (0.00%)	0 (0.00%)	835 (18.56%)	453 (23.18%)	355 (34.60%)	
Hypertension (%)						<0.001
No	846 (100.00%)	1,545 (100.00%)	1,888 (41.96%)	911 (46.62%)	236 (23.00%)	
Yes	0 (0.00%)	0 (0.00%)	2,612 (58.04%)	1,043 (53.38%)	790 (77.00%)	
Current smoking (%)						<0.001
No	458 (54.14%)	922 (59.68%)	2,215 (49.22%)	1,056 (54.04%)	364 (35.48%)	
Yes	388 (45.86%)	623 (40.32%)	2,285 (50.78%)	898 (45.96%)	662 (64.52%)	
Alcohol (%)						<0.001
Yes	674 (79.67%)	1,168 (75.60%)	3,398 (75.51%)	1,281 (65.56%)	743 (72.42%)	
No	172 (20.33%)	377 (24.40%)	1,102 (24.49%)	673 (34.44%)	283 (27.58%)	
CHF (%)						<0.001
Yes	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	382 (37.23%)	
No	846 (100.00%)	1,545 (100.00%)	4,500 (100.00%)	1,954 (100.00%)	644 (62.77%)	
CHD (%)						<0.001
Yes	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	509 (49.61%)	
No	846 (100.00%)	1,545 (100.00%)	4,500 (100.00%)	1,954 (100.00%)	517 (50.39%)	
Stroke (%)						<0.001
Yes	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	417 (40.64%)	
No	846 (100.00%)	1,545 (100.00%)	4,500 (100.00%)	1,954 (100.00%)	609 (59.36%)	
CVD (%)						<0.001
Yes	846 (100.00%	1,545 (100.00%	4,500 (100.00%	1,954 (100.00%	0 (0.00%)	

	)	)	)	)		
No	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1,026 (100.00%)	
CKD (%)						<0.001
Yes	843 (99.65%)	1,537 (99.48%)	4,059 (90.20%)	1,815 (92.89%)	801 (78.07%)	
No	3 (0.35%)	8 (0.52%)	441 (9.80%)	139 (7.11%)	225 (21.93%)	
TC (mg/dl)	197.07 ± 36.222	197.17 ± 36.015	197.20 ± 41.213	215.99 ± 46.277	181.47 ± 45.225	<0.001
TG (mg/dl)	79.94 ± 26.649	89.00 ± 25.500	178.64 ± 107.106	231.18 ± 222.736	177.47 ± 129.568	<0.001
Scr (mg/dl)	0.84 ± 0.174	0.83 ± 0.184	0.95 ± 0.401	0.83 ± 0.306	1.07 ± 0.522	<0.001
HBA1C (%)	5.37 ± 0.469	5.53 ± 0.421	5.98 ± 1.136	6.20 ± 1.422	6.34 ± 1.377	<0.001
HDL-C (mg/dl)	66.87 ± 18.760	59.83 ± 15.999	51.55 ± 15.398	47.34 ± 13.107	48.40 ± 14.655	<0.001
eGFR (ml/（min×1.73m²）	95.51 ± 13.861	94.01 ± 14.513	86.39 ± 19.026	90.82 ± 19.362	75.95 ± 21.903	<0.001
Klotho (pg/ml)	900.68 ± 291.009	887.83 ± 308.353	860.98 ± 310.800	860.62 ± 299.499	814.01 ± 282.900	<0.001

Abbreviations: BMI - Body Mass Index; PIR Poverty income ratio; CHF - Congestive Heart Failure; CHD- Coronary Heart Disease; CVD - Cardiovascular Disease; CKD - Chronic Kidney Disease; TC - Total Cholesterol; TG – Triglycerides; Scr - Serum Creatinine; HDL-C - High-Density Lipoprotein Cholesterol; eGFR - Estimated Glomerular Filtration Rate

Table 2 Levels of Klotho in Relation to CKM Stage 0 to 4

Continuous or categories	Model1 OR (95%CI)	*P*	Model2 OR (95%CI)	*P*	Model3 OR (95%CI)	*P*
Continuous variable (per SD)	0.89(0.86, 0.93)	<0.001	0.91(0.87, 0.94)	<0.001	0.90(0.86, 0.93)	<0.001
High (⩾722)						
Low (< 722)	1.23(1.14, 1.32)	<0.001	1.19(1.11, 1.28)	<0.001	1.20(1.11, 1.30)	<0.001

Abbreviations: OR, odds ratio; CI, confidence interval. Adjusted Mode: Model 1 (unadjusted), Model 2 (adjusted for sex, age, and race), and Model 3 (further adjusted for sex, age, race, education, PIR, BMI, smoking, alcohol consumption, hypertension, and diabetes). Bold indicates P value < 0.05.

Table 3 Regression analysis of Klotho and all-cause mortality, cardiovascular mortality among CKM stage 0-3 and stage 4 populations

Characteristic	Model 1 HR (95% CI)	*P*	Model 2 HR (95% CI)	*P*	Model 3 HR (95% CI)	*P*
All-cause mortality						
Continuous variable (per SD)	0.92(0.87, 0.98)	0.008	0.94(0.88, 0.99)	0.031	0.94(0.89, 1.00)	0.044
High (⩾722)						
Low (< 722)	1.29 (1.15, 1.44)	<0.001	1.24 (1.10, 1.39)	<0.001	1.21(1.08, 1.36)	<0.001
Cardiovascular mortality						
Continuous variable (per SD)	0.63(0.55, 0.72)	<0.001	0.68(0.60, 0.78)	<0.001	0.65 (0.57, 0.75)	<0.001
High (⩾722)						
Low (< 722)	1.84 (1.48, 2.28)	<0.001	1.79 (1.44, 2.22)	<0.001	1.76 (1.41, 2.19)	<0.001

Abbreviations: HR, hazard ratio; CI, confidence interval. Adjusted Mode: Model 1 (unadjusted), Model 2 (adjusted for sex, age, and race), and Model 3 (further adjusted for gender, age, race, education, PIR, BMI, smoking, alcohol consumption, hypertension, and diabetes). Bold indicates P value < 0.05.

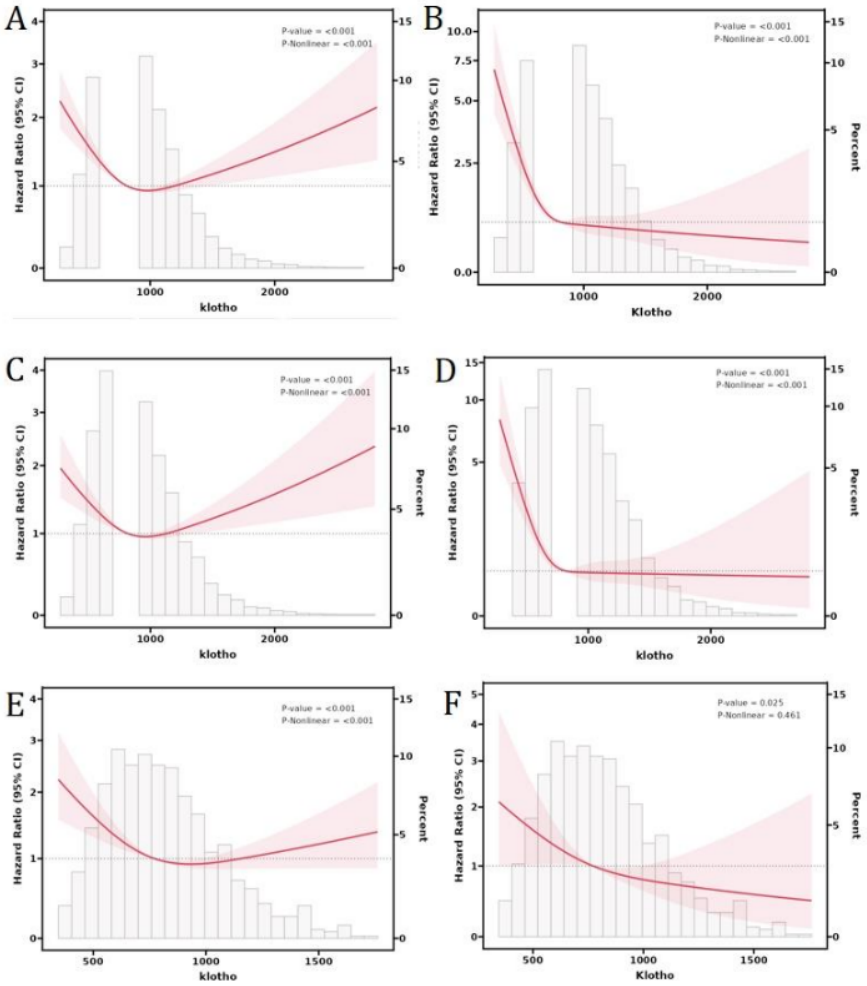


Figure 1 Nonlinear associations Klotho and all-cause mortality, cardiovascular mortality among CKM stage 0-3 and stage 4 populations

A: The relationship between Klotho and all-cause mortality among CKM stage 0-4 populations (fully adjusted RCS Model). B: The relationship between Klotho and cardiovascular mortality among CKM stage 0-4 populations (fully adjusted RCS Model). C: The relationship between Klotho and all-cause mortality among CKM stage 0-3 populations (fully adjusted RCS Model). D: The relationship between Klotho and cardiovascular mortality among CKM stage 0-3 populations (fully adjusted RCS Model). E: The relationship between Klotho and all-cause mortality among CKM stage 4 populations (fully adjusted RCS Model). F: The relationship between Klotho and cardiovascular mortality among CKM stage 4 populations (fully adjusted RCS Model). Adjustments in the model accounted for the following variables: age, gender, educational level, ethnicity, marital status, family PIR, smoking status, drinking status, BMI, diabetes, and hypertension.

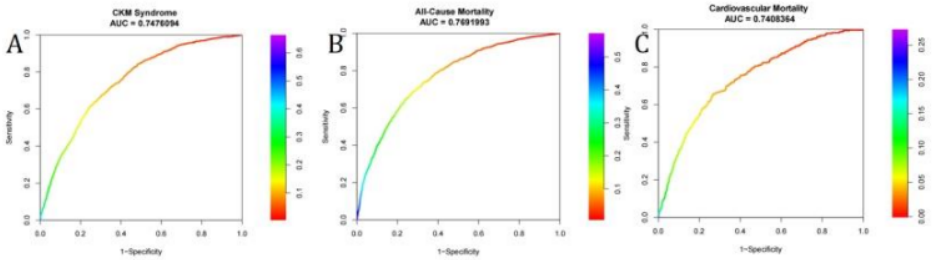
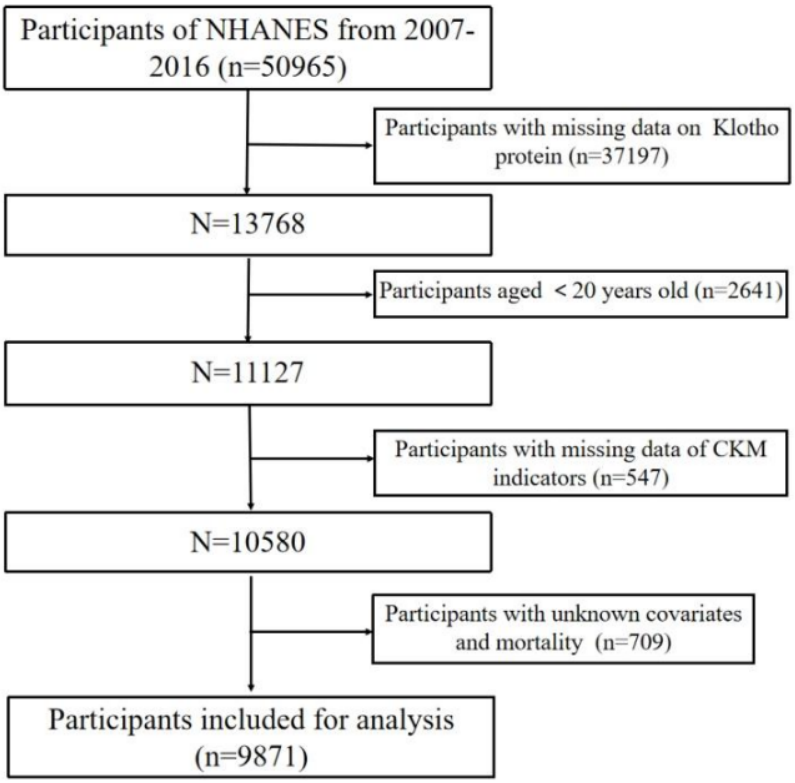


Figure 2 ROC curve analysis of Klotho with age, gender, race, and BMI in predicting CKM Stage 0 to 4, all-cause or cardiovascular mortality. The AUC for CKM syndrome, all-cause and cardiovascular mortality were 0.748, 0.769, and 0.741, respectively.



Supplementary Figure 1 Flowchart of the study population Abbreviations: NHANES National Health and Nutrition Examination Survey, CKM Cardiovascular- Kidney- Metabolic Syndrome

$$eGFR = 142 \times \left(\frac{Scr}{A} \right)^{B} \times (0.9938)^{Age} \times C$$

(1) $Female: C = 1.012; Scr \le 0.7 mg/dL: A = 0.7, B = -0.241$

$Scr > 0.7 mg/dL: A = 0.7, B = -1.2$

(2) $Male: C = 1.000; Scr \le 0.7 mg/dL: A = 0.9, B = -0.302$

$Scr > 0.7 mg/dL: A = 0.9, B = -1.2$

Supplementary Figure 2 eGFR calculation formula Abbreviation: Scr-Serum Creatinine; eGFR estimated glomerular filtration rate

Supplementary Table 1 Detailed algorithm of the simplified 10-year CVD risk models

Gender	10-year CVD risk
Female	**log-Odds** = -3.307728 + 0.7939329 × (age – 55) /10 + 0.0305239 × (TC – HDL-C – 3.5) – 0.1606857 × (HDL-C –1.3) /0.3 – 0.2394003 × (min(SBP, 110) – 110) /20 + 0.360078 × (max(SBP, 110) – 130) /20 + 0.8667604 × (if diabetes) + 0.5360739 × (if current smoker) + 0.6045917 × (min(eGFR, 60) – 60) / -15 + 0.0433769 × (max(eGFR, 60) – 90) / -15 + 0.3151672 × (if using anti-hypertensive medication) – 0.1477655 × (if using statin) – 0.0663612 × (if using anti-hypertensive medication) × (max(SBP, 110) – 130) /20 + 0.1197879 × (if using statin) × (TC – HDL-C – 3.5) – 0.0819715 × (age – 55) /10 × (TC – HDL-C – 3.5) + 0.0306769 × (age – 55) /10 × (HDL-C – 1.3) /0.3 – 0.0946348 × (age – 55) /10 × (max (SBP, 110) – 130) /20 – 0.27057 × (age – 55) /10 × (if diabetes) – 0.078715 × (age – 55) /10 × (if current smoker) – 0.1637806 × (age – 55) /10 × (min (eGFR, 60) – 60) / -15 **Risk** = exp(log-Odds) / (1 + exp(log-Odds))
Male	**log-Odds** = -3.031168 + 0.7688528 × (age – 55) /10 + 0.0736174 × (TC – HDL-C – 3.5) – 0.0954431 × (HDL-C – 1.3) /0.3 – 0.4347345 × (min(SBP, 110) – 110) /20 + 0.3362658 × (max(SBP, 110) – 130) /20 + 0.7692857 × (if diabetes) + 0.4386871 × (if current smoker) + 0.5378979 × (min(eGFR, 60) – 60) / -15 + 0.0164827 × (max(eGFR, 60) – 90) / -15 + 0.288879 × (if using anti-hypertensive medication) – 0.1337349 × (if using statin) – 0.0475924 × (if using anti-hypertensive medication) × (max(SBP, 110) – 130) /20 + 0.150273 × (if using statin) × (TC – HDL-C – 3.5) – 0.0517874 × (age – 55) /10 × (TC – HDL-C – 3.5) + 0.0191169 × (age – 55) /10 × (HDL-C – 1.3) /0.3 – 0.1049477 × (age – 55) /10 × (max(SBP, 110) – 130) /20 – 0.2251948 × (age – 55) /10 × (if diabetes) – 0.0895067 × (age – 55) /10 × (if current smoker) – 0.1543702 × (age – 55) /10 × (min(eGFR, 60) – 60) / -15 **Risk** = exp(log-Odds) / (1 + exp(log-Odds))

Abbreviations: eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol.

Supplementary Table 2 Methods for evaluating each CKM stage

CKM health stages	Definition
Stage 0: No CKM health risk factors	Individuals with normal BMI and waist circumference, normoglycemia, normotension, a normal lipid profile, and no evidence of CKD or subclinical or clinical CVD. The predicted 10-year CVD risk＜20%.
Stage 1: Excess and/or dysfunctional adiposity	Individuals with overweight/obesity, abdominal obesity, or dysfunctional adipose tissue, without the presence of other metabolic risk factors or CKD. The predicted 10-year CVD risk＜20%.
Stage 2: Metabolic risk factors and CKD	Individuals with metabolic risk factors (hypertriglyceridemia, hypertension, MS, diabetes), or CKD The predicted 10-year CVD risk＜20%.
Stage 3: Subclinical CVD in CKM	Subclinical CVD among individuals with excess/dysfunctional adiposity, other metabolic risk factors, or CKD The predicted 10-year CVD risk＜20%.
Stage 4: Clinical CVD in CKM	Clinical CVD among individuals with excess/dysfunctional adiposity, other metabolic risk factors, or CKD

Abbreviations: BMI, body mass index; CKD, chronic kidney disease; CKM, cardiovascular-kidney-metabolic; CVD, cardiovascular disease

Supplementary Table 3 The Cut-point is found by the maximum Youden index

AUC	Cut-point	Metric Score: Yonden's index	Sensitivity	Specificity	PPV	NPV	Accuracy	Precision
0.556	772	0.08863038	42.70%	66.20%	12.80%	90.90%	63.70%	12.80%

Supplementary Table 4 The values of Receiver operating characteristic (ROC) curves of CKM Stages 0 to 4, all-cause and cardiovascular mortality

character	AUC (95%CI)	Accuracy (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)
CKM	0.748 (0.733-0.763)	0.668 (0.659-0.677)	0.663 (0.653 - 0.673)	0.710 (0.682 - 0.737)	0.952 (0.946 - 0.957)	0.196 (0.184 - 0.209)
all-cause mortality	0.769 (0.755-0.783)	0.685 (0.676-0.694)	0.679 (0.669 - 0.689)	0.733 (0.707 - 0.758)	0.949 (0.943 - 0.954)	0.238 (0.224 - 0.252)
CVD mortality	0.741 (0.715-0.767)	0.720 (0.711-0.729)	0.722 (0.713 - 0.731)	0.667 (0.616 - 0.717)	0.984 (0.981 - 0.987)	0.079 (0.069 - 0.089)

Supplementary Table 5 Subgroup analysis between klotho protein and all-cause mortality

Subgroup	≥ 772[1]	< 772[1]	Crude HR (95% CI)	*P* value	*P* for interaction
Overall	5,013/5,612 (89.3)	3,661/4,251 (86.1)	0.91 (0.87-0.95)	<0.001	
gender					0.895
Male	2,292/2,639 (86.9)	1,876/2,233 (84.0)	0.91 (0.86-0.97)	0.003	
Female	2,721/2,973 (91.5)	1,785/2,018 (88.5)	0.91 (0.86-0.97)	0.002	
Diabetes					0.448
No	4,252/4,665 (91.1)	3,116/3,555 (87.7)	0.90 (0.86-0.95)	<0.001	
Yes	761/947 (80.4)	545/696 (78.3)	0.95 (0.85-1.06)	0.323	
Hypertension					0.747
No	2,103/2,465 (85.3)	1,583/1,977 (80.1)	0.92 (0.86-0.98)	0.009	
Yes	2,910/3,147 (92.5)	2,078/2,274 (91.4)	0.90 (0.85-0.96)	<0.001	
Alcohol					0.558
No	1,413/1,583 (89.3)	861/1,022 (84.2)	0.89 (0.82-0.97)	0.008	
Yes	3,600/4,029 (89.4)	2,800/3,229 (86.7)	0.91 (0.87-0.96)	<0.001	
Smoking					0.989
No	2,798/3,030 (92.3)	1,815/1,981 (91.6)	0.92 (0.86-0.97)	0.004	
Yes	2,215/2,582 (85.8)	1,846/2,270 (81.3)	0.91 (0.86-0.97)	0.004	

[1]no. of events / total no. (%)

Supplementary Table 6 Subgroup analysis between klotho protein and cardiovascular mortality

Subgroup	≥ 772[1]	< 772[1]	Crude HR (95% CI)	*P* value	*P* for interaction
Overall	140/5,611 (2.5)	199/4,251 (4.7)	1.84 (1.48-2.28)	<0.001	
gender					0.423
Male	75/2,638 (2.8)	126/2,233 (5.6)	1.94 (1.46-2.59)	<0.001	
Female	65/2,973 (2.2)	73/2,018 (3.6)	1.61 (1.15-2.25)	0.005	
Diabetes					0.317
No	99/4,664 (2.1)	152/3,555 (4.3)	1.97 (1.53-2.54)	<0.001	
Yes	41/947 (4.3)	47/696 (6.8)	1.55 (1.02-2.35)	0.041	
Hypertension					0.762
Yes	81/2,465 (3.3)	121/1,977 (6.1)	1.88 (1.42-2.50)	<0.001	
No	59/3,146 (1.9)	78/2,274 (3.4)	1.74 (1.24-2.44)	0.001	
Alcohol					0.281
No	50/1,583 (3.2)	51/1,022 (5.0)	1.55 (1.05-2.29)	0.028	
Yes	90/4,028 (2.2)	148/3,229 (4.6)	2.01 (1.54-2.61)	<0.001	
Smoking					0.263
No	53/3,030 (1.7)	75/1,981 (3.8)	2.07 (1.46-2.95)	<0.001	
Yes	87/2,581 (3.4)	124/2,270 (5.5)	1.62 (1.23-2.13)	0.001	

[1]no. of events / total no. (%)

List of abbreviations

NHANES	National Health and Nutrition Examination Survey
CKM	Cardiovascular-Kidney-Metabolic
RCS	Restricted cubic spline
ROC	Receiver operating characteristic
CKD	chronic kidney disease
CVD	cardiovascular disease
CHF	Congestive Heart Failure
CHD	Coronary Heart Disease
AUC	area under the curve
ELISA	enzyme-linked immunosorbent assay
BMI	Body mass index
OR	odds ratio
HR	hazard ratio
95% CI	95 % confidence interval
MS	metabolic syndrome
T2DM	type 2 diabetes mellitus
FGF23	fibroblast growth factor 23
FGFR	fibroblast growth factor receptor
PIR	Poverty income ratio
TC	Total Cholesterol
TG	Triglycerides
eGFR	estimated glomerular filtration rate
HDL-C	high-density lipoprotein
Scr	Serum Creatinine

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