

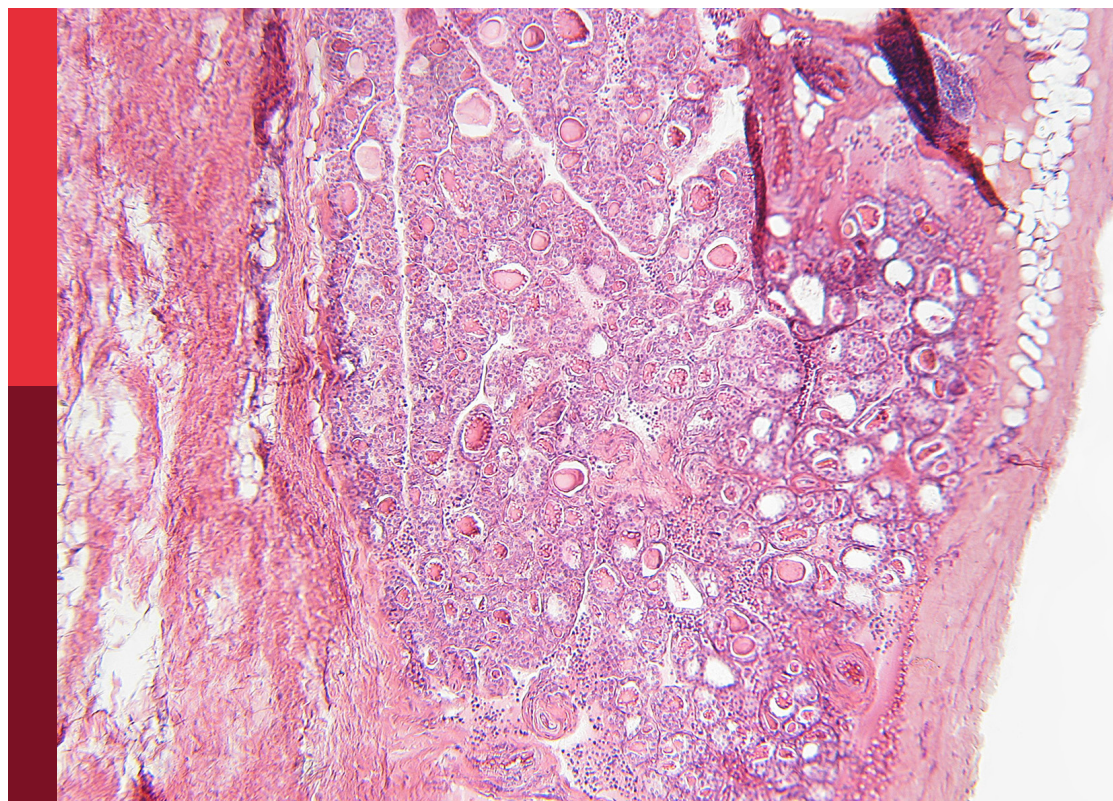
Endocrine imbalances of mineral ions and vitamins in chronic disease pathogenesis

Edited by

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Endocrine imbalances of mineral ions and vitamins in chronic disease pathogenesis

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Table of contents

- 05 **Editorial: Endocrine imbalances of mineral ions and vitamins in chronic disease pathogenesis**
Mor-Li Hartman, Hosam G. Abdelhady and Mohammed S. Razzaque
- 09 **Impact of dietary selenium and blood concentration on liver function: a population-based study**
Qiaoli Liang, Ruihua Huang, Ziming Peng and Menglong Zou
- 22 **The relationship between 25-hydroxy vitamin D and serum asprosin in patients with type 2 diabetes in the community**
Junfang Cui, Zhengqian Wang, Jianhong Yin, Mina Li, Qianqian Wu, Ming Liu, Hong Su, Huijuan Ren, Minggang Xu, Jing Yang and Linxin Xu
- 28 **The role of magnesium in pancreatic beta-cell function and homeostasis**
Nuraly S. Akimbekov, Seval Ozkan Coban, Azeddine Atfi and Mohammed S. Razzaque
- 35 **Neuroprotective effects of magnesium: implications for neuroinflammation and cognitive decline**
Veer Patel, Nuraly S. Akimbekov, William B. Grant, Carolyn Dean, Xiaoqian Fang and Mohammed S. Razzaque
- 43 **Association of dietary and circulating antioxidant vitamins with metabolic syndrome: an observational and Mendelian randomization study**
Qian Sun, Zhixing Fan, Fangfang Yao, Xiaojing Zhao, Min Jiang, Mudan Yang, Menglu Mao and Chaojun Yang
- 57 **U-shaped association between dietary niacin intake and chronic kidney disease among US elderly: a nationwide cross-sectional study**
Zhouzhou Xie, Shansen Peng, Gejun Ou, Xiaoqi Zhou, Guihao Zhang, Huiming Jiang, Tianhui Zhang and Nanhui Chen
- 67 **Effects of probiotic supplementation on bone health in postmenopausal women: a systematic review and meta-analysis**
Fang Wang, Wei Wei and Peng Ju Liu
- 80 **Predictive value of bone metabolism markers in the progression of diabetic kidney disease: a cross-sectional study**
Yi Kang, Qian Jin, Mengqi Zhou, Zirong Li, Huijuan Zheng, Danwen Li, Weijing Liu, Yaoxian Wang and Jie Lv
- 95 **Nonlinear relationship between serum Klotho and chronic kidney disease in US adults with metabolic syndrome**
Xiaobin Lin and Lin Yang
- 110 **The role of vitamin D in slipped capital femoral epiphysis in children and adolescents: a retrospective case-control study**
Peng Ning, Shuting Lin, Huiyu Geng and Tianjing Liu

- 119 **A novel LC-MS/MS analysis of vitamin D metabolites in mice serum and hair: impact of diet and light exposure**
Muhammad K. Hakeem, Asma Al-Menhali, Sampath K. Elangovan and Iltaf Shah
- 137 **Association between magnesium depletion score and prevalence of hyperuricemia in American adults: a study based on NHANES 2007-2018**
Yongchun Xiao, Yong Mou, Ping Wu, Ke Wang, Tianjun Chen, Zhiwan Chen, Hongsheng Lin, Hua Yang and Zhisheng Ji
- 147 **Adults with Phenylketonuria have suboptimal bone mineral density apart from vitamin D and calcium sufficiency**
Beatrice Hanusch, Anne Schlegtendal, Corinna Grasemann, Thomas Lücke and Kathrin Sinning
- 160 **The inflection point: α -Klotho levels and the risk of all-cause mortality**
Jianling Song, Hong Li and Xiangdong Fang
- 173 **Advancing patient evidence in XLH (APEX): rationale and design of a real-world XLH global data unification program**
Maria Luisa Brandi, Thomas O. Carpenter, Seiji Fukumoto, Dieter Haffner, Erik A. Imel, Masanori Kanematsu, Keith P. McCullough and Keiichi Ozono



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Editorial: Endocrine imbalances of mineral ions and vitamins in chronic disease pathogenesis

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Editorial on the Research Topic

Endocrine imbalances of mineral ions and vitamins in chronic disease pathogenesis

Chronic diseases represent a major global health burden, accounting for nearly two-thirds of all deaths worldwide (1). In the United States, poor dietary habits and insufficient physical activity, both strongly associated with obesity, are leading contributors to this burden (1). Among the many factors implicated in the onset and progression of chronic diseases, disruptions in mineral and vitamin homeostasis play a critical yet often underappreciated role. Essential mineral ions such as calcium, magnesium, phosphate, zinc, selenium, and iron are fundamental to human physiology. They participate in metabolic regulation, immune responses, neuromuscular function, and bone health (2, 3). Both deficiency and excess of these micronutrients can lead to significant health complications. For example, dysregulation of calcium and phosphate levels is linked to osteoporosis, cardiovascular events, and neuromuscular dysfunction (2, 4, 5). Similarly, chronic kidney disease (CKD) is influenced by imbalances in minerals, vitamins, and other metabolites, with evidence supporting their potential therapeutic roles in modulating inflammation and oxidative stress (5). Micronutrient deficiencies are also frequently observed in metabolic disorders such as type 2 diabetes, obesity, cardiovascular disease, and metabolic syndrome. Notably, patients with type 2 diabetes often exhibit reduced levels of zinc, magnesium, and chromium, which may exacerbate insulin resistance and impair glucose metabolism (6–9). Recent work also suggests that salivary phosphate levels could serve as early biomarkers for obesity risk in children, linking oral biomarkers with systemic metabolic changes (10). Despite these insights, the mechanistic relationships between micronutrient status and chronic disease progression remain incompletely understood. Emerging evidence further highlights the protective effects of a nutrient-rich diet, particularly in younger populations. Adolescents consuming diets high in fruits, vegetables, legumes, and whole grains show significantly lower risk of developing chronic diseases compared to those adhering to Western dietary patterns (11). These findings underscore the importance of early dietary interventions and the need to investigate how micronutrient dynamics contribute to disease etiology, prevention, and treatment across the lifespan.

Key contributions to this Research Topic

In this Research Topic, [Sun et al.](#) examined the relationship between antioxidant vitamins and metabolic syndrome, employing Mendelian randomization (MR) to explore potential causal links. Their findings suggest that adequate intake of antioxidant vitamins A, C, and carotenoids may contribute to a reduced risk of developing metabolic syndrome. Additionally, magnesium, an essential micronutrient involved in numerous physiological processes, remains a critical component of metabolic health. Complementing this, [Akimbekov et al.](#) explained the role of magnesium in glucose homeostasis, with particular emphasis on pancreatic β -cell function. They highlighted evidence linking magnesium deficiency to impaired β -cell activity and heightened insulin resistance in individuals with type 2 diabetes. The authors underscore the essential role of magnesium in facilitating glucose utilization and insulin signaling, emphasizing that adequate magnesium status is vital for metabolic health and the prevention of related disorders. [Patel et al.](#) elaborated the role of magnesium in neurodegenerative diseases among the aging population. Their review highlights the neuroprotective properties of magnesium and examines how magnesium deficiency contributes to neuroinflammation. The authors discuss the potential of magnesium as a therapeutic agent for mitigating cognitive decline and delaying the progression of neurodegenerative disorders. [Xiao et al.](#) examined the association between magnesium deficiency and hyperuricemia, a chronic metabolic disorder (12), using cross-sectional data from the 2007–2018 National Health and Nutrition Examination Survey (NHANES). They introduced the Magnesium Depletion Score (MDS) as a novel marker of magnesium status and found that higher MDS was significantly associated with increased prevalence of hyperuricemia, suggesting a potential contributory role of magnesium deficiency. The authors emphasize the need for prospective studies to confirm these results. Moreover, they noted magnesium's role in vitamin D biosynthesis, an essential hormone for glucose metabolism and insulin sensitivity.

[Cui et al.](#) analyzed data from 463 patients with type 2 diabetes in China to examine the relationship between Asprosin, an adipokine associated with insulin resistance (13), and vitamin D. Previous studies have shown that Asprosin levels correlate positively with insulin resistance (14). In this study, the authors found an inverse relationship between serum Asprosin and 25-hydroxyvitamin D, the primary circulating form of vitamin D. These findings underscore the need for further research into vitamin D metabolism and its broader implications for human health (6, 15, 16). [Hakeem et al.](#) examined the metabolism of vitamin D and the various factors influencing its levels, with a particular focus on validating a novel LC-MS/MS method for analyzing vitamin D and its metabolites in mouse hair samples. The use of hair as a biomatrix for assessing vitamin D status represents an innovative approach. Their findings highlight the roles of dietary intake, light exposure, and metabolic regulation in maintaining adequate vitamin D levels in mice. The study supports the importance of sufficient vitamin D and sunlight exposure for optimal vitamin D status. This

complements existing research emphasizing the critical role of vitamin D in bone health, particularly in calcium and phosphate homeostasis (15, 17, 18).

Bone health was another major focus. [Ning et al.](#) examined the relationship between vitamin D levels and the risk of slipped capital femoral epiphysis (SCFE) in children and adolescents. Their analysis revealed that higher vitamin D levels were associated with a lower risk of SCFE, indicating a possible protective effect of sufficient vitamin D intake. However, no link was found between the severity of vitamin D deficiency and the occurrence of SCFE. These findings lay the groundwork for future studies to further investigate and clarify the role of vitamin D in preventing SCFE. [Hanusch et al.](#) explored factors influencing bone health in adults with phenylketonuria (PKU), a population known to have reduced bone mineral density (BMD) (19). Their study focused on lifestyle and dietary adherence but found no specific nutritional or lifestyle factors associated with the observed decrease in BMD among PKU patients. The authors emphasized the need for further research to better understand the early onset of bone loss in this population and to develop targeted interventions. They also proposed investigating additional contributors, such as alterations in the gut microbiome, low muscle mass, or chronic low-grade inflammation, which may affect bone formation and resorption. Bone health remains especially critical for postmenopausal women, who are at increased risk of osteoporosis due to the natural decline in estrogen, a hormone with protective effects on BMD (20). Emerging evidence suggests that the gut microbiota can influence bone metabolism by modulating the balance between osteoclast and osteoblast activity (21–23). [Wang et al.](#) conducted a systematic review and meta-analysis of randomized controlled trials, focused on the effects of probiotic supplementation on bone health in postmenopausal women. By analyzing data from randomized controlled trials (RCTs), the study assessed changes in BMD and bone turnover markers (BTMs). The findings revealed that probiotic supplementation was associated with improved BMD in the lumbar spine and hip, with stronger effects observed in women with osteopenia compared to those with osteoporosis. These results highlight the potential of probiotics as a supportive strategy for maintaining bone health and underscore the value of bone metabolism markers in early diagnosis and targeted intervention.

[Kang et al.](#) investigated the relationship between bone metabolism markers and the progression of diabetic kidney disease (DKD) in patients with type 2 diabetes mellitus. The study analyzed various stages of DKD and assessed serum levels of key markers, including Klotho, fibroblast growth factor 23 (FGF23), 25-hydroxyvitamin D3 [25(OH)D3], intact parathyroid hormone (iPTH), calcium, and phosphorus. Their findings revealed significant alterations in these markers across DKD stages, suggesting that changes in FGF23, 25(OH)D3, iPTH, and calcium levels are associated with disease progression and may serve as potential therapeutic targets. As DKD resulting from metabolic disease poses a major global health challenge, further research is needed to better understand its underlying mechanisms (24), emphasizing consequences of abnormal regulation of FGF23, vitamin D and phosphate metabolism in chronic diseases (25, 26).

Lin and Yang investigated the association between serum Klotho levels and the risk of CKD in middle-aged and older adults with metabolic syndrome. Using cross-sectional data from NHANES, they identified a non-linear, L-shaped relationship between Klotho levels and CKD risk, with an inflection point at 9.88 pg/mL. Their findings suggest that both abnormally low and high Klotho concentrations are associated with increased CKD risk, underscoring the importance of maintaining Klotho within an optimal range. The study highlights the potential of Klotho as both a serum biomarker and a target for preventive strategies in this high-risk population.

Song et al. examined the relationship between circulating alpha-Klotho levels and all-cause mortality, addressing the current lack of a defined normal reference range for this biomarker (27). Their analysis revealed a U-shaped association, with an inflection point at 2.89 pg/mL, indicating that both low and high alpha-Klotho levels may be linked to increased mortality risk. However, further research is needed to elucidate the underlying mechanisms driving this relationship. Xie et al. reported a U-shaped association between dietary niacin intake and CKD, with an inflection point at 38.83 mg/day, indicating that both low and high dietary niacin levels may be linked to disease burden of CKD. Drawing on cross-sectional data from the 2003–2018 NHANES, the study investigated the link between niacin, recognized for its renal protective effects, and CKD in the elderly U.S. population. The findings underscore the importance of early prevention and intervention strategies in this at-risk group. However, due to the cross-sectional design, causality cannot be established, emphasizing the need for confirmation through large-scale prospective cohort studies. Liver health is a critical concern due to the liver's central role in metabolism, detoxification, and biochemical synthesis (28). Selenium, an essential micronutrient obtained through the diet, has garnered attention for its potential impact on liver function, given its key role in enzymatic activity and antioxidant defense mechanisms (29). Liang et al. described the relationship between selenium intake, blood selenium levels, and liver function to inform future dietary guidelines and interventions. Their cross-sectional study focused on a population with relatively high selenium intake and blood concentrations, but without significant liver impairment. The findings suggest that both dietary and circulating selenium levels influence liver function parameters. The authors emphasize the need for further research to clarify the interplay between selenium status and liver health across diverse populations, to improve preventive care strategies.

Finally, this Research Topic includes a study protocol by Brandi et al., designed to address critical knowledge gaps in X-linked hypophosphatemia (XLH), a rare genetic disorder. Through the Advancing Patient Evidence in XLH (APEX) program, the study will collect and analyze comprehensive data on the natural history, treatment, and outcomes of individuals with XLH. By aggregating data beyond individual or regional studies, APEX aims to generate insights that can inform clinical practice and enhance patient care. The findings are expected to advance understanding of XLH and support the development of more effective management strategies.

Collectively, the articles in this Research Topic provide valuable insights into the multifaceted roles of vitamins, minerals, and other

micronutrients in chronic disease prevention, diagnosis, and management. By examining their influence across metabolic, renal, neurological, hepatic, and skeletal systems, these studies highlight both emerging biomarkers and potential therapeutic targets that merit further investigation. The diversity of methodologies, including observational studies, meta-analyses, and protocol development, underscores the complexity of micronutrient-disease interactions and the need for integrative, interdisciplinary research approaches. Importantly, these findings reinforce the significance of early nutritional interventions, routine micronutrient monitoring in clinical practice, and global collaboration to address gaps in knowledge, especially for underserved populations and rare diseases. Moving forward, advancing our understanding of micronutrient biology will be essential for developing targeted strategies to reduce the global burden of chronic diseases and improve long-term health outcomes.

Author contributions

MH: Writing – original draft, Writing – review & editing. HA: Writing – review & editing. MR: Writing – review & editing.

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Impact of dietary selenium and blood concentration on liver function: a population-based study

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Background: Evidence on the association between selenium and liver function parameters is limited and controversial.

Methods: Data on dietary selenium intake, blood selenium concentration, and liver function parameters were obtained from the National Health and Nutrition Examination Survey (NHANES) 2017–2020. Associations between selenium (dietary intake and blood concentration) and liver function parameters [alanine aminotransferase (ALT), aspartate aminotransferase (AST), the ALT/AST ratio, gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP)] were assessed using multivariate linear regression models. Subgroup analyses and interaction tests were conducted to examine differences in associations according to age, gender, body mass index (BMI), diabetes, and physical activity.

Results: The study included 6,869 participants after screening. The multivariate linear regression model revealed that dietary selenium intake was positively associated with ALT ($\beta = 0.112$, 95% CI = 0.041, 0.183) and the ALT/AST ratio ($\beta = 0.002$, 95% CI = 0.001, 0.004) after adjustment for covariates. Results of blood selenium concentration also showed that higher blood selenium levels were positively associated with ALT ($\beta = 0.436$, 95% CI = 0.308, 0.564), AST ($\beta = 0.112$, 95% CI = 0.015, 0.208), and the ALT/AST ratio ($\beta = 0.012$, 95% CI = 0.009, 0.015). However, ALP decreased with increasing blood selenium concentration ($\beta = -0.207$, 95% CI = -0.414 , -0.000). In addition, we found significant differences in the effect of selenium on liver function parameters according to age, gender, and BMI.

Conclusion: Dietary selenium intake and blood concentration affect liver function parameters. These findings suggest that further research is needed to explore these associations to promote liver health and disease prevention.

KEYWORDS

dietary selenium intake, blood selenium concentration, liver function, cross-sectional study, NHANES

1 Introduction

The liver, being the largest metabolic organ in the human body, is essential for metabolism, detoxification, and biochemical synthesis (1). Liver injury can occur due to a variety of factors, including viral infections, excessive alcohol consumption, drug-induced hepatotoxicity, and metabolic disorders (2). Liver function tests (LFTs) are crucial for assessing liver status and the extent of injury. These tests include measurements of alanine aminotransferase (ALT), aspartate aminotransferase (AST), the ALT/AST ratio, gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP). The liver is not only sensitive to a variety of factors but is also intimately involved in the metabolism of numerous trace elements in the body (3, 4). In the multifaceted study of liver health, the role of trace minerals has received increasing attention (5–8).

Selenium is a vital micronutrient that is absorbed by the human body through the food chain, primarily from seafood, plant sources, and animal products (9). In the human body, selenium is not only a component of several important proteins, such as glutathione peroxidases and selenoprotein P, but also plays a role in antioxidant defense and maintaining cellular redox balance (10). Because of these important functions, selenium intake is closely linked to overall health, impacting cardiovascular disease (11, 12), immune function (13, 14), and thyroid health (15, 16). Blood selenium concentration is a crucial and reliable indicator of an individual's overall selenium nutritional status. However, the relationship between selenium intake and blood concentration is not linear and is influenced by individual absorption, metabolism, and excretion processes (17). Both inadequate and excessive selenium intake can have adverse health effects. Therefore, understanding the relationship between dietary selenium intake and blood selenium concentration and how these factors influence human health, particularly in maintaining liver health, is critical for developing dietary guidelines and preventive strategies.

The role of selenium in liver health has received increasing attention due to its essential functions in enzymatic processes and antioxidant defense mechanisms (18). Selenium contributes significantly to the ability of the liver to mitigate oxidative stress, a known contributor to liver injury (19–21). Oxidative stress in the liver can be caused by several factors, including environmental toxins, metabolic imbalances, and chronic alcohol use, all of which can lead to liver cell damage and subsequent liver disease (22, 23). Research has shown that selenium deficiency may exacerbate liver injury by impairing the liver's antioxidant defense system, thereby increasing susceptibility to oxidative damage (24, 25). However, the relationship between selenium and liver health is complex and dose-dependent, with both selenium deficiency and excess posing risks to liver function. This study aims to elucidate the relationship between dietary selenium intake, blood selenium concentration, and liver damage in US adults using data from the 2017–2020 National Health and Nutrition Examination Survey (NHANES). By examining these associations, this research aims to provide insight into the range of selenium intake and blood selenium concentrations for liver health and to contribute to the development of dietary guidelines and interventions aimed at preventing liver injury.

2 Materials and methods

2.1 Study population

The NHANES is an annual, nationally representative survey conducted in the United States by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC). NHANES has been utilizing an advanced multistage probability sampling design since 1999 to gather data biennially from a diverse, non-institutionalized section of the United States population. Ethical approval for NHANES was secured from the NCHS Research Ethics Review Board, and each participant willingly provided written informed consent. NHANES data from 2017 to 2020 were used in this study. From the initial pool of 15,560 eligible participants, several exclusions were made to ensure the integrity of the analysis: 4,697 individuals were excluded due to missing data on selenium intake or blood selenium levels, and 2,230 individuals were excluded due to missing LFTs results. The primary objective of this study is to investigate the impact of selenium on liver function in the general population. Including hepatitis-positive individuals could complicate the interpretation of results, as their abnormal liver function parameters may be attributable to the disease itself rather than selenium levels. Therefore, we excluded 82 participants who were positive for hepatitis B antibody and 118 participants who were positive for hepatitis C antibody. We excluded 1,498 individuals under the age of 20 because they are in a developmental stage, and their liver function parameters and selenium metabolism may differ significantly from adults, affecting the comparability and interpretability of the results. This exclusion also helps to enhance the homogeneity and data consistency of the study sample, and facilitates the analysis of the impact of educational level on the study results. We also excluded 66 individuals who reported being pregnant to refine the cohort for more accurate and relevant results. The study ultimately encompassed 6,869 participants (Figure 1).

2.2 Measurement of liver function

In NHANES, fasting blood samples were systematically collected from participants at a mobile examination center, ensuring consistency and standardization in the sample collection process. These samples were then immediately refrigerated and sent to a central laboratory for careful analysis. In the laboratory, serum liver function biomarkers were quantitatively assessed using the advanced Beckman Coulter DxC800 Synchron clinical system. ALT, primarily found in liver cells, is an enzyme involved in amino acid metabolism (26, 27). When liver cells are damaged or die, ALT is released into the blood. Elevated serum ALT levels are often seen as a sign of liver damage. AST, found in liver cells, cardiomyocytes, muscle cells, and other tissues, is not as liver-specific as ALT (26, 27). However, AST levels increase in response to liver injury. The ALT to AST ratio is used to differentiate between various types of liver disease. GGT and ALP are indicators of cholestasis (26, 27). Specifically, in this cross-sectional study, ALT, AST, ALT/AST ratio, GGT, and ALP were used to assess liver function impairment.

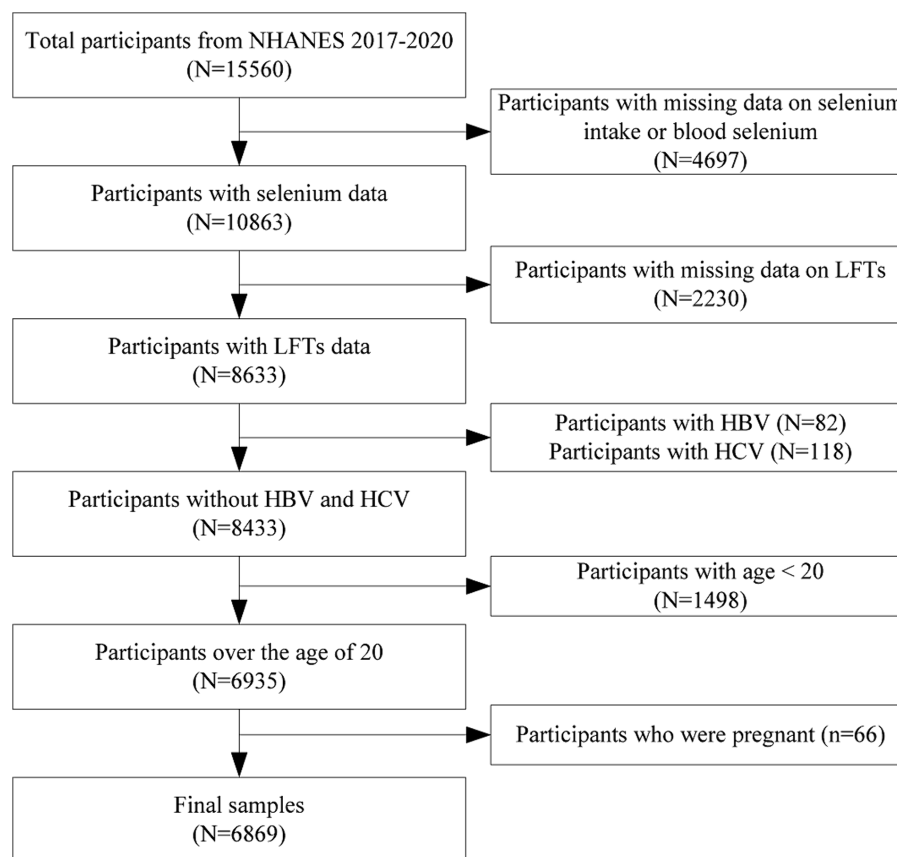


FIGURE 1

Flowchart of participants selection. NHANES, National health and nutrition examination survey; LFTs, Liver function tests; HBV, Hepatitis B virus; and HCV, Hepatitis C virus.

2.3 Measurement of dietary selenium intake and blood selenium level

The process for gathering dietary selenium data has been carefully designed to ensure accuracy and reliability. Dietary intake data were collected over two non-consecutive days, beginning with a face-to-face interview in a mobile examination center and followed by a telephone interview on a subsequent day. Participants were instructed to provide a detailed account of all food and beverages consumed in the previous 24 h, detailing specific meal contents and quantities. These details were then analyzed using the USDA's Food and Nutrient Database for Dietary Studies, enabling an accurate estimation of selenium intake. To enhance individual dietary data accuracy, this study averaged selenium intake from two recall days, minimizing day-to-day dietary variation. In NHANES 2017–2020, whole blood selenium concentrations were measured using inductively coupled plasma mass spectrometry. Detailed measurements can be found in a previously published study (28).

2.4 Measurement of covariates

Covariates were identified as confounders based on previously published studies (29–31). Demographic characteristics, including

gender, education, age, ethnicity, and family income-to-poverty ratio (PIR), were considered. Health-related factors included body mass index (BMI), alcohol consumption (at least 12 alcoholic beverages per year), smoking (at least 100 cigarettes in a lifetime), and physical activity (engaging in moderate or vigorous exercise or recreational activities weekly). Clinical factors included diabetes mellitus, fasting glucose, and total cholesterol (TC).

2.5 Statistical analysis

Dietary selenium intakes and blood selenium concentrations were divided into quartiles, with quartiles 1–4 representing progressively increasing intakes or concentrations. In the sample characteristics, continuous variables were expressed as means $\pm$ standard deviation, and categorical variables as count (number) and percentage. To compare means between groups, one-way ANOVA was used, while chi-squared tests were employed for percentages. Associations between selenium (dietary intake and blood concentration) and liver function parameters (ALT, AST, ALT/AST, GGT, and ALP) were calculated using multivariate linear regression models. The multivariate analysis utilized three models: crude model with no adjustment; Model 1 adjusted for age, gender, and race; and Model 2 included adjustments for the full range of

covariates. Subgroup analyses and interaction tests were conducted to explore variations in the associations by age, gender, race, education, and BMI. *p* values less than 0.05 were considered statistically significant. The statistical analyses and graphical representations in this study were generated using R (version 4.1.3) and EmpowerStats (version 2.0).

3 Results

3.1 Baseline characteristics

Table 1 presents a comparison of baseline characteristics of participants across four quartiles of dietary selenium intake. A total of 6,869 participants with an average age of 50.788 ± 17.384 were included in this study, of which 48.62% were males and 51.38% were females. Notably, selenium intakes were higher than international recommendations and blood concentrations were also quite high. In addition, liver enzymes as well as cholesterol were within reference ranges. Blood selenium concentrations increased with each quartile of dietary selenium intake ($p < 0.05$). Significant differences were observed among the quartiles in age, gender, race, education level, family income-to-poverty ratio (PIR), BMI, fasting glucose, drinking habits, physical activity, and various liver function markers (all $p < 0.05$). Participants in the highest quartile were more likely to be male, consume alcohol, have above high school education, engage in physical activity, and have higher BMI, fasting glucose, ALT, AST, GGT, and ALT/AST levels, but lower ALP levels compared to those in the lowest quartile.

Table 2 compares the baseline characteristics of participants according to four quartiles of blood selenium concentration. With increasing dietary selenium intake, the quartiles of the participants' blood selenium concentrations increased ($p < 0.05$). Significant differences in age, gender, race, education level, fasting glucose, drinking habits, physical activity, ALT, AST, GGT, ALT/AST, and TC (all $p < 0.05$). Participants in the highest quartile were more likely to be male, to have above high school education, to be physically active, and to have higher levels of fasting glucose, ALT, AST, GGT, ALT/AST, and TC, compared to participants in the lowest quartile.

3.2 Relationship between dietary selenium intake and liver function parameters

To better observe the relationship between selenium intake and liver function parameters, the unit of selenium intake was converted to $10 \mu\text{g}$ per day. The results of multivariate linear regression analyses examining the association between dietary selenium intake and liver function parameters are shown in Table 3. The present study found a positive association between dietary selenium intake and ALT and the ALT/AST ratio whatever the model. There was no robust association with other liver function parameters. The effect of selenium intake on liver function parameters was illustrated through a smoothed curve fit (Figure 2). Dietary selenium intake was then categorized into quartile variables to examine the linear relationship (Table 4). In model 2, higher selenium intake was associated with higher effect values for ALT and ALT/AST.

3.3 Relationship between blood selenium concentration and liver function parameters

Similarly, blood selenium concentrations were converted to $10 \mu\text{g/L}$. Results from multivariate linear regression analyses examining the association between blood selenium concentration and liver function parameters are presented in Table 5. Consistent with the findings for dietary selenium intake, blood selenium concentrations were positively correlated with ALT and the ALT/AST ratio. Additionally, blood selenium concentrations were positively correlated with AST and negatively correlated with ALP in model 2. The effect of blood selenium on liver function parameters was illustrated through a smoothed curve fit (Figure 3). Blood selenium concentration was then categorized into quartile variables to examine the linear relationship (Table 6). In model 2, similar trend test results were observed for ALT and the ALT/AST ratio (p for trend < 0.001), but not for AST and ALP.

3.4 Subgroup analysis

Based on the above results, we found correlations between dietary selenium and blood concentrations with ALT. Subgroup analyses further explored the effects of age, gender, BMI, diabetes, and physical activity on these associations. The present results showed that correlations between selenium intake and blood concentrations with ALT were not consistent. Significant associations were found between dietary selenium and ALT in participants aged 20–40 years, female, BMI > 30 , non-diabetic, and with or without physical activity (Figure 4). The results of interaction tests indicated that there were no statistically significant variations in the association between dietary selenium intake and ALT across different stratifications, implying that age, gender, BMI, diabetes, and physical activity did not have a significant impact on this positive correlation. However, interaction tests revealed that the association between selenium blood concentrations and ALT levels is significantly influenced by age, gender, and BMI stratifications (Figure 5). Higher selenium concentrations were associated with a significant increase in ALT levels across all age and gender stratifications, with a more pronounced effect in individuals aged 20–40 and in males. The increase in ALT levels was also significant in obese individuals. In addition, although the interaction test for physical activity and diabetes status was not significant, higher selenium concentrations still significantly increased ALT levels in all stratifications of physical activity and diabetes status.

4 Discussion

In this cross-sectional population-based study, dietary selenium intake was consistently positively associated with ALT and the ALT/AST ratio before and after adjustment for covariates. In addition, results indicated that high blood selenium was positively associated with ALT, AST, and ALT/AST ratio. However, ALP levels decreased with increasing blood selenium concentrations. In conclusion, this cross-sectional study demonstrated that dietary selenium intake and blood selenium concentration affect liver function parameters. Selenium is an essential trace element, and adequate intake is crucial

TABLE 1 Baseline characteristics of participants according to dietary selenium intake.

Variables	Dietary selenium intake (μg/day)				p value
	Q1 (<65.4) N = 1,717	Q2 (65.4–93.3) N = 1,717	Q3 (93.3–127.8) N = 1,715	Q4 (>127.8) N = 1,720	
Age, year, Mean (SD)	49.01 (17.70)	49.45 (17.90)	49.10 (17.16)	45.96 (16.02)	<0.001
Gender, N (%)					<0.001
Male	584 (34.01%)	687 (40.01%)	881 (51.37%)	1,188 (69.07%)	
Female	1,133 (65.99%)	1,030 (59.99%)	834 (48.63%)	532 (30.93%)	
Race, N (%)					<0.001
Mexican American	175 (10.19%)	205 (11.94%)	218 (12.71%)	231 (13.43%)	
Other Hispanic	202 (11.76%)	158 (9.20%)	168 (9.80%)	179 (10.41%)	
Non-Hispanic White	598 (34.83%)	671 (39.08%)	658 (38.37%)	593 (34.48%)	
Non-Hispanic Black	445 (25.92%)	442 (25.74%)	428 (24.96%)	423 (24.59%)	
Other race-including multi-racial	297 (17.30%)	241 (14.04%)	243 (14.17%)	294 (17.09%)	
Education level, N (%)					<0.001
Above high school	873 (50.84%)	986 (57.43%)	1,092 (63.67%)	1,058 (61.51%)	
Completed high school	391 (22.77%)	299 (17.41%)	267 (15.57%)	259 (15.06%)	
Less than high school	453 (26.38%)	432 (25.16%)	356 (20.76%)	403 (23.43%)	
Ratio of family income to poverty, Mean (SD)	2.81 (1.55)	3.10 (1.52)	3.35 (1.53)	3.21 (1.55)	<0.001
BMI, kg/m ² , Mean (SD)	29.44 (7.36)	29.46 (6.93)	29.88 (6.90)	30.51 (7.69)	<0.001
Diabetes, N (%)					0.948
Yes	287 (16.72%)	260 (15.14%)	265 (15.45%)	239 (13.90%)	
No	1,393 (81.13%)	1,409 (82.06%)	1,389 (80.99%)	1,428 (83.02%)	
Borderline	37 (2.15%)	48 (2.80%)	61 (3.56%)	53 (3.08%)	
Fasting glucose, mg/dL, Mean (SD)	110.96 (20.45)	110.48 (20.27)	112.75 (24.68)	112.63 (25.18)	0.004
Drink, N (%)					<0.001
Yes	647 (37.68%)	755 (43.97%)	839 (48.92%)	896 (52.09%)	
No	1,070 (62.32%)	962 (56.03%)	876 (51.08%)	824 (47.91%)	
Smoke, N (%)					0.105
Yes	693 (40.36%)	702 (40.89%)	710 (41.40%)	772 (44.88%)	
No	1,024 (59.64%)	1,015 (59.11%)	1,005 (58.60%)	948 (55.12%)	
Physical activity, N (%)					<0.001
Yes	723 (42.11%)	793 (46.19%)	864 (50.38%)	913 (53.08%)	
No	994 (57.89%)	924 (53.81%)	851 (49.62%)	807 (46.92%)	
ALT, U/L, Mean (SD)	20.27 (14.59)	20.93 (14.38)	22.98 (16.46)	25.13 (16.64)	<0.001
ALP, U/L, Mean (SD)	77.18 (25.49)	75.31 (24.40)	73.64 (25.44)	75.44 (23.88)	0.016
AST, U/L, Mean (SD)	20.55 (10.12)	21.03 (12.49)	22.18 (12.17)	22.15 (9.20)	<0.001
GGT, U/L, Mean (SD)	27.05 (32.73)	27.39 (31.75)	29.35 (43.50)	31.61 (35.50)	<0.001
ALT/AST, Mean (SD)	0.96 (0.34)	0.98 (0.33)	1.01 (0.35)	1.11 (0.37)	<0.001
TC, mmol/L, Mean (SD)	4.89 (1.06)	4.88 (1.05)	4.86 (1.01)	4.79 (1.05)	0.192
Blood selenium concentration, μg/L, Mean (SD)	187.66 (26.07)	187.52 (28.38)	188.87 (26.45)	190.76 (27.99)	<0.001

Significant results highlighted in bold. BMI, Body mass index; TC, Total cholesterol; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; ALP, Alkaline phosphatase.

TABLE 2 Baseline characteristics of participants according to blood selenium concentration.

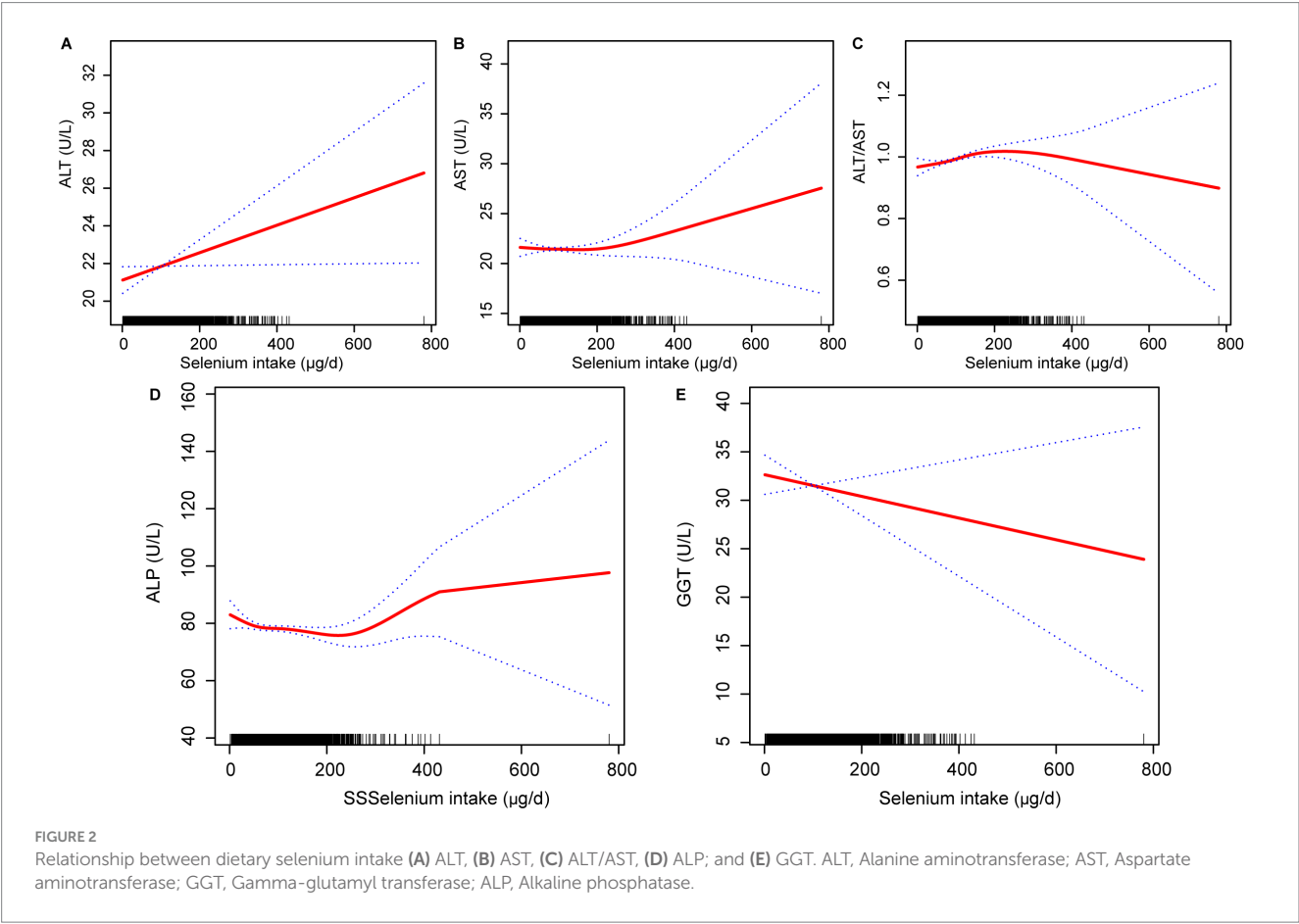
Variables	Blood selenium concentration (μg/L)				p value
	Q1 (<168.8)	Q2 (168.8–183.75)	Q3 (183.75–200.55)	Q4 (>200.55)	
	N = 1,716	N = 1,717	N = 1,718	N = 1,718	
Age, year, Mean (SD)	49.29 (18.08)	47.50 (17.31)	47.91 (17.07)	48.78 (16.63)	0.014
Gender, N (%)					<0.001
Male	758 (44.17%)	820 (47.76%)	836 (48.66%)	926 (53.90%)	
Female	958 (55.83%)	897 (52.24%)	882 (51.34%)	792 (46.10%)	
Race, N (%)					<0.001
Mexican American	157 (9.15%)	212 (12.35%)	242 (14.09%)	218 (12.69%)	
Other Hispanic	189 (11.01%)	185 (10.77%)	159 (9.25%)	174 (10.13%)	
Non-Hispanic White	604 (35.20%)	621 (36.17%)	666 (38.77%)	629 (36.61%)	
Non-Hispanic Black	559 (32.58%)	470 (27.37%)	367 (21.36%)	342 (19.91%)	
Other race-including multi-racial	207 (12.06%)	229 (13.34%)	284 (16.53%)	355 (20.66%)	
Education level, N (%)					<0.001
Above high school	916 (53.38%)	993 (57.83%)	1,051 (61.18%)	1,049 (61.06%)	
Completed high school	459 (26.75%)	430 (25.04%)	368 (21.42%)	387 (22.53%)	
Less than high school	341 (19.87%)	294 (17.12%)	299 (17.40%)	282 (16.41%)	
Ratio of family income to poverty, Mean (SD)	3.05 (1.60)	3.15 (1.54)	3.16 (1.57)	3.14 (1.50)	0.173
BMI, kg/m ² , Mean (SD)	29.65 (7.64)	29.83 (7.28)	29.97 (7.01)	29.87 (7.10)	0.662
Diabetes, N (%)					0.444
Yes	266 (15.50%)	247 (14.39%)	249 (14.49%)	289 (16.82%)	
No	1,407 (81.99%)	1,419 (82.64%)	1,418 (82.54%)	1,375 (80.03%)	
Borderline	43 (2.51%)	51 (2.97%)	51 (2.97%)	54 (3.14%)	
Fasting glucose, mg/dL, Mean (SD)	112.00 (27.17)	110.48 (18.44)	110.91 (20.36)	113.50 (25.04)	<0.001
Drink, N (%)					<0.001
Yes	728 (42.42%)	798 (46.48%)	838 (48.78%)	773 (44.99%)	
No	988 (57.58%)	919 (53.52%)	880 (51.22%)	945 (55.01%)	
Smoke, N (%)					0.056
Yes	737 (42.95%)	726 (42.28%)	700 (40.75%)	714 (41.56%)	
No	979 (57.05%)	991 (57.72%)	1,018 (59.25%)	1,004 (58.44%)	
Physical activity, N (%)					0.021
Yes	741 (43.18%)	825 (48.05%)	868 (50.52%)	859 (50.00%)	
No	975 (56.82%)	892 (51.95%)	850 (49.48%)	859 (50.00%)	
ALT, U/L, Mean (SD)	19.55 (12.01)	21.81 (15.33)	23.27 (15.48)	24.33 (18.20)	<0.001
ALP, U/L, Mean (SD)	75.93 (28.87)	74.44 (23.69)	76.96 (24.17)	74.07 (22.97)	0.235
AST, U/L, Mean (SD)	20.80 (10.00)	21.30 (11.02)	21.74 (9.63)	22.06 (13.11)	<0.001
GGT, U/L, Mean (SD)	28.75 (45.82)	27.06 (31.40)	29.21 (34.06)	30.49 (34.67)	0.044
ALT/AST, Mean (SD)	0.93 (0.32)	0.99 (0.34)	1.04 (0.36)	1.08 (0.37)	<0.001
TC, mmol/L, Mean (SD)	4.59 (0.93)	4.83 (1.05)	4.86 (1.02)	5.06 (1.09)	<0.001
Dietary selenium intake, μg/day, Mean (SD)	157.12 (10.20)	176.47 (4.26)	191.56 (4.86)	220.48 (25.55)	<0.001

Significant results highlighted in bold. BMI, Body mass index; TC, Total cholesterol; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; and ALP, Alkaline phosphatase.

TABLE 3 Multiple linear regression associations of dietary selenium intake (continuous) with liver function parameters.

Exposure	Outcome	β (95%CI), <i>p</i> value		
		Crude	Model 1	Model 2
Selenium intake	ALT	0.416 (0.345, 0.488) <0.001	0.163 (0.090, 0.236) <0.001	0.112 (0.041, 0.183) 0.002
	AST	0.162 (0.111, 0.213) <0.001	0.058 (0.005, 0.112) 0.032	0.049 (−0.005, 0.102) 0.075
	ALT/AST	0.010 (0.009, 0.012) <0.001	0.004 (0.002, 0.006) <0.001	0.002 (0.001, 0.004) 0.005
	ALP	−0.135 (−0.249, −0.020) 0.021	−0.079 (−0.197, 0.039) 0.190	−0.039 (−0.153, 0.076) 0.507
	GGT	0.266 (0.098, 0.433) 0.002	−0.048 (−0.223, 0.126) 0.588	−0.060 (−0.232, 0.111) 0.492

Crude: no covariates were adjusted. Model 1: age, gender, and race were adjusted. Model 2: age, gender, race, education, ratio of family income to poverty, diabetes, BMI, drink, smoke, physical activity, and TC were adjusted. Significant results highlighted in bold. BMI, Body mass index; TC, Total cholesterol; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; and ALP, Alkaline phosphatase.



for maintaining liver health. However, the use of selenium supplements should be approached with caution in light of our findings. While selenium has health benefits, excessive intake can lead to adverse effects, including potential effects on liver function. Therefore, clinicians should consider patients' total selenium intake when recommending supplements to avoid overconsumption.

The role of selenium as a micronutrient essential for various bodily functions has been well-documented over the decades. However, supplementation, especially in the context of the Selenium and Vitamin E Cancer Prevention Trial, has raised concerns due to adverse outcomes like increased incidence of type 2 diabetes and prostate cancer (32, 33). Reports from the 1930s about selenium's

potential hepatotoxic effects in overexposure cases set a precedent for careful selenium dosage consideration in supplementation practices (34). In a recent study, Li et al. (35) associated blood selenium with elevated AST in 15,328 samples from NHANES 2011–2018, which is consistent with our findings. However, they did not explore dietary selenium intake, subgroup analyses, or BMI's impact on selenium metabolism. Yang et al.'s cross-sectional study in China with 8,550 participants found significant ALT and AST increases with rising plasma selenium concentrations (36). They observed a linear correlation between plasma selenium and ALT and AST when converting plasma selenium concentrations into a categorical variable. Moreover, our results align with Longnecker et al. (37), who noted a

TABLE 4 Multiple linear regression associations of dietary selenium intake (quartile) with liver function parameters.

LFTs	Model	Dietary selenium intake (quartile)				<i>p</i> for trend
		Q1	Q2 β (95% CI)	Q3 β (95% CI)	Q4 β (95% CI)	
ALT	Crude	Reference	0.660 (−0.417, 1.738)	2.710 (1.651, 3.769)	4.862 (3.799, 5.924)	<0.001
	Model 1	Reference	0.046 (−0.995, 1.087)	1.102 (0.069, 2.134)	1.589 (0.524, 2.654)	<0.001
	Model 2	Reference	−0.064 (−1.073, 0.945)	0.660 (−0.348, 1.668)	0.897 (−0.140, 1.934)	0.039
AST	Crude	Reference	0.484 (−0.282, 1.250)	1.636 (0.882, 2.389)	1.606 (0.851, 2.361)	<0.001
	Model 1	Reference	0.198 (−0.562, 0.957)	0.922 (0.169, 1.675)	0.219 (−0.558, 0.996)	0.277
	Model 2	Reference	0.136 (−0.622, 0.893)	0.809 (0.052, 1.565)	0.113 (−0.666, 0.891)	0.439
ALT/AST	Crude	Reference	0.021 (−0.003, 0.045)	0.056 (0.032, 0.079)	0.149 (0.126, 0.173)	<0.001
	Model 1	Reference	0.006 (−0.018, 0.029)	0.016 (−0.007, 0.039)	0.068 (0.045, 0.092)	<0.001
	Model 2	Reference	0.003 (−0.018, 0.024)	0.001 (−0.021, 0.022)	0.042 (0.021, 0.064)	<0.001
ALP	Crude	Reference	−1.873 (−3.584, −0.161)	−3.536 (−5.218, −1.853)	−1.734 (−3.421, −0.047)	0.016
	Model 1	Reference	−1.831 (−3.509, −0.153)	−3.240 (−4.905, −1.575)	−0.735 (−2.452, 0.982)	0.220
	Model 2	Reference	−0.818 (−2.443, 0.808)	−1.858 (−3.482, −0.234)	−0.050 (−1.721, 1.620)	0.704
GGT	Crude	Reference	0.337 (−2.174, 2.848)	2.301 (−0.168, 4.770)	4.557 (2.080, 7.033)	<0.001
	Model 1	Reference	−0.556 (−3.043, 1.931)	0.271 (−2.196, 2.738)	0.716 (−1.828, 3.260)	0.453
	Model 2	Reference	−0.198 (−2.635, 2.239)	0.405 (−2.029, 2.840)	0.741 (−1.764, 3.246)	0.476

Crude: no covariates were adjusted. Model 1: age, gender, and race were adjusted. Model 2: age, gender, race, education, ratio of family income to poverty, diabetes, BMI, drink, smoke, physical activity, and TC were adjusted. Significant results highlighted in bold. BMI, Body mass index; TC, Total cholesterol; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; and ALP, Alkaline phosphatase.

TABLE 5 Multiple linear regression associations of blood selenium concentration (continuous) with liver function parameters.

Exposure	Outcome	β (95%CI), <i>P</i> value		
		Crude	Model 1	Model 2
Blood selenium	ALT	0.644 (0.509, 0.780) <0.001	0.527 (0.397, 0.658) <0.001	0.436 (0.308, 0.564) <0.001
	AST	0.205 (0.109, 0.301) <0.001	0.154 (0.058, 0.250) 0.002	0.112 (0.015, 0.208) 0.023
	ALT/AST	0.017 (0.014, 0.020) <0.001	0.014 (0.011, 0.017) <0.001	0.012 (0.009, 0.015) <0.001
	ALP	−0.228 (−0.443, −0.013) 0.038	−0.211 (−0.423, 0.000) 0.051	−0.207 (−0.414, −0.000) 0.049
	GGT	0.215 (−0.101, 0.530) 0.182	0.099 (−0.214, 0.413) 0.535	−0.109 (−0.420, 0.201) 0.489

Crude: no covariates were adjusted. Model 1: age, gender, and race were adjusted. Model 2: age, gender, race, education, ratio of family income to poverty, diabetes, BMI, drink, smoke, physical activity, and TC were adjusted. Significant results highlighted in bold. BMI, Body mass index; TC, Total cholesterol; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; and ALP, Alkaline phosphatase.

positive correlation between selenium intake and ALT levels in United States selenium-enriched regions. Such corroborative findings reinforce the theoretical link between selenium and liver health. The major antioxidant effects of selenium are mediated by selenium-dependent glutathione peroxidase (GPx) and selenoproteins (38–40). Maximum selenoprotein levels in the blood were in the range of 70–90 $\mu\text{g/L}$ (41). The increase in blood selenium above these levels does not reflect an increase in selenoprotein levels but primarily reflects non-specific binding of selenomethionine to albumin instead of methionine (41). The mean serum selenium level of the participants was 186.11 $\mu\text{g/L}$, and the increase in selenium levels may have resulted in the non-specific binding of selenomethionine. A previous report also showed that the dietary selenium intake of United States adults far exceeds the amount of selenium needed to achieve maximal blood selenoprotein levels (42). Adequate selenium intake regulates the activity of selenoproteins, such as thioredoxin reductase (TrxR), thereby enhancing the body's antioxidant capacity and mitigating the adverse effects of oxidative stress (43). TrxR and GPx, as core

components of the thioredoxin (Trx) and glutathione (GSH) systems, constitute key thiol/selenol-dependent antioxidant systems. These systems maintain redox balance within the body and protect cells from oxidative damage (44–46). Selenium deficiency leads to decreased TrxR activity (47, 48). Compared to low selenium levels, selenium supplementation increases TrxR activity but stabilizes at normal levels. Notably, excessive selenium does not further increase TrxR activity. Conversely, in an acetaminophen (APAP)-induced liver injury model, selenium-enriched mice exhibit more severe liver damage than mice with normal selenium levels (49). Typical selenium compounds, such as sodium selenite, have been widely used at high concentrations to inhibit tumor cell growth (50). In mammals, selenite is reduced by TrxR to produce hydrogen selenide, which is highly reactive with oxygen, thereby generating reactive oxygen species (ROS). Selenomethionine, an organic selenium compound, is one of the predominant forms of selenium in nature. It is metabolized through various pathways within cells. Compared to selenite, higher selenium doses generates hydrogen selenide as a metabolic product,

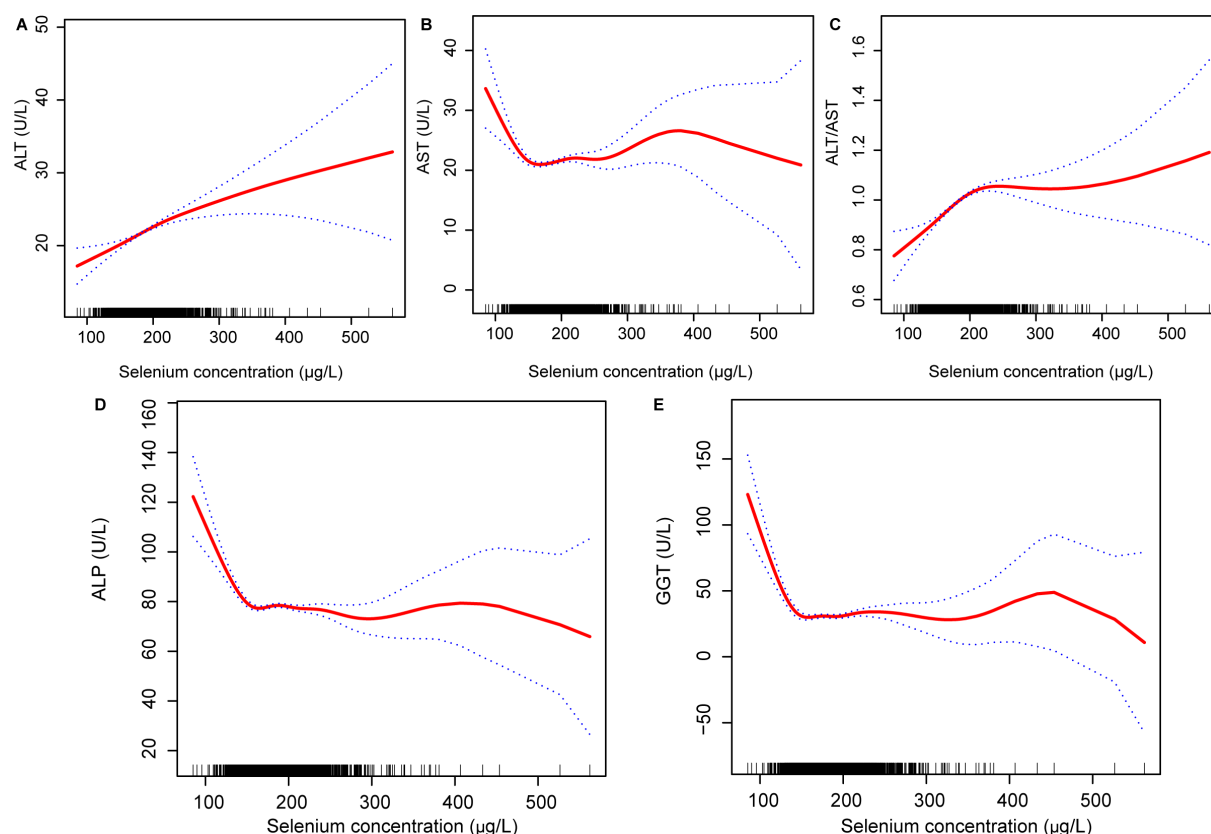


FIGURE 3
Relationship between blood selenium concentration (A) ALT, (B) AST, (C) ALT/AST, (D) ALP; and (E) GGT. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; and ALP, Alkaline phosphatase.

leading to oxidative stress (51). In summary, excessive selenium significantly increases oxidative stress levels within hepatocytes, causing cellular damage, which may be the underlying biological mechanism for the observed elevation in ALT levels induced by excessive selenium.

In subgroup analyses, significant associations were found between dietary selenium and ALT in participants aged 20–40 years, female, BMI >30, and non-diabetes. The results of interaction tests indicated that there were no statistically significant variations in the association between dietary selenium intake and ALT across different stratifications. However, interaction tests revealed that the association between selenium blood concentrations and ALT levels was significantly influenced by age, gender, and BMI stratifications. Higher selenium concentrations were associated with a significant increase in ALT levels across all age and gender stratifications, with a more pronounced effect in individuals aged 20–40 and in males. The increase in ALT levels was also significant in obese individuals. Adults between the ages of 20 and 40 are in a period of high metabolic activity, which may make them more sensitive to selenium intake, especially if the intake exceeds the body's needs, which may have adverse effects on liver function (52). Individuals with higher body weight, especially those who are obese, may have altered selenium distribution and action owing to metabolic activities in adipose tissue, which could amplify adverse effects on liver function (53). These findings underscore the need to consider individual variability in research on the relationship between selenium intake and liver health.

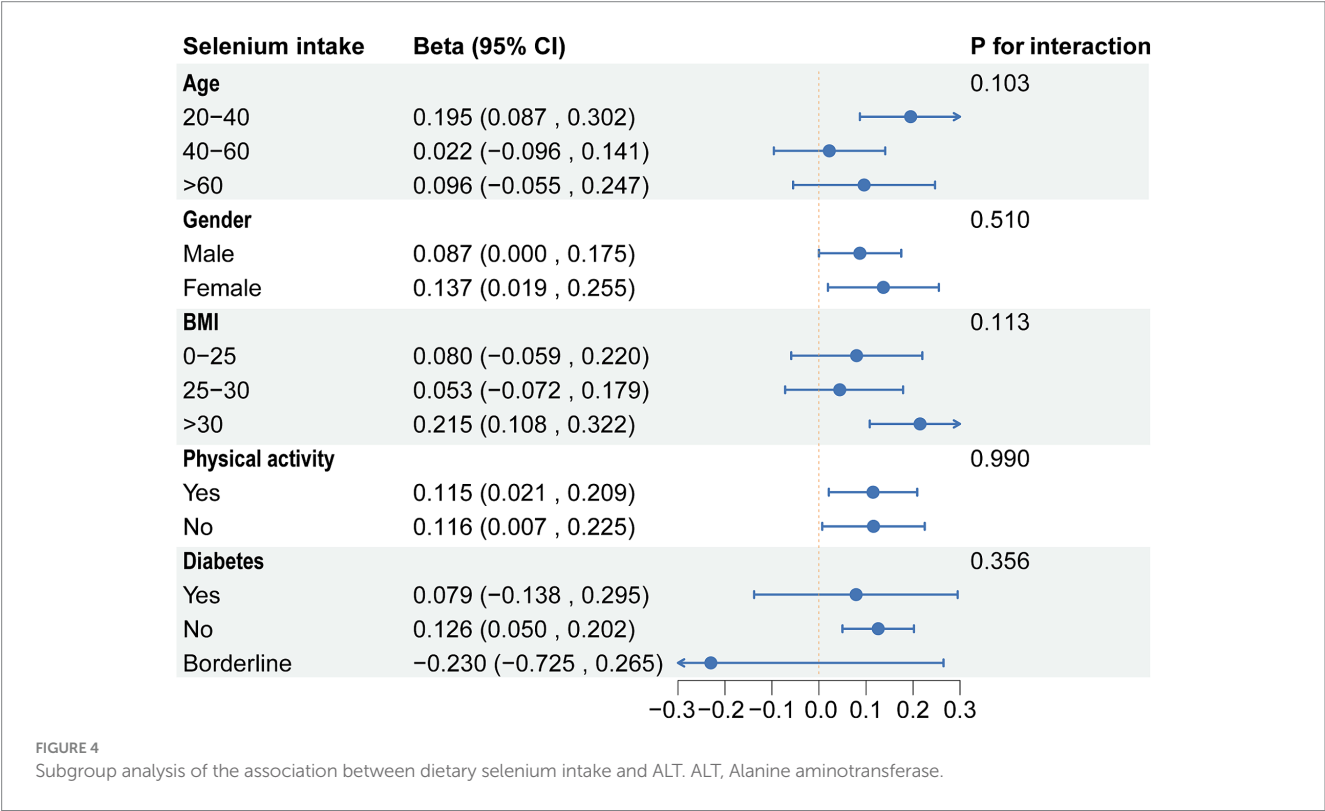
Future strategies and guidelines should be personalized, accounting for the unique needs and risks of diverse populations to ensure beneficial selenium intake levels for liver health.

In this cross-sectional study, the effects of dietary selenium intake on ALT, ALT/AST, and GGT were consistent with the results for blood selenium concentration. However, the findings regarding AST and ALP were inconsistent. Methodologically, the differences might be due to the nature of the data collection. Dietary selenium intake data is typically based on 24-h dietary recalls, which can be affected by recall bias and reporting accuracy. Blood selenium levels, on the other hand, reflect the actual storage and utilization of selenium in the body, which may have a time lag and individual variability. Additionally, the bioavailability of dietary selenium can vary depending on the type of food and an individual's absorption capacity, leading to different impacts of dietary selenium intake and blood selenium levels on AST and ALP. Biologically, the metabolism and function of selenium are complex and varied. Selenium primarily exerts its antioxidant effects through GPx and selenoprotein P, which have different expression and activity levels in various tissues, possibly resulting in differential impacts on liver enzymes. ALT is predominantly found in hepatocytes, while AST is expressed in multiple tissues including the liver, heart, and muscles, which might explain the different mechanisms of selenium's effects on ALT and AST. Furthermore, selenium might influence ALP levels through its role in regulating apoptosis and inflammatory responses, which can vary depending on an individual's genetic background and health status.

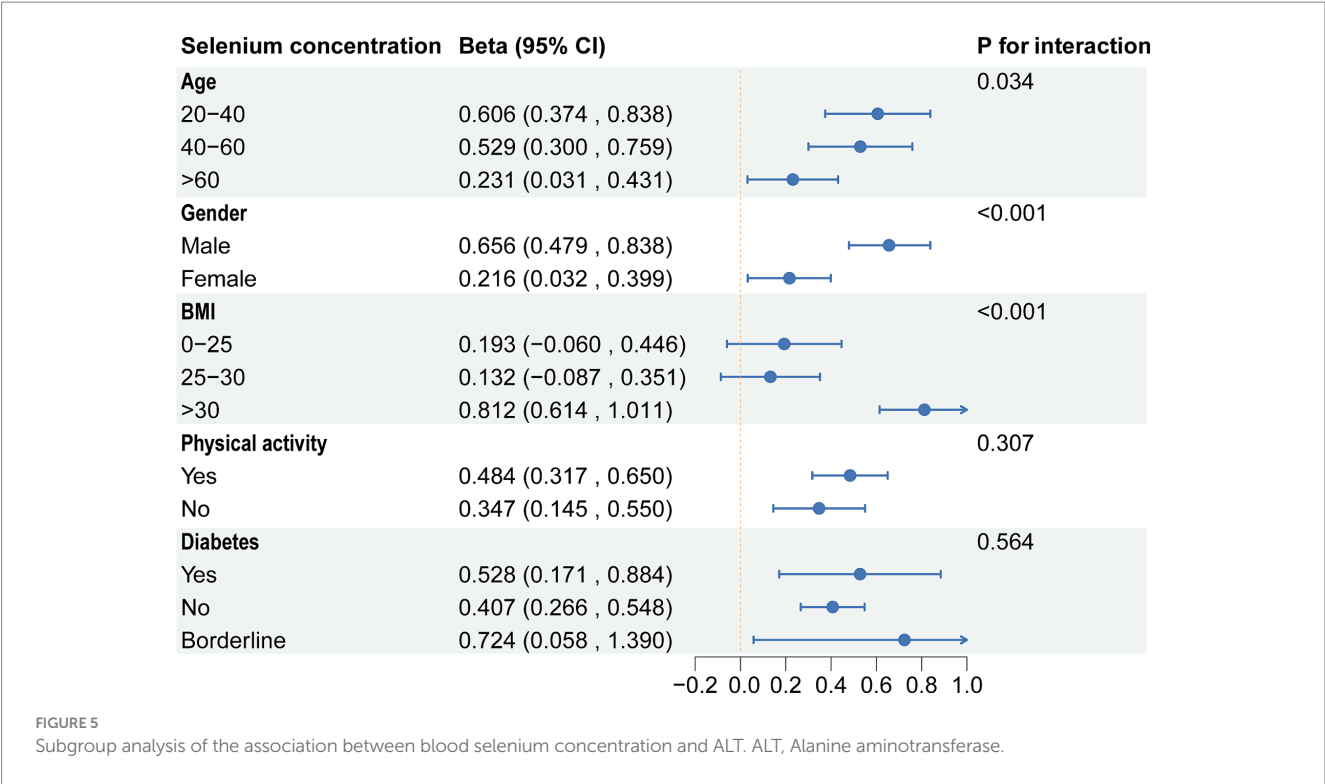
TABLE 6 Multiple linear regression associations of blood selenium concentration (quartile) with liver function parameters.

LFTs	Model	Blood selenium concentration (quartile)				p for trend
		Q1	Q2 β (95% CI)	Q3 β (95% CI)	Q4 β (95% CI)	
ALT	Crude	Reference	2.263 (1.161, 3.364)	3.723 (2.643, 4.803)	4.779 (3.711, 5.848)	<0.001
	Model 1	Reference	1.947 (0.889, 3.004)	3.191 (2.153, 4.230)	3.772 (2.742, 4.802)	<0.001
	Model 2	Reference	1.468 (0.441, 2.495)	2.591 (1.579, 3.602)	2.783 (1.771, 3.795)	<0.001
AST	Crude	Reference	0.501 (−0.282, 1.284)	0.942 (0.174, 1.710)	1.256 (0.496, 2.016)	<0.001
	Model 1	Reference	0.432 (−0.341, 1.206)	0.784 (0.024, 1.544)	0.862 (0.108, 1.616)	0.018
	Model 2	Reference	0.217 (−0.556, 0.990)	0.504 (−0.257, 1.266)	0.432 (−0.330, 1.194)	0.209
ALT/AST	Crude	Reference	0.064 (0.039, 0.089)	0.107 (0.082, 0.131)	0.145 (0.121, 0.169)	<0.001
	Model 1	Reference	0.054 (0.031, 0.078)	0.091 (0.068, 0.114)	0.119 (0.096, 0.141)	<0.001
	Model 2	Reference	0.044 (0.023, 0.066)	0.078 (0.057, 0.099)	0.097 (0.075, 0.118)	<0.001
ALP	Crude	Reference	−1.495 (−3.242, 0.253)	1.022 (−0.692, 2.735)	−1.866 (−3.561, −0.170)	0.235
	Model 1	Reference	−1.008 (−2.718, 0.703)	1.622 (−0.058, 3.303)	−1.448 (−3.115, 0.218)	0.455
	Model 2	Reference	−0.952 (−2.608, 0.704)	1.990 (0.359, 3.621)	−1.841 (−3.473, −0.209)	0.259
GGT	Crude	Reference	−1.694 (−4.260, 0.872)	0.456 (−2.060, 2.972)	1.740 (−0.749, 4.230)	0.044
	Model 1	Reference	−1.550 (−4.083, 0.984)	0.509 (−1.980, 2.997)	0.978 (−1.490, 3.447)	0.167
	Model 2	Reference	−2.733 (−5.219, −0.248)	−0.823 (−3.271, 1.625)	−1.525 (−3.976, 0.925)	0.590

Crude: no covariates were adjusted. Model 1: age, gender, and race were adjusted. Model 2: age, gender, race, education, ratio of family income to poverty, diabetes, BMI, drink, smoke, physical activity, and TC were adjusted. Significant results highlighted in bold. BMI, Body mass index; TC, Total cholesterol; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma-glutamyl transferase; and ALP, Alkaline phosphatase.



While our study has revealed associations between blood selenium levels, dietary selenium intake, and liver function parameters, it is important to further investigate the different forms of selenium in the blood and their varying effects on liver function. Selenium exists in multiple forms in the blood, including inorganic selenium (such as selenite and selenate) and organic selenium (such as selenomethionine



and selenocysteine). These different forms of selenium have distinct biological activities and metabolic pathways, which may impact liver function in various ways. For instance, organic forms of selenium like selenomethionine can be non-specifically incorporated into body proteins as a long-term selenium storage, whereas inorganic selenium is mainly involved in the synthesis of selenoproteins like GPx. These different forms of selenium may differentially affect antioxidant capacity, cellular protection, and inflammatory responses, thus exhibiting varying effects on liver function parameters. Therefore, future studies should consider selenium speciation analysis to differentiate between these various forms of selenium and their respective impacts on liver function. This will provide a more comprehensive and detailed understanding of the biological roles of selenium and its mechanisms in liver function regulation, thereby offering a scientific basis for developing more precise public health strategies.

This study assessed the impact of dietary selenium intake and blood selenium concentration on liver function parameters among US adults using the latest NHANES dataset. A key strength of this study is its large sample size, coupled with a detailed characterization of the study population. Additionally, the adjustment for covariates enhances the reliability of results. Finally, we performed subgroup analyses and interaction tests to explore differences in these associations by age, gender, race, education level, and BMI.

Although this study provided a comprehensive analysis of dietary selenium intake and blood selenium concentration's effects on liver function, it has several limitations. First, blood selenium concentration, a direct biomarker of exposure, can be influenced by short-term dietary changes, individual metabolic differences, and physiological conditions, potentially not reflecting long-term intake accurately. Additionally, the relationship between dietary intake and

blood concentration can be complicated by individual differences in absorption and metabolism. Second, despite adjusting for numerous potential confounders, the study cannot eradicate all confounding biases. For instance, dietary records and questionnaires depend on self-reporting, susceptible to recall or reporting bias. Furthermore, this observational study identified associations between dietary selenium intake, blood concentrations, and liver function, but it cannot establish causality. Future research should confirm these associations via prospective or interventional studies. Third, despite this study exploring the association between selenium levels and various liver function indicators (such as ALT, AST, ALP, and GGT), we did not include bilirubin in our analysis. Bilirubin is an important indicator of liver metabolic capacity and biliary system patency, and its levels are influenced by multiple factors including hemolysis, liver metabolic function, and biliary tract patency. While ALT, AST, ALP, and GGT are also affected by various factors, the metabolic pathway of bilirubin is more complex and involves additional potential confounders. Therefore, we chose not to include bilirubin in the analysis for this study. We acknowledge that this decision introduces certain limitations, making the interpretation of selenium's impact on overall liver function less comprehensive. Future research should incorporate bilirubin into the analysis to more accurately assess the multifaceted effects of selenium on liver function. Lastly, given that the NHANES database does not provide the exact dates of dietary recall interviews and blood sample collections, we cannot directly analyze the temporal relationship between these two variables. This hinders our ability to assess the time-dependent effects of dietary selenium intake and blood selenium concentrations on liver function parameters. Future studies also should consider the temporal relationship between dietary intake and biospecimen collection in their design to improve the accuracy and reliability of the findings.

5 Conclusion

This population-based, cross-sectional study demonstrated that dietary selenium intake and blood selenium concentration impact liver function parameters in a population with high selenium intakes and blood concentrations and without major liver disturbances. Public health strategies should consider promoting moderate rather than excessive selenium intake as part of liver disease prevention efforts. Further research should also focus on the relationship between dietary selenium intake and blood selenium concentration with liver function in diverse populations to develop more precise prevention strategies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by the National Center for Health Statistics Research Ethics Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

QL: Conceptualization, Writing – original draft. RH: Funding acquisition, Investigation, Writing – review & editing. ZP:

Conceptualization, Supervision, Writing – review & editing. MZ: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The relationship between 25-hydroxy vitamin D and serum asprosin in patients with type 2 diabetes in the community

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Objectives: This study aimed to investigate the link between 25-hydroxy vitamin D and serum asprosin in individuals with type 2 diabetes within the community. The goal was to provide a foundation for clinical interventions.

Methods: Between November 2019 and July 2021, data from 463 patients with type 2 diabetes were consistently gathered at a community health service station in Southeast Shanxi Province. General information and laboratory metrics were compiled, including serum asprosin levels. The participants were categorized based on three serum asprosin quantiles, allowing for a comparison of various factors among the groups. The correlation between serum asprosin levels and other factors was analyzed. Employing a general linear model, the connection between 25-hydroxy vitamin D and serum asprosin levels was studied. Utilizing three quantiles of 25-hydroxy vitamin D, serum asprosin was treated as the dependent variable, while 25-hydroxy vitamin D served as the independent variable for linear regression analysis.

Results: As serum asprosin increased, there were gradual increments in age, disease duration, SBP, BMI, WC, creatinine, and SUA levels ($P < 0.05$). Conversely, HbA1c, HDL-C, GFR, and 25-hydroxy vitamin D levels exhibited gradual declines ($P < 0.05$). Age, 25-hydroxy vitamin D, SUA, creatinine, and LDL-C emerged as independent influencing factors for serum asprosin. Across the 1st to 3rd 25-hydroxy vitamin D quantiles, elevated 25-hydroxy vitamin D levels correlated with a gradual reduction in mean serum asprosin ($P < 0.05$).

Conclusion: Serum asprosin levels demonstrate an inverse correlation with 25-hydroxy vitamin D levels in community-dwelling individuals with type 2 diabetes. Serum asprosin levels might independently contribute to 25-hydroxy vitamin D levels.

KEYWORDS

diabetes, type 2, asprosin, 25-hydroxy vitamin D, community, insulin resistance

Introduction

In 2016, Romere et al. made the initial discovery of asprosin, encoded by two exons (exon 65 and exon 66) of the fibrillin 1 (FBN1) gene, as a novel adipokine protein involved in glucose production (1). This finding emerged from a study on patients with Neonatal Progeroid Syndrome (NPS). Individuals with NPS, who exhibit asprosin deficiency due to truncated FBN1 mutations, maintain normal blood glucose levels despite reduced plasma insulin levels (1). Extensive research has shown a relationship between asprosin concentration and insulin resistance (IR) as well as diabetes in both mice and humans. A cross-sectional analysis involving 143 subjects demonstrated elevated asprosin levels in Impaired Glucose Regulation (IGR) and Type 2 Diabetes Mellitus (T2DM) groups in comparison to the Normal Glucose Regulation (NGR) group. Asprosin exhibited positive correlation with insulin resistance homeostatic model assessment (HOMA-IR) and negative correlation with β -cell function (HOMA- β) (2). Moreover, a case-control study encompassing 170 subjects identified higher T2DM-associated asprosin levels in adults compared to the control group. Notably, an independent relationship between fasting plasma glucose and asprosin in T2DM patients was observed, in alignment with numerous other studies (3). Animal investigations have also indicated heightened liver asprosin levels in Type 1 Diabetes Mellitus (T1DM) mice (4). Consequently, serum asprosin could serve as a biomarker for early diabetes diagnosis.

The principal form of vitamin D in the body, 25-hydroxy vitamin D, contributes to preserving pancreatic islet β -cell function and inhibiting β -cell apoptosis (5). Studies have revealed that deficiency in 25-hydroxy vitamin D impacts glucose metabolism in individuals with Type 2 Diabetes Mellitus (T2DM) and is presumed to associate with insulin resistance (6). A link between adipocytokines and blood 25-hydroxy vitamin D levels has also been established. Among patients with T2DM, fasting serum asprosin levels increase and demonstrate a strong association with bone mineral density (3). Additionally, research involving prenatal screening for Trisomy 21 demonstrated an inverse correlation between amniotic fluid 25-hydroxy vitamin D and asprosin levels (7). Despite these findings, the intricate relationship among 25-hydroxy vitamin D, asprosin, and T2DM necessitates further elucidation. One plausible hypothesis is that 25-hydroxy vitamin D might influence the production and/or secretion of adipocytokines. Whether a linear negative correlation exists between these factors in T2DM patients remains uncertain. This study thus collected clinical data from community-based T2DM patients to analyze the connection between 25-hydroxy vitamin D and serum asprosin levels.

Materials and methods

Research participants

The subjects of the present report were those of the previous study (Xu et al, 2022) with the same inclusion criteria. Clinical and biochemical data were taken from the previous study (methods outlined in Xu et al, 2022) (8), possession of complete clinical data,

and willingness to cooperate with the study. Exclusion criteria encompassed patients with renal insufficiency characterized by creatinine levels exceeding 178 μ mol/L, type 1 diabetes, diabetes resulting from other endocrine disorders, diabetic ketoacidosis, diabetic hyperosmolar coma, severe liver or kidney dysfunction, severe infection, malignant tumors, individuals with communication disorders like mental illness, and those unable to collaborate with the study's requirements. After excluding 35 participants due to incomplete clinical data, this investigation analyzed clinical information from 463 patients.

Research methods

General data collection

General data collection involved measuring patients' height and weight to calculate the body mass index (BMI). Patient demographic information, such as gender, age, abdominal circumference, systolic and diastolic blood pressure, and the usage of hypoglycemic medications and insulin, was gathered. Fasting venous blood samples were obtained to measure fasting plasma glucose (FPG), 2-hour postprandial plasma glucose (2hPG), serum uric acid (SUA), creatinine (CRE), total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using Beckman Automatic Biochemical Analyzer (USA, BK-200). High-pressure liquid phase assessment (Roche 501, Switzerland) was conducted to determine glycated hemoglobin A1c (HbA1c) levels. Roche Diagnostic Products (Shanghai) Co. Ltd provided serum 25-hydroxy vitamin D concentration determination kits, utilizing chemiluminescence methods with within-batch CV < 8% and between-batch CV < 10%.

Measurement of serum asprosin

After fasting for 8–10 hours, venous blood samples were collected the next morning, followed by centrifugation at 3000 rpm for 15 minutes. Serum asprosin levels were determined using enzyme-linked immunosorbent assay (ELISA) provided by Hepeng (Shanghai) Biotechnology Co. Ltd. All samples were stored at -80°C . Double-well duplication was employed for measurement, ensuring batch-to-batch difference < 11% and intra-batch difference < 8%. Strict adherence to kit and instrument manuals was maintained. Patients with T2DM were categorized into three groups based on serum asprosin tertiles: T1 (asprosin < 295.0 pg/ml, 153 cases), T2 (asprosin 295.0–370.5 pg/ml, 153 patients), and T3 (asprosin > 370.5 pg/ml, 157 cases).

Statistical processing

Statistical analysis was performed using SPSS 22.0 software. Normally distributed metric data were expressed as Mean $\pm$ SD. One-way ANOVA was used for group comparisons, followed by LSD tests for pairwise comparisons. Non-normally distributed measurement data were expressed as M(Q1, Q3), and Mann-Whitney rank sum tests were applied for intergroup comparisons. Pearson correlation analysis was conducted for normally distributed data, while Spearman correlation analysis was used for data not conforming to bivariate normal distribution.

The relationship between serum asprosin and 25-hydroxyvitamin D tertiles was evaluated using a general linear model, with statistical significance defined as $P < 0.05$.

Results

The clinical characteristics of the patient

Among the 463 patients with type 2 diabetes, 266 were male with a mean age of 54.3 ± 13.0 years, and 197 were female with a mean age of 59.6 ± 12.1 years. The median duration of diabetes was 10 years, and

the average glycated hemoglobin was 8.8%. Patients were categorized into three groups based on serum asprosin quantiles. As serum asprosin levels increased, patient age, disease duration, SBP, BMI, WC, creatinine, and SUA levels also increased, while levels of HbA1c, HDL-C, GFR, and 25-hydroxy vitamin D decreased (Table 1).

The correlation between serum asprosin and other variables

Serum asprosin displayed positive correlations with disease duration, age, SBP, BMI, WC, TG, creatinine, SUA, AST, and

TABLE 1 Comparison of clinical data for 463 community-based patients with type 2 diabetes.

Serum asprosin (pg/ml)	Gender (male /female, e.g)	Course of disease* (years)	Age (years)	SBP (mmHg)	DBP (mmHg)	BMI (kg/m ²)	WC (cm)	TC (mmol/L)	TG* (mmol/L)	LDL-C (mmol/L)
<295	153 (71/82)	9.0 (4.0,15.5)	53.5 $\pm$ 13.1	130.1 $\pm$ 17.4	78.8 $\pm$ 10.4	25.1 $\pm$ 3.6	90.78 $\pm$ 10.56	4.6 $\pm$ 1.1	1.5 (1.0,2.2)	2.7 $\pm$ 0.8
295~370.5	156 (96/60)	10.0 (5.0,16.0)	57.3 $\pm$ 12.5	132.5 $\pm$ 17.3	79.8 $\pm$ 10.9	25.8 $\pm$ 3.7	93.32 $\pm$ 10.06	4.7 $\pm$ 1.2	1.5 (1.1,2.1)	2.8 $\pm$ 1.0
>370.5	154 (99/55)	12.0 (16.0,17.0)	58.8 $\pm$ 12.5	135.5 $\pm$ 17.8	80.4 $\pm$ 10.7	26.8 $\pm$ 4.0	98.26 $\pm$ 11.35	4.6 $\pm$ 1.2	1.7 (1.2,2.5)	2.7 $\pm$ 0.8
χ^2 /F value	6.099	5.984	7.138	3.700	0.885	8.554	19.278	0.404	6.163	1.688
P-value	0.002	0.050	0.001	0.025	0.413	<0.001	<0.001	0.668	0.046	0.186

Serum asprosin (pg/ml)	HDL-C (mmol/L)	AST* (mmol/L)	ALT* (mmol/L)	CRE* (μ mol/L)	GFR (mL/min)	FPG (mmol/L)	PBG (mmol/L)	HbA1c (%)	SUA (μ mol/L)	25-hydroxy vitamin D (nmol/L)
<295	1.0 $\pm$ 0.3	17.0 (14.0,21.5)	16.0 (12.5,26.5)	56.0 (46.0,66.0)	126.77 $\pm$ 35.61	8.2 $\pm$ 3.0	13.2 $\pm$ 4.2	9.3 $\pm$ 2.1	262.4 $\pm$ 60.8	31.0 (25.5,39.4)
295~370.5	0.98 $\pm$ 0.25	18.0 (15.0, 24.0)	17.0 (13.0,29.0)	63.0 (52.0,72.5)	113.34 $\pm$ 31.44	7.5 $\pm$ 2.8	12.0 $\pm$ 3.9	8.7 $\pm$ 1.8	329.6 $\pm$ 54.3	30.7 (24.9,37.6)
>370.5	0.9 $\pm$ 0.2	20.0 (16.0,27.0)	22.0 (14.0,34.0)	68.0 (58.0,80.0)	107.31 $\pm$ 67.26	7.5 $\pm$ 2.4	12.1 $\pm$ 4.0	8.4 $\pm$ 1.7	392.7 $\pm$ 83.0	29.2 (21.6,37.5)
χ^2 /z/t value	3.421	2.915	1.680	51.195	6.726	3.857	3.996	9.224	145.285	3.542
P-value	0.034	0.055	0.188	<0.001	0.001	0.022	0.019	<0.001	<0.001	0.030

Serum asprosin (pg/ml)	taking metformin (n,%)	taking glycosidase inhibitors (n,%)	taking sulfonylureas (n,%)	taking DPPIV enzyme inhibitors (n,%)	taking thiazolidinedione (n,%)	using insulin (n,%)
<295	78 (51.0)	42(27.5)	34(22.2)	0(0)	2(1.3)	69(45.1)
295~370.5	69(45.1)	47(30.7)	33(21.6)	3(2.0)	7(4.6)	82(53.6)
>370.5	77(49.0)	58(36.9)	39(24.8)	4(2.5)	1(0.6)	77(49)
χ^2 /z/t value	1.061	1.130	0.869	0.856	0.450	1.060
P-value	0.328	0.178	0.857	0.881	1.000	0.331

Continuous data are presented as mean $\pm$ standard deviation. Count data are presented as percentages. Skewed distribution data are expressed as median (25th, 75th) percentiles. SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; TG, Triglycerides; LDL-C, Low-Density Lipoprotein Cholesterol; HDL-C, High-Density Lipoprotein Cholesterol; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; CRE, Serum Creatinine; GFR, Glomerular Filtration Rate; FPG, Fasting Plasma Glucose; PBG, 2-Hour Postprandial Blood Glucose; HbA1c, Glycated Hemoglobin; SUA, Serum Uric Acid.
*Means non normal distribution.

ALT ($r = 0.114, 0.168, 0.126, 0.189, 0.276, 0.104, 0.332, 0.622, 0.205$, and 0.144 , respectively; $P < 0.05$). Conversely, serum asprosin exhibited negative correlations with HDL-C, GFR, FPG, HbA1c, and 25-hydroxy vitamin D ($r = -0.121, -0.165, -0.113, -0.194$, and -0.122 , respectively; $P < 0.05$) (Table 2).

TABLE 2 Correlation analysis results between serum asprosin and other variables.

variable	Groups	
	<i>r</i>	<i>P</i>
Duration of the disease* (years)	0.114	0.015
Age (years)	0.168	<0.001
SBP(mmHg)	0.126	0.007
DBP(mmHg)	0.061	0.190
BMI(kg/m ²)	0.189	<0.001
WC(cm)	0.276	<0.001
TC(mmol/L)	0.008	0.870
TG*(mmol/L)	0.104	0.026
LDL-C(mmol/L)	0.015	0.752
HDL-C(mmol/L)	-0.121	0.009
CRE*(μmol/L)	0.332	<0.001
GFR(mL/min)	-0.165	<0.001
SUA(μmol/L)	0.622	<0.001
FPG(mmol/L)	-0.113	0.015
HbA1c(%)	-0.194	<0.001
AST*(U/L)	0.205	<0.001
ALT*(U/L)	0.144	<0.001
25-hydroxy vitamin D	-0.122	0.009

Continuous data are presented as Pearson correlation coefficient (*r*). Skewed distribution data are expressed as median (25th, 75th) percentiles. SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; BMI, Body Mass Index; WC, Waist Circumference; TC, Total Cholesterol; TG, Triglycerides; LDL-C, Low-Density Lipoprotein Cholesterol; HDL-C, High-Density Lipoprotein Cholesterol; CRE, Serum Creatinine; GFR, Glomerular Filtration Rate; SUA, Serum Uric Acid; FPG, Fasting Plasma Glucose; HbA1c, Glycated Hemoglobin; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase.

TABLE 3 Multiple stepwise linear regression analysis of factors influencing serum asprosin in patients with type 2 diabetes.

independent variable	β value	SE value	β'	t value	<i>P</i> value	95%CI
age	0.956	0.228	0.137	4.187	0.000	0.507~1.404
25-hydroxyvitamin D	-0.932	0.202	-0.149	-4.626	0.000	-1.328~-0.536
SUA	0.686	0.036	0.660	19.251	0.000	0.616~0.757
creatinine	0.394	0.158	0.086	2.488	0.013	0.083~0.705
LDL-C	7.053	3.308	0.070	2.132	0.034	0553~13.553

LDL-C, Low-Density Lipoprotein Cholesterol; CRE, Serum Creatinine; SUA, Serum Uric Acid; 25-hydroxyvitamin D.

The results of multiple stepwise linear regression analysis of serum asprosin level and related indexes

Utilizing multiple stepwise linear regression analysis with serum asprosin as the dependent variable and age, BMI, SBP, HbA1c, creatinine, LDL-C, 25-hydroxy vitamin D, and SUA as independent variables, the study found that age, 25-hydroxy vitamin D, SUA, creatinine, and LDL-C were independent influencing factors for serum asprosin ($P < 0.05$, Table 3).

Correlation analysis of 25-hydroxy vitamin D and serum asprosin levels indifferent groups

By grouping patients into three quantiles of 25-hydroxy vitamin D (All patients: T1 < 26.2 nmol/L, T2 26.2~35.5 nmol/L, T3 > 35.5 nmol/L; Male: T1 < 263.3nmol/L, T2 23.3~36.7 nmol/L, T3 > 36.7 nmol/L;Female:T1 < 25.3 nmol/L, T2 25.3~40.8 nmol/L, T3 > 40.8 nmol/L);, a general linear regression model was used with serum asprosin as the dependent variable. Mean serum asprosin levels (95% CI) for T1, T2, and T3 were 360.6 (346.2, 375.5), 328.4 (315.5, 341.3), and 324.0 (309.8, 338.2) in all patients, Mean serum asprosin levels (95% CI) for T1, T2, and T3 were 374.1 (352.1, 396.0, 337.1 (323.4, 350.8), and 351.6 (330.5 372.6) in male patients, Mean serum asprosin levels (95% CI) for T1, T2, and T3 were 354.8 (324.2, 385.4), 322.8 (306.6, 338.9), and 284.6 (259.1, 309.9) in female patients, respectively. Compared to the T1 group, serum asprosin levels were significantly reduced in the other two groups between all patients and female patients ($P < 0.01$). As 25-hydroxy vitamin D levels increased from T1 to T3, mean serum asprosin gradually decreased (Figure 1). Compared to T2 groups, serum asprosin levels were significantly increased in male patients (Figure 1).

Discussion

The findings of this study demonstrated that in community-dwelling patients with type 2 diabetes, there was a gradual increase in age, disease duration, SBP, BMI, WC, creatinine, and SUA levels with the rise in serum asprosin levels. Conversely, serum asprosin

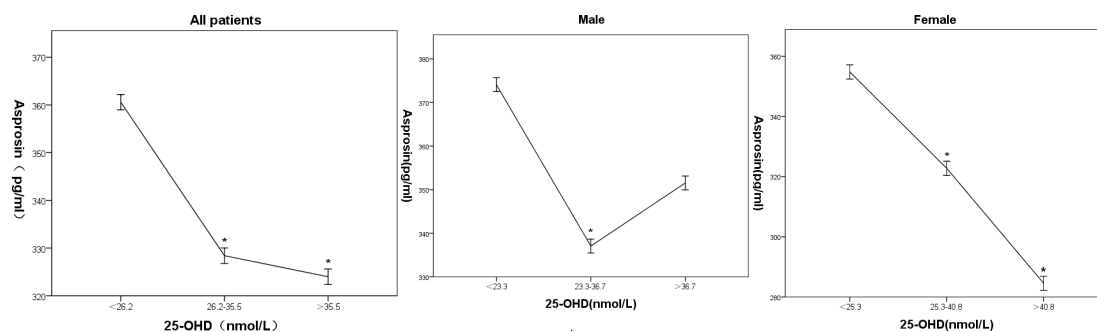


FIGURE 1

Association of 25-hydroxy Vitamin D with Serum Asprosin in Patients with Type 2 Diabetes in all, male and female patients. Mean serum asprosin and 95% confidence intervals corresponding to three quantiles of 25-hydroxyvitamin D; * indicates statistical significance compared to the first quantile array (* $P < 0.05$).

displayed an inverse correlation with 25-hydroxy vitamin D levels, indicating a gradual decrease in serum asprosin as 25-hydroxy vitamin D levels increased.

25-hydroxy vitamin D deficiency has been identified as a common underlying factor for metabolic disorders like obesity, type 2 diabetes, and hypertension (9–12). Asprosin, a novel protein hormone found in white adipose tissue, has been shown in animal studies to exhibit elevated serum levels in mammals with insulin resistance (1, 13). In population-based investigations involving polycystic ovary syndrome patients and women with type 2 diabetes, higher serum asprosin levels were identified compared to healthy individuals (14). The present study revealed that heightened serum asprosin levels were associated with indicators linked to insulin resistance, including elevated BMI and WC levels, as well as decreased blood glucose and 25-hydroxy vitamin D levels. Correlation analysis further indicated a positive association between serum asprosin and BMI, as well as TG, while a negative correlation existed between serum asprosin and 25-hydroxy vitamin D. These findings suggest a potential connection between increased serum asprosin levels, decreased 25-hydroxy vitamin D, and insulin resistance in community-based patients with type 2 diabetes. Notably, patients with serum asprosin > 370.5 pg/ml displayed a higher proportion receiving oral hypoglycemic drugs and insulin-lowering therapy, possibly contributing to lower blood glucose levels, which aligns with findings by Wang (15).

The potential mechanism underlying the link between 25-hydroxy vitamin D and asprosin can be understood as follows (16–21). Firstly, Adipokines and adipose tissue may be a direct target for vitamin D. The expression of both the vitamin D receptor and 25-hydroxyvitamin D 1 α -hydroxylase (CYP27B1) genes has been demonstrated in murine and human adipocytes. Secondly, Vitamin D metabolites might influence an increased asprosin production. Furthermore, Vitamin D may also be involved in the regulation of asprosin levels through modification of insulin sensitivity. The study's findings revealed a negative correlation between serum asprosin and 25-hydroxy vitamin D levels in patients with type 2 diabetes, but this correlation was observed only within a certain range of 25-hydroxy vitamin D levels. The underlying cause remains uncertain and necessitates confirmation

through further investigations. One hypothesis is that low stimulation of fat cells by 25-hydroxy vitamin D might contribute to conditions like obesity and insulin resistance. This could lead to elevated inflammatory factors like tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1), ultimately causing an imbalance in adipocytokine levels (22).

Type 2 diabetes and obesity have a comorbidity mechanism, which is related to 25 hydroxyvitamin D deficiency, and may affect the synthesis and metabolism of 25 hydroxyvitamin D through inflammation and oxidative stress. White adipose tissue is mainly produced by white adipocytes, and we speculate that obesity may indirectly affect the level of 25 hydroxyvitamin D by affecting the production of white adipose tissue.

Several limitations should be acknowledged in this study. Firstly, geographic factors, sample size, and population characteristics could have introduced confounding influences, and there might be unaccounted variables. Additionally, this study has a cross-sectional design, the relationship between 25-hydroxy vitamin D and serum asprosin might be bidirectional and interacting, necessitating further confirmation through prospective studies. Therefore, causal relationships between 25-hydroxy vitamin D and serum asprosin cannot be established solely from this study. Secondly, the assessment of islet function and insulin resistance index was lacking in this community-based study of patients with type 2 diabetes. Lastly, diet control and sunlight conditions during the research period were not evaluated in this study, so the effect on asprosin could not be more clearly reflected, which is also a limitation of this study".

In addition, we found that with an increase in 25-OHD levels, the trend of serum asprosin levels in males and females was different. Currently, there are no reports on 25-OHD and asprosin levels, and we believe it may be related to changes in estrogen levels. Further confirmation is needed in other studies to determine whether there is a gender difference in the effect of 25-OHD on asprosin levels.

In conclusion, further investigation is warranted to understand the intricate regulatory role of 25-hydroxy vitamin D on adipocytokines, particularly its potential variations between health and disease states. This study underscored an inverse correlation between serum asprosin and 25-hydroxy vitamin D levels among patients with type 2 diabetes in the community.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the First Hospital of Shanxi Medical University (Approval number: 2019 [K056]). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JC: Conceptualization, Formal analysis, Methodology, Software, Validation, Writing – original draft. ZW: Data curation, Formal analysis, Methodology, Software, Writing – original draft. JYi: Data curation, Writing – original draft. MLi: Methodology, Writing – original draft. QW: Investigation, Writing – original draft. MLiu: Investigation, Writing – original draft. HS: Validation, Writing – original draft. HR: Visualization, Writing – original draft. MX: Investigation, Writing – original draft. JYa: Funding acquisition, Supervision, Writing – review & editing. LX: Conceptualization,

Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The role of magnesium in pancreatic beta-cell function and homeostasis

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Magnesium plays an essential role in glucose utilization and insulin signaling. Recent advances have revealed a greater prevalence of hypomagnesemia in general, and low intracellular magnesium levels in individuals with diabetes contribute to β -cell dysfunction and insulin resistance. This article describes the documented effects of magnesium on various aspects of β -cells and glucose homeostasis. Studies have demonstrated that magnesium deficiency is associated with reduced pancreatic β -cell activity and increased insulin resistance in patients with type 2 diabetes. Additionally, magnesium is involved in many cellular events, including energy homeostasis, protein synthesis, and DNA stability. Furthermore, magnesium is critical for proper glucose utilization and insulin signaling, and magnesium deficiency can lead to the dysregulation of ATP-sensitive potassium (KATP) channels in pancreatic β -cells, impairing insulin secretion. Therefore, maintaining adequate magnesium levels is crucial for maintaining overall health and preventing of metabolic disorders such as type 2 diabetes.

KEYWORDS

magnesium, diabetes, insulin, glucose, β -cells

Introduction

Magnesium is one of the most abundant minerals in the human body. It is essential for many biochemical reactions and is indispensable for preserving normal cellular and organ functions, ranging from cellular energy metabolism to skeletal mineralization (1, 2). Maintaining an optimal magnesium balance is essential, as any deviation may lead to dysregulated glucose turnover. Magnesium plays a vital role in regulating glucose homeostasis by modulating the functions of pancreatic β -cells. In type 2 diabetes, magnesium deficiency can increase insulin resistance, and insulin resistance can also contribute to magnesium deficiency. Intracellular free magnesium levels are lower in patients with type 2 diabetes than in nondiabetic individuals. Intracellular magnesium concentrations are essential for optimal insulin receptor bioactivity and downstream signaling events in target cells. Low magnesium levels contribute to impaired tyrosine kinase activities of insulin receptors, causing altered cellular glucose utilization and eventual insulin resistance. An increased incidence of hypomagnesemia has been reported in patients with type 2 diabetes. Numerous clinical studies have documented the benefits of magnesium supplementation on the metabolic profiles of patients with diabetes. Based on the existing evidence, the potential effects of magnesium on various cellular aspects of glucose homeostasis are briefly discussed.

Pancreatic β -cells are clumped together in groups called islets. β -cells generate insulin to regulate blood glucose homeostasis. In response to elevated blood glucose levels, β -cells secrete increasing amounts of insulin to increase the cellular uptake of glucose from the blood to store it as glycogen, primarily in liver and muscle cells. Depending on the body's needs, stored glycogen can be broken down into glucose for further use. β -cells can also release the hormone amylin, which reduces the amount of glucose entering the bloodstream to decrease blood glucose elevation after meals. In certain disease conditions, such as type 1 diabetes mellitus, when pancreatic β -cells are damaged or destroyed, insulin synthesis is impaired, resulting in elevated blood glucose levels. In type 2 diabetes, despite pancreatic β -cells producing enough or even excess insulin, the body develops insulin resistance, where effector tissues (mainly adipose tissue, liver, and muscle) are unable to convert bloodstream glucose into glycogen. Insulin resistance reduces glucose utilization, causing a compensatory increase in the production of insulin by β -cells. In situations where blood glucose levels are consistently high, physiological communication or sensing between the glucose load and the pancreatic β -cell response is lost, and exhausted β -cells gradually lose their ability to generate the required amounts of insulin to control the blood glucose balance. Chronic insulin resistance can eventually lead to type 2 diabetes mellitus (3). In addition to β -cells, the islets also contain pancreatic α -cells, which store and release glucagon to maintain optimal blood glucose balance by stimulating liver and muscle cells to convert glycogen back into glucose for energy utilization when necessary. Fine-tuning between insulin and glucagon stabilizes the sugar balance. The development of effective nutritional intervention methods for maintaining pancreatic β -cell functions is an intense area of research for diseases related to increased glucose burden. Magnesium is one of the most important players in β -cells and is necessary for adequate glucose utilization and optimal insulin signaling. Importantly, studies have shown a greater prevalence of hypomagnesemia and lower intracellular concentrations of magnesium in patients with diabetes (4). Dysregulation of the concentration of cellular magnesium, which acts as a second messenger for insulin bioactivity, is likely to contribute to insulin resistance (5). This brief article discusses the cellular effects of magnesium on various aspects of glucose homeostasis.

Magnesium

In humans, magnesium is the second most abundant intracellular cation after potassium. Magnesium is also the fourth most common mineral in the human body (after calcium, potassium, and sodium) (1, 6). According to enzymatic databases, magnesium acts as a cofactor or activator for more than 800 enzymes (6). Most magnesium is present in bone and teeth (~60%), while its concentrations in intracellular compartments and extracellular fluids constitute ~40% and <1%, respectively (2, 6–9). The normal blood range of magnesium is 1.8–2.3 mg/dl (0.75–0.95 mmol/L). However, studies have shown that serum magnesium concentrations <0.85 mmol/L are associated with increased health risks; hence, it is recommended that the lower limit of the reference range be increased to 0.85 mmol/L (2.07 mg/dL)

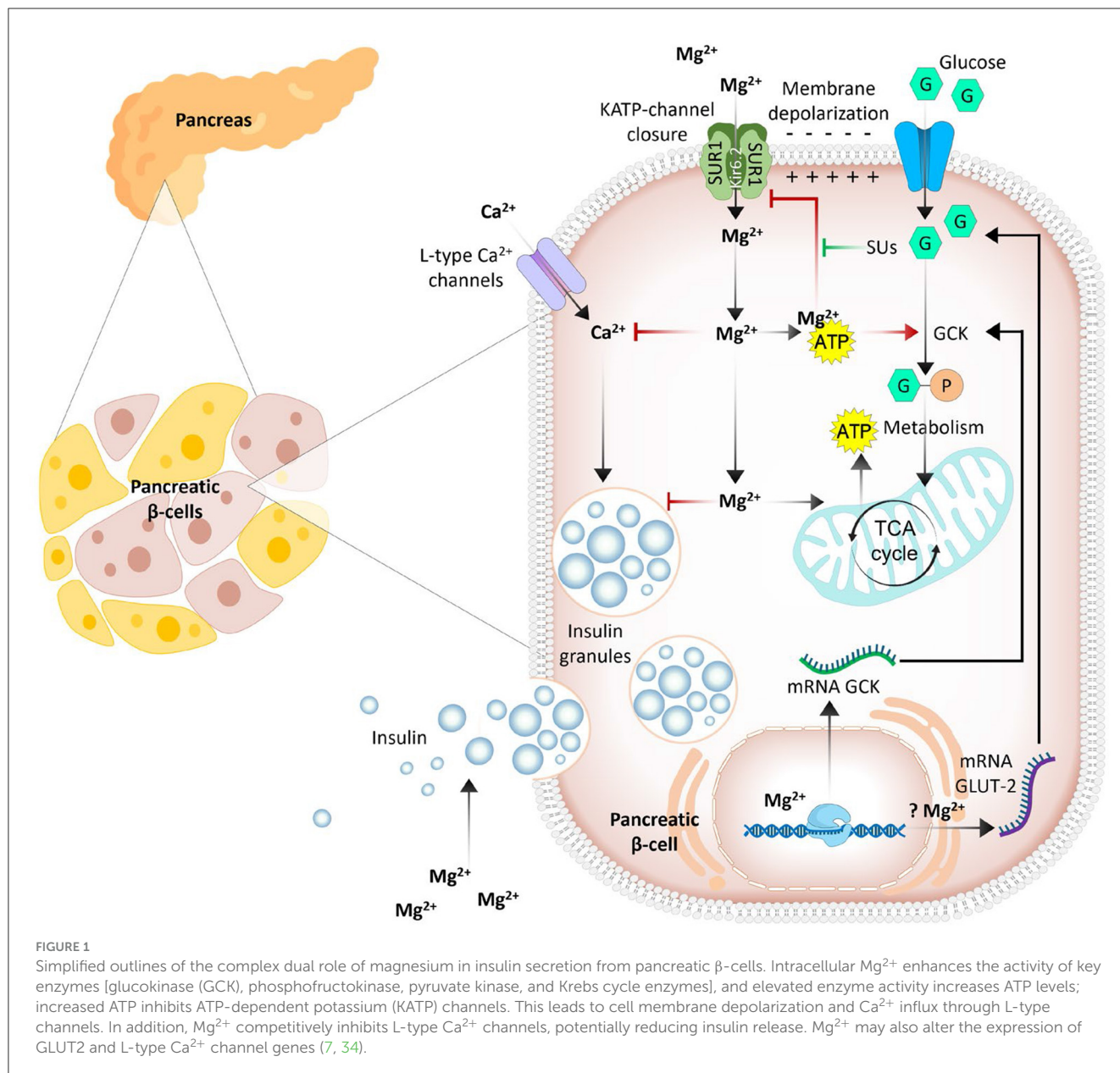
(10). Approximately 0.3% of total body magnesium is present in blood, and consequently, the serum level of magnesium is not a good predictor of intracellular magnesium content or total body magnesium content (1, 6). The recommended dietary allowance (RDA) for magnesium is 420 mg/day for men and 320 mg/day for women. Studies in Europe and the U.S. have shown that Western diets have low magnesium content; magnesium intake in the U.S. has decreased from ~500 mg/day to 175–225 mg/day in the last 100 years (11).

The magnesium balance is controlled by delicate organ crosstalk and is regulated by intestinal absorption and renal reabsorption (6). In addition to passive paracellular intestinal absorption, a small fraction of magnesium is transported by transient receptor potential melastatin (TRPM) subfamily ion channels (TRPM6 and TRPM7). Reduced consumption of magnesium from food or drinking water, increased renal loss of magnesium, and chronic use of certain medications (including bumetanide, furosemide, and ethacrynic acid) can induce magnesium inadequacy, including hypomagnesemia (7, 12, 13). Certain nephrotoxic drugs, including cyclosporine, cisplatin, and tacrolimus, can also increase urinary magnesium wasting (14). In addition, chronic intestinal diseases, including Crohn's disease, ulcerative colitis, celiac disease, and Whipple's disease, can impair magnesium absorption (11). Skeletal magnesium is not easily exchanged, and any rapid need for magnesium is contributed by the magnesium present in the intracellular compartment.

Metformin, a commonly used drug to treat type 2 diabetes, can cause hypomagnesemia due to gastrointestinal wasting of magnesium (13) and may be one of the reasons for the increased incidence of magnesium inadequacy among patients with type 2 diabetes. An inverse relationship between magnesium consumption and the risk of diabetes has been reported in a large cohort of 37,309 participants (15). A prospective Black Women's Health Study cohort with 41,186 participants revealed that a diet with high magnesium (particularly whole grains) can substantially lower the risk of type 2 diabetes (16). Magnesium supplementation can improve the ability of pancreatic β -cells to secrete insulin and compensate for variations in insulin sensitivity in nondiabetic individuals to regulate glucose homeostasis (17).

Magnesium and pancreatic β -cell function

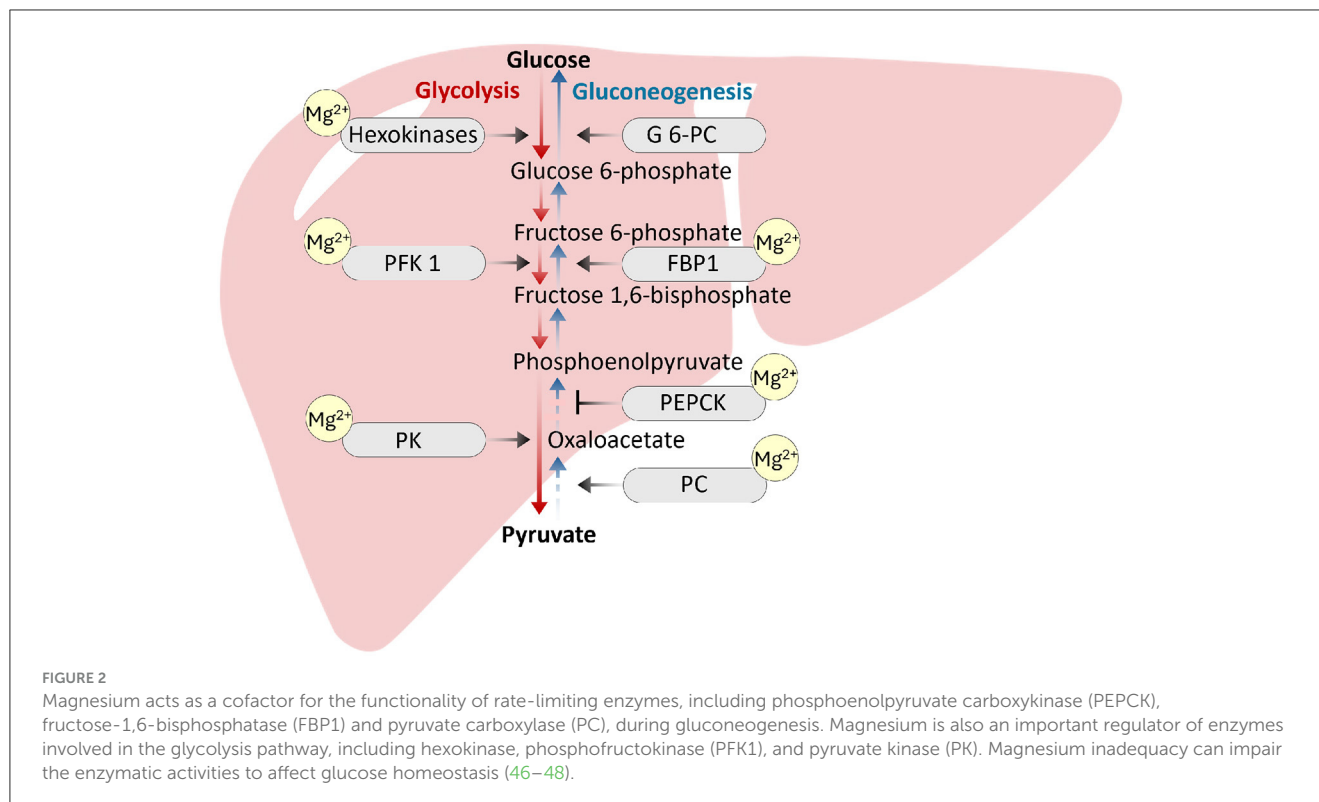
Magnesium deficiency is associated with pancreatic β -cell dysfunction and insulin resistance, culminating in an increased risk of developing type 2 diabetes and metabolic syndrome (7, 18). An inadequate cellular magnesium balance may promote insulin resistance and alter cellular glucose influx by modulating the functions of glucokinase (5, 18). Glucokinase apparently acts as a glucose sensor in β -cells and controls the rate of cellular glucose entry (19). Magnesium plays a role in the mitochondrial synthesis of adenosine triphosphate (ATP) to form the Mg-ATP complex and can regulate glucokinase functions by acting as a cofactor (for Mg-ATP) (20). Furthermore, the ATP-sensitive potassium channel KATP (consisting of the Kir6.2 and SUR1 subunits) promotes insulin secretion by pancreatic β -cells (20).



In magnesium deficiency, reduced intracellular levels of the Mg-ATP complex inhibit the opening of KATP channels and delay insulin responses to glucose. The Mg-ATP complex also activates phosphoglucomutase, an enzyme that plays a role in glucose metabolism in adipose tissue (21). In addition, cell signaling utilizes Mg-ATP for protein phosphorylation and the activation of cyclic adenosine monophosphate (cAMP), which is involved in numerous biochemical processes, including the amplification of insulin secretion in β -cells (Figure 1) (22, 23). cAMP also plays a role in regulating glucagon secretion from α -cells in the pancreas to fine-tune glucose homeostasis (23).

An adequate intracellular magnesium concentration is also critical for the phosphorylation of insulin receptors, and dysregulation of magnesium balance can induce peripheral insulin resistance, partly by desensitizing insulin receptors (18). Moreover, the affinity of insulin receptor tyrosine kinase for

Mg-ATP increases when the free magnesium concentration increases, whereas the affinity of insulin receptor tyrosine kinase for free magnesium increases when the Mg-ATP concentration increases (18), implying that magnesium inadequacy could impact kinase activities to impair insulin receptor signaling and ultimately affect peripheral insulin sensitivity. Insulin can modulate the shift of magnesium from the extracellular to the intracellular compartment. The intracellular level of magnesium is tightly controlled by a few factors, including insulin (24). For example, when uterine smooth muscle cells were treated with insulin, a net intracellular surge of magnesium and potassium was detected, leading the authors to propose that insulin, after interacting with its cell surface receptors, could affect an ATPase pump to increase the cellular entry of magnesium and potassium (24). In a similar study with human erythrocytes collected from nondiabetic healthy individuals, after the ingestion of glucose,



an insulin-induced shift of magnesium from the extracellular to the intracellular compartment was detected, which was associated with a reduced plasma magnesium level and a transition elevation of magnesium content in erythrocytes (25). Reverse changes in magnesium distribution were noted in the plasma and erythrocyte compartments during the euglycemic hyperinsulinemic glucose clamp (25). *In vitro* studies have suggested that in magnesium-deficient adipocytes, insulin-dependent glucose uptake is reduced by approximately 50% compared with that in control adipocytes exposed to magnesium-containing media (26). Magnesium supplementation increases insulin sensitivity and reduces glucose levels in individuals with or without diabetes (27, 28). Notably, the prevalence of hypomagnesemia in individuals with type 2 diabetes is approximately tenfold greater than that in the healthy population (29–32).

Magnesium is also an important regulator of enzymes involved in glycolysis and the Krebs cycle, as documented through the ability of the Mg-ATP complex to influence the activities of several glycolytic enzymes, including hexokinase, phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase, presumably by acting as cofactors for these enzymes (33). Magnesium also plays a critical role as a cofactor for the rate-limiting enzymes of gluconeogenesis, a process of generating glucose from noncarbohydrate molecules such as fatty acids and glucogenic amino acids. Magnesium acts as a cofactor for the functionality of rate-limiting enzymes, including phosphoenolpyruvate carboxy kinase (PEPCK), fructose-1,6-bisphosphatase and pyruvate carboxylase, during gluconeogenesis (Figure 2). Studies have shown that magnesium deficiency is strongly associated with increased PEPCK activity, resulting in

increased gluconeogenesis in the liver (34). An association between genetic variations in specific magnesium channels or transporters and a greater risk of type 2 diabetes has also been reported (20). For example, a single nucleotide polymorphism in the TRPM6 and solute carrier family 41 member 1 (SLC41A1) genes is associated with an increased risk of type 2 diabetes (20). Additionally, genetic changes in pancreatic β -cell KATP channels can play a key role in impairing insulin secretion and the eventual development of type 2 diabetes (35). Similarly, the role of NIPA-like domain containing 1 (NIPAL1), a magnesium influx transporter, in pancreatic β -cell function and insulin secretion has been studied (36). The investigators reported that NIPAL1 expression is a magnesium-dependent process and is localized to the Golgi in β -cells. NIPAL1 knockdown decreases basal insulin secretion and total insulin content, whereas NIPAL1 overexpression increases total insulin content. These results suggest that NIPAL1 plays a crucial role in insulin production and secretion, particularly under hypomagnesemia conditions, which are common in type 2 diabetes patients.

Hyperglycemia and hyperinsulinemia can increase urinary magnesium loss and contribute to magnesium inadequacy (37). In animals with diabetes, magnesium supplementation has been shown to increase insulin sensitivity and decrease insulin resistance (38). Similar benefits of magnesium supplementation in reducing insulin resistance have been reported in individuals with hypomagnesemia presenting with insulin resistance (39). Increased serum magnesium is related to increased insulin sensitivity (40). A meta-analysis reported a linear relationship between dietary magnesium intake and metabolic syndrome, with an increase of 150 mg/day leading to a 12% reduction in the risk of metabolic

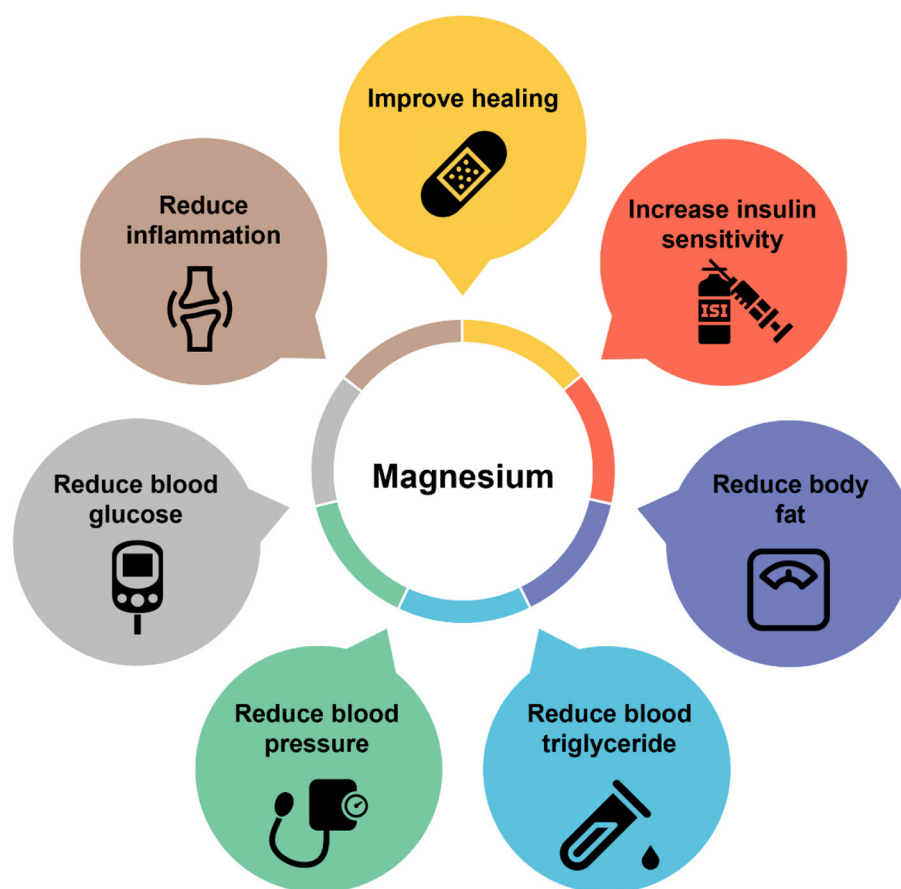


FIGURE 3
Partial list of the wide range of health-promoting functions of magnesium.

syndrome (41). Another study involving 4,637 U.S. adults aged 18–30 years reported that the risk of metabolic syndrome was reduced by 31% among individuals in the highest quartile of dietary magnesium consumption (42). It is important to maintain an optimal magnesium balance, along with other minerals and vitamins, to support normal physiological functions, including metabolic health (43–45).

Conclusions

Adequate magnesium balance is necessary for a wide range of functions, including pancreatic β -cell function and optimal insulin secretion, as magnesium inadequacy is associated with β -cell dysfunction and insulin resistance, which are prominent hallmarks of diabetes mellitus (type 2) (Figure 3). In type 2 diabetes mellitus patients, the intracellular magnesium concentration is low, and an inverse association exists between the plasma level of magnesium and insulin resistance (5). A low intracellular magnesium concentration may impact subcellular insulin signaling activity to modify insulin sensitivity. In pancreatic β -cells, glucose

metabolism is initiated by the conversion of glucose to glucose-6-phosphate, which is catalyzed by the enzyme glucokinase. This reaction is a crucial first step that eventually leads to an increase in intracellular ATP levels. The inadequacy of magnesium can impair the glucose metabolism process in pancreatic β -cells by reducing glucokinase activity due to limited Mg-ATP availability, disrupting the translocation of glucokinase from the nucleus to the cytoplasm and potentially altering the affinity of glucokinase for glucose, resulting in decreased glucose binding and reduced catalytic activity. Studies on patients with type 2 diabetes have shown that magnesium supplementation improves insulin sensitivity. There are dualistic interactions between magnesium metabolism and glucose metabolism, with magnesium being a cofactor of numerous enzymes involved in glucose metabolism that can also influence the bioactivities of insulin, while insulin stimulates magnesium uptake in insulin-sensitive tissues (5). Future prospective studies to minimize magnesium inadequacy before and during the appearance of the cluster of conditions associated with metabolic syndrome are needed to determine the potential benefits of magnesium on cardiometabolic risk in the general population.

Author contributions

NA: Writing – review & editing, Visualization. MR: Conceptualization, Writing – original draft, Supervision. SC: Writing – review & editing. AA: Writing – review & editing.

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Neuroprotective effects of magnesium: implications for neuroinflammation and cognitive decline

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Neurodegenerative diseases, which are characterized by progressive neuronal loss and cognitive decline, are a significant concern for the aging population. Neuroinflammation, a shared characteristic of these diseases, is implicated in their pathogenesis. This article briefly summarizes the role of magnesium, an essential mineral involved in numerous enzymatic reactions and critical for neuronal bioactivity, in the context of neuroinflammation and cognitive decline. The potential neuroprotective effects of magnesium, including the mechanisms of neuroprotection by magnesium through maintaining neuronal ion homeostasis, reducing inflammation, and preventing excitotoxicity, are also described. Additionally, we discuss the impact of inadequate magnesium on neuroinflammation and its potential as a therapeutic agent for attenuating cognitive decline to improve neurodegenerative conditions.

KEYWORDS

magnesium, neuroinflammation, neurodegenerative disease, cognitive decline, neuroprotection

Introduction

As the global population ages, neurodegenerative diseases, which are characterized by an ongoing loss of neuron structure and function, are becoming increasingly public health burdens. These disorders, including dementia (along with vascular dementia), amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, and Huntington's disease, often result in cognitive decline, which severely impacts the quality of life of affected individuals. The insidious nature of these diseases and the lack of curative interventions highlight the need for novel therapeutic strategies. The global prevalence of dementia,

primarily Alzheimer's disease, is expected to double every 20 years, reaching 81.1 million by 2040 (1, 2). Similarly, Parkinson's disease, the second most common neurodegenerative disorder, affects 2-3% of the population aged ≥ 65 years (3, 4). These statistics highlight the escalating public health challenge of neurodegenerative diseases.

Neuroinflammation, a common feature of neurodegenerative diseases, is recognized as a critical player in the pathogenesis of these disorders (5, 6). The inflammatory response in the brain is a double-edged sword. Whereas acute inflammation can be beneficial for neuronal repair and recovery, chronic inflammation can lead to persistent neuronal damage and eventually to neurodegeneration (7). Inflammatory processes in the brain are primarily mediated by microglia shown in Figure 1. Upon activation, microglia release proinflammatory cytokines, including interleukin 1β (IL- 1β), tumor necrosis factor α (TNF- α), IL-6, IL-18, and IL-12, reactive oxygen species (ROS), and other neurotoxic substances, including nitric oxide, glutamate, and prostaglandins, as well as enzymes such

as matrix metalloproteinases (MMPs) (7). Given the critical role of neuroinflammation in neurodegeneration, modulating the inflammatory response could reduce disease progression and is likely to improve clinical outcomes.

We conducted a literature search using PubMed, Google Scholar, and Scopus databases. The search was performed using the keywords: "neuroinflammation", "magnesium", "cognitive function" and "neurodegenerative diseases". We included peer-reviewed articles in English published between 2000 and 2023.

Neuroinflammation and neurodegeneration

Neuroinflammation is partly mediated by the activation of glial cells and the release of proinflammatory mediators in the brain (8). It plays a crucial role in the pathogenesis of neurodegenerative diseases.

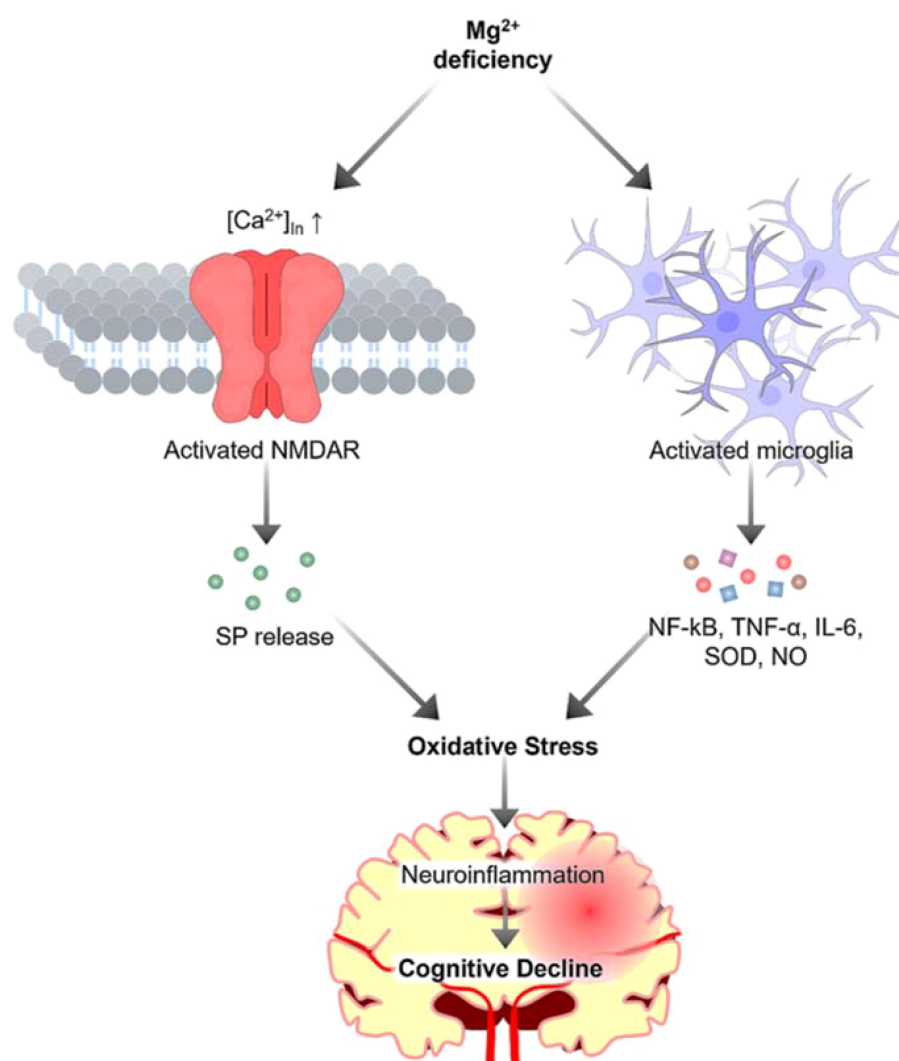


FIGURE 1

The role of magnesium in neuroinflammation. Magnesium deficiency activates microglia, resulting in the release of proinflammatory cytokines and toxic substances, which contribute to oxidative stress. Additionally, magnesium deficiency triggers calcium influx, inducing the release of substance P (SP), further exacerbating oxidative stress to increase neuroinflammation and ultimately contributes to cognitive decline.

Magnesium has been shown to modulate neuroinflammation (9, 10). It is recognized for its diverse roles in maintaining human health, specifically in modulating inflammatory signaling pathways within the neurological landscape. Magnesium plays a crucial role in over 600 enzymatic reactions in the human body (11). According to Worker, “Magnesium is a critical mineral in the human body and is involved in ~80% of known metabolic functions” (12). The concentrations of magnesium in serum and cerebrospinal fluid (CSF) are regulated to maintain normal physiological function. Normal serum magnesium levels typically range from 0.75 to 0.95 mmol/L (13), while in CSF, they range between 0.77 and 1.17 mmol/L (14). Magnesium levels are generally higher in CSF as compared to the serum levels, perhaps due to the active transport of magnesium across the blood-brain barrier (15); the blood-brain barrier and the choroid plexus help regulate magnesium levels in the CSF. In magnesium deficiency state, CSF concentrations decline, although such reduction lags behind and is usually less pronounced than the changes noted in plasma levels of magnesium (15). Serum magnesium levels are crucial for neuromuscular function, enzyme activity, and bone structure (16). Magnesium in CSF plays a vital role in supporting various functions of the central nervous system. Decreased CSF magnesium levels correspond with reduced concentrations of extracellular brain magnesium and have been associated with epilepsy (14). Additionally, magnesium is well known for its implication in multiple neurological disorders (17). For instance, magnesium sulfate supplementation has been associated with reduced neuroinflammation in a rat model of Alzheimer’s disease (10). Studies involving animal models suggest that magnesium deficiency may trigger greater recruitment of phagocytic cells (18). These cells could lead to generation of more ROS, leading to the production of various cytokines, such as TNF- α , which are key players in the inflammatory response (18). In Alzheimer’s disease, neuroinflammation is a pathological feature exacerbated by the accumulation of amyloid-beta plaques through the activation of inflammatory proteins including IL-1, IL-6, and TNF- α . Interestingly, magnesium supplementation has been shown to reduce the levels of these proinflammatory cytokines and increase the levels of anti-inflammatory mediators in the hippocampus of a rat model of Alzheimer’s disease, suggesting modulation of an inflammatory responses (19). However, due to the complexity of the immune system in the brain, with the involvement of microglia, astrocytes, and various cytokines and chemokines, dampening inflammation alone might not be sufficient. Chronic neuroinflammation results in an adverse cascade of events, causing neuronal damage, disrupting synaptic functionality, and leading to cognitive impairment. When this inflammatory response is sustained, it results in the overproduction of proinflammatory cytokines. This hyperreactive state disrupts the delicate balance of synaptic plasticity (the ability of synapses to strengthen or weaken over time) thereby diminishing key cognitive functions like memory retention and learning (20).

Furthermore, prolonged inflammation triggers oxidative stress, wherein excess free radicals lead to neurotoxicity and cellular damage (9). This accelerates the progression of neurodegenerative processes, as observed in diseases such as Alzheimer’s disease and Parkinson’s disease, which are characterized by the accumulation of disease-specific proteins in the brain, amyloid-beta and alpha-

synuclein, respectively (21). Additionally, inflammation-induced oxidative stress and resultant neuronal damage have been identified as significant contributors to cognitive decline following traumatic brain injury. These findings illustrate the detrimental link between chronic neuroinflammation and cognitive decline.

Magnesium deficiency syndromes

Hypomagnesemia (typically below 0.61 mmol/L) can cause a wide range of disorders and has significant neurological consequences. The causes of hypomagnesemia can be related to gastrointestinal disorders, including chronic diarrhea, malabsorption syndromes (e.g., celiac disease, inflammatory bowel disease), chronic pancreatitis, and excessive vomiting. Similarly, renal disorders, including tubular dysfunction, diabetic nephropathy (leading to increased urinary magnesium loss), and the use of certain medications (e.g., diuretics, proton pump inhibitors, and some antibiotics), can cause hypomagnesemia. Alcoholism, severe burns, chronic stress, hyperaldosteronism, and prolonged parenteral fluid administration without magnesium supplementation can also lead to hypomagnesemia. Magnesium plays a key role in neural function, and its inadequacy can lead to various neurological symptoms and complications, including neuromuscular hyperexcitability, muscle twitches and cramps, tremors, and seizures. Of clinical importance, the severity of neurological symptoms often correlates with the severity of magnesium deficiency. Patients with mild hypomagnesemia (below 0.61 mmol/L) may cause subtle symptoms, while severe hypomagnesemia (below 0.49 mmol/L) can lead to more pronounced neurological manifestations. Severe magnesium deficiency syndromes can be associated with cognitive and mood disturbances, headaches, migraines, and neuropathy (numbness and tingling sensations, particularly in the extremities). The long-term complications of severe magnesium deficiency have also been linked to nystagmus (involuntary eye movements) and neurodegenerative diseases, possibly mediated by neuroinflammation. It is essential to maintain an optimal balance of magnesium, along with other minerals and vitamins, throughout life to support normal physiologic functions, including neurological health (22–25).

Role of magnesium in neuroinflammation

In the nervous system, magnesium is essential for maintaining neuronal ion homeostasis, modulating synaptic plasticity, and regulating neurotransmitter release (26). Kang et al. highlighted the integral role of this mineral in managing the activity of N-methyl-D-aspartate (NMDA) receptors (27). Their findings emphasize the significance of this interaction in maintaining the balance of glutamate, an excitatory neurotransmitter. If left unchecked, glutamate can potentially tip the scale toward inflammation. Kramer et al. suggested the aftereffects of magnesium deficiency (28). According to their findings, insufficient magnesium can trigger an increase in substance P, a neuropeptide that propagates inflammatory pain (28).

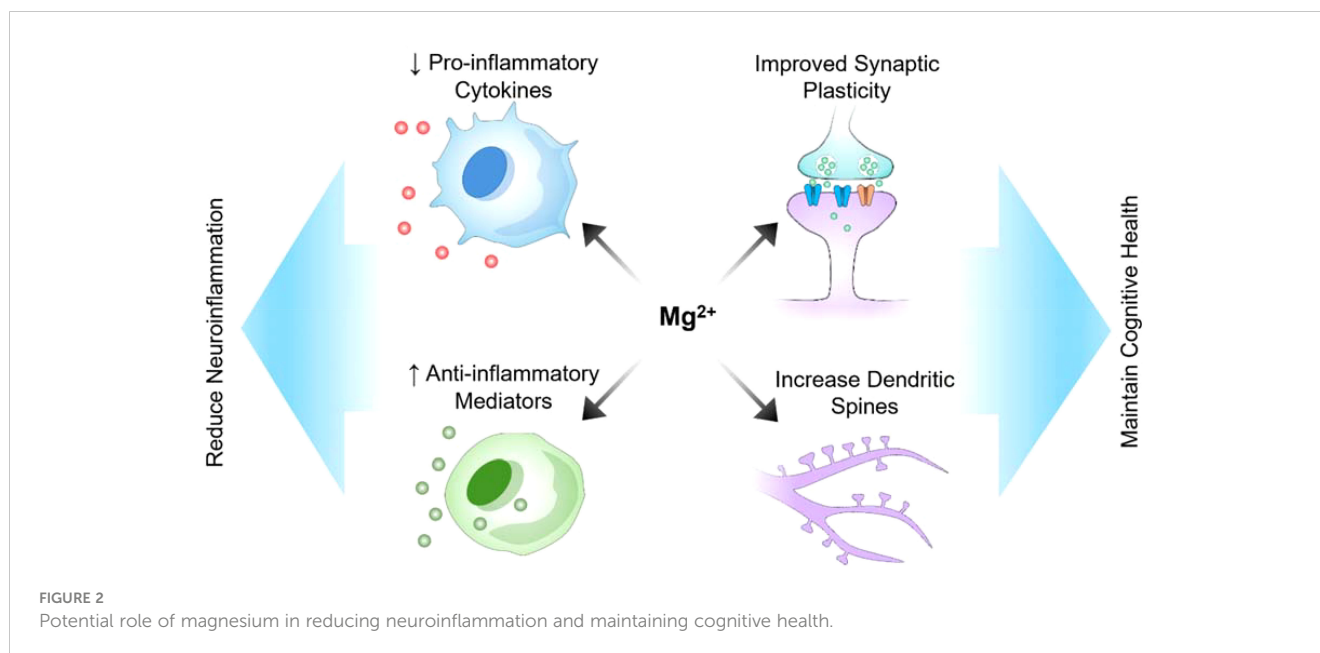
Other researchers have highlighted the complex interplay between magnesium and calcium within neurons (9). By restraining calcium influx into neurons, magnesium helps prevent events that could otherwise lead to intensified inflammation and neuronal injury. Whereas low levels of this mineral are associated with chronic inflammation, restoring magnesium balance has been shown to potentially counteract this condition (29). Apart from managing neurotransmitter activity, magnesium has been found to play a crucial role in modulating immune responses, particularly by interacting with nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (19). This research provides compelling evidence of the role of magnesium as an NF- κ B inhibitor, a transcription factor that regulates the expression of pro-inflammatory cytokines, including TNF- α and IL-6 (19). By inhibiting NF- κ B activation, magnesium can dampen the resultant proinflammatory gene expression, thereby reducing the overall inflammatory response within the brain (19). A meta-analysis by Veronese et al. revealed magnesium's anti-inflammatory effects, marked by reductions in plasma fibrinogen and other markers, such as tartrate-resistant acid phosphatase type 5 (TRACP 5) and tumor necrosis factor-ligand superfamily member 13B (TNFSF13B) (30). Additionally, it was also noticed that ST2 protein and IL-1 levels went down. However, the study revealed no significant changes in IL-6 or total antioxidant capacity levels, indicating a selective impact of magnesium on various inflammatory markers (30). Of clinical significance, measuring circulating ionized magnesium appears to be a more accurate indicator of magnesium supplement bioavailability compared to assessing total magnesium levels in plasma (31). Although the role of magnesium in regulating neurotransmission and immune responses is well established, it also plays a crucial role in maintaining brain health by acting as an antioxidant. Research findings suggest that magnesium may contribute to neutralizing ROS to delay the progression of neurodegenerative disorders (32).

Although magnesium is not considered a component of the antioxidant defense system, research indicates that magnesium deficiency may increase oxidative stress markers. These markers encompass oxidative modification products of lipids, proteins, and DNA. Furthermore, a significant association was observed between magnesium deficiency and weakened antioxidant defense mechanisms. This relationship between magnesium deficiency and oxidative stress involves multifaceted mechanisms at both the systemic and cellular levels, including inflammation, endothelial dysfunction, mitochondrial dysfunction, and excessive fatty acid production (32). The studies suggest that magnesium may possess inherent antioxidant properties, although not as a conventional antioxidant molecule such as vitamin C or vitamin E. One notable mechanism highlighted is magnesium's role in stabilizing the critical antioxidant enzyme superoxide dismutase (SOD) (32). SOD substantially mitigates oxidative damage by converting harmful superoxide radicals into less reactive molecular species. This stabilization of SOD by magnesium provides a unique and essential link between magnesium and the antioxidant defense system (32).

Magnesium and neuroprotection

Neuroprotective agents are substances that can potentially preserve neuronal structure and function. These substances help prevent or slow the progression of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. These agents work through various mechanisms, including reducing neuroinflammation, shielding against oxidative stress, and modulating neurotransmission (33).

Many preclinical and clinical studies have suggested the potential of magnesium as a neuroprotective agent (Figure 2). Magnesium is present both intracellularly and extracellularly, with its intracellular presence in compartments such as the nuclei, mitochondria, and endoplasmic reticulum being crucial for central nervous system functions, including synaptic connectivity (34). Intracellular magnesium can modify synaptic properties, influencing various neuronal processes. For instance, recent research by Liu's group reported that presynaptic intracellular magnesium plays a crucial role in mediating the transition between two synaptic configurations: one involved in information encoding and learning, and the other in information storage and memorization (35). Research has demonstrated that magnesium can enhance cognitive function and synaptic plasticity in animal models of Alzheimer's disease, offering optimism for addressing cognitive decline (10). Additionally, a study conducted on a rat model of Alzheimer's disease demonstrated that magnesium sulfate supplementation improved cognitive function, synaptic plasticity, and dendritic spine morphology (10). Moreover, intracellular magnesium levels have been shown to correlate with Parkinson's disease activity. In 1-methyl-4-phenylpyridinium (MPP+) model of Parkinson's disease, the application of MPP+ induced an increase in intracellular magnesium concentration, which inhibited cellular ROS production, maintained ATP generation, and preserved cell viability, thereby protecting neurons from MPP+ toxicity (36). In demyelination rat models, a mutation in the mitochondrial magnesium uptake gene disrupted magnesium homeostasis in oligodendrocytes, affecting ATP production and leading to axonal demyelination (37). Besides supporting myelin formation, intracellular magnesium also enhanced oligodendrocytes' tolerance against cellular stress, increasing resistance to a hypoxic-ischemic injury (38). Although preclinical studies suggest that magnesium has potential neuroprotective effects, translating these findings to humans presents numerous challenges. Differences in metabolism, blood-brain barrier permeability, and magnesium bioavailability between humans and animal models may affect its efficacy in clinical settings. Additionally, the optimal dosage, duration of treatment, and form of magnesium (e.g., magnesium sulfate, magnesium citrate, etc.) that are both effective and safe for humans require rigorous clinical trials. A gap exists between demonstrating neuroprotection under controlled laboratory conditions and achieving measurable, meaningful outcomes in diverse human populations with varying stages of neurological conditions. Supplementation with magnesium sulfate increased brain magnesium contents and attenuated memory deficits induced by intracerebroventricular administration of streptozocin (ICV-STZ). Furthermore, magnesium reduces tau hyperphosphorylation, a hallmark of Alzheimer's disease, and modulates the PI3K/Akt signaling pathway (10). Additionally,



magnesium supplementation has been associated with improved neurological outcomes in models of acute brain injury, demonstrating its relevance in central nervous system injuries (39). Moreover, in an experimental setting involving a rat model of sciatic nerve injury, a diet rich in magnesium was found to stimulate neurological function recovery and enhance nerve regeneration, revealing its potential in the treatment of peripheral nerve disorders (39). The neuroprotective effects of magnesium are believed to stem from its capacity to regulate neuronal calcium homeostasis, thus reducing excitotoxicity, and its ability to modulate neuroinflammatory processes (10). The mechanisms by which magnesium exerts its effects (e.g., calcium homeostasis regulation, reduction in excitotoxicity, anti-inflammatory actions) suggest that its neuroprotective properties could be applicable to a wide range of neurological conditions. However, this also raises questions about specificity and targeted therapy. For instance, although reducing tau hyperphosphorylation is promising for treating Alzheimer's disease, it is unclear how these mechanisms interact in the presence of other neurodegenerative disorders or comorbidities. The multifunctional nature of magnesium might mean that its efficacy could vary greatly depending on the specific pathological context. Additionally, magnesium appears to influence nitric oxide production; nitric oxide is a molecule critical for regulating cerebral blood flow and neuronal damage.

Between 2002 and 2008, several randomized clinical trials explored the potential of magnesium sulfate for neuroprotection in preterm births and its effects on cerebral palsy (40). Although these studies did not consistently achieve statistical significance for their primary outcomes, they indicated that magnesium sulfate exposure significantly reduced the likelihood of cerebral palsy in preterm infants. A similar clinical study by Temkin et al., 2007 involving 499 participants aimed to test whether magnesium treatment favorably affects outcomes in head-injured patients (41). The results show that participants who were randomly assigned to the lower dose magnesium group performed significantly worse than those in the placebo group. Therefore, there was greater mortality

with the magnesium dose than with the placebo. These findings highlight a discrepancy between preclinical expectations and clinical observations, suggesting that the magnesium infusions given to patients within 8 hours of traumatic brain injury did not have a neuroprotective effect on traumatic brain injury (41). However, other studies have claimed that intravenous magnesium infusion and hyperbaric oxygen therapy could reduce the clinical symptoms of brain injury (42–44). Therefore, additional pre-clinical and clinical research is needed to provide stronger scientific validation.

Another study investigated the combined effects of magnesium supplementation and treadmill exercise on memory deficits in aged rats (45); combined approach led to improved memory function in the aged rats. In the context of central nervous system injury, a comprehensive review highlighted the significant decrease in blood and brain (free) magnesium concentrations following both direct and indirect neurotrauma (46). A decrease in magnesium was associated with neurological deficits and oxidative stress, emphasizing the importance of magnesium homeostasis in central nervous system injury. The administration of magnesium salts, such as magnesium sulfate and magnesium chloride, increased brain (free) magnesium concentrations and improved functional outcomes (46).

The cognitive lifeline: magnesium supplementation

Research has demonstrated that magnesium supplementation can effectively increase extracellular magnesium levels, particularly in the serum, which may help inhibit the aggregation of calciprotein particles and reduce vascular calcifications, helping manage conditions such as chronic kidney disease (47). However, the effects on intracellular magnesium levels are more complex and require a long-term, consistent approach to supplementation. This slow adjustment is necessary because of the body's regulatory

mechanisms, which ensure that cellular functions remain stable and effective.

In neurological disorders such as Alzheimer's disease and Parkinson's disease, the neurodegenerative process has occurred for many years, potentially reducing the responsiveness of these disorders to the benefits of magnesium. Magnesium impacts calcium regulation and neurotransmitter functions, which are implicated in the pathophysiology of these diseases. In Parkinson's disease, abnormal magnesium levels are linked to transporter dysfunctions, suggesting that supplementation could stabilize these transport mechanisms and potentially slow disease progression (48).

Conversely, in acute neurological conditions such as stroke or traumatic brain injury, rapid onset and progression do not allow magnesium levels to be corrected in a timeframe that influences immediate outcomes. In these cases, emergency treatments focus on restoring blood flow or reducing inflammation rather than correcting metabolic imbalances. The slow cellular uptake and regulatory effects of magnesium are less practical here because the therapeutic window is very narrow, and the rapid physiological changes postinjury require immediate interventions that go beyond magnesium supplementation. Therefore, while chronic neurological disorders could benefit from sustained magnesium research owing to their slow progression, acute disorders would receive minimal benefit from such research. This is due to need for immediate and aggressive treatment in acute conditions, where the timing and rapid action are critical.

Magnesium supplementation varies significantly in form and administration, each tailored for specific clinical scenarios. Oral magnesium, available in forms such as oxide, citrate, and glycinate, is commonly used for long-term management of conditions such as cardiovascular health and migraine prophylaxis. These forms are preferred for their high bioavailability and ease of administration, making them ideal for ongoing, nonemergency supplementation. Conversely, intravenous magnesium, primarily known as magnesium sulfate, is used in emergency settings where rapid correction of magnesium levels is critical. This form is used in acute medical conditions such as severe asthma, eclampsia, or life-threatening arrhythmias. Direct administration into the bloodstream provides an immediate therapeutic effect, which is crucial in life-saving interventions. Topical magnesium, often in the form of oils or gels, is used for local applications such as muscle soreness and cramps. While it offers the advantages of bypassing the gastrointestinal system and avoiding some side effects associated with oral forms, its systemic absorption and overall efficacy are less documented.

The relationship between magnesium intake and cognitive function is a promising research area. A study from the National Health and Nutrition Examination Survey (NHANES) 2011 to 2014 investigated the associations of vitamin D status and magnesium with cognitive status in older adults (49). The study found that higher serum 25-hydroxyvitamin D [25(OH)D] levels, linked with magnesium metabolism, were associated with reduced risk of declining cognitive function. Specifically, an inverse association of higher serum 25(OH)D levels with cognitive function was observed primarily among participants with a daily total magnesium intake

of <254 mg or ≤ 375 mg. Essential roles of magnesium in the activation of vitamin D has been explained in various research publications (49–53). The associations between serum 25(OH)D and risk of mortality may be modified by the intake level of magnesium (49). Nevertheless, some studies reported that there were no clear associations for cognitive function with overall magnesium intake (54). Although not directly focused on magnesium, research has highlighted the potential cognitive benefits of other dietary components. For instance, a study conducted in Qatar revealed that habitual consumption of nuts (almonds, cashews, Brazil nuts, and walnuts), which are rich in magnesium, is positively associated with cognitive function, especially among older adults (55).

Furthermore, a multicenter study of hemodialysis patients revealed a U-shaped association between serum magnesium levels and mild cognitive impairment. Both lower and higher serum magnesium levels were observed to increase the risk of mild cognitive impairment in this specific population. The optimal range of magnesium levels for the lowest risk of mild cognitive impairment was identified as 1.12–1.24 mmol/L (56). This discrepancy suggests that while the current reference range for serum magnesium (0.75–0.95 mmol/L) may be adequate for typical physiological functions, higher levels would be necessary for optimal cognitive health. This indicates that standard ranges might not fully address the specific needs of the brain and neurological health. Therefore, maintaining serum magnesium levels at the higher end of the range could provide potential neuroprotective benefits. The empirical data from specialized populations like hemodialysis patients delineate magnesium's potential as a cognitive lifeline. The observed associations between magnesium levels and cognitive outcomes highlight the significance of this mineral and raise questions about optimal intake levels for cognitive preservation.

Magnesium glycinate, known for its high bioavailability, ensures that magnesium is efficiently absorbed into the bloodstream and, consequently, available to the body and brain (57). Although direct studies on the impact of magnesium glycinate on cognitive function are limited, its role in enhancing sleep quality and reducing anxiety could indirectly support cognitive health by promoting restorative sleep and lowering stress levels, both of which are beneficial for cognitive performance and neuroprotection (58). Magnesium L-threonate has been specifically studied for its unique ability to increase magnesium concentrations in the brain, thus directly influencing cognitive functions. Rats supplemented with magnesium L-threonate showed a significant increase in synaptic density in regions of the brain associated with memory and learning, translating to a 15% improvement in maze navigation tasks compared to controls. This study demonstrated that this form of magnesium could reverse certain aspects of brain aging and improve synaptic density, suggesting that magnesium has promising implications for delaying and treating cognitive decline associated with aging and neurodegenerative diseases (59).

The existing body of research underscores the need for more rigorous, long-term clinical trials to provide conclusive evidence. A study by Nosheny et al. emphasized the role of dyadic cognitive reports and subjective cognitive decline in early Alzheimer's disease

research and trials (60). Although this study did not focus on magnesium directly, it highlighted the importance of long-term monitoring and the complexities in data interpretation, suggesting that similar rigorous methodologies should be applied to studies on magnesium. Furthermore, research by Planche et al. on brain atrophy subtypes during aging indicated that certain atrophy patterns might predict long-term cognitive decline and future Alzheimer's disease (61).

Conclusion

The role of magnesium in cognitive health and neuroprotection is both compelling and complex, a testament to the sophisticated nature of the nervous system and its interplay with essential nutrients. Research has revealed that magnesium is a critical player in maintaining and regulating neurobiological behaviors. In fact, its ability to mediate inflammatory signaling pathways and inhibit the activation of NF- κ B provides a basis for its potent anti-inflammatory effects. By reducing oxidative burden and inflammation (two phenomena significantly contributing to cognitive decline), magnesium helps to preserve neuronal integrity. Epidemiological and clinical research consistently stresses the importance of adequate magnesium levels for improving cognitive health. Studies have shown a direct correlation between magnesium intake and cognitive function in healthy individuals. Although existing studies have laid a substantial foundation, they also highlight the need for further in-depth research, including more comprehensive, long-term clinical trials to determine the therapeutic potency of magnesium in improving cognitive health to provide safe and compassionate patient care (62), to reduce the burden of neurodegenerative diseases.

Author contributions

MR: Conceptualization, Supervision, Writing – review & editing. VP: Writing – original draft. NA: Visualization, Writing

– review & editing. WG: Writing – review & editing. CD: Writing – review & editing. XF: Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Association of dietary and circulating antioxidant vitamins with metabolic syndrome: an observational and Mendelian randomization study

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Aims: The objective of this study was to investigate the associations of dietary and circulating antioxidant vitamins with metabolic syndrome (MetS), and to assess causality using Mendelian randomization (MR).

Methods: This study included 10,308 participants from the National Health and Nutrition Examination Survey. The associations of vitamins A, C, E and carotenoids with MetS were assessed using multivariable weighted logistic regression analysis. Subsequently, the MR approach was employed to test the causal associations, with inverse variance weighted (IVW) serving as the primary analysis.

Results: Observationally, dietary vitamin A (OR=0.852, 95%CI: 0.727-0.999), C (OR=0.802, 95%CI: 0.675-0.952), carotene (OR=0.832, 95%CI: 0.706-0.982), and β -carotene (OR=0.838, 95%CI: 0.706-0.995) in quartile 4 had lower incidents of MetS, when compared to quartile 1. Circulating vitamin C and carotene were also present inversely associated with MetS, while the vitamin A and E both increased this risk. IVW-MR confirmed the associations of dietary vitamin A (OR=0.920, 95%CI: 0.861-0.984), vitamin C (OR=0.905, 95%CI: 0.836-0.979) and carotene (OR=0.918, 95%CI: 0.865-0.974) with MetS. However, there was only circulating β -carotene (OR=0.909, 95%CI: 0.857-0.965) was found to be causally associated with MetS.

Conclusions: Observational and MR studies have shown that adequate dietary intake of vitamin A, C and carotenoids may help to reduce the risk of MetS.

KEYWORDS

antioxidant vitamins, metabolic syndrome, obesity, hypertension, NHANES, Mendelian randomization

1 Introduction

Metabolic syndrome (MetS) is a group of metabolic abnormalities characterized by high blood glucose, abnormal blood lipids, high blood pressure, and abdominal obesity (1). The global prevalence of MetS ranges from 12.5% to 31.4%, with higher prevalence in the Eastern Mediterranean region and the Americas, and it increases with increasing national income levels (2). From 1999 to 2018, cardiovascular and metabolic indicators (blood glucose, lipids, blood pressure, and adiposity) continued to deteriorate in the United States, and the prevalence of MetS increased from 28.23% to 45.9% (3–5). Due to the complex state of metabolic dysregulation, MetS is an important risk factor for cardiovascular disease, diabetes, stroke, and death (6–9). MetS impose a heavy health economic burden on global public health systems, requiring urgent intervention.

Antioxidant vitamins are a class of vitamins with antioxidant properties, mainly including vitamin A, vitamin C, vitamin E and carotenoids, which can reduce the damage caused to cells by oxidative stress by scavenging oxygen free radicals. A study have shown that a prudent dietary pattern in adolescence, comprising fruits and vegetables, cereals, and legumes, has been associated with a significantly reduced risk of MetS in middle age, in comparison to a Western dietary pattern focused on meat, refined grains, processed, and fried foods (10). Fruits and vegetables in a prudent dietary pattern are rich in antioxidant vitamins, which may play an important role in reducing the incidence of MetS (11). However, the association between antioxidant vitamins and MetS is currently controversial. Some studies have suggested that taking antioxidant vitamins may reduce the risk of MetS (12, 13), while others have found no association (14). Some studies have found that people with MetS have significantly lower blood levels of antioxidant vitamins than healthy people (15, 16), while others have found the opposite (17, 18). In addition, due to the body's metabolism of nutrients, serum antioxidant vitamins do not directly represent dietary intake. Whether they are consistent with metabolic syndrome remains unknown. Therefore, it is necessity to study the dietary and serum antioxidant vitamins separately in relation to metabolic syndrome.

Observational studies may be subject to confounding and reverse causality due to non-randomization, which limits the extrapolation and application of research findings. Mendelian randomization (MR) is a method of causal inference based on Mendelian segregation and the principle that alleles are randomly assigned in the process of gamete formation, using genetic variation as an instrumental variable (19). Because genetic variation is associated with outcomes in a causal temporal order and is unaffected by common confounders such as postnatal environment and social factors, MR can accurately infer the causal relationship between exposure and outcome (20). Li et al. (21) analyzed the relationship between antioxidant vitamins and MetS using MR, but less association was found. Therefore, this study aims to analyse the relationship between dietary and

circulating antioxidant vitamins and MetS using an observational study and MR analysis.

2 Materials and methods

2.1 Overall study design and data sources

The present study was conducted in two stages, as illustrated in Figure 1. In stage 1, using data deposited in the National Health and Nutrition Examination Survey (NHANES) database, we performed multivariable regression analysis to determine the observational association of dietary and serum antioxidant vitamins with MetS. In stage 2, we employed a two-sample MR analysis of summary statistics data from the genome-wide association study (GWAS) to assess the causal effect of genetically determined dietary and serum antioxidant vitamins on MetS.

The NHANES is a complex, multistage, nationally representative survey of the civilian noninstitutionalized population in the USA, conducted by the National Center for Health Statistics (NCHS). The survey includes household interviews, physical examinations, and laboratory tests. NHANES research protocols and data collection procedures were approved by the NCHS Research Ethics Review Board, and written informed consent was obtained from the participants. The additional ethical review was no longer required for the present study due to the usage of publicly available data without identifiable personal information. Participants in NHANES were selected from 2001 to 2006 who were 20 years of age or older ($n = 16,299$). A total of 5,991 participants were excluded (Supplementary Figure S1): (1) Without 24-h dietary recall or missing dietary antioxidant vitamins ($n=917$); (2) Had no information of serum antioxidant vitamins ($n=1,285$); (3) Unable to be diagnosed as MetS or not ($n=815$); (3) Had no information of covariables ($n = 2904$).

The design of MR analysis needs to satisfy the following assumptions (Supplementary Figure S2) (22): (1) The SNPs employed as IVs are related to antioxidant vitamins; (2) IVs are not associated with the confounders; (3) IVs affect the risk of MetS only by antioxidant vitamins. The available summary GWAS data for MR analysis were obtained from the IEU open GWAS project [<https://gwas.mrcieu.ac.uk/>](accessed on 1 March 2024)], GWAS Catalog [<https://www.ebi.ac.uk/gwas/>](accessed on 1 March 2024)], or references. Further details on the GWAS datasets are provided in Supplementary Table S1. The summary data on dietary antioxidant vitamins were derived from the UK Biobank with 9,851,867 single-nucleotide polymorphisms (SNPs). The SNPs that were significantly associated with the absolute circulating antioxidants and circulating metabolite's concentrations were obtained from published literature (23–28). The most comprehensive GWAS analysis was employed to obtain summary-level data for MetS, which included 291,107 individuals (59,677 cases and 231,430 controls) (29). Genetic information on components of MetS was also obtained, including fasting blood glucose (FBG), waistline, hypertension, triglycerides, high-density lipoprotein cholesterol (HDL-C) (30, 31).

2.2 Measurement of antioxidant vitamins

The intake of antioxidant vitamins was quantified through dietary interviews, including vitamin A, vitamin C, vitamin E, carotene, α -carotene, and β -carotene. In NHANES, dietary data were collected by 2–24 h dietary recalls (DRs), including the first day (Day 1) and second day (Day 2) which were collected in via the MEC and telephone, respectively. The in-person interview was conducted in the MEC dietary interview room, with participants reporting the amounts of foods consumed by means of a set of measuring guides (various glasses, bowls, mugs, household spoons, measuring cups and spoons, a ruler, thickness sticks, bean bags and circles). Telephone dietary interviews were self-reported by telephone, occurring 3 to 10 days after the MEC dietary interview. In UK Biobank, dietary data were from on-line dietary questionnaire, based on a 24-hour dietary recall of the previous day.

The serum levels of antioxidant vitamins were accessed using isocratic high-performance liquid chromatography (HPLC) with electrochemical detection. This consisted of vitamins A (retinol), vitamin C (ascorbate), vitamin E (α -tocopherol) and carotenoids. Sample collection, transformation, storage and analysis were conducted in accordance with the laboratory procedure manual.

2.3 Definition of metabolic syndrome

MetS was defined based on the National Cholesterol Education Program's Adult Treatment Panel III (ATP III) as having 3 or more of the following (32): 1) FBG ≥ 5.6 mmol/L (or 100 mg/dL) or drug treatment for elevated blood glucose; 2) HDL-C < 40 mg/dL (or 1.0 mmol/L) in men or < 50 mg/dL (or 1.3 mmol/L) in women or drug treatment for low HDL; 3) triglyceride level > 150 mg/dL (or 1.7 mmol/L) or drug treatment for elevated triglyceride; 4) waist circumference > 102 cm in men or > 88 cm in women; 5) systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 85 mmHg or taking hypertension medications.

2.4 Assessment of covariates

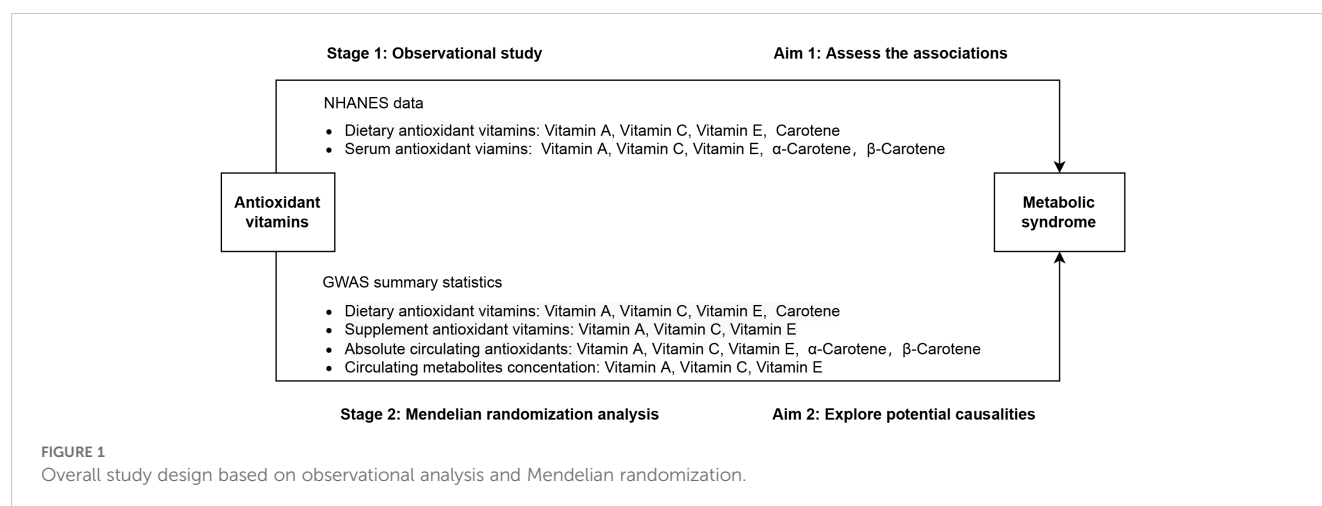
Information on demographic characteristics, lifestyle factors, and health conditions was obtained in NHANES. Sociodemographic characteristics consisted of age (20–40 years and 40–60 years), gender (women and men), race (non-Hispanic white, non-Hispanic black, Mexican American, other Hispanic and others), education (less than high school, high school graduate, some college and college graduate or above), marital status (married and others), poverty-income ratio (PIR: < 1 , 1–1.8, ≥ 1.8), health insurance (uninsured and any insurance). Lifestyle information was obtained from a series of questionnaires, including smoking status (never, former, now), drinking condition (yes and no), and physical activity (no, moderate activity and vigorous activity). The health conditions included in arteriosclerotic cardiovascular disease (ASCVD), consisted of coronary heart disease, angina, heart attack and stroke), chronic kidney disease (CKD), liver condition, thyroid disease, and cancer. All of these disease conditions were self-

reported by the subjects, except for CKD, which was diagnosed according to KDIGO 2021 (33) (urine albumin to creatinine ratio of 30 mg/g or higher, or estimated glomerular filtration rate less than 60 mL/min/1.73 m²).

2.5 Statistical analysis

NHANES data were extracted and pre-processed by the “nhanesR package” for the observational association of each antioxidant vitamins with MetS. Sample weights were calculated according to NHANES tutorials for combining NHANES data from three cycles. A survey-weighted algorithm was employed for statistical analysis, considering complex sampling. The distribution of social demographics, lifestyle, and health conditions was expressed by numbers (unweighted) and percentage (weighted). The dietary intake and serum level of antioxidant vitamins were presented as the median and interquartile range [M, (P25, P75)] due to a skewed distribution. The difference between the groups was compared by Kruskal-Wallis' test for continuous variables with non-normal distribution, and χ^2 test for categorical variables. The correlation between dietary antioxidants and serum levels was analyzed by Spearman. Dietary and serum antioxidant vitamins were divided into four groups according to quartile. A survey-weighted Logistic regression model was employed to evaluate the odds ratio (OR) and 95% confidence interval (CI) for the relationship between each antioxidant vitamins and MetS. Four statistical models were fitted, with Model 1 not adjusting for any factors, Model 2 adjusting for social demographic factors, Model 3 further adjusting for lifestyle factors, and Model 4 additionally adjusting for the health conditions. We performed tests for linear trend by entering the median value of each quartile of antioxidant vitamins as a continuous variable in the models. To investigate dose-response association between each antioxidant vitamins and MetS, a restricted cubic spline (RCS) regression model was fitted by Logistic regression model with three knots (10th, 50th, and 90th percentile). Tests for nonlinearity were performed using the likelihood ratio test. The relationship between antioxidant vitamins and components of MetS was also analyzed.

Two-sample MR analyses were performed using the “TwoSampleMR package” to investigate the causal analyses. In order to satisfy the core assumption of tool variables, three steps were carried out to filter IVs. Firstly, $P < 5 \times 10^{-6}$ was used as the primary screening criterion to ensure sufficient SNPs associated with exposure. Secondly, in order to ensure the independence of IVs, SNPs with linkage disequilibrium (LD) ($r^2 < 0.001$) were eliminated based on European ancestry reference data from the 1000 Genomes Project. Thirdly, IVs that showed genome-wide association with outcomes at a significance level of 5×10^{-8} were excluded. Additionally, palindromic SNPs were excluded after conducting harmonizing processes. *F* statistics were employed to assess the strength of weak instrumental variables ($F > 10$ indicating the stronger instrument strength). Subsequently, the inverse variance weighted (IVW) method was utilized as the primary



statistical method in MR to ascertain the causal relationship between antioxidant vitamins and MetS. Additionally, the MR Egger, weighted median, simple mode, and weighted mode were applied as sensitivity analyses. The MR-Egger method was employed to assess heterogeneity and pleiotropy. If the *P*-values of heterogeneity were greater than 0.05 without evidence of heterogeneity, the fixed-effect IVW approach was considered; conversely, the random effects IVW approach was utilized if there was substantial heterogeneity ($P < 0.05$). The causal relationship between antioxidant vitamins and components of MetS was also analyzed. Additionally, in the causal association of dietary antioxidant vitamins with MetS, multivariate MR analysis was performed by adjustment for supplement antioxidant vitamins.

All statistical analyses were performed using R version 4.3.3 software. Statistical significance was determined by a two-sided *P* value < 0.05 . Because of multiple comparisons, the significance level was corrected using the Bonferroni method. *P* value $< 0.008 (0.05/6)$ was considered a strong association, a *P* value between 0.008 and 0.05 was considered a potential association, and the *P*-values were two-sided.

3 Results

3.1 NHANES general population study

A total of 10,308 adults from the US with data on exposure (dietary and serum vitamins) and outcome (MetS) were eligible for analysis during the study period. The main characteristics of these participants are shown in Table 1. Of these participants, 2,613 (25.36%) met the diagnostic criteria for MetS. In comparison to the healthy controls, individuals with MetS were more likely to be older, have a lower education level and income, smoke, engage in no physical activity, and to have a history of ASCVD, CKD, liver condition, thyroid disease, and cancer. The dietary intake of antioxidant vitamins (vitamin A, vitamin C, vitamin E, carotene, α -carotene, and β -carotene) was significantly lower in the MetS group than that of the control group (Supplementary Table S2).

With regard to serum levels of antioxidant vitamins, the distribution between the two groups was consistent with dietary intake, with the exception of vitamins A and E, which exhibited opposite results. (Supplementary Table S2).

Correlation analysis revealed that dietary vitamin C ($r=0.347$, $P<0.001$), carotene ($r=0.345$, $P<0.001$), α -carotene ($r=0.363$, $P<0.001$), and β -carotene ($r=0.329$, $P<0.001$) were moderately correlated with serum levels, while vitamin A ($r=0.161$, $P<0.001$) and vitamin E ($r=0.078$, $P<0.001$) exhibited relatively low correlation with serum levels (Figure 2).

3.2 Associations between antioxidant vitamins and metabolic syndrome in NHANES

The results of the weighted logistic regression indicated that dietary intake of vitamin A (OR=0.852, 95%CI: 0.727-0.999), vitamin C (OR=0.802, 95%CI: 0.675-0.952), carotene (OR=0.832, 95%CI: 0.706-0.982), and β -carotene (OR=0.838, 95%CI: 0.706-0.995) in quartile 4 had lower incidence of MetS compared to quartile 1, after adjustment for demographic characteristics, lifestyle factors, and health conditions (Figure 3A; Supplementary Table S3). The intake of four antioxidant vitamins was found to be significantly and inversely associated with lower risk of MetS (*P* for trend=0.023, 0.006, 0.015, 0.024, respectively). Moreover, the results of the restricted cubic spline (RCS) model indicated a dose-response relationship between dietary vitamin A (*P*-nonlinear=0.096), carotene (*P*-nonlinear=0.284), and β -carotene (*P*-nonlinear=0.355) and MetS (Figure 4). Furthermore, the relationship between dietary vitamin C and MetS was nonlinear (*P*-nonlinear=0.007), exhibiting a rapid decrease and then a gradual, stable in the risk of MetS as dietary vitamin A increase. Nevertheless, there were no significant associations between vitamin E and α -carotene intake with the risk of MetS across all the quartile categories.

For each component of MetS, the association of dietary antioxidant vitamins with HDL and obesity was mainly found

TABLE 1 Baseline characteristics of the participants in NHANES.

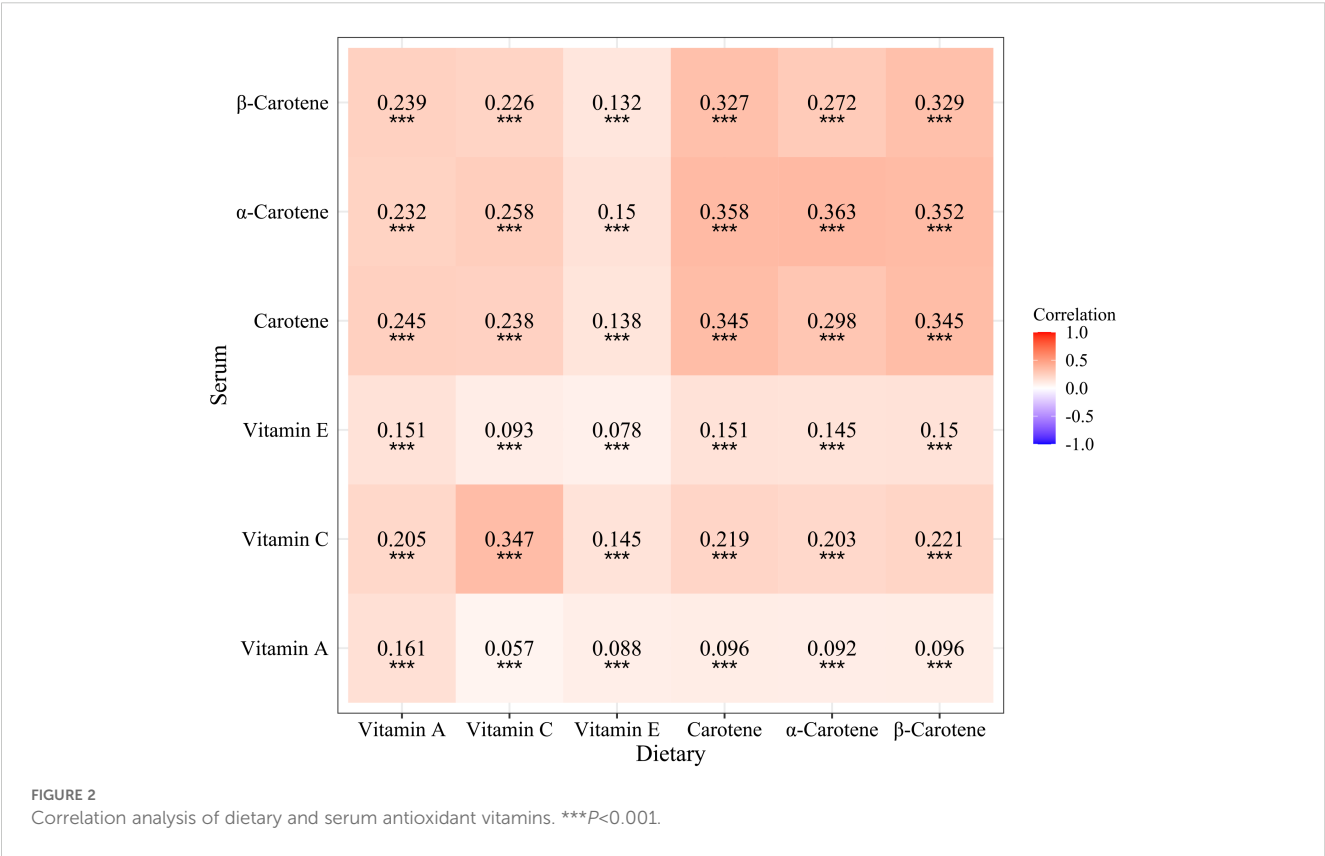
Characteristic	Total (n=10308)	Control (n=7695)	Metabolic syndrome (n=2613)	P
Age (years)				< 0.001
18-39	3330(32.31)	2901(42.47)	429(20.73)	
40-59	3390(32.89)	2443(38.93)	947(47.14)	
60-85	3588(34.81)	2351(18.60)	1237(32.12)	
Sex				0.172
Female	4996(48.47)	3617(50.00)	1379(52.10)	
Male	5312(51.53)	4078(50.00)	1234(47.90)	
Race				0.051
Non-Hispanic white	5569(54.03)	4100(73.31)	1469(76.91)	
Non-Hispanic black	1986(19.27)	1565(10.53)	421(8.07)	
Mexican American	2057(19.96)	1488(7.32)	569(6.84)	
Other Hispanic	340(3.30)	261(4.09)	79(4.18)	
Other race	356(3.45)	281(4.75)	75(4.01)	
Education				< 0.001
Less than high school	2815(27.31)	1993(15.67)	822(20.09)	
High school graduate	2521(24.46)	1802(24.45)	719(30.05)	
Some college	2868(27.82)	2168(31.11)	700(31.49)	
College graduate or above	2104(20.41)	1732(28.77)	372(18.37)	
Marital status				0.174
No	4483(43.49)	3382(40.95)	1101(38.72)	
Yes	5825(56.51)	4313(59.05)	1512(61.28)	
PIR				0.004
Low income	1660(16.1)	1194(11.06)	466(12.38)	
Middle income	2182(21.17)	1587(15.27)	595(17.75)	
High income	6466(62.73)	4914(73.66)	1552(69.87)	
Health insurance				0.003
No	2016(19.56)	1601(18.28)	415(14.66)	
Yes	8292(80.44)	6094(81.72)	2198(85.34)	
Smoking				< 0.001
Never	5148(49.94)	3928(50.69)	1220(46.71)	
Former	2794(27.11)	1946(23.99)	848(30.04)	
Now	2366(22.95)	1821(25.32)	545(23.25)	
Alcohol				< 0.001
No	3059(29.68)	2093(23.59)	966(34.51)	
Yes	7249(70.32)	5602(76.41)	1647(65.49)	
Physical activity				< 0.001
No	4032(39.12)	2794(29.42)	1238(42.06)	
Moderate	3133(30.39)	2273(30.75)	860(34.67)	

(Continued)

TABLE 1 Continued

Characteristic	Total (n=10308)	Control (n=7695)	Metabolic syndrome (n=2613)	P
Vigorous	3143(30.49)	2628(39.83)	515(23.27)	
ASCVD				< 0.001
No	9211(89.36)	7065(94.32)	2146(85.81)	
Yes	1097(10.64)	630(5.68)	467(14.19)	
CKD				< 0.001
No	8371(81.21)	6546(89.73)	1825(76.75)	
Yes	1937(18.79)	1149(10.27)	788(23.25)	
Liver condition				0.020
No	9969(96.71)	7464(96.91)	2505(95.76)	
Yes	339(3.29)	231(3.09)	108(4.24)	
Thyroid disease				< 0.001
No	9324(90.45)	7059(91.10)	2265(86.63)	
Yes	984(9.55)	636(8.90)	348(13.37)	
Cancer				< 0.001
No	9358(90.78)	7042(92.13)	2316(89.26)	
Yes	950(9.22)	653(7.87)	297(10.74)	

PIR, poverty-income ratio; ASCVD, arteriosclerotic cardiovascular disease; CKD, chronic kidney disease.



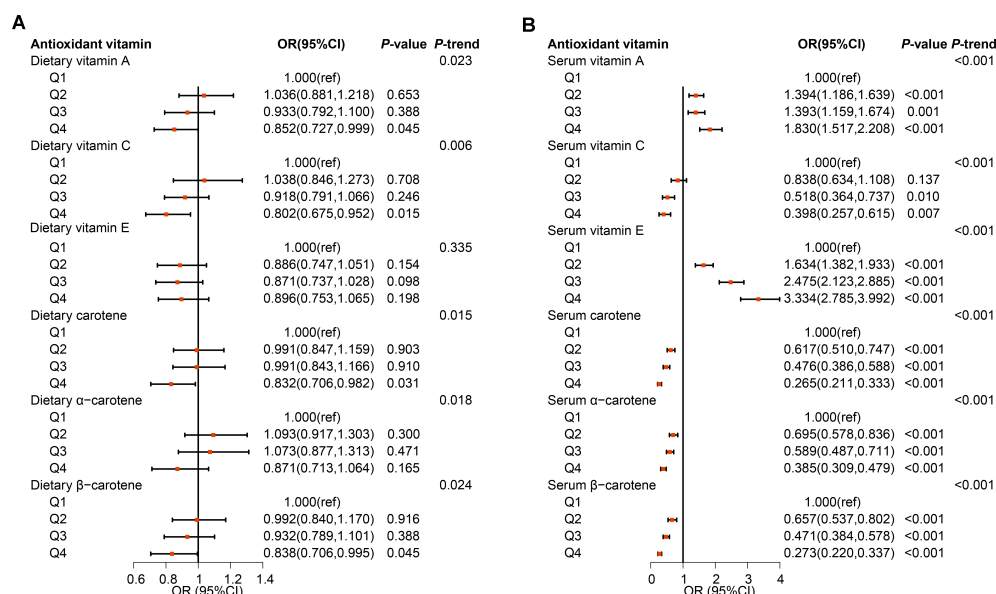


FIGURE 3

Observational associations of dietary (A) and serum (B) antioxidant vitamins on metabolic syndrome in NHANES population. The models were adjusted for demographic characteristics (age, gender, race, education, marital status, poverty-income ratio, and health insurance), lifestyle factors (smoking status, drinking condition, and physical activity), and health conditions (arteriosclerotic cardiovascular disease, chronic kidney disease, liver condition, thyroid disease, and cancer).

(Supplementary Figures S3-S7; Supplementary Tables S4-S8). The intake of vitamin A (OR=0.808, 95%CI: 0.699-0.934), vitamin E (OR=0.787, 95%CI: 0.668-0.926), carotene (OR=0.806, 95%CI: 0.677-0.959), and β-carotene (OR=0.793, 95%CI: 0.659-0.954) in quartile 4, and vitamin C (OR=0.850, 95%CI: 0.724-0.998) in quartile 3 were inversely associated with the risk of HDL decreasing compared to quartile 1 (Supplementary Figure S4; Supplementary Table S5). Meanwhile, the higher the dietary intake of vitamin A (OR=0.847, 95%CI: 0.735-0.976), vitamin C (OR=0.777, 95%CI: 0.653-0.925), carotene (OR=0.719, 95%CI: 0.606-0.853), α-carotene (OR=0.858, 95%CI: 0.743-0.991), and β-carotene (OR=0.741, 95%CI: 0.622-0.882), the lower the risk of obesity (Supplementary Figure S7; Supplementary Table S9). In addition, dietary vitamin C in quartile 4 decreased the risk of blood glucose elevating (OR=0.837, 95%CI: 0.703-0.996), and dietary vitamin A in quartile 4 decreased the risk of blood pressure elevating (OR=0.820, 95%CI: 0.686-0.981).

The serum vitamin C, carotene, α-carotene and β-carotene in quartile 2 to 4 also present inversely associated with the risk of MetS when compared to quartile 1 with all *P* for trend <0.001 (Figure 3B; Supplementary Table S3). Furthermore, the relationship between vitamin C and MetS was linear (*P*-nonlinear=0.363), while the relationship between carotenes and MetS was non-linear (all *P*-nonlinear<0.001) with a rapid decrease and then slow decrease in the risk of MetS as serum carotenes increase (Figure 4). However, the higher level of serum vitamin A and vitamin E both increased the risk of MetS. For each component analysis, serum vitamin C and carotenes were also demonstrated to significantly reduce the risk of components of MetS (Supplementary Figures S3-S7; Supplementary Tables S4-S9).

3.3 Causality between antioxidant vitamins and metabolic syndrome in MR

The results showed that the MR effect estimates of dietary vitamin A (OR=0.920, 95%CI: 0.861-0.984), vitamin C (OR=0.905, 95%CI: 0.836-0.979) and carotene (OR=0.918, 95%CI: 0.865-0.974) were significantly associated with the risk of MetS (Figure 5; Supplementary Table S9). While, MR analyses of the dietary vitamin E (OR=0.978, 95%CI: 0.906-1.056) instrument did not show evidence for associations with MetS. Each antioxidant vitamins have different degree of causal association with each components of MetS, vitamin C, vitamin A, carotene and vitamin E reduced the 5, 4, 2, and 2 components risks, respectively (Figure 6; Supplementary Figures S8-S12; Supplementary Tables S10-S14). The MR-Egger intercept tests showed that for all *P*-pleiotropy >0.05, suggesting that there was no horizontal pleiotropy.

To avoid the effect of antioxidant vitamin supplements on MetS, we performed a multivariate MR. After adjusting for antioxidant vitamin supplements, dietary vitamin A (OR=0.912, 95%CI: 0.845-0.984) and vitamin C (OR=0.906, 95%CI: 0.829-0.989) were still found to reduce the risk of MetS (Supplementary Table S15).

For the serum antioxidant vitamins, there was only significance of the MR effect estimates between absolute circulating antioxidants β-carotene (OR=0.909, 95%CI: 0.857-0.965) and MetS (Figure 5; Supplementary Table S9). What's more, β-carotene mainly decreased the level of triglyceride (OR=0.977, 95%CI: 0.961-0.992) (Supplementary Figure S10; Supplementary Table S12). We did not find causal relationships between other serum antioxidant vitamins and MetS. Nonetheless, genetically predicted circulating

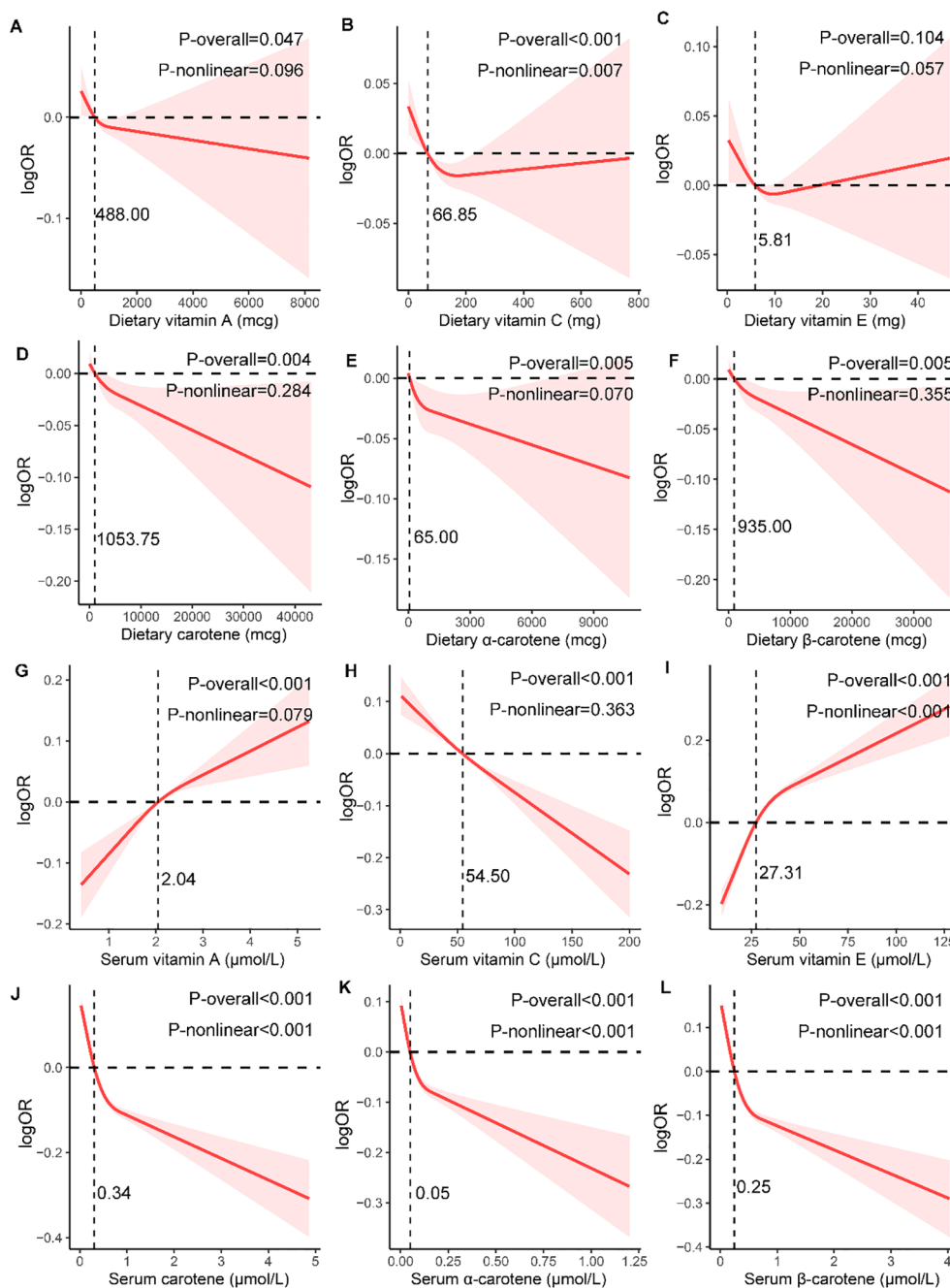


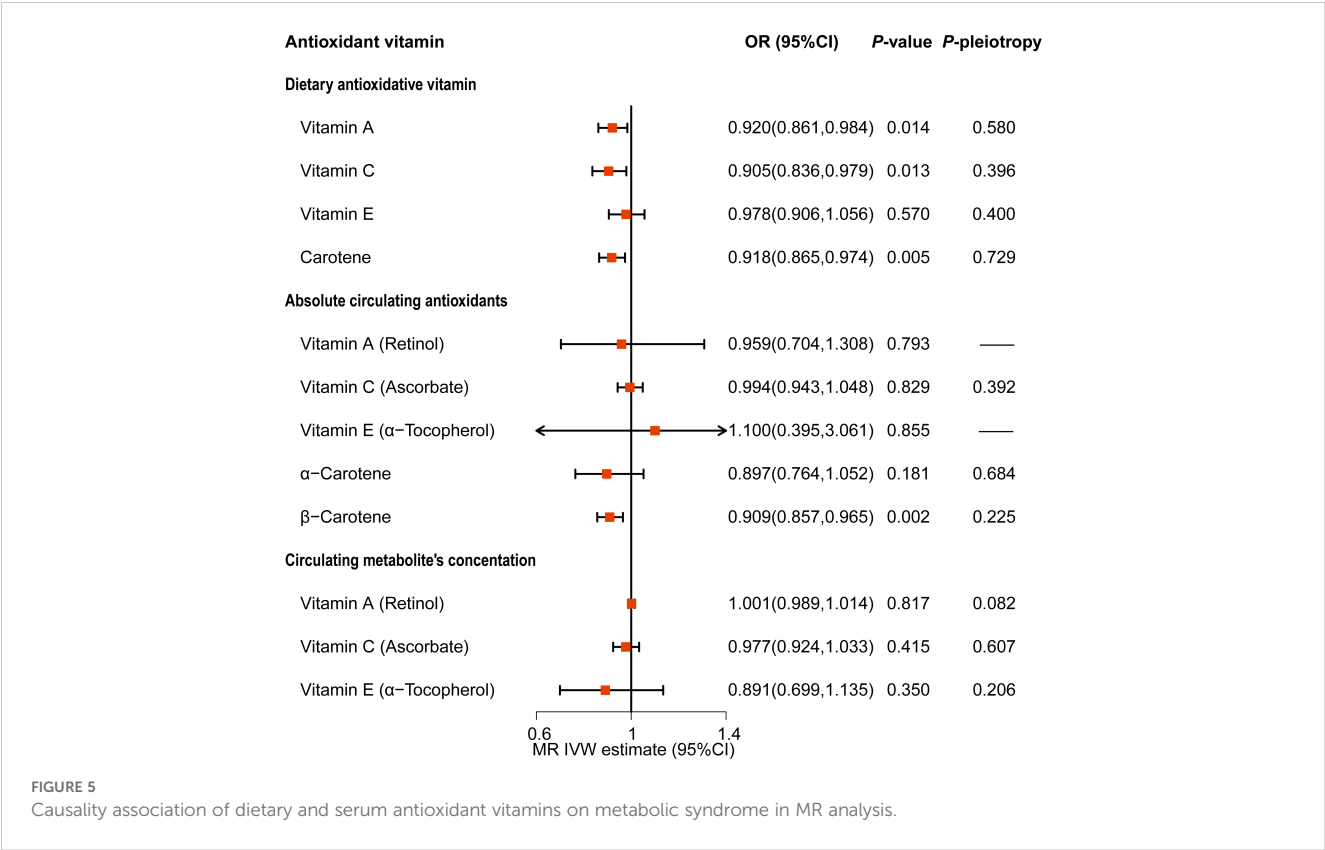
FIGURE 4

Dose-response association of dietary (A–F) and serum (G–L) antioxidant vitamins on metabolic syndrome in NHANES population. The models were adjusted for demographic characteristics (age, gender, race, education, marital status, poverty-income ratio, and health insurance), lifestyle factors (smoking status, drinking condition, and physical activity), and health conditions (arteriosclerotic cardiovascular disease, chronic kidney disease, liver condition, thyroid disease, and cancer).

vitamin A decreased level of HDL (OR=0.911, 95%CI: 0.984–0.998) and increased the levels of glucose (OR=1.004, 95%CI: 1.001–1.006) and triglyceride (OR=1.012, 95%CI: 1.006–1.019) (Figure 6; Supplementary Figures S9, S10; Supplementary Tables S11, S12). Besides, absolute circulating vitamin E was associated with the increased risk of hypertension (OR=1.059, 95%CI: 1.028–1.090) and decreased risk of obesity. (OR=0.936, 95%CI: 0.894–0.979) (Figure 6; Supplementary Figures S11, S12; Supplementary Tables S13, S14).

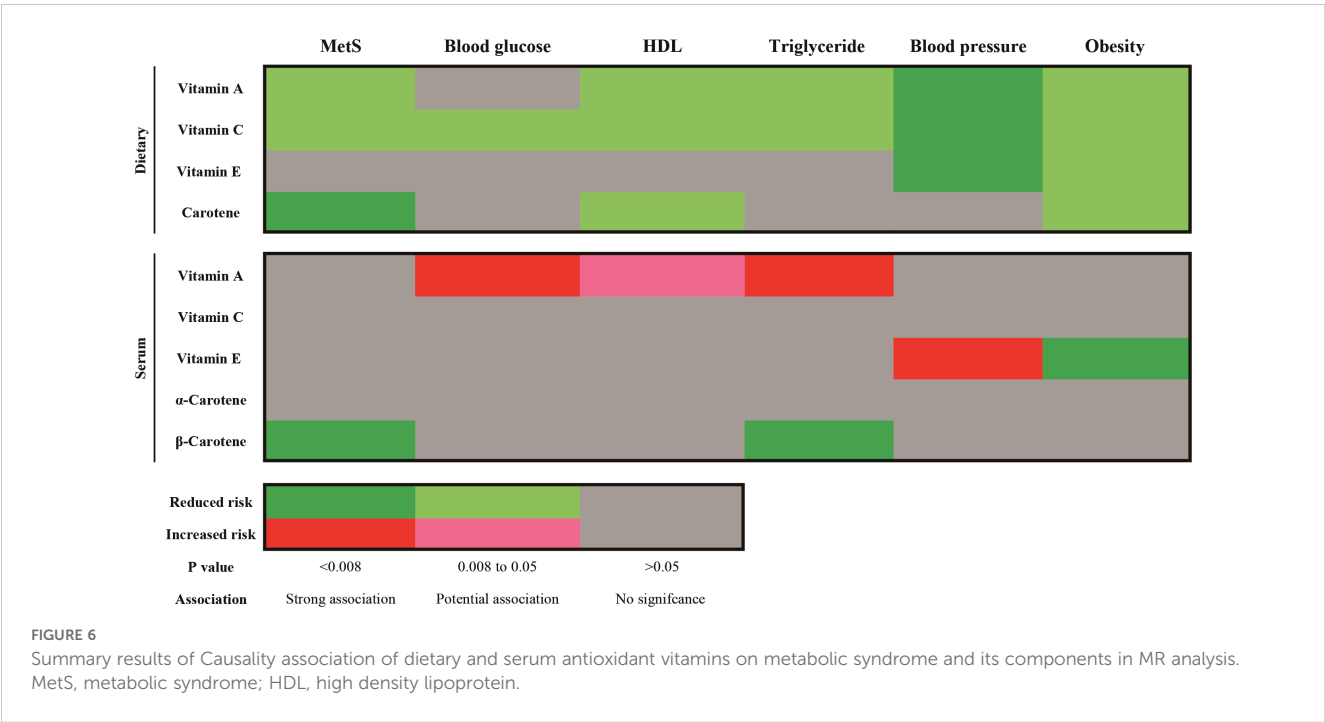
4 Discussion

Our results showed that dietary vitamin A, vitamin C and carotenoids were causally associated with a reduced risk of MetS, whereas no association was found between dietary vitamin E intake and MetS. In the circulation, only an elevated β-carotenoid concentration was associated with a reduced risk of MetS. In addition, although circulating vitamin A and vitamin E levels did not show a causal relationship with MetS, their elevation may



increase the risk of its components. This is evidenced by the fact that circulating vitamin A decreases HDL levels and increases glucose and triglyceride levels, while circulating vitamin E increases the risk of hypertension. Our study is an extension of Li et al. (21), and is also consistent with them. In their study, 18 circulating antioxidants nutrients, including vitamin A, C E, β-

carotenoids and so on, were analyzed in relation to the MetS, and the protective effect of β-carotenoids was also found. But in this study, only serum antioxidant vitamins were studied, no dietary antioxidant vitamins. It is our understanding that our study is the inaugural investigation to provide a comprehensive analysis of the correlation between dietary and serum antioxidant vitamins and



MetS. This analysis is based on a combination of large-scale observational study data and MR analysis of large-scale genetic data.

This study shows that dietary vitamin C is beneficial in reducing the risk of all components of the MetS, which is consistent with previous studies (34). Vitamin C, a widely used free radical scavenger, is a water-soluble vitamin that is completely dependent on dietary intake (35). Previous epidemiological studies have shown that vitamin C deficiency increases the risk of MetS in adults and leads to an increased incidence of diabetes (36). Vitamin C deficiency not only reduces cholesterol excretion and impairs hepatic lipid homeostasis, but also reduces the expression and activity of various antioxidant enzymes and increases markers of oxidative stress, thereby impairing the protective effects of hepatic protein and lipid oxidation (37). As a result of impaired gut barrier function due to overnutrition, MetS can repeatedly develop metabolic endotoxaemia, a vicious cycle that reduces vitamin C absorption while increasing inflammation and oxidative damage (38). A recent RCT showed that a micronutrient supplement high in vitamin C did not significantly reduce inflammation in patients with MetS, but did improve metabolic health indices (39). Currently, there is evidence that dietary vitamin C can improve obesity and diabetes by improving insulin sensitivity and myxophage abundance, regulating glucose and lipid metabolism (40–42); It may also help maintain vascular elasticity and promote healthy blood pressure levels by increasing the ability to synthesize NO and improving endothelial function (43).

The results of our study indicate that dietary vitamin A and carotenoids reduce the risk of MetS, and a beneficial effect of β -carotenoids on MetS was also found in serum. Beydoun et al. (44) also highlighted a negative association between β -carotenoids and MetS. Vitamin A is a fat-soluble vitamin that encompasses all compounds with the biological activity of retinol, including formed vitamin A (retinol) and pro-vitamin A (carotenoids) (45). β -carotene is a vital precursor of vitamin A, which can produce retinol after metabolism in the body and has significant biological activity of vitamin A. The antioxidant activity of vitamins A and β -carotene is conferred by the hydrophobic chain of the polyene unit, which can quench singlet oxygen, neutralize sulphur radicals and bind to and stabilize peroxy radicals (46). Vitamin A and its metabolite retinoic acid are thought to be important in maintaining normal white adipose tissue (WAT) and brown adipose tissue (BAT) physiology, and studies have shown that retinoic acid may contribute to the transition from WAT to BAT, thereby having a beneficial effect in preventing the accumulation of excess triglycerides (47). Carotenoids may also play an important role in adipose tissue biology, regulating adipocyte physiology by inhibiting peroxisome proliferation-activated receptors (PPAR), influencing the distribution of central obesity and the development of insulin resistance (48, 49). In addition, both vitamin A and β -carotenoids have been shown to play a protective role in blood pressure by modulating inflammatory responses and regulating NO pathways (50–52).

However, circulating vitamin A increased the risk of hyperglycemia and dyslipidemia in MR analysis, which was the opposite of the effect of dietary vitamin A. Our study also found a weak correlation between dietary vitamin A and serum levels, suggesting that circulating vitamin A does not represent dietary intake. The reason for the inconsistent

effects of dietary and circulation vitamin A on metabolic indexes may be related to its absorption and transportation. Vitamin A is a fat-soluble vitamin that must be absorbed into the human body with lipids, so its biological function is closely related to lipid synthesis and metabolism, in addition to its antioxidant effect (47). Dietary vitamin A is decomposed into free retinol by the pancreatic fluid in the intestine or retinoesterase in the brush margin of villi, which is absorbed, combined with chylomicrons and transported to the liver by the lymphatic system, and stored in the form of fat droplets in the fat cells (53). Study shows increased vitamin A absorption in people with MetS compared to healthy adults (54). This increase may be due to increased activity of phospholipase B, pancreatic lipase-related protein 2, and phospholipase A2 group IB (54). Therefore, it is more likely that people with abnormal lipid metabolism in MetS will absorb more vitamin A than healthy people, resulting in increased blood concentrations, than that circulation vitamin A increased metabolic markers. Regarding the transportation of vitamin A, it enters the circulation when retinol binds to retinol-binding protein 4, which plays an important role in insulin resistance, dyslipidaemia, obesity and diabetes (55–57). Consequently, dietary intake of the antioxidant vitamin A is a means of reducing the risk of MetS, rather than circulating vitamin A.

Dietary vitamin E did not show an association or causality with reduced risk of MetS, possibly because the above three antioxidant vitamins reduced the risk of more than three components, whereas vitamin E reduced the risk of only two components, blood pressure and obesity. A meta-analysis of observational studies showed an inverse association between dietary vitamin E levels and MetS (58). Vitamin E is a potent peroxy radical scavenger that can block the propagation of free radicals in cell membranes and plasma lipoproteins, thereby reducing oxidative stress and lowering the risk of hypertension (59). It also increases the production of the vasodilators prostaglandins I₂ and E₂ in a dose-dependent manner by increasing the expression of intracellular phospholipase A₂ and the release of the substrate AA, as well as inhibiting cyclooxygenase activity, thereby maintaining endothelial function (60). In addition, vitamin E and its metabolites regulate blood pressure by inhibiting diuretic potassium and calcium channels (61). At the same time, vitamin E may reduce adipose tissue fibrosis and collagen deposition through anti-inflammation and oxidative stress, and improve obesity and metabolic status (including hepatic steatosis, hypertriglyceridemia and insulin sensitivity) (62).

The findings of our research have significant implications for public health and practical applications. Firstly, we elucidated the relationship between antioxidant vitamins and MetS. The risk of MetS can be reduced by dietary intake of antioxidant vitamins, especially vitamins A, C and carotenoids, which are beneficial for many components (Figure 7). Furthermore, we conducted a separate analysis of the associations between dietary and serum antioxidant vitamins with MetS, which revealed partial inconsistent associations, mainly for vitamin A. This is because the absorption and transport of fat-soluble vitamin are affected by lipid metabolism, so the serum concentrations of vitamin A do not absolutely reflect dietary intake. Individuals with abnormally elevated serum vitamins A should be mindful of the potential for metabolic abnormalities. Therefore, we recommend reducing MetS

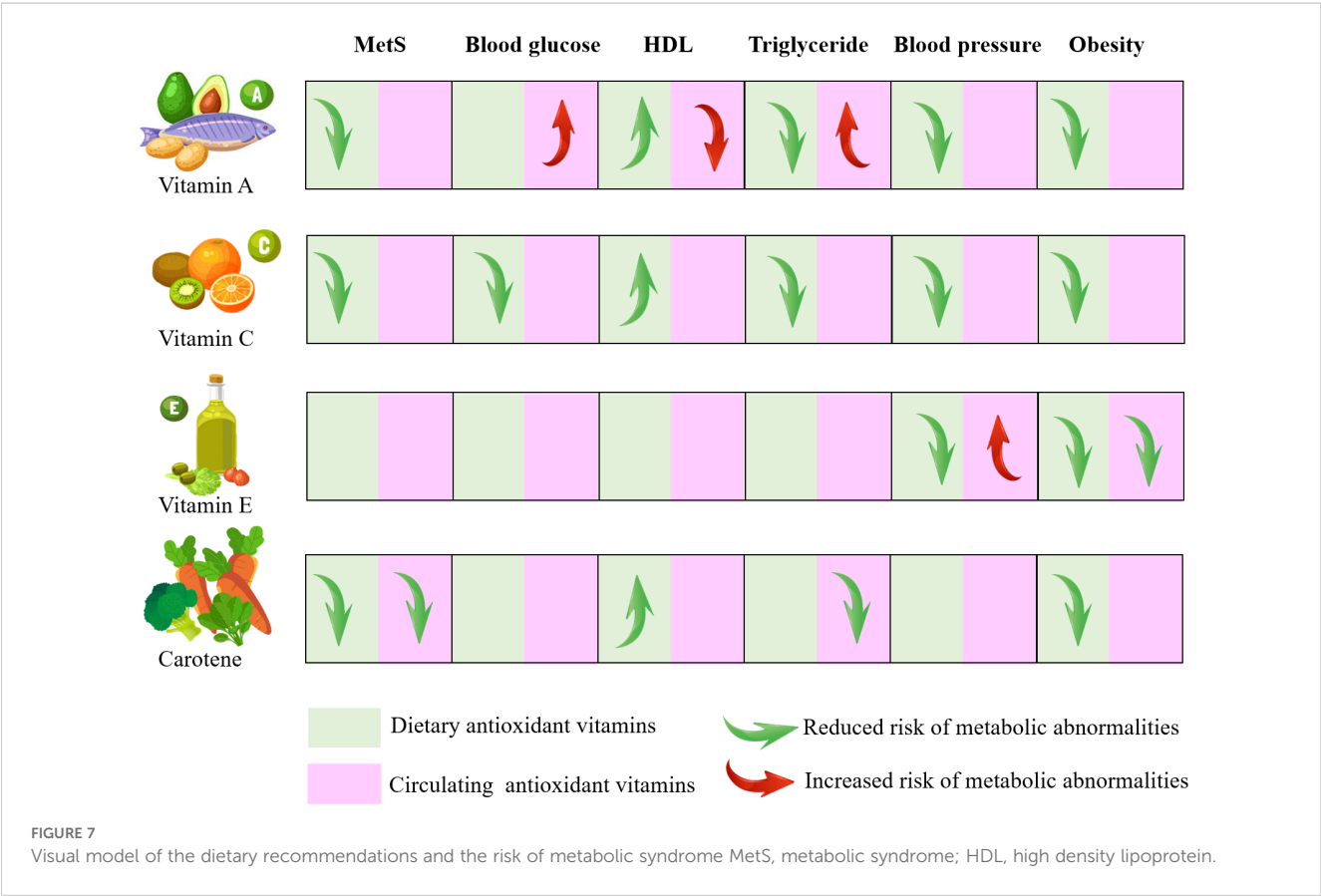
risk by increasing dietary intake rather than circulating concentration. Additional, Individuals with abnormal lipid metabolism should exercise caution when consuming vitamin A. It is recommended to monitor vitamin A levels through blood tests to prevent it accumulation that may lead to adverse effects. Before starting vitamin A supplementation, they should first manage their dyslipidemia with medication or other appropriate methods to ensure metabolic balance.

The major strength of our study was the use of MR analyses combined with the observational study design in NHANES. In a nationally representative sample of NHANES, the strategy of weighting algorithms and full adjustment for confounders was used to analyze the association between antioxidant vitamins and MetS. The consistency of findings between the MR analyses and the observational study made the results more robust. There are also several limitations to this study. First, antioxidant vitamin intake was measured by 24-hour DR, which may be subject to recall bias due to self-report. Second, due to the lack of data on vitamin supplementation in NHANES, this study did not analyze the effect of antioxidant vitamin supplementation on MetS. Third, in the MR study, we cannot completely rule out horizontal pleiotropy, an association between the outcome of interest and the MR instrument through pathways other than the proposed exposure, although the MR Egger intercepts in the statistical analysis showed no evidence of pleiotropy. Fourth, in order to obtain sufficient SNPs as instrumental variables, we relaxed the P-value setting of the association between exposure and SNPs. Although F-statistics

were used to assess the instrumental variables, the problem of weak instrumental variable bias may still arise. Fifth, our observational data suggest a possible non-linear association between some antioxidant vitamins and MetS; however, only linear causality was examined in the MR study, so non-linear causality cannot be ruled out. Sixth, our observational and genetic data were not from the same sample, as we used a multiracial US population in the cross-sectional study and individuals of European descent in the MR study. Last, although we adjusted confounders sufficiently to reduce the effect of confounders and used MR analysis to simulate the environment of randomized controlled trial, control group was not truly set up to explore the effect of antioxidant vitamins on MetS. In the future, we will accurately quantify dietary antioxidant vitamin intake to carry out interventional studies, evaluate the relationship between dietary vitamin intake at different levels and MetS and the improvement of metabolic indicators, and further explore the mechanism of the difference in biological effects of dietary and circulating antioxidant vitamins.

5 Conclusions

Based on observational and MR studies, we find that adequate dietary intake of vitamins A, C and carotenoids may reduce the risk of MetS, whereas vitamin E only reduces two metabolic components. In the circulation, only increased β -carotenoid concentration is associated with a reduced risk of MetS. In



addition, circulating vitamin A was associated with the increased risk of hyperglycaemia and dyslipidaemia. This suggests that eating more fruits and vegetables rich in antioxidant vitamins may help reduce the risk of MetS, but be wary of abnormally high concentrations of fat-soluble vitamins, such as vitamin A. If a high concentration of vitamin A is detected, it is not necessarily the result of increased intake, but most likely an increase in concentration due to abnormal absorption and transport caused by metabolic abnormalities.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The data were publicly available from NHANSE (<https://www.cdc.gov/nchs/nhanes/>). All data generated or analyzed during this study are included in this published article. The available summarized GWAS data for MR analysis were obtained from the IEU open GWAS project (<https://gwas.mrcieu.ac.uk/>), GWAS Catalog (<https://www.ebi.ac.uk/gwas/>), or references introduced in manuscript.

Ethics statement

NHANES study was conducted in accordance with the Declaration of Helsinki and approved by the NCHS Research Ethics Review Board (#98-12 and #2005-06). In MR analysis, all relevant ethics approvals are from original genome-wide association studies. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Our research was also exempted from ethical review (202406021722000305292) by the Affiliated LiHuiLi Hospital of Ningbo University.

Author contributions

QS: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. ZF: Data curation, Funding acquisition, Writing – original draft, Writing – review & editing. FY: Investigation, Methodology, Writing – review & editing. XZ:

Writing – review & editing. MJ: Writing – review & editing. MY: Writing – review & editing. MM: Writing – review & editing. CY: Conceptualization, Supervision, Writing – review & editing, Software.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1446719/full#supplementary-material>

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U-shaped association between dietary niacin intake and chronic kidney disease among US elderly: a nationwide cross-sectional study

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Background: In addition to hypertension or diabetes, elderly people are also considered one of the high-risk groups for chronic kidney disease (CKD). Although niacin is recognized for its renal protective properties, the link between dietary niacin intake and CKD remains uncertain. This study investigated this relationship in the elderly.

Methods: We included participants aged 60 and older from the National Health and Nutrition Examination Survey (NHANES) for the years 2003–2018. Dietary niacin intake was assessed through two non-consecutive 24-hour dietary recalls. CKD was diagnosed in individuals with a urine albumin-to-creatinine ratio exceeding 30 mg/g or an estimated glomerular filtration rate below 60 mL/min per 1.73 m². The study cohort comprised 4,649 participants, 1,632 of whom had CKD. Propensity score matching (PSM) was utilized to adjust for baseline differences between the groups.

Results: Our analysis, using smooth curve fitting and generalized additive models both before and after PSM, found a U-shaped curve depicting the relationship between dietary niacin intake and CKD risk, confirmed by a log-likelihood ratio test ($P < 0.05$). Threshold effect analysis (after PSM) indicated a reduced risk of CKD in older adults with a niacin intake below 38.83 mg per day [odds ratio (OR) = 0.99, 95% confidence interval (CI) 0.97–1.00]. In contrast, higher intake levels significantly increased the risk (OR = 1.03, 95% CI 1.00–1.06). Subgroup analysis indicated that these associations were consistent across different stratification variables (P for interaction > 0.05).

Conclusion: Our findings suggested a U-shaped association between dietary niacin intake and CKD risk among older Americans. However, further prospective cohort studies are needed to confirm this finding.

KEYWORDS

dietary niacin, chronic kidney disease, NHANES, elderly, u-shaped

1 Introduction

Chronic kidney disease (CKD) constitutes a major public health issue worldwide, afflicting more than 10% of the adult population in the United States and resulting in an annual financial burden exceeding \$50 billion (1). Not only does CKD elevate the risk of cardiovascular diseases, but it also leads to renal insufficiency and end-stage renal disease, necessitating interventions such as dialysis or kidney transplantation (2). Individuals aged 60 years and older are particularly susceptible to CKD, a concern that was exacerbated by the aging population dynamics, thereby underlining the critical need for strategies to prevent CKD in the elderly (3, 4).

Among the range of controllable lifestyle factors, dietary habits were particularly crucial. Appropriate nutritional intake can not only mitigate the risk of CKD but also enhances the overall health status of individuals (5). Niacin, or Vitamin B3, is an essential nutrient prevalent in a diverse array of foods such as meats, fish, grains, and dairy products (6). It functions as a precursor for synthesizing nicotinamide adenine dinucleotide (NAD), playing a crucial role in cellular metabolic processes, redox reactions, and energy metabolism (7). Niacin mitigates endothelial dysfunction by elevating intracellular NAD levels, enhancing glutathione synthesis, and diminishing reactive oxygen species production in endothelial cells (8). Current research suggests that supplementation with NAD precursors might represent a promising preventative and therapeutic strategy against renal damage (9, 10). Abnormal levels of myeloperoxidase, lipids and hyperphosphatemia, to varying degrees, indirectly or directly compromise the body's ability to counteract oxidative stress and inflammation, thereby exacerbating renal dysfunction (11–13). There were indications that niacin could improve dyslipidemia, oxidative stress, inflammatory responses, endothelial function, and serum phosphate levels, thus potentially delaying the progression of CKD (14). Studies suggested that a high dietary intake of niacin might benefit renal function in the elderly to some extent (15). However, some metabolites of niacin, such as prostaglandin D2, homocysteine, and 5-hydroxytryptamine, may also adversely affect renal function. Given niacin's potential dual effects on kidney health, it is crucial to establish an optimal daily intake range to maximize its protective benefits (16–18).

As far as we know, the association between dietary niacin intake and CKD in the elderly continues to be poorly understood, especially the effects of excessive intake. Considering this, exploring the curvilinear association between the two, and determining the optimal intake threshold, is of paramount importance for shaping public health policies and managing individual health among elderly. In this study, we utilized data spanning from 2003 to 2018 from the National Health and Nutrition Examination Survey (NHANES) to conduct a cross-sectional analysis.

2 Materials and methods

2.1 Study participants

NHANES, overseen by the National Center for Health Statistics at the Centers for Disease Control and Prevention, utilizes a

stratified, multistage sampling approach for its continuous survey efforts. NHANES is a nationwide cross-sectional study. This initiative seeks to thoroughly evaluate the health and nutritional conditions of U.S. adults and children via structured interviews and physical exams. The data required for this analysis were obtained directly from the NHANES official website (<https://www.cdc.gov/nchs/nhanes>). Written consent was obtained from all NHANES participants for their participation.

This study is a cross-sectional analysis utilizing continuous NHANES data collected over eight cycles from 2003 to 2018. Over this 16-year span, an initial pool of 80,312 participants was considered. Firstly, 64,931 individuals under the age of 60 were excluded from the analysis. Subsequently, 2,151 individuals with unclear CKD diagnoses and 1,969 participants with missing dietary niacin intake data were also excluded. Furthermore, 6,612 participants lacking other relevant covariate information were removed from the analysis. Ultimately, this study included 4,649 participants who met all the inclusion criteria (Figure 1).

2.2 Dietary niacin intake

Dietary niacin intake is evaluated using the dietary interview section of NHANES. All individuals eligible to participate in NHANES are required to complete two rounds of 24-hour dietary recall interviews. These interviews record the dietary intake for the day prior to the interview, from midnight to midnight. To more accurately estimate long-term nutrient intake, it is recommended to compute an average based on at least two non-consecutive days of dietary data (19). This methodological approach enhances the reliability of the nutrient intake estimates by mitigating the day-to-day variability in intake patterns.

2.3 Definition of CKD

CKD is diagnosed when at least one of the following criteria is met: 1) a ratio of albumin to creatinine in the urine exceeding 30 mg/g; 2) the estimated glomerular filtration rate (eGFR) estimated to be less than 60 mL/min per 1.73 m² (20). The measurement of albumin in the urine utilizes a solid-phase fluorescent immunoassay approach. Creatinine levels in the urine are assessed using the Jaffé method, which involves a kinetic reaction (21). Calculations of eGFR are based on the creatinine formula from the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (22).

2.4 Selection of covariates

To mitigate potential confounding factors, this study considered several covariates: age, body mass index (BMI), gender, race, marital status, educational level, poverty index ratio (PIR), smoking status, drinking status, activity, hypertension, diabetes, and various biochemical markers. The biochemical markers analyzed included uric acid, serum phosphate, total cholesterol (TC), triglycerides (TG), low-density lipoprotein

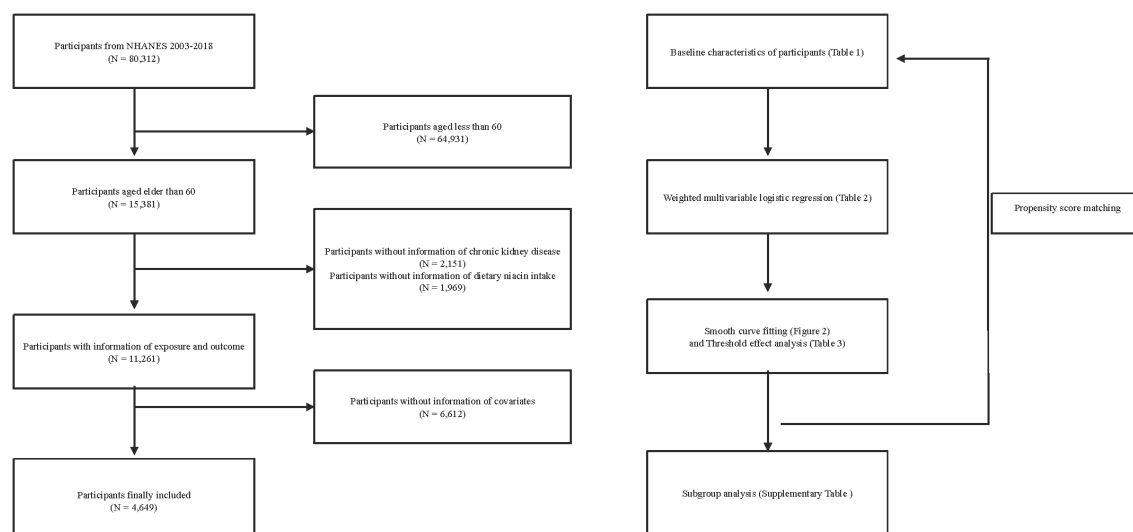


FIGURE 1

Flow chart for inclusion and exclusion of study participants. NHANES, National Health and Nutrition Examination Survey. .

cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C).

2.5 Statistical analysis

Considering the complexities of the multistage sampling design, we employed the sample weighting data and combination methods recommended by NHANES to ensure national representativeness in our analyses. For baseline characteristics, continuous variables were described using weighted means (95% confidence intervals), while categorical variables were presented as the number of observed (weighted percentage). To assess differences among CKD populations, the study employed weighted linear regression analysis for the appraisal of continuous variables and utilized weighted Chi-square tests to examine categorical variables. To investigate the relationship between dietary niacin intake and CKD, we employed weighted multivariate logistic regression models, establishing three different analytical models: 1) Model 1, which adjusted for no covariates; 2) Model 2, which included preliminary adjustments for age, gender, race, marital status, educational level, and PIR; 3) Model 3, which comprehensively adjusted for all covariates. Initially, dietary niacin intake was examined as a continuous variable. To preliminarily explore potential dose-response relationships, we divided niacin intake into four equally spaced quartiles (Q1, Q2, Q3, Q4) and conducted tests for trend.

To corroborate the robustness of our initial findings, we conducted a series of sensitivity analyses. We utilized smoothing spline fits and generalized additive models (GAM) to probe the non-linear relationship between dietary niacin intake and CKD. GAMs can explore the non-linear relationship between independent and dependent variables through smooth functions, offering greater expressive power than linear models (23). Based on previous literature, we set the smoothness parameter to 3 to ensure

the curve's smoothness (24). Subsequently, a piecewise linear regression approach was adopted to delineate two distinct segments and compute threshold effects. We contrasted this with a traditional linear model, applying a Log-likelihood ratio test to verify the presence of any thresholds. The threshold size was identified using a two steps recursive method (25). Additionally, subgroup analyses were conducted to explore whether the impact of dietary niacin intake on CKD varied across different strata, assessing interaction effects through likelihood ratio tests. Lastly, given the baseline differences between CKD and non-CKD participants (Table 1), propensity score matching (PSM) was used to align baseline characteristics between the two groups, thereby controlling for confounding and preventing bias. All baseline characteristics in Table 1, except for dietary niacin intake, were included in the PSM model, employing a 1:1 nearest neighbor matching technique with a caliper width allowing for a 0.05 difference in propensity scores. A smaller caliper width helps to improve the balance between matching pairs, thus better controlling for the effect of confounding variables (26).

R software (version 4.3.2) and EmpowerStats (X&Y Solutions, Inc., Boston, MA) were the tools utilized for the statistical computations and graphical representations in this study. We defined statistical significance at a cutoff of $p < 0.05$ using a two-sided test.

3 Results

3.1 Baseline characteristics of participants

Our research encompassed 4,649 individuals aged 60 and above, all subject to detailed inclusion and exclusion criteria outlined (Figure 1). Within this group, 1,632 were identified as having CKD. Before PSM, significant statistical differences were observed

TABLE 1 Baseline characteristics of participants between 2003 and 2018 before and after PSM.

Characteristic	Before PSM			After PSM		
	Non-CKD	CKD	P	Non-CKD	CKD	P
	(N = 3017)	(N = 1632)		(N = 1369)	(N = 1369)	
Age, years	67.82 (67.54, 68.11)	72.74 (72.28, 73.20)	<0.0001	70.47 (69.99, 70.95)	71.89 (71.39, 72.38)	<0.0001
BMI, kg/m ²	29.00 (28.67, 29.33)	29.65 (29.25, 30.04)	0.0125	29.13 (28.71, 29.55)	29.52 (29.08, 29.96)	0.1734
Gender, n (%)			0.0158			0.0794
Male	1506 (46.46)	786 (41.59)		689 (47.03)	673 (42.81)	
Female	1511 (53.54)	846 (58.41)		680 (52.97)	696 (57.19)	
Race, n (%)			0.4038			0.7087
Mexican American	694 (6.84)	276 (6.19)		278 (6.80)	241 (6.35)	
Non-Hispanic White	1582 (81.40)	990 (81.15)		750 (79.37)	812 (80.66)	
Non-Hispanic Black	558 (7.58)	285 (8.68)		254 (8.88)	243 (8.67)	
Other	183 (4.17)	81 (8.68)		87 (4.95)	73 (4.31)	
Marital status, n (%)			<0.0001			0.015
Married/with partner	1934 (69.83)	912 (58.23)		822 (65.99)	796 (60.79)	
Single	1083 (30.17)	720 (41.77)		547 (34.01)	573 (39.21)	
Education level, n (%)			<0.0001			0.3031
Less than high school	812 (15.98)	498 (21.03)		410 (20.75)	409 (20.30)	
High school or equivalent	720 (24.62)	446 (29.94)		315 (24.61)	349 (28.07)	
College or above	1485 (59.40)	688 (49.04)		644 (54.64)	611 (51.63)	
PIR, n (%)			<0.0001			0.682
< = 1.30	750 (14.05)	465 (19.46)		375 (17.18)	385 (18.84)	
> 1.30, < = 3.50	1259 (39.39)	762 (47.17)		620 (46.78)	625 (45.58)	
> 3.50	1008 (46.56)	405 (33.37)		374 (36.04)	359 (35.58)	
Smoking status, n (%)			0.7			0.5159
No	1420 (47.34)	798 (48.19)		648 (45.87)	668 (47.57)	
Yes	1597 (52.66)	834 (51.81)		721 (54.13)	701 (52.43)	
Drinking status, n (%)			<0.0001			0.1035
No	876 (25.20)	580 (34.13)		436 (28.75)	467 (32.52)	
Yes	2141 (74.80)	1052 (65.87)		933 (71.25)	902 (67.48)	
Activity, n (%)			<0.0001			0.1156
Inactive or moderate	2654 (86.28)	1535 (93.16)		1239 (89.88)	1277 (92.22)	
Vigorous	363 (13.72)	97 (6.84)		130 (10.12)	92 (7.78)	
Hypertension, n (%)			<0.0001			0.0075
No	1365 (47.80)	445 (27.09)		482 (36.92)	424 (30.60)	
Yes	1652 (52.20)	1187 (72.91)		887 (63.08)	945 (69.40)	
Diabetes, n (%)			<0.0001			0.0003
No	2391 (82.82)	1047 (68.28)		990 (76.20)	908 (70.99)	
Yes	525 (13.93)	535 (28.29)		306 (18.70)	426 (26.13)	

(Continued)

TABLE 1 Continued

Characteristic	Before PSM			After PSM		
	Non-CKD	CKD	P	Non-CKD	CKD	P
	(N = 3017)	(N = 1632)		(N = 1369)	(N = 1369)	
Borderline	101 (3.24)	50 (3.43)		73 (5.10)	35 (2.88)	
Uric acid, mg/dL	5.46 (5.40, 5.53)	6.27 (6.18, 6.36)	<0.0001	5.76 (5.67, 5.86)	6.03 (5.94, 6.12)	0.0003
Phosphorus, mg/dL	3.63 (3.59, 3.66)	3.72 (3.69, 3.76)	0.0004	3.65 (3.61, 3.68)	3.70 (3.66, 3.74)	0.0376
HDL-C, mg/dL	57.54 (56.54, 58.53)	55.73 (54.34, 57.12)	0.0257	56.27 (55.21, 57.33)	56.54 (54.96, 58.81)	0.7634
LDL-C, mg/dL	115.12 (113.36, 116.88)	106.13 (103.72, 108.55)	<0.0001	111.55 (109.39, 113.71)	107.49 (104.73, 110.25)	0.0177
TG, mg/dL	122.21 (118.25, 126.16)	130.90 (126.39, 135.41)	0.0035	127.93 (123.06, 132.80)	128.54 (123.55, 133.53)	0.8644
TC, mg/dL	197.09 (194.97, 199.21)	188.04 (185.26, 190.82)	<0.0001	193.40 (190.84, 195.95)	189.73 (186.54, 192.92)	0.0588
Dietary niacin intake, mg	23.76 (23.02, 24.50)	20.78 (20.12, 21.43)	<0.0001	22.34 (21.69, 22.99)	21.03 (20.26, 21.79)	0.0025

CKD, chronic kidney disease; PSM, propensity score matching; BMI, body mass index; PIR, poverty income ratio; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol; N/n, number of observed; %, survey-weighted percentage.
For continuous variables: numerical value with survey-weighted mean (95% CI), P was by survey-weighted linear regression.
For categorical variables: number of observed with survey-weighted percentage, P was by survey-weighted Chi-square test.

across most baseline characteristics between the groups categorized by CKD status (Table 1). After applying a 1:1 PSM, the number of participants reduced to 2,738, evenly distributed between the CKD and non-CKD groups. After PSM, significant reductions were observed in the statistical differences between the two groups regarding BMI, gender, educational level, PIR, drinking status, activity, HDL-C, TG, and TC.

3.2 Associations between dietary niacin intake and CKD in older adults

Findings from the logistic regression revealed a notable inverse correlation between dietary niacin intake and the occurrence of CKD among older adults (Table 2). The link was statistically significant in the unadjusted Model 1 [odds ratio (OR) = 0.97, 95% confidence interval (CI) 0.96-0.98], showed persistence in the minimally adjusted Model 2 (OR = 0.98, 95% CI 0.97-0.99), and remained stable in the fully adjusted Model 3 (OR = 0.99, 95% CI 0.97-1.00). The robustness of these findings was corroborated by the Model 4 (after PSM: OR = 0.99, 95% CI 0.98-1.00).

In Model 3, a negative association was found between dietary niacin intake and CKD when the lowest quartile of intake was used as a reference. OR for the second, third, and highest quartiles were 0.86 (95% CI 0.66-1.13), 0.64 (95% CI 0.48-0.85), and 0.70 (95% CI 0.51-0.95) respectively, with a trend significance (P for trend = 0.0075). It is of particular interest that the most pronounced protective effect of niacin was evident in the third quartile, implying a potential threshold effect. This pattern aligns with results from the Model 4, suggesting a non-linear link between niacin and CKD risk among the elderly.

3.3 The non-linear relationship between dietary niacin intake and CKD prevalence in older adults

After multivariate adjustments in Model 3, smoothing spline fitting and GAM elucidated a U-shaped association between these variables (Figures 2A, C). Using a two-piecewise linear regression model, we demonstrated a significant non-linear relationship between dietary niacin intake and CKD risk both before and after PSM (P for log likelihood ratio test < 0.05) in Table 3.

The turning points of dietary niacin intake were calculated using the two steps recursive method, identified at 40.35 mg/d before PSM and 38.83 mg/d after PSM. For most participants, daily dietary niacin intake was below these turning points (Figures 2B, D). Before reaching these turning points, an increase in dietary niacin intake was significantly associated with a reduced risk of CKD (before PSM: OR = 0.99, 95%CI 0.98-1.00; after PSM: OR = 0.99, 95%CI 0.97-1.00). However, beyond these turning points, the relationship inverted, and higher dietary niacin intake appeared to correlate with an increased risk of CKD, particularly after PSM where the association became more pronounced (OR = 1.03, 95%CI 1.00-1.06).

3.4 Subgroup analysis

Further investigation through subgroup analysis was conducted to explore if this curvilinear association differed across various participant characteristics. The findings, as detailed in Supplementary Table 1, were very stable, showing no significant interactions in any stratification variables (P for interaction > 0.05).

TABLE 2 Before and after PSM, weighted multivariable logistic regression for the association between dietary niacin intake and CKD.

Before PSM	OR (95%CI)			After PSM	OR (95%CI)
	Model 1	Model 2	Model 3		Model 4
Niacin continuous, mg	0.97 (0.96, 0.98)	0.98 (0.97, 0.99)	0.99 (0.97, 1.00)	Niacin continuous, mg	0.99 (0.98, 1.00)
Niacin quartile, mg				Niacin quartile, mg	
Q1 (< 14.90)	1.00 (reference)	1.00 (reference)	1.00 (reference)	Q1 (< 14.58)	1.00 (reference)
Q2 (14.90 - 20.09)	0.83 (0.66, 1.03)	0.92 (0.72, 1.18)	0.86 (0.66, 1.13)	Q2 (14.59 - 19.68)	0.80 (0.61, 1.06)
Q3 (21.10 - 26.38)	0.57 (0.45, 0.71)	0.66 (0.51, 0.86)	0.64 (0.48, 0.85)	Q3 (19.69 - 25.95)	0.64 (0.47, 0.89)
Q4 (> 26.38)	0.48 (0.38, 0.62)	0.67 (0.51, 0.89)	0.70 (0.51, 0.95)	Q4 (> 25.96)	0.68 (0.51, 0.90)
P for trend	<0.0001	0.0012	0.0075	P for trend	0.0043

OR: odds ratio; 95%CI: 95% confidence interval; Q: quartile; PSM: propensity score matching.
Model 1: No covariates were adjusted.
Model 2: Age, gender, race, education level, marital status, and PIR were adjusted.
Model 3: Age, gender, race, educational level, marital status, PIR, smoking status, drinking status, activity, hypertension, diabetes, BMI, uric acid, phosphorus, HDL-C, LDL-C, TG and TC were adjusted.
Model 4: After PSM, the covariates were adjusted in accordance with model 3.

Consistency in results was maintained after PSM, as indicated in [Supplementary Table 2](#).

4 Discussion

In this nationally representative cross-sectional study, a U-shaped relationship was identified between dietary niacin intake and CKD prevalence among individuals aged 60 and older. We determined the inflection points for dietary niacin intake to be 40.35

mg/d before PSM and 38.83 mg/d after PSM. These thresholds were near the upper intake level of 35 mg/d recommended by the Institute of Medicine for adults (27), suggesting a close alignment with our findings. However, it was notable that the majority of elderly individuals did not reach these niacin intake thresholds. The findings of this study have potential implications for clinical practice, aiding doctors in recommending appropriate niacin intake for elderly. It is crucial to avoid excessive or insufficient niacin intake to maintain a safe range. For public health, these findings underscore the importance of building public awareness

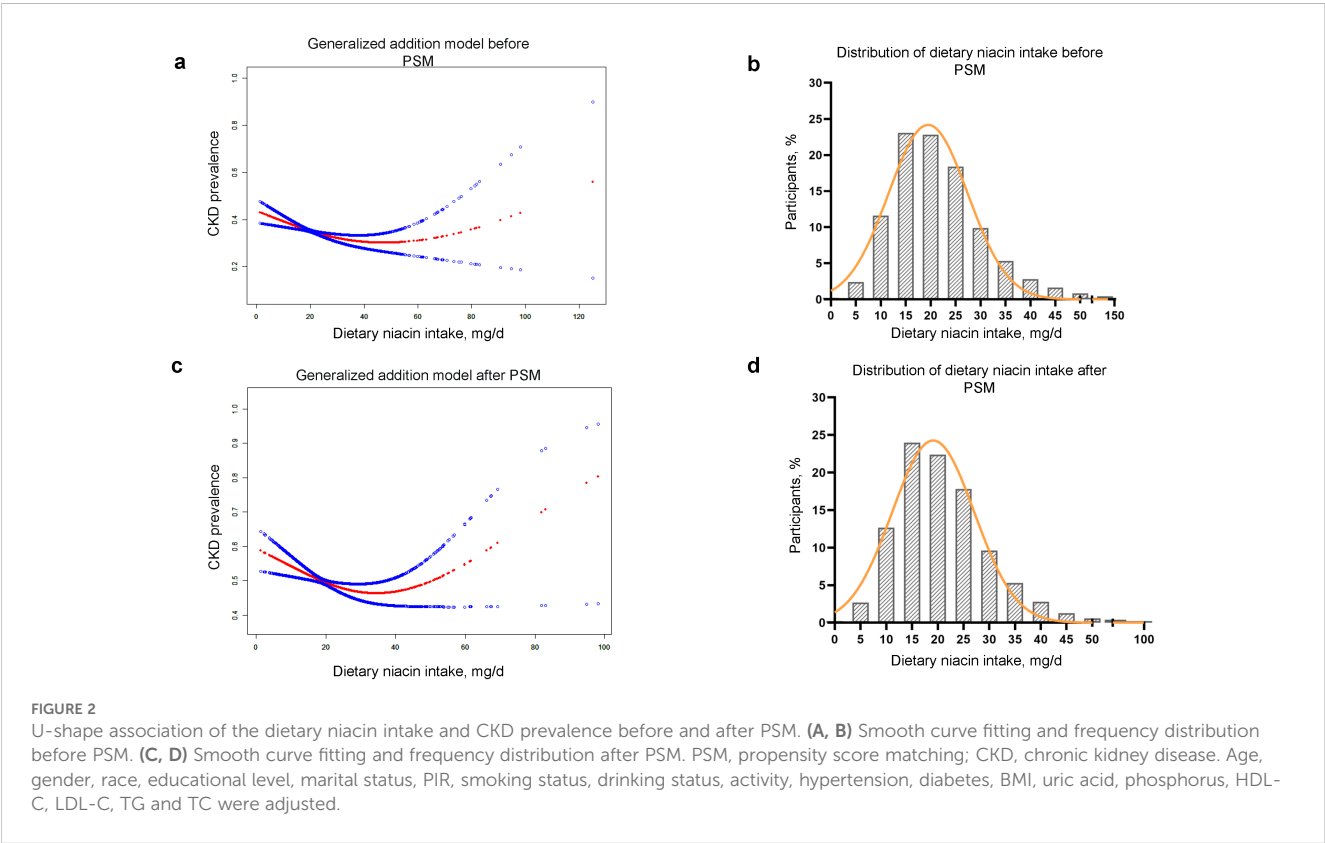


TABLE 3 Threshold effect analysis using two-piecewise linear regression based on dietary niacin intake and CKD before and after PSM.

Dietary niacin intake, mg	OR (95%CI)	P
Before PSM		
Fitting model by two-piecewise linear regression		
Inflection point		
< 40.35	0.99 (0.98, 1.00)	0.0054
> = 40.35	1.02 (0.99, 1.04)	0.1563
P for log likelihood ratio test		0.04
After PSM		
Fitting model by two-piecewise linear regression		
Inflection point		
< 38.83	0.99 (0.97, 1.00)	0.0055
> = 38.83	1.03 (1.00, 1.06)	0.0426
P for log likelihood ratio test		0.007

PSM, propensity score matching; OR, odds ratio; 95%CI, 95% confidence interval. Age, gender, race, educational level, marital status, PIR, smoking status, drinking status, activity, hypertension, diabetes, BMI, uric acid, phosphorus, HDL-C, LDL-C, TG and TC were adjusted.

about appropriate nutrient intake, developing policies for dietary supplement regulation to prevent excessive intake, updating dietary guidelines for elderly, and emphasizing both upper and lower limits of niacin intake to prevent CKD and other related health issues. In conclusion, this study not only informs individual clinical practice but also provides an empirical basis for public health policy-making. This finding can help reduce the prevalence of CKD in elderly and improve overall health.

Niacin has been reported to contribute to the slowing of eGFR decline, demonstrating significant potential as a renal protective agent (14). However, research linking dietary niacin intake with CKD risk remained limited. A study involving children demonstrated a noteworthy positive correlation between dietary niacin intake and eGFR (28). However, the study did not quantify the niacin intake, and the sample size was limited to 19 participants. The results suggest a potential association between niacin and kidney function in children, but not in the elderly. Moreover, a recent cross-sectional study conducted in Japan discovered a significant association between higher dietary niacin intake and reduced CKD risk among individuals aged 40 and older who were homozygous for the rs883484 allele (OR = 0.74, 95% CI 0.57–0.96) (29).. However, the findings are specific to individuals homozygous for the RS883484 allele, who constitute only 15% of the population. It is also worth noting that the study participants had a mean age of 62.1 ± 10.8 years, which somewhat represents the older population. To a certain extent, this supported some of our findings and underscored the potential preventive benefits of dietary niacin against CKD. Additionally, this study introduced the concept of dietary niacin indications for specific genetic types, which could be an interesting direction for future research. Earlier prospective cohort studies also aligned

with our findings, demonstrating that higher dietary niacin intake (14.7 mg/d) was associated with a reduced risk of renal function decline in individuals aged 65 and over compared to the lowest quartile (7.9 mg/d) [Relative Risk (RR) = 0.728, 95% CI 0.548–0.966] over an average follow-up of 3.2 years, highlighting the benefits of niacin for elderly renal function (15). However, compared to our study, the baseline levels of dietary niacin intake were relatively low in this research. For instance, the lowest quartile had an intake of 7.9 mg/d and the highest 14.7 mg/d, which might explain why the previous study did not observe significant threshold effects associated with excessive niacin intake.

Our study offered a new insight into the prevention of CKD among individuals aged 60 and older. We found that for participants with dietary niacin intake below the threshold of 38.83 mg/d (after PSM), an increase in niacin intake was significantly associated with a decreased risk of CKD (OR = 0.99, 95% CI 0.97–1.00). The pathogenesis of CKD was mediated by various risk factors such as oxidative stress, inflammatory responses, and endothelial dysfunction (30). Research in animal models of CKD demonstrated that niacin could mitigate conditions like hypertension, proteinuria, and glomerulosclerosis in mice, reducing kidney oxidative stress, inflammation, and tissue damage (31). Additionally, niacin assisted in mitigating endothelial dysfunction by elevating cellular NAD levels, replenishing glutathione, and decreasing reactive oxygen species production in endothelial cells (8). Recent studies had established a close link between disruptions in NAD synthesis and kidney damage (32), suggesting that modulating NAD metabolism could be a novel therapeutic approach to combat renal cell damage (9). Furthermore, myeloperoxidase (MPO) served as a critical mediator linking oxidative stress, inflammation, and endothelial dysfunction. Elevated MPO activity was linked to the advancement of glomerular lesions (11). Niacin effectively inhibited neutrophil MPO release and treated MPO-mediated inflammatory lesions (33). An increasing number of prospective cohort studies had demonstrated an association between diverse abnormalities in the lipid profile and the progression of CKD (14). This association was particularly significant given the kidney’s highly vascular nature; lipid abnormalities generally exerted harmful effects on renal function. Abnormal lipid levels could impair the body’s antioxidative and anti-inflammatory capacities, subsequently damaging renal vascular endothelial function (12). In summary, dyslipidemia was identified as a predictive factor for the progression of CKD. Niacin had been proven to reduce lipid and serum phosphate levels in the short term, improving the eGFR of CKD patients (34). However, further research is required to evaluate the long-term effects of niacin. Serum phosphate might play an independent pathogenic role in the progression of CKD (13). Hyperphosphatemia was linked with several key regulators of renal vascular calcification, such as elevated levels of fibroblast growth factor 23 (FGF23) and decreased expression of Klotho (35). Niacin not only reduced phosphate absorption in the gut but also promoted its excretion in the urine, helping to control serum phosphate levels (36). A randomized controlled trial confirmed that niacin significantly

reduced serum phosphate levels by approximately 0.40 mg/dL, regardless of whether eGFR was <60 or ≥60 ml/min compared to placebo (37). Additional studies also indicated that niacin could significantly improve serum phosphate levels in CKD patients, providing strong evidence for niacin's role in ameliorating the progression of CKD (34, 38, 39).

In participants with dietary niacin intake exceeding 38.83 mg/d (after PSM), an increase in niacin intake was associated with a significant rise in CKD risk (OR = 1.03, 95%CI 1.00-1.06). This might be attributed to increased levels of metabolites such as prostaglandin D2 (PGD2) (16), homocysteine (HCY) (17), and serotonin (5-HT) (18) following excessive niacin intake. PGD2 was reported to promote renal fibrosis in CKD via CTRH2-mediated activation of Th2 lymphocytes (40). A prospective cohort study identified elevated serum homocysteine (HCY) levels as an independent risk factor for CKD in the general population (41). Hyperhomocysteinemia mediated several key pathogenic mechanisms in CKD, such as oxidative stress, endoplasmic reticulum stress, inflammation, and hypomethylation (42). Afolabi et al. found that 5-HT impaired renal function by activating the transient receptor potential vanilloid 4 channels in smooth muscle cells of the renal microvasculature (43). Using antagonists to inhibit 5-HT expression helped reduce renal fibrosis and inflammation (44). Additionally, recent research indicated that the end metabolite produced from excessive niacin intake, N1-methyl-4-pyridone-3-carboxamide, had biological activity and could cause vascular inflammation and leukocyte adhesion (45). This metabolite was previously considered to be an obsolete uremic toxin (46). Therefore, when consuming dietary niacin, it was crucial to consider the dosage to avoid potential renal side effects. Our study identified a threshold of 38.83 mg/d (after PSM), closely aligning with the tolerable upper intake limit set at 35 mg/d (27). However, considering the adverse effects of exceeding the tolerable intake limit, this threshold might need to be optimized.

Our study had several strengths. Firstly, it was the first to comprehensively assess the association between dietary niacin intake and CKD among the elderly. Secondly, our research considered the complex sampling design and weights of NHANES, ensuring that the data was representative of the national population. Additionally, we adjusted for multiple covariates and used PSM to minimize bias from confounding factors. Moreover, to check the stability of the results, we conducted subgroup analyses. Finally, we fitted smoothing curves and identified a nonlinear relationship between dietary niacin intake and CKD, including determining the threshold effects through analysis.

Nonetheless, our study also encountered some limitations. Firstly, its cross-sectional design precluded definitive establishment of causality. Secondly, despite obtaining dietary data through two 24-hour dietary recalls, the potential for recall bias could not be entirely eliminated. Niacin is predominantly found in animal-derived foods; however, the absence of specific data on vegetarian dietary patterns in the NHANES may introduce a degree of bias that could potentially impact the results. Moreover, there might have been confounding factors that were not fully accounted for. These limitations may impact the accuracy of the study's findings. However, as a preliminary study, this work will provide a theoretical foundation for future prospective research. Lastly, our study was limited to the population aged 60 and

over in the United States and extrapolating the results to other populations necessitates further investigation. In the future, prospective cohort studies of dietary niacin intake in different populations could be conducted to explore the effects of niacin on CKD.

5 Conclusions

Our study demonstrates a U-shaped relationship between dietary niacin intake and CKD among the U.S. population aged 60 and older, with a curve inflection point at 38.83 mg/d. This finding offers new perspectives and scientific evidence for early prevention and intervention strategies for CKD in elderly Americans. However, due to the inherent limitations of the cross-sectional study design, it is important to interpret the findings cautiously, as they cannot establish causality or long-term effects. Therefore, further validation through large-scale prospective cohort studies is needed.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author/s.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

ZX: Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing – original draft, Writing – review & editing. SP: Writing – original draft, Writing – review & editing. GO: Writing – original draft, Writing – review & editing. XZ: Writing – original draft, Writing – review & editing. GZ: Writing – original draft, Writing – review & editing. HJ: Writing – original draft, Writing – review & editing. TZ: Writing – original draft, Writing – review & editing. NC: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1438373/full#supplementary-material>

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Effects of probiotic supplementation on bone health in postmenopausal women: a systematic review and meta-analysis

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Context: The beneficial effects of probiotic supplementation on bone health in postmenopausal women require further validation.

Objective: This study systematically reviewed and conducted a meta-analysis of randomized controlled trials (RCTs) to assess the relationship between probiotic supplementation and changes in bone mineral density (BMD) and bone turnover markers (BTMs) among postmenopausal women.

Methods: A systematic search was conducted across four databases to retrieve data on lumbar spine BMD, hip BMD, collagen type 1 cross-linked C-telopeptide (CTX), receptor activator of nuclear factor- κ B ligand (RANKL), osteocalcin (OC), osteoprotegerin (OPG), N-terminal propeptide of type 1 procollagen (P1NP), and bone-specific alkaline phosphatase (BALP) in postmenopausal women. Eligible RCTs were quantitatively analyzed using random-effects meta-analyses. Additional analyses, including subgroup, sensitivity, and meta-regression analyses, were performed.

Results: Twelve RCTs involving 1183 postmenopausal women were included. Compared with the control group, postmenopausal women who received probiotic supplementation showed significantly greater BMD in both the lumbar spine (standardized mean difference [SMD] = 0.60, 95% confidence interval [CI] 0.14 to 1.05) and the hip (SMD = 0.74, 95%CI 0.15 to 1.33). Additionally, probiotic supplementation was associated with reduced levels of CTX (SMD = -1.51, 95%CI -1.88 to -0.41) and BALP (SMD = -1.80, 95%CI -2.78 to -0.81). No significant differences were found between the probiotic and control groups in terms of other BTMs. Subgroup analyses revealed that the increase in BMD due to probiotic supplementation was more significant in postmenopausal women with osteopenia than in those with osteoporosis. The meta-analysis results for both lumbar spine and hip BMD remained robust after conducting sensitivity analyses and meta-regressions.

Conclusion: Supplementation with probiotics may increase BMD among postmenopausal women, with stronger evidence in women with osteopenia than osteoporosis. Further RCTs are suggested to confirm and refine these findings.

Systematic review registration: <https://www.crd.york.ac.uk/PROSPERO/>, identifier CRD42024576764.

KEYWORDS

probiotic, postmenopausal women, osteoporosis, osteopenia, bone, bone turnover marker

Introduction

Osteoporosis is a common bone disease characterized by reduced bone mass and density, leading to an increased risk of fractures (1). Globally, it affects a significant proportion of the population, particularly among older adults. The prevalence of osteoporosis is higher in women than in men, with approximately one in three women over the age of 50 being affected (2). Postmenopausal women are particularly at risk for osteoporosis and fractures due to the natural decline in endogenous estrogen production, which is known to have a protective effect on bone mineral density (BMD). This decline in estrogen leads to a substantial reduction of bone mineral density (generally ranging from 2% to 5% per year) during the late perimenopausal period as well as in the first postmenopausal years (3). Moreover, the reduction in estrogen adversely impacts the bone's microarchitecture, making the bones more susceptible to fractures.

Despite pharmaceutical interventions being available, adherence rates remain strikingly low, with less than 50% of patients continuing treatment beyond the first year (4, 5). This has been attributed to various factors, including a preference for alternative treatments and concerns over medication side effects (6). There is a clear trend toward seeking low-risk strategies to counteract the effects of osteoporosis, with dietary supplements like calcium and vitamin D gaining popularity. However, their impact on osteoporosis management may be less significant than initially thought (7), prompting a search for additional interventions (8).

Existing evidence has shown that there are remarkable changes in gut microbiota or its metabolites in postmenopausal women, and such changes are notably correlated with postmenopausal osteoporosis (PMO) (9–12). These correlations offer novel insights into the underlying mechanism of PMO and new

strategies for treatment that could improve bone health in postmenopausal women.

Probiotics, gaining popularity as dietary interventions, are beneficial live microorganisms that can provide health advantages when consumed in sufficient quantities. There is a growing body of research highlighting the gut microbiota's substantial influence on bone health through various interconnected mechanisms. This influence may involve the regulation of pro-inflammatory cytokines, which can increase bone resorption, the stimulation of intestine-derived estrogen production, the preservation of intestinal barrier integrity to prevent endotoxin translocation, and the increased production of short-chain fatty acids to inhibit osteoclast differentiation and promote the formation of osteoblastic cells as well as nutrient absorption essential for bone formation and maintenance (13–17). In animal models that mimic postmenopausal osteoporosis, supplementation with probiotics, including both *Lactobacillus* and *Bifidobacterium* species, has been demonstrated to significantly enhance BMD and bone volume in ovariectomized subjects (18). However, in human studies, particularly those focused on postmenopausal women, there exists a scarcity of comprehensive meta-analyses. The existing meta-analysis (19) is limited by a small number of randomized controlled trials (RCTs) included—specifically, only five studies (20–24)—and a focus on English-language publications, which restricts the generalizability of the findings.

Recently, additional available trials focusing on postmenopausal women (25–28), plus the prior literature from China (29–31), has expanded the available data, effectively doubling the number of studies considered compared to previous meta-analysis (19). This has prompted a new systematic review and meta-analysis of a broader range of RCTs. Consequently, we embarked on a systematic review and meta-analysis that encompassed a range of RCTs, with the objective to assess the potential skeletal benefits of probiotic interventions specifically in postmenopausal women.

Materials and methods

This review was conducted in accordance with the PRISMA guidelines for reporting systematic reviews (32). The protocol has

Abbreviations: BALP, bone-specific alkaline phosphatase; BMD, bone mineral density; BTM, bone turnover marker; CTX, collagen type 1 cross-linked telopeptide; OC, osteocalcin; OPG, osteoprotegerin; P1NP, N-terminal propeptide of type 1 procollagen; PMO, postmenopausal osteoporosis; RANKL, receptor activator of nuclear factor- κ B ligand.

been registered in PROSPERO (<https://www.crd.york.ac.uk/PROSPERO/>, identifier: CRD42024576764).

Data sources and searches

We systematically searched four electronic databases—*MEDLINE (PubMed)*, *Embase*, *Web of Science*, and *China National Knowledge Infrastructure (CNKI)*—from inception through 5 August 2024 for published RCTs evaluating the effects of probiotic supplementation (versus control or placebo) on bone mineral density (BMD) and bone turnover markers (BTMs) in postmenopausal women. We used the following search terms: ‘probiotics’, ‘probiotic*’, ‘lactobacillus’, ‘bifidobacterium’, ‘enterococcus’, ‘bone’, ‘bone mineral density’, ‘bone loss’, ‘bone turnover’, ‘osteoporosis’, ‘osteopenia’, ‘osteopor*’, ‘osteopeni*’, ‘postmenopausal’, ‘post menopause’, and ‘postmenopause’ (see [Supplementary Table 1](#) for details on the search strategy). Reference lists of original trials were manually examined to obtain additional relevant data. The language was restricted to English and Chinese.

Inclusion and exclusion criteria

The details regarding the PICOTS criteria are provided in [Supplementary Table 2](#).

Inclusion criteria are as follows: 1) RCTs focusing on postmenopausal women; 2) the use of probiotic (multiple-strain or single-strain) supplementation as interventions and use of placebo (or control) as a comparison and consideration of the change of BMD and/or BTMs as outcomes, or trials with multiple interventions (e.g., coadministered probiotics and vitamin D or calcium) were eligible if the study groups differed only by the use of probiotics; 3) trials utilized dual-energy X-ray absorptiometry (DXA) for the measurement of BMD in the lumbar spine and hip at baseline and trial’s end. Concurrently, BTMs were identified via blood analysis at the same time points; 4) probiotic supplementation for at least 3 months and 5) original articles are written in English or Chinese. In addition, when results from a study population were reported more than once, the results with the longest follow-up time were utilized.

The following types of studies were excluded: 1) cross-sectional, cohort or case-control studies, reviews or meta-analyses, case reports, and animal or cell experiments; 2) articles only reporting protocols, editorials, comments, letters, conferences or abstracts of meeting presentations and 3) absence of expected data for meta-analysis.

Data extraction

Two reviewers (FW and PJJ) extracted independently the following information from each trial: the first author, year of publication, country, main participants’ characteristics (sample size, age, and body mass index), type of probiotics, intervention duration, other treatments, adherence with intervention, adverse

effects, main outcomes including BMD in the lumbar spine and hip, collagen type 1 cross-l inked C-telopeptide (CTX), receptor activator of nuclear factor- κ B ligand (RANKL), osteocalcin (OC), osteoprotegerin (OPG), N-terminal propeptide of type 1 procollagen (P1NP), and bone-specific alkaline phosphatase (BALP). Additionally, the descriptions of the evidence of gut coloization for included studies after probiotic supplementation were presented in [Supplementary Table 3](#). In the current study, BMD in the lumbar spine and hip were defined as the primary outcomes, while BTMs were utilized as secondary outcomes.

Risk of bias assessment

Methodological quality was independently assessed by two investigators (W.W. and F.W.) by using the Cochrane Collaboration tool (33). All disagreements were resolved through consultation with a third investigator (P.J.L.). Bias in studies was appraised as low, high, or unclear, based on an evaluation of sequence generation, allocation concealment, participant and staff blinding, outcome assessor blinding, handling of incomplete data, selective outcome reporting, and other potential biases ([Supplementary Table 4](#), [Supplementary Figures 1A, B](#)).

Statistical analysis

Statistical analyses for this review were performed using STATA 14.0, and Review Manager 5.3 software. The impact of probiotic supplementation on bone status and BTMs was evaluated by examining the mean relative change from baseline to the conclusion of the intervention, alongside its standard deviation (SD). Direct usage of means and SDs of changes from baseline was prioritized; where not available, data were transformed using established methods (34–37). In cases where trials had multiple intervention arms of the same nature, they were combined into a single arm as per previous methods (36).

The pooled effects of the studies were expressed as standardized mean differences (SMD) with 95% confidence intervals (CIs). Heterogeneity among the studies was assessed using Cochrane’s Q test and quantified with the I^2 statistic, with thresholds for low, moderate, and high heterogeneity set at <25%, 25%–50%, and >50%, respectively (38). A random-effects model was employed for calculating pooled effect measures in the presence of any heterogeneity ($I^2 > 0\%$). Sensitivity analyses were conducted to test the robustness of the results, involving the sequential omission of individual studies and further removal of studies with a high risk of bias to observe changes in heterogeneity. For the primary outcomes of lumbar spine and hip BMD, subgroup analyses were conducted to explore potential interactions based on the types of probiotic supplements, dosage of probiotic supplementation, intervention duration, geographical region, and participants’ baseline BMD indicators, including the presence or absence of osteoporosis (defined by a T-score of ≤ -2.5). Additionally, meta-regression analysis was utilized to determine if heterogeneity could be attributed to specific baseline characteristics such as age and

BMI. Publication bias was assessed in meta-analyses with at least 10 studies using funnel plots and the Egger test (39). The trim-and-fill method was applied to identify and adjust for potential publication bias in the effect estimates.

Results

Search results

Our initial search strategies across four databases yielded a total of 346 papers. After the removal of 112 duplicate records and screening the titles or abstracts of the remaining 234 records, we excluded 199 records that were obviously not relevant. The full text of the 35 eligible reports was read, which helped us to identify one additional article. Finally, we identified twelve RCTs that involved 1183 postmenopausal women (635 in the intervention group and 548 in the control group) as eligible for meta-analyses (20–31). A detailed overview of the selection process is provided in the PRISMA flow diagram in Figure 1.

Characteristics of the included studies

The general characteristics of the included trials published between 2017 and 2024 were presented in Table 1. These studies

were parallel-design, single-center trials conducted in the China (25, 29–31), Denmark (20), Iran (21), Japan (22), Poland (26), Sweden (23, 24, 27), and Thailand (28). Of these studies, four trials (22, 24, 26, 27) employed a single-strain probiotic as their intervention approach, in contrast to the rest that used multiple-strain probiotic formulations (20, 21, 23, 25, 28–31). The duration (3–24 months) of intervention varied across the studies, with three studies implementing interventions for a period of three months (25, 26, 28), five studies with interventions spanning six months (21, 22, 29–31), and four studies conducting interventions for a timeframe exceeding one year (20, 23, 24, 27). Additionally, eleven trials reported the results of lumbar spine BMD (20–27, 29–31), and ten studies described the results of hip BMD (20–27, 29, 30). According to the dosage of probiotics, seven studies were defined as the high-dose group ($\geq 1 \times 10^9$ CFU/d) (20–27) and three as the low-dose group ($< 1 \times 10^8$ CFU/d) (29–31).

Main outcomes

Effects of probiotic supplementation on BMD

The meta-analysis investigating the effects of probiotics on BMD in the lumbar spine involved eleven trials (20–27, 29–31), while that concerning the hip BMD comprised ten trials (20–27, 29, 30). The pooled results using the random effects model showed that probiotic supplementation had a positive effect on both lumbar

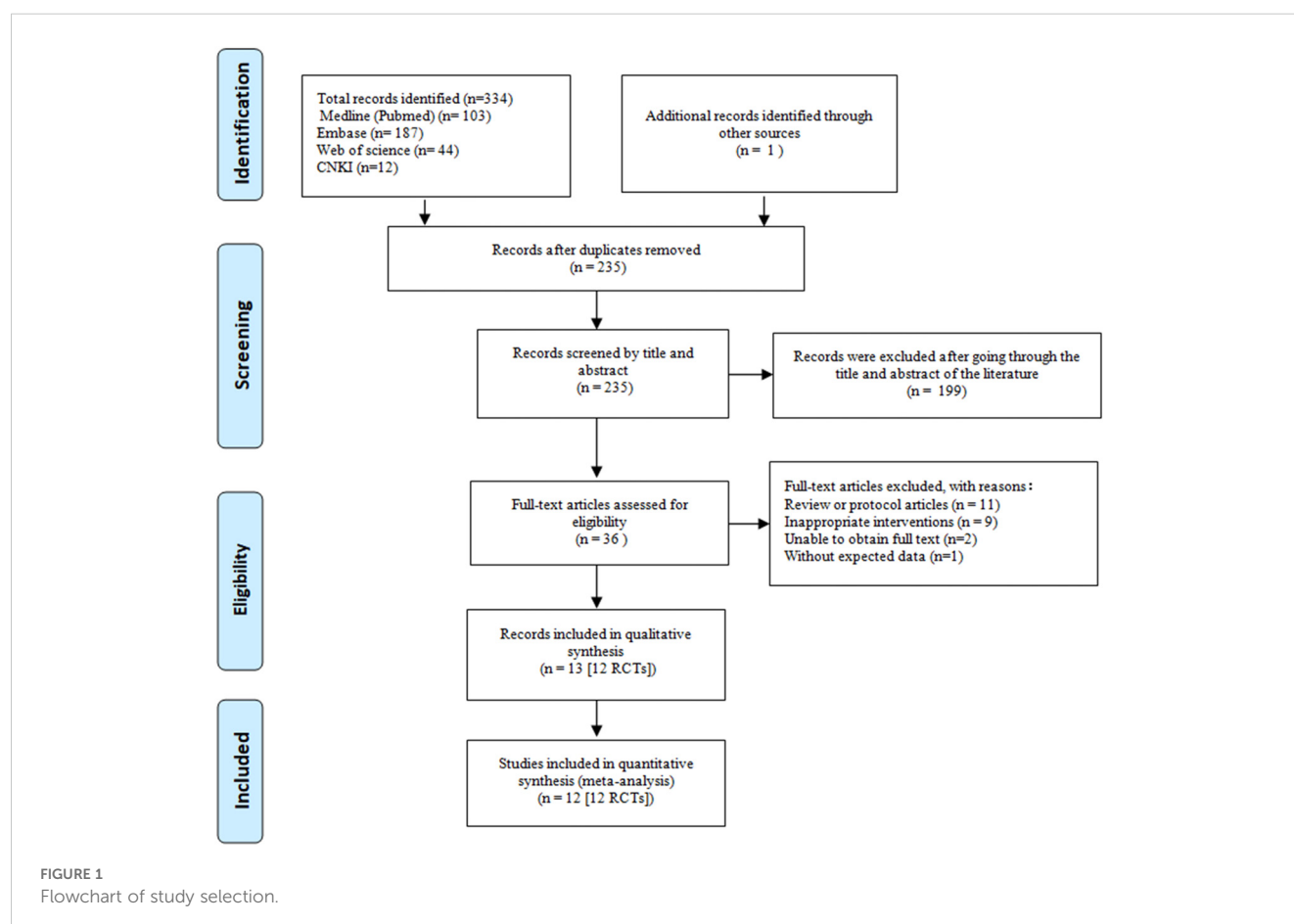


TABLE 1 Characteristics of included randomized controlled trials in the meta-analysis.

Study (Author, year)	Country	Sample size (C/I group)	Blinding	Mean age, years (mean $\pm$ SD)	BMI kg/m ² (mean $\pm$ SD)	Descriptions of intervention	Comparison intervention	Duration (months)	Adherence with intervention	Outcomes	adverse events
Jansson (2019)	Sweden	123/126	Double blind	C: 58.1 $\pm$ 4.3 I: 59.1 $\pm$ 3.8	C: 23.9 $\pm$ 2.6 I: 24.2 $\pm$ 2.7	Three lactobacillus strains: L. paracasei 8700:2 (DSM 13434), L. plantarum heal 9(DSM 15312), and L. plantarum heal 19(DSM 15313)	Placebo	12	NR	BMD (lumbar spine and total hip)	The number of adverse events considered to be related to the treatment were similar between the groups (24% in Lactobacillus and 26% in placebo). No treatment-related serious adverse events reported
Takimoto (2018)	Japan	30/31	Double blind	C: 57.8 $\pm$ 5.4 I: 57.5 $\pm$ 4.3	C: 23.9 $\pm$ 2.6 I: 24.2 $\pm$ 2.7	Probiotic Bacillus subtilis C-3102 (C-3102)	Placebo	6	The overall mean compliance rate was 99.5% $\pm$ 0.1% (placebo = 99.6% $\pm$ 0.2%, C-3102 = 99.5% $\pm$ 0.2%)	BMD (lumbar spine and total hip)	No adverse effects were reported during the study period
Lambert (2017)	Denmark	40/38	Double blind	C: 62.9 $\pm$ 1 I: 60.8 $\pm$ 1.1	C: 26.7 $\pm$ 0.8 I: 24.8 $\pm$ 0.6	Lactic acid bacteria and soffavones Other treatments: twice daily red clover extract (RCE) plus vitamin and mineral tablets containing 1040 mg Ca, 487 mg Mg, and 25 mg vitamin D/d	Placebo	12	The mean compliance rate was 96.44% $\pm$ 0.40%. There were no significant intergroup differences in compliance rates.	BMD (lumbar spine and hip [femoral neck]), CTX, OPG, RANKL, OC	Three participants (one in control group and two in intervention group) dropped out of the study due to gastrointestinal issues, and there was no significant difference between groups
Jafarnejad (2017)	Iran	21/20	Double blind	C: 57.3 $\pm$ 0.7 I: 68.9 $\pm$ 0.7	C: 23.8 $\pm$ 0.4 I: 24.9 $\pm$ 0.4	Multispecies probiotic supplement (GeriLact capsule): L. casei, Bifidobacterium longum, L. acidophilus, L. rhamnosus, L. bulgaricus, Bifidobacterium breve and Streptococcus thermophilus Other treatments: 500 mg calcium plus 200 IU vitamin D daily	Placebo+other treatments identical to those in the intervention group	6	NR	BMD (lumbar spine and hip), CTX, RANKL, OPG, OC, BALP	There were no significant adverse effects reported directly attributed to the treatment
Nilsson (2018)	Sweden	36/32	Double blind	C: 76.3 $\pm$ 1.1 I: 76.4 $\pm$ 1.0	C: 25.3 $\pm$ 3.3 I: 25.5 $\pm$ 3.5	Freeze-dried L. reuteri 6475 (BioGaia AB, Stockholm, Sweden)	Placebo	12	NR	BMD (lumbar spine and total hip), CTX, BALP	Adverse events considered to be related to the treatment were similar between the groups (40% in L. reuteri 6475 and 44% in placebo)

(Continued)

TABLE 1 Continued

Study (Author, year)	Country	Sample size (C/I group)	Blinding	Mean age, years (mean $\pm$ SD)	BMI kg/m ² (mean $\pm$ SD)	Descriptions of intervention	Comparison intervention	Duration (months)	Adherence with intervention	Outcomes	adverse events
Vanitchanont (2024)	Thailand	20/20	Double blind	C: 64.1 $\pm$ 3.6 I: 62 $\pm$ 5.1	C: 24.2 $\pm$ 2.8 I: 23.4 $\pm$ 3.8	Lactobacillus reuteri GL-104, Lactobacillus paracasei MP-137, Lactobacillus rhamnosus MP108, Lactobacillus rhamnosus F-1, Lactobacillus rhamnosus BV77, Bifidobacterium animalis ssp. lactis CP-9, Bifidobacterium longum ssp. longum OLP-01, and Bacillus coagulans Other treatments: at least 1200 mg of calcium daily and 20,000 IU of vitamin D2 per week	Placebo+other treatments identical to those in the intervention group	3	NR	CTX, PINP	Four participants in the placebo group and two in the multispecies probiotic group reported adverse reactions during the study period. There were no significant differences between groups in these adverse reactions.
Gregori (2024)	Sweden	79/160	Double blind	C: 55(53-56) High-dose: 55 (52-56) Low-dose: 55 (53-56)	C:23.7 (21.4-28.3) High-dose:23.9 (22.1-27.5) Low-dose:24.5 (21.9-27.9)	High-dose: L reuteri 6475 (BioGaia AB) (5×10^9 colony-forming units) Low-dose: L reuteri 6475 (BioGaia AB) (5×10^8 colony-forming units) Other treatments: 200 IU of cholecalciferol per day	Placebo+other treatments identical to those in the intervention group	24	Overall, mean (SD) adherence to the study product was high, ranging from 87.5% (24.6%) in the high-dose L reuteri group to 93.6% (12.9%) in the placebo group	BMD (lumbar spine and total hip), CTX, PINP	No significant adverse effects were observed.
Harahap (2024)	Poland	32/32	Double blind	Overall: 45-70	C: 28.6 $\pm$ 4.4 I: 25.3 $\pm$ 4.8	L. acidophilus UALa-01	Placebo	3	NR	BMD (lumbar spine and hip [femoral neck]), PINP, CTX, BALP	A significant increase in glucose concentration was observed in the probiotic group
Zhao (2023)	China	20/20	Double blind	C:61.6 $\pm$ 7.9 I: 62.8 $\pm$ 6.0	C: 23.4 $\pm$ 2.5 I: 23.1 $\pm$ 2.2	Bifidobacterium animalis subsp. lactis Probio-M8, Probio-M8 Other treatments: daily 600 mg of calcium and 0.25 μ g of calcitriol	Placebo+other treatments identical to those in the intervention group	3	NR	BMD (lumbar spine and hip [femoral neck]), PINP, CTX, OC	NR
Li (2021)	China	73/73	Unclear	C: 69.8 $\pm$ 21.5 I: 68.2 $\pm$ 22.4	C: 24.9 $\pm$ 7.4 I: 26.3 $\pm$ 8.4	Bifidobacterium quadruple viable bacteria tablets 0.5 g Tid + oral alendronate sodium 10 mg Qd + subcutaneous or intramuscular injection of salmon calcitonin 50 IU Qd.	Oral alendronate sodium 10 mg Qd + subcutaneous or intramuscular injection of	6	NR	BMD (lumbar spine and hip [femoral neck]), CTX, OC, BALP	There were no significant differences between groups in adverse reactions.

(Continued)

TABLE 1 Continued

Study (Author, year)	Country	Sample size (C/I group)	Blinding	Mean age, years (mean ± SD)	BMI kg/m ² (mean ± SD)	Descriptions of intervention	Comparison intervention	Duration (months)	Adherence with intervention	Outcomes	adverse events
							salmon calcitonin 50 IU Qd.				
Guo (2020)	China	24/30	Double blind	C: 63.4 ± 5.7 I: 61.9 ± 6.4	C: 23.9 ± 3.2 I: 23.6 ± 3.4	Dry Probio-M8 lactic acid bacteria	placebo	6	NR	BMD (lumbar spine and hip [femoral neck]), CTX, OC	NR
Zhang (2018)	China	50/53	Unclear	C: 58.6 ± 7.5 I: 56.8 ± 6.2	C: 25.9 ± 3.7 I: 25.9 ± 3.0	Bifidobacterium quadruple viable bacteria tablets 0.5 g Tid+ calcium carbonate 600mg/d+vitamin D3 (125IU/d)	calcium carbonate 600mg/d+vitamin D3 125IU/d	6	NR	BMD (lumbar spine), CTX, BALP	NR

C, control; CTX, collagen type 1 cross-linked C-telopeptide; I, intervention; BMD, bone mineral density; RANKL, Receptor activator of nuclear factor-κ B ligand; OC, osteocalcin; OPG, osteoprotegerin; PINP, N-terminal propeptide of type I procollagen; BSAP, bone-specific alkaline phosphatase; NR, not reported; BALP, bone-specific alkaline phosphatase.

spine BMD (SMD=0.60, 95%CI [0.14, 1.05], P=0.01; $I^2 = 92.1\%$; **Figure 2A**) and hip BMD (SMD=0.74, 95%CI [0.15, 1.33], P=0.013; $I^2 = 94.5\%$; **Figure 2B**) when compared with control.

Heterogeneity, subgroup, sensitivity analyses and meta-regressions

For the primary outcomes included in the meta-analysis, there was obvious heterogeneity ($I^2 > 50\%$). Subsequently, subgroup, sensitivity analyses and meta-regressions were performed to try to identify potential sources of heterogeneity.

The sensitivity analysis for lumbar spine BMD showed that the I^2 statistic did not fall below 50% after excluding any single trial from the eleven, and the hip BMD analysis also resulted in a similar finding. In addition, the I^2 statistic for both lumbar spine and hip BMD continued to exceed 50% even after the removal of trials that were reported in the Chinese language. Despite this, the meta-analysis results for both lumbar spine and hip BMD were robust.

Subgroup analyses revealed that probiotic supplementation, regardless of whether it involves single- or multiple-strain formulations, resulted in significant increases in both lumbar spine and hip BMD in trials that specifically included postmenopausal women with osteopenia. This enhancement in BMD was notably more pronounced in comparison to trials involving women who have osteoporosis (characterized by a T-score of more than or equal to -2.5) (**Tables 2, 3**). Furthermore, our results indicated that high-dose supplementation of probiotics could improve both lumbar spine and hip BMD more effectively than low-dose supplementation (**Tables 2, 3**). In addition, our findings showed that extended probiotic supplementation (at least one year) indicated greater benefits for enhancing lumbar spine BMD (SMD=1.03, 95%CI [0.06, 2.00], P=0.037; $I^2 = 96.4\%$) compared to supplementation regimens that last for six months or less (**Table 2**). Meta-regressions did not reveal any significant correlation between the effects of probiotics on BMD and the age and BMI of postmenopausal women (**Supplementary Figures 2A, B**).

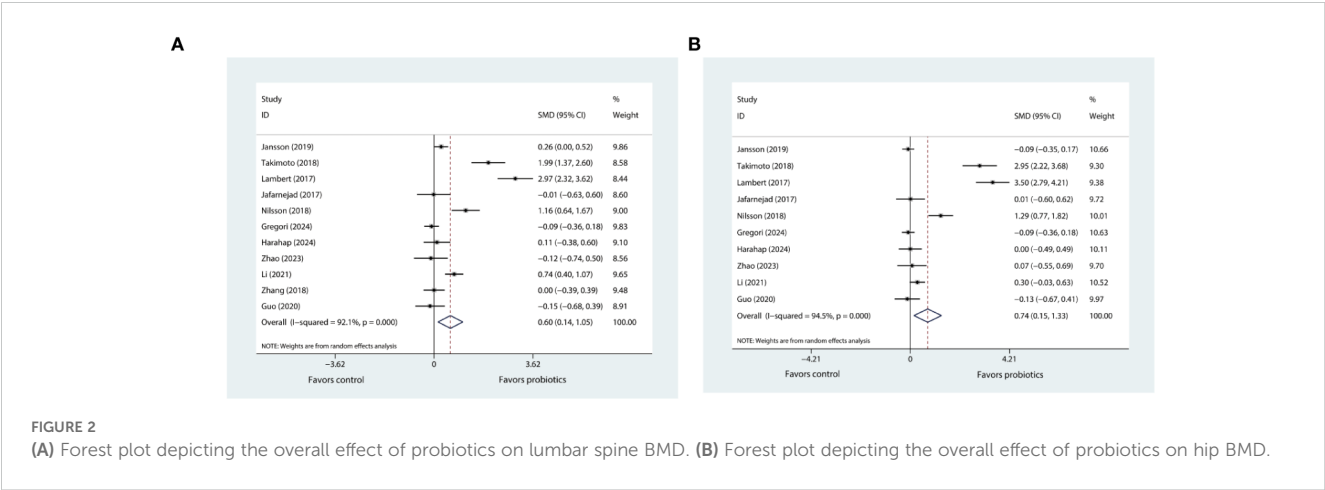
Effects of probiotic supplementation on bone turnover markers and sensitivity analyses

CTX, BALP, and PINP

According to data pooled from nine eligible trials (20, 21, 25–31), when compared with control, probiotic supplementation significantly reduced CTX levels (SMD= -1.51, 95%CI [-1.88, -0.41], P= 0.002; **Figure 3A**). The degree of heterogeneity was high ($I^2 = 95.2\%$).

There were five eligible trials reporting the results of BALP (21, 24, 26, 30, 31). Upon consolidating the data from these studies, it was determined that the supplementation with probiotics was associated with a significant reduction in BALP levels (SMD= -1.80, 95%CI [-2.78, -0.81], P < 0.001; $I^2 = 94.3\%$; **Figure 3B**) when compared to the control group.

The sensitivity analysis, in which we sequentially removed each of the nine trials from the meta-analysis, showed that the I^2 statistic



did not decrease below 50%. Despite this persistent heterogeneity, the overall meta-analysis results for CTX-1 maintained their robustness. The similar findings were observed in the sensitivity analysis of BALP.

Based on the pooled data from seven trials (20, 24–29), our results indicated that probiotic supplementation did not exert any significant influence on P1NP levels (SMD= 0.59, 95%CI [-0.14, 1.32], P= 0.112; I^2 = 92.5%; Figure 3C) in comparison to control group. After conducting sensitivity analysis, it was observed that the results of meta-analysis for P1NP remained stable and did not show any significant alteration. Also, the heterogeneity across the studies, as indicated by the I^2 statistic, remained high, exceeding 50%.

OC, OPG and RANKL

Six trials (20, 21, 25, 29–31) reported changes in OC levels before and after the intervention. Pooled analysis showed that probiotic supplementation did not exert any significant influence

on OC levels (SMD= -0.16, 95%CI [-0.53, 0.22], P= 0.416; I^2 = 77.1%; Figure 3D) when compared to control. Through further sensitivity analysis, we did not observe any change in the meta-analysis results or heterogeneity. Only two trials reported the results pertaining to OPG (20, 21) (Supplementary Figure 3A) and RANKL (20, 21) (Supplementary Figure 3B), respectively. The pooled results indicated that use of probiotic supplementation had no significant effect on these two markers.

Publication bias

Potential publication bias was detected using funnel plots and the Egger test. The funnel plots of lumbar spine BMD (Supplementary Figure 4A) and hip BMD (Supplementary Figure 4B) were displayed in the Supplementary Materials. Funnel plots were not created for the other markers as there were fewer

TABLE 2 Results of subgroup analyses of the effects of probiotics on lumbar spine BMD.

Subgroup		Lumbar spine BMD			
		Effect size	95% CI	I^2	P value
T score < -2.5	No (n = 8)	0.76	0.17, 1.36	93.9%	0.012
	Yes (n = 3)	0.19	-0.46, 0.84	80.9%	0.559
Intervention duration	≤ 6 months (n = 7)	0.36	-0.14, 0.86	86.1%	0.155
	> 6 months (n = 4)	1.03	0.06, 2.00	96.4%	0.037
Types of probiotics	Single-strain (n = 4)	0.77	-0.13, 1.66	93.7%	0.095
	Multiple-strain (n = 7)	0.51	-0.08, 1.10	92.3%	0.091
Region	Asia (n =6)	0.40	-0.18, 0.98	88.0%	0.173
	Europe (n = 5)	0.84	0.05, 1.63	95.2%	0.037
Dosage of probiotics	≥ 1 x 10 ⁹ CFU/d (n = 8)	0.76	0.13, 1.39	93.9%	0.018
	< 1 x 10 ⁸ CFU/d (n = 3)	0.22	-0.35, 0.79	82.5%	0.451

BMD, bone mineral density; CFU, colony forming unit; CI, confidence interval.

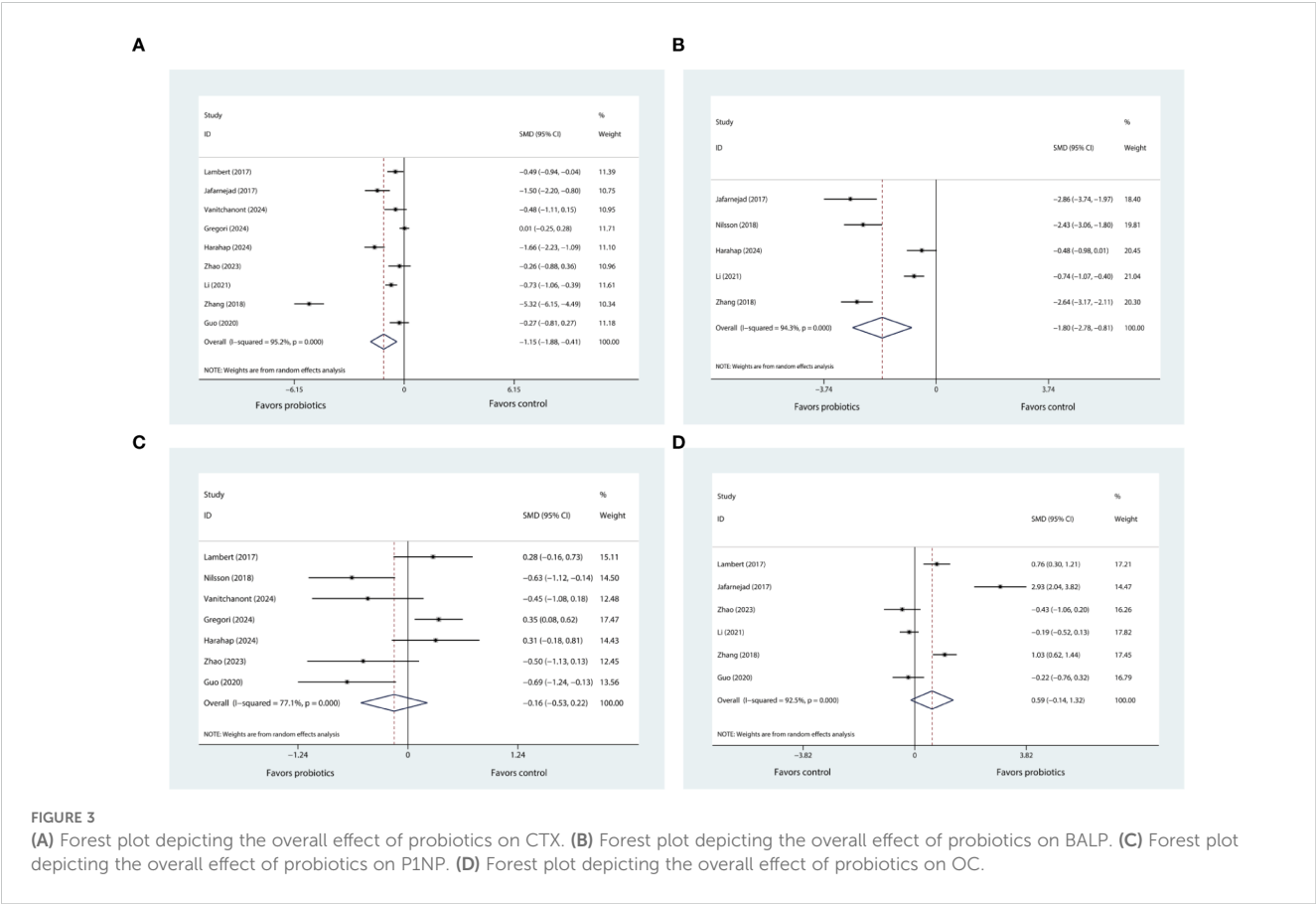
TABLE 3 Results of subgroup analyses of the effects of probiotics on hip BMD.

Subgroup		Hip BMD			P value
		Effect size	95% CI	I ²	
T score < -2.5	No (n = 7)	1.04	0.19, 1.89	96.3%	0.016
	Yes (n = 3)	0.17	-0.09, 0.42	0.0%	0.201
Intervention duration	≤ 6 months (n = 6)	0.50	-0.21, 1.22	91.0%	0.165
	> 6 months (n = 4)	1.10	-0.02, 2.22	97.2%	0.054
Types of probiotics	Single-strain (n = 4)	1.00	-0.15, 2.16	95.9%	0.088
	Multiple-strain (n = 6)	0.58	-0.20, 1.35	94.4%	1.144
Region	Asia (n =5)	0.61	-0.26, 1.50	92.5%	0.169
	Europe (n = 5)	0.87	-0.03, 1.78	96.3%	0.059
Dosage of probiotics	≥1 x 10 ⁹ CFU/d (n = 7)	1.04	0.19, 1.89	96.3%	0.016
	< 1 x 10 ⁸ CFU/d (n = 3)	0.17	-0.09, 0.42	0.0%	0.201

BMD, bone mineral density; CFU, colony forming unit; CI, confidence interval.

than ten included trials in their meta-analyses (39). The Egger test results revealed publication bias for the effects of probiotic supplementation on hip BMD (t=2.42, P=0.042; [Supplementary Figure 5B](#)), while the results regarding lumbar spine BMD did not show publication bias (t=1.52, p=0.162; [Supplementary Figure 5A](#)).

We used the trim-and-fill method to detect and adjust for publication bias regarding the results of hip BMD, but the updated overall effect estimate did not show significant changes (z=2.945, P=0.003; [Supplementary Figure 5C](#)). Consequently, more studies with large number are further needed.



Discussion

Main findings

This meta-analysis of twelve RCTs involving 1183 postmenopausal women sheds new light on the influence of probiotic supplementation on bone health in this demographic. The analysis revealed that supplementation with probiotics, especially at a dose of at least 1×10^9 CFU per day, positively impacted bone health, with stronger evidence in women with osteopenia than osteoporosis, as indicated by improvements in lumbar spine and hip BMD. Additionally, probiotic supplementation correlated with reduced levels of CTX and BALP, pointing to a potential anti-osteoporotic effect of probiotics. Despite significant heterogeneity across the included studies, the findings are supported by objective primary and secondary outcome measures and a robust random-effects analysis model. The credibility of the results is further supported by the stability of the meta-analysis outcomes following sensitivity analysis.

Postmenopausal women are disproportionately affected by osteoporosis due to estrogen deficiency (40, 41). The current understanding of osteoporosis is not yet sufficient to develop pharmaceuticals capable of completely preventing or stopping the disease's progression (42). However, research has shown a link between the gut microbiota and bone mass reduction, as well as osteoporosis prevalence (13–16, 43, 44). The gut microbiota is believed to influence bone metabolism by affecting the balance between osteoclast and osteoblast activity, thereby impacting the host's metabolism and immune system (9, 10, 45) (The diagram illustrating the potential impact of gut microbiota regulation on promoting bone health is presented in Figure 4). This has led to the exploration of modulating the intestinal microbiome, such as through probiotic supplementation, as a treatment for osteoporosis or osteopenia in postmenopausal women (20–31). However, the effects of probiotics on the bone health in postmenopausal women are still inconsistent.

To our knowledge, only one previous meta-analysis has specifically examined the effects of probiotic supplementation on bone health in postmenopausal women (19). That study found positive effects on BMD in the spine but no observed benefits in the hip region. Another meta-analysis (46) evaluated the impact of probiotics on bone health among postmenopausal women and other individuals suffering from senile or diabetic osteoporosis, and it reported similar findings regarding BMD in the lumbar spine and hip among postmenopausal women as Yu et al.'s study (19). The discrepancy in the effects of probiotics on BMD in different areas among postmenopausal women may be due to the small sample size of studies included in these two meta-analyses (19, 46). Our current meta-analysis reaffirms the previous findings on lumbar spine BMD and further expands on these results by demonstrating positive effects of probiotics on hip BMD as well.

Additionally, subgroup analyses were conducted and revealed that the improvement in BMD was more pronounced in postmenopausal women with mild bone loss (osteopenia) (20–24, 26–31), when treated with probiotics, as opposed to those with osteoporosis (25, 29, 30). This may be due to the limited inclusion of studies in the osteoporosis group. Therefore, further research is needed on probiotic intervention for postmenopausal osteoporosis. When the included trials were divided into subgroups according to intervention duration (≤ 6 months or > 12 months), we found extended durations of probiotic intervention (> 12 months) (20, 23, 24, 27), in contrast to brief periods of treatment, demonstrated superior enhancement in lumbar spine BMD ($P=0.037$), suggesting that longer-term probiotic supplementation may yield more benefits in bone health. Additionally, the improvement in hip BMD did not reach statistical significance ($SMD= 1.10$, 95%CI $[-0.02, 2.22]$, $P=0.054$). We also conducted subgroup analysis based on study region (Europe and Asia), and found that probiotics appear to be more effective in enhancing lumbar spine BMD in postmenopausal women from Europe compared to those from Asian backgrounds (21, 22, 25, 28–31). This observation could be attributed to potential

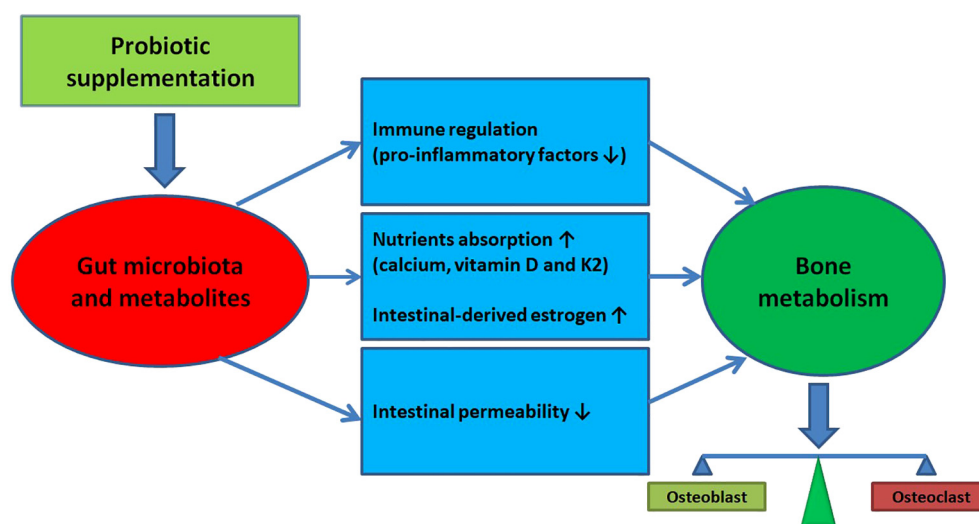


FIGURE 4
The potential impact of gut microbiota regulation on promoting bone health.

differences in gut microbiome composition, genetic factors, lifestyle, or dietary habits between these populations, although further research is needed to clarify these regional disparities in response to probiotic treatment. Of note, our subgroup analysis also suggested that there was no significant difference in the impact on BMD between single- and multiple-strain probiotics.

CTX and P1NP are both widely recognized bone turnover markers (BTMs) in clinical use, with CTX indicating bone resorption and P1NP indicating bone formation (47). Our study's findings that probiotic supplementation can lead to a reduction in CTX levels are in line with two previous meta-analyses (19, 46), neither of which reported the effects of probiotics on P1NP due to the limited data. By synthesizing our findings on both P1NP and CTX, it is clear that the mechanism through which probiotic supplementation may help in preventing bone loss is likely due to its effect on inhibiting bone resorption by suppressing osteoclast activity (22). BALP, a marker traditionally associated with osteoblast proliferation, is recognized as a bone formation indicator (45). However, there is a growing body of evidence suggesting that BALP should be reclassified as a marker of bone turnover rather than solely bone formation (48, 49). In our study, it was found that levels of BALP could be decreased by supplementation with probiotics. This is consistent with the findings of other studies, which have reported a decrease in BALP following the consumption of symbiotic products (50) or specific probiotic strains such as *Lactobacillus reuteri* (51). These collective findings suggest that the classification of BALP as a bone formation marker may need to be reconsidered in light of its association with the broader process of bone turnover. However, the meta-analysis by Yu et al. showed no significant changes in BALP (19). This is most likely due to the limited number of studies included.

In addition our study also examined other bone turnover markers, including OC, OPG, and RANKL. Our findings are in line with previous meta-analyses (19, 46), which did not find any significant differences in the levels of these markers between postmenopausal women who received probiotics and those in the control group. However, it is important to highlight a preclinical study that demonstrated the supplementation of heat-killed *Lactocaseibacillus paracasei* GMNL-653 could lead to a specific reduction in the mRNA level of RANKL in ovariectomized mice; Whole-genome sequencing and comparative genomics analysis indicated that genes associated with the transport and metabolism of carbohydrates, as well as the biogenesis of cell walls, membranes, and envelopes, might play a role in the anti-osteoporotic effects of GMNL-653 (52). This observation prompts the hypothesis that the impact of different probiotic strains on bone turnover markers might vary. To substantiate these preliminary findings, further research is essential to explore the potential variability in effects among different strains of probiotics on BTMs.

Limitations and strengths

Our study has several limitations. Firstly, there is high heterogeneity between the included studies, although random-effects model was used to calculate the results, complemented by

suitable subgroup analyses and meta-regressions. Secondly, we had to calculate SMD rather than the weighted mean difference due to the inconsistent units describing BMD change among the included studies. Third, in several trials (20–22, 25, 27, 28, 30, 31), co-interventions were used as treatment methods, rather than probiotics alone. This might lead to potential bias; however, after we conducted sensitivity analysis, the robustness of the meta-analysis findings was maintained. Lastly, based on the current trials included, it was not possible for us to discern which probiotic strains specifically improve BMD or BTMs.

On the other hand, this study also has several strengths. Firstly, most of the included trials were of high quality. Secondly, we performed adequate subgroup analyses; sensitivity analysis and meta-regressions were also conducted to minimize heterogeneity between the included studies. Thirdly, compared with previous meta-analyses and reviews, this meta-analysis includes a larger number of trials and a wider population.

Conclusion

Our systematic review and meta-analysis found that probiotic supplementation in postmenopausal women was associated with improved BMD in the lumbar spine and hip, with stronger evidence in women with osteopenia than osteoporosis. This suggests that probiotic supplementation may serve as an alternative approach to decelerate bone mass deterioration in postmenopausal women with osteopenia. In addition, administration of probiotics could decrease levels of CTX and BALP. In the future, more research is needed to validate these findings, and specific strains beneficial for bone health in postmenopausal women need to be further explored.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding author.

Author contributions

FW: Conceptualization, Funding acquisition, Writing – review & editing, Methodology. WW: Formal analysis, Investigation, Methodology, Software, Validation, Writing – review & editing. PL: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1487998/full#supplementary-material>

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Predictive value of bone metabolism markers in the progression of diabetic kidney disease: a cross-sectional study

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Objective: This study aimed to investigate the relationship between bone metabolism markers, including serum klotho, fibroblast growth factor 23 (FGF23), 25(OH)D3, iPTH, calcium (Ca), and PHOS and the progression of diabetic kidney disease (DKD) in patients with type 2 diabetes mellitus (T2DM). Additionally, the predictive value of these markers for DKD progression was evaluated.

Methods: This study involved 126 patients with T2DM between May 2021 and March 2023. DKD staging was assessed based on urinary protein excretion rates and estimated glomerular filtration rate (eGFR). The study evaluated serum concentrations of klotho, FGF23, 25(OH)D3, iPTH, Ca and PHOS across various stages and examined their relationships with clinical parameters. Receiver operating characteristic (ROC) curve analysis was utilized to determine the predictive accuracy of these bone metabolism markers for DKD. Multivariate linear and logistic regression analyses identified risk factors linked to DKD severity.

Results: Among the 126 participants, 30 had non-DKD with normal proteinuria, while 96 had DKD, categorized as 31 with stage III DKD (microproteinuria), 34 with stage IV DKD, and 31 with stage V DKD (massive proteinuria). With advancing DKD from stage III to V, levels of klotho, 25(OH)D3, and Ca decreased significantly, whereas FGF23, iPTH and PHOS levels increased markedly. Klotho is significantly positively correlated with eGFR ($r = 0.285$, $P = 0.001$) and negative correlations with serum creatinine (Scr) and UACR ($r = -0.255$, $P = 0.004$; $r = -0.260$, $P = 0.011$). FGF23 was positively related to systolic blood pressure (SBP) ($r = 0.224$, $P = 0.012$), but negatively with eGFR ($r = -0.294$, $P = 0.001$). Additionally, 25(OH)D3 exhibited significant negative correlations with several adverse clinical biomarkers, and both iPTH, Ca and PHOS were strongly associated with DKD progression ($P < 0.05$). ROC analysis showed high predictive accuracy for DKD using these bone metabolism markers, with a combined area under the curve (AUC) of 0.846. Multivariate logistic regression analysis reinforced the significance of these markers in DKD progression.

Conclusion: Bone metabolism markers, such as klotho, FGF23, 25(OH)D3, iPTH, Ca and PHOS are intricately linked to DKD progression and may function as valuable predictive biomarkers.

KEYWORDS

type 2 diabetes mellitus, diabetic kidney disease, bone metabolism markers, predictive value, progression

1 Introduction

Diabetic kidney disease (DKD) presents a substantial public health concern due to its increasing prevalence and the absence of effective interventions to prevent its progression. As a complication arising from diabetes, DKD inflicts damage on various renal components, including the glomeruli, tubules, interstitium, and vasculature (1). Clinically, DKD manifests through a spectrum of proteinuria and a continuous decline in renal function, eventually progressing to end-stage renal disease (ESRD). Although renal biopsy is the definitive diagnostic method for DKD, its invasive nature limits its widespread application in clinical settings. Consequently, clinicians rely on key indicators such as the urinary albumin excretion rate and estimated glomerular filtration rate (eGFR) to diagnose and monitor DKD progression. However, these parameters are subject to numerous influencing factors, leading to insufficient sensitivity in detecting renal injury. Therefore, the quest for more reliable biomarkers for DKD screening and prediction remains ongoing (2). Historically, DKD research has predominantly viewed the kidney as an isolated organ, concentrating on its molecular pathological mechanisms. However, emerging evidence underscores the significance of inter-organ communication in disease pathogenesis, with the “bone-kidney axis” becoming a pivotal concept in DKD studies. Factors such as hyperglycemia, accumulation of advanced glycation end products (AGEs), and the progression of chronic kidney disease (CKD) contribute to disruptions in calcium-phosphorus metabolism, abnormal parathyroid hormone (PTH) levels, and impaired bone mineralization, all of which can precipitate bone disease (3). Diabetes is an independent risk factor for skeletal health (4). In the context of DKD, the interactions among key bone regulatory factors (FGF23, klotho, and iPTH) are further disrupted, accelerating the progression of bone disease (5). The skeletal system, now recognized as an endocrine organ, secretes bone-derived hormones that are intricately involved in key pathogenic processes of DKD, such as energy metabolism (6), insulin resistance (7), inflammation (8), and epithelial-mesenchymal transition (9). Moreover, disruptions in bone metabolism may further accelerate DKD progression (10).

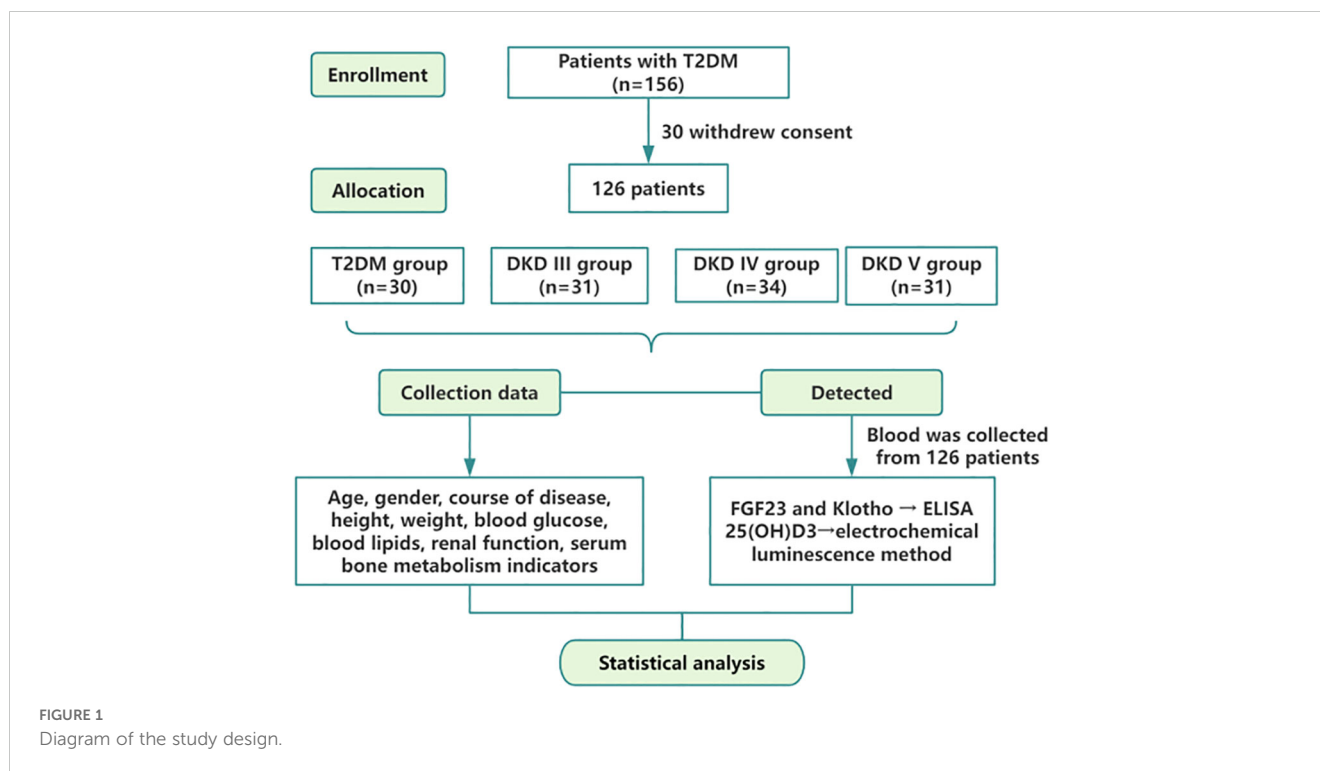
Previous research on mineral and bone disorders in CKD has largely concentrated on the later stages, such as end-stage renal disease (ESRD) and dialysis, where abnormalities in mineral and

bone metabolism are more pronounced (11). However, emerging studies have highlighted that bone-derived hormone abnormalities can occur early in CKD (12, 13). Identifying these abnormalities early is vital for disease management. For example, a prospective study involving T2DM patients found that elevated FGF23 levels independently predict all-cause mortality and are closely linked with an increased risk of progressing to ESRD (14–16). The interaction between bone and kidney function is crucial, as FGF23, secreted mainly by osteoblasts and osteocytes (17), relies on renal Klotho protein for its biological effects (18). FGF23 regulates renal sodium, calcium, and phosphate balance, vitamin D metabolism, immune-inflammatory responses, and the renin-angiotensin-aldosterone system (RAAS), and affects Klotho, angiotensin-converting enzyme 2, and erythropoietin expression (19, 20). Klotho is also a potential biomarker for early DKD detection (21). These insights suggest that bone metabolism disturbances significantly impact DKD progression and renal function. Investigating the communication between bone and kidney may clarify DKD pathogenesis (22). FGF23 and klotho are involved in phosphate and vitamin D metabolism and calcium-phosphate balance in CKD patients. This study examines the correlation between serum levels of klotho, FGF23, 25(OH)D3, iPTH, calcium (Ca) and PHOS with DKD progression in clinical patients, evaluating the predictive value of these bone metabolism markers. By analyzing different DKD stages in T2DM patients, the study aims to offer new reference markers for the early diagnosis and intervention of DKD.

2 Methods

2.1 Study design and population

This study encompassed patients diagnosed with type 2 diabetes mellitus (T2DM) and DKD who were admitted to Dongzhimen Hospital, Dongcheng Campus, Beijing University of Chinese Medicine, from May 2021 to March 2023. The study was sanctioned by the Ethics Committee of Dongzhimen Hospital, Beijing University of Chinese Medicine (2022DZMEC-062-03), and all participants provided written informed consent. The flowchart is depicted in [Figure 1](#).



2.2 Inclusion and exclusion criteria

The inclusion criteria were (1): age between 30 and 90 years (2); diagnosis of T2DM conforms to the “Chinese Guidelines for the Prevention and Treatment of Type 2 Diabetes” (2020 edition) (23); diagnosis of DKD based on the 2007 National Kidney Foundation (NKF-KD/OQI) guidelines (24); the 2020 Clinical Practice Guideline for the Evaluation and Management of CKD by the Kidney Disease: Improving Global Outcomes (KDIGO) organization (25) and the 2021 Chinese Clinical Guidelines for the Diagnosis and Treatment of Diabetic Kidney Disease (26). Staging criteria followed the 2007 NKF-KD/OQI guidelines (24) and the Mogensen staging criteria (27).

The DKD staging criteria are defined as follows: Stage III DKD: Urinary albumin-to-creatinine ratio (UACR) consistently ranges from 30–300 mg/g, with a eGFR remaining near normal or slightly elevated. Stage IV DKD: UACR exceeds 300 mg/g, or 24-hour urine protein levels are greater than 0.5 g, accompanied by a significant reduction in eGFR; Stage V DKD (end-stage renal failure, uremia stage): eGFR drops below 15 ml/min/1.73 m². Patients are classified based on proteinuria as follows: Non-DKD patients have normal proteinuria with UACR less than 30 mg/g; DKD patients are divided into two groups: the microproteinuria group with UACR ranging from 30–300 mg/g, and the massive proteinuria group with UACR exceeding 300 mg/g.

Exclusion criteria included (1): patients who had used medications such as active vitamin D, calcitonin, bisphosphonates, or other agents affecting calcium and phosphorus metabolism within the past six months; (2) patients who had undergone parathyroidectomy; (3)

patients who had received dialysis treatment; (4) those with primary or secondary kidney diseases; (5) patients with urinary tract infections or other acute or chronic inflammatory conditions; (6) patients with liver function abnormalities, autoimmune diseases, malignant tumors, hematologic disorders, or mental illnesses; and (7) patients who had experienced trauma, surgery, or psychological stress within the last six months.

2.3 Data collection and measurement

2.3.1 Participant characteristics

Patient clinical data, including age, gender, height, weight, systolic blood pressure, and diastolic blood pressure, were collected. Body mass index (BMI) was calculated as height/weight².

2.3.2 Laboratory tests

Fasting venous blood samples were collected from all participants on the second morning after hospital admission. The samples were analyzed using a Mindray CL1000i automatic chemiluminescence analyzer for 25(OH)D3 and iPTH. Additionally, an AU680 automatic biochemical analyzer (Beckman Coulter, USA) was used to measure serum Ca, PHOS, glucose (GLU), total protein (TP), urea (UREA), serum creatinine (CREA), uric acid (UA), 24-hour urine protein quantification (24-UTP), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). The estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI formula.

For females:

$$eGFR = 144 \times \left(\frac{\text{Serum Creatinine}}{88.4} \right)^{-0.329} \times 0.993^{\text{Age}} \text{ (if Serum Creatinine} \leq 61.88 \mu\text{mol/L)}$$
$$eGFR = 144 \times \left(\frac{\text{Serum Creatinine}}{88.4} \right)^{-1.209} \times 0.993^{\text{Age}} \text{ (if Serum Creatinine} > 61.88 \mu\text{mol/L)}$$

For Males:

$$eGFR = 141 \times \left(\frac{\text{Serum Creatinine}}{88.4} \right)^{-0.411} \times 0.993^{\text{Age}} \text{ (if Serum Creatinine} \leq 79.56 \mu\text{mol/L)}$$
$$eGFR = 141 \times \left(\frac{\text{Serum Creatinine}}{88.4} \right)^{-1.209} \times 0.993^{\text{Age}} \text{ (if Serum Creatinine} > 79.56 \mu\text{mol/L)}$$

2.3.3 Detection of bone metabolism-related biomarkers

On the second day of hospitalization, fasting venous blood samples (2 mL) were collected from each participant in the morning. After standing at room temperature for 30 minutes, the samples were centrifuged at 3000 rpm for 10 minutes at 4°C. The resulting serum was carefully separated, aliquoted into labeled EP tubes, and stored at -80°C for future analysis. Intact FGF23 and klotho levels were measured using enzyme-linked immunosorbent assay (ELISA) kits provided by Elabscience (Hubei, China).

2.4 Statistical methods

SPSS 26.0 was employed for statistical analysis. Quantitative data following a normal distribution were presented as mean ± standard deviation ($\bar{x} \pm s$), whereas non-normally distributed data were shown as median with interquartile range. Categorical variables were reported as frequencies and percentages (%). For normally distributed quantitative data, comparisons between groups were conducted using one-way analysis of variance. For non-normally distributed quantitative data, comparisons between groups were performed using non-parametric tests, specifically the Kruskal-Wallis test. Chi-square tests were used for categorical data analysis. The Bonferroni correction was applied to adjust p-values for multiple comparisons within groups. Pearson or Spearman rank correlation tests assessed correlations. Multiple linear regression evaluated the independent

associations of eGFR with serum levels of FGF23, klotho, 25(OH)D3, iPTH, Ca and PHOS. Multinomial logistic regression was used to examine the link between DKD severity and bone metabolism-related markers. $P < 0.05$ was deemed statistically significant.

3 Results

3.1 Clinical characteristics of study participants

A total of 126 T2DM patients participated in this study, including 80 males and 46 females, with a mean age of 59.50 ± 11.29 years. The cohort consisted of 30 patients with T2DM alone (normal proteinuria [NP]) and 96 patients with DKD, divided into 31 with DKD stage III (microproteinuria [MP]), 34 with DKD stage IV, and 31 with DKD stage V (massive proteinuria [MAP]). The findings reveal no notable differences across the four groups regarding age, gender, BMI, DBP, and lipid profiles. Conversely, significant increases in SBP and UA levels were observed in the DKD IV and V groups. Additionally, a marked decrease in TP levels was noted. The DKD V group demonstrated a considerable reduction in FPG levels. Analyses of Scr and eGFR indicated that Scr levels were significantly elevated in both the DKD IV and V groups. Simultaneously, eGFR exhibited a substantial decline ($P < 0.001$), pointing to a pronounced worsening of renal function. Moreover, UACR and 24-UTP displayed a rise ($P < 0.001$), suggesting a close association between disease progression and the severity of proteinuria (Table 1).

3.2 Changes in bone metabolism markers across different stages of DKD and T2DM

Compared with the T2DM group, klotho levels were significantly lower in DKD stage IV (0.373 ± 0.177) and stage V (0.321 ± 0.135) ($P < 0.05$). Similarly, klotho levels in DKD stage V showed a declining trend compared to stage III (0.451 ± 0.244) ($P < 0.05$) (Figure 2A). Compared with the T2DM group (219.95 ± 93.48) and DKD stage III (227.23 ± 100.45), FGF23 levels were

TABLE 1 Baseline Characteristics of Participants.

Variables	T2DM (n = 30)	DKD III(n=31)	DKD IV (n=34)BD268	DKD V(n=31)	P
	Normal proteinuria (n=30)	Microproteinuria (n=31)	Massive proteinuria (n =65)		
Age (years)	58.80 ± 11.62	58.87 ± 11.58	60.41 ± 11.57	59.81 ± 10.85	0.93
Male (%)	17 (56.67)	17 (54.84)	21 (61.76)	25 (80.65)	0.136
Female (%)	13 (43.33)	14 (45.16)	13 (38.23)	6 (19.35)	
BMI (kg/m ²)	24.6 (22.63,26.91)	25.54 (23.41,29.9)	25.56 (22.82,27.91)	26.05 (24.17,29.50)	0.922
SBP (mmHg)	134.80 ± 18.36	132.10 ± 18.85	141.38 ± 19.96 [#]	146.90 ± 17.26 ^{*#}	0.010
DBP (mmHg)	81.03 ± 14.37	78.13 ± 14.44	78.56 ± 10.90	75.81 ± 9.71	0.444

(Continued)

TABLE 1 Continued

Variables	T2DM (n = 30)	DKD III(n=31)	DKD IV (n=34)BD268	DKD V(n=31)	P
	Normal proteinuria (n=30)	Microproteinuria (n=31)	Massive proteinuria (n =65)		
FPG (mmol/L)	10.83 (8.38,15.86)	10.07(7.06,14.49)	8.83 (6.97,12.20)	6.64 (6.00,9.15)*	0.002
TG (mmol/L)	1.70 (1.15,2.76)	1.59(1.05,2.53)	1.90 (1.24,3.38)	1.40 (1.05,2.00)	0.115
LDL-C (mmol/L)	2.56 (2.07,3.67)	2.78(1.99,4.04)	3.14 (2.02,3.74)	2.35 (1.66,3.06)	0.206
HDL-C (mmol/L)	1.18 ± 0.27	1.21 ± 0.30	1.28 ± 0.39	1.14 ± 0.35	0.379
TP (g/L)	71.35 (66.25,76.55)	73.30(65.80,75.30)	63.50 (59.25,66.35)**	60.50 (56.30,66.60)**##	<0.001
ALP (U/L)	85.09 ± 18.54	77.17 ± 24.61	87.05 ± 31.80	85.14 ± 23.93	0.422
UA (μmol/L)	342.05 (284.35,410.48)	295.40(266.80,407.40)	396.00 (329.70,434.43)	451.80 (395.90,502.40)**##	<0.001
Scr (μmol/L)	63.25 (53.40,83.15)	65.40(60.70,73.00)	138.00 (102.80,248.80)**##	488.50 (406.40,635.30)**##▲	<0.001
eGFR (ml/ min/1.73m ²)	98.34 (83.83,106.88)	97.25(85.28,116.87)	39.73 (22.57,54.25)**##	9.86 (7.50,12.03)**##▲	<0.001
UACR (mg/g)	15.00 (10.00,16.25)	150.00(100.00,180.00)**	800.00 (800.00,1500.00)**##	-----	<0.001
24h-UTP (mg)	87.00 (55.00,131.50)	174.00(143.00,246.00)*	3754.00 (1567.25,6861.00)**##	4466.00 (2831.00,6697.00)**##	<0.001

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, Fasting Plasma Glucose; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TP, total protein; ALP, alkaline phosphatase; UA, serum uric acid; Scr, serum Scleritin; eGFR, estimated glomerular filtration rate; UACR, urine albumin to Scleritin ratio; 24h-UTP, 24-hour Urine Total Protein. Compared with the T2DM group, * $p < 0.05$, ** $p < 0.001$; compared with the DKD III group, # $p < 0.05$, ## $p < 0.001$; compared with the DKD IV group, ▲ $p < 0.05$, ▲▲ $p < 0.001$. Bold values indicate $P < 0.05$.

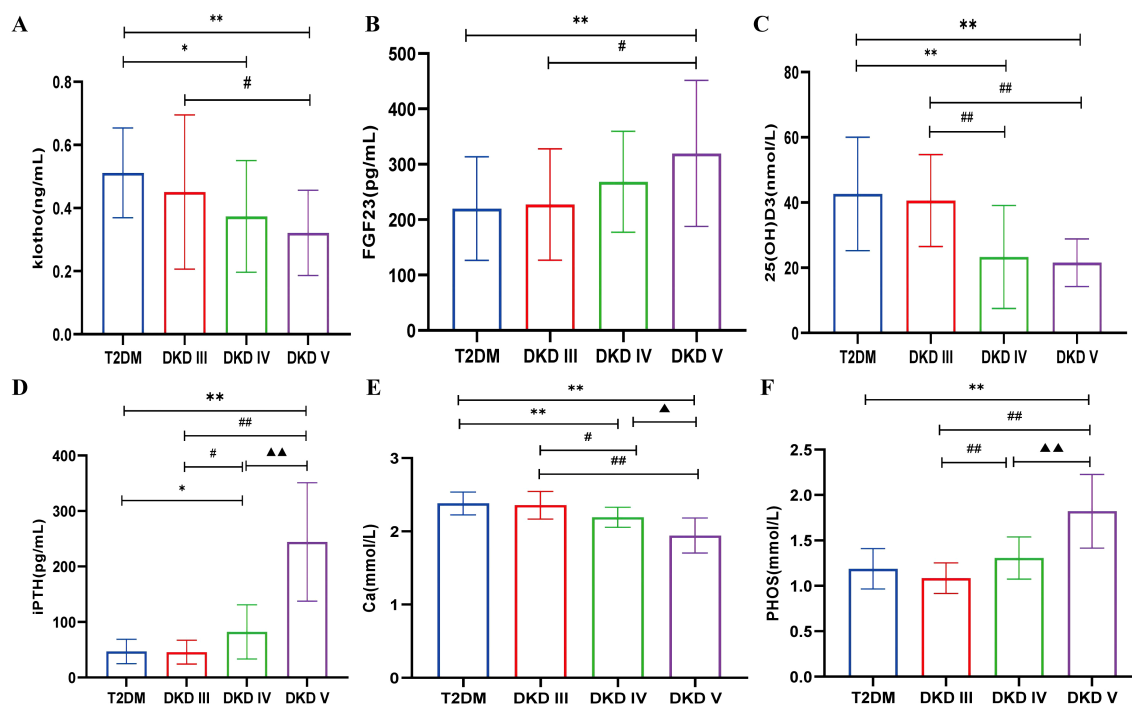


FIGURE 2

The levels of bone metabolism markers among different stages of DKD and T2DM group. (A) serum klotho. (B) serum FGF23. (C) serum 25(OH)D3. (D) serum iPTH. (E) serum Ca. (F) serum PHOS. Compared with the T2DM group, * $p < 0.05$, ** $p < 0.001$; compared with the DKD III group, # $p < 0.05$, ## $p < 0.001$; compared with the DKD IV group, ▲ $p < 0.05$, ▲▲ $p < 0.001$.

significantly elevated in DKD stage V (319.54 ± 131.93) ($P < 0.05$) (Figure 2B). Compared with the T2DM group, 25(OH)D3 levels were significantly lower in DKD stage IV (23.30 ± 15.80) and stage V (21.54 ± 7.31) ($P < 0.05$). A similar downward trend was observed in DKD stages IV and V compared to stage III (40.58 ± 14.10) (Figure 2C). Compared with the T2DM group (46.85 ± 22.08), iPTH levels were significantly higher in DKD stage IV (82.06 ± 48.76) and stage V (244.05 ± 106.66) ($P < 0.05$). Compared to DKD stage III, iPTH levels also showed an increasing trend in DKD stages IV and V, with stage V levels significantly higher than stage IV ($P < 0.05$) (Figure 2D). Compared with the T2DM group (2.38 ± 0.16), Ca levels were significantly lower in DKD stage IV (2.38 ± 0.16) and stage V (1.94 ± 0.24) ($P < 0.05$). A similar decreasing trend was observed in DKD stages IV and V compared to stage III (2.36 ± 0.19), with Ca levels in stage V significantly lower than in stage IV ($P < 0.05$) (Figure 2E). The PHOS level in the DKD V group (1.82 ± 0.40) was significantly higher compared to the T2DM group (1.19 ± 0.22), DKD III group (1.08 ± 0.17), and DKD IV group (1.31 ± 0.23) ($P < 0.05$). Additionally, compared to the DKD III group, the PHOS level in the DKD IV group showed an increasing trend ($P < 0.05$) (Figure 2F).

3.3 Changes in bone metabolism markers among different proteinuria groups

Patients were categorized into NP, MP, and MAP groups based on proteinuria levels. There were no significant differences in bone metabolism markers between the NP and MP groups. However, significant differences were observed in the MAP group compared to both NP and MP groups, indicating that alterations in bone metabolism occur predominantly in the later stages of DKD with

massive proteinuria. klotho, 25(OH)D3, and Ca levels were lower, while FGF23, iPTH and PHOS levels were higher in the MAP group compared to the NP and MP groups ($P < 0.05$) (Figures 3A–F).

3.4 Correlation analysis between bone metabolism markers and clinical indicators in DKD patients

A correlation analysis involving 126 patients identified significant relationships between bone metabolism indicators and clinical parameters. Klotho levels showed a positive correlation with eGFR ($r = 0.285$, $P < 0.001$) and negative correlations with Scr and UACR ($r = -0.255$, $P = 0.004$; $r = -0.260$, $P = 0.011$), suggesting that lower klotho levels may link to worsening renal function (Figures 4A–C). FGF23 levels positively correlated with SBP ($r = 0.224$, $P = 0.012$) and negatively with eGFR ($r = -0.294$, $P = 0.001$), indicating that higher FGF23 levels might be associated with increased blood pressure and declining renal function (Figures 4D, E). The level of 25(OH)D3 negatively correlated with SBP, ALP, UA, Scr, UACR, and 24h-UTP ($P < 0.05$), while positively correlating with TP and eGFR (Figures 4F–I). iPTH levels positively correlated with UA, Scr, UACR, and 24h-UTP ($r = 0.328$, $P < 0.001$; $r = 0.788$, $P < 0.001$; $r = 0.432$, $P < 0.001$; $r = 0.520$, $P < 0.001$) and negatively with eGFR ($r = -0.708$, $P < 0.001$), implying that elevated iPTH levels are closely tied to renal function decline and metabolic issues (Figures 4J–M). Ca levels exhibited positive correlations with eGFR ($r = 0.608$, $P < 0.001$) and negative correlations with UA ($r = -0.315$, $P < 0.001$), Scr ($r = -0.663$, $P < 0.001$), UACR ($r = -0.430$, $P < 0.001$), and 24h-UTP ($r = -0.589$, $P < 0.001$), suggesting that fluctuations in Ca levels may indicate the severity of renal impairment and

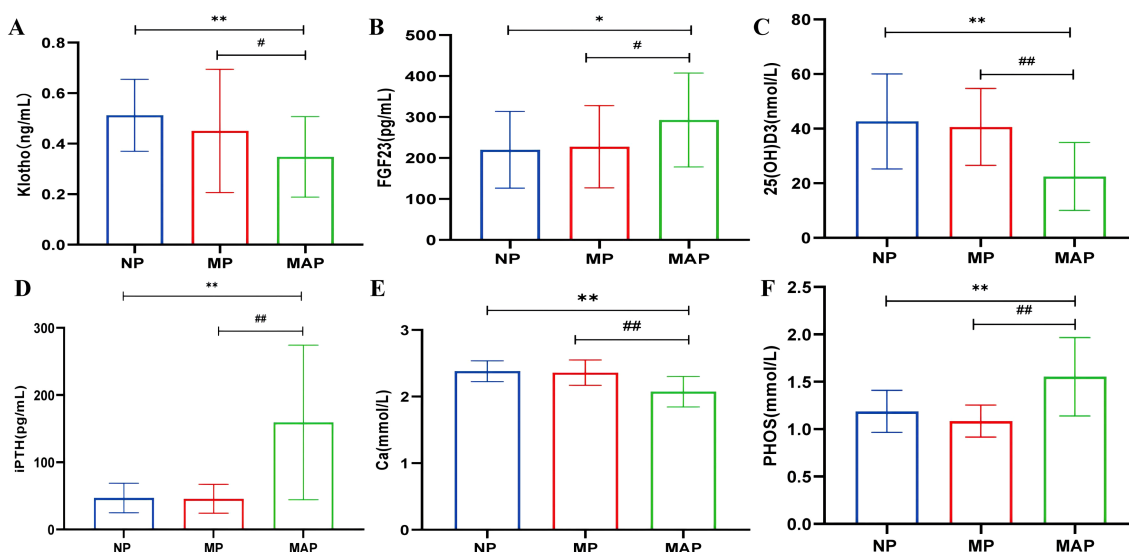


FIGURE 3

The levels of bone metabolism markers among different proteinuria groups. (A) serum klotho. (B) serum FGF23. (C) serum 25(OH)D3. (D) serum iPTH. (E) serum Ca. (F) serum PHOS. Compared with the NP group, * $p < 0.05$, ** $p < 0.001$; compared with the MP group, # $p < 0.05$, ## $p < 0.001$.

proteinuria (Figures 4N–Q). PHOS levels were negatively correlated with eGFR ($r = -0.637$, $P < 0.001$) and positively with UA ($r = 0.364$, $P < 0.001$), Scr ($r = 0.779$, $P < 0.001$), UACR ($r = 0.330$, $P = 0.001$), and 24h-UTP ($r = 0.398$, $P < 0.001$), indicating that PHOS levels notably rise during renal function deterioration (Figures 4R–U) (Table 2).

3.5 Clinical predictive value of bone metabolism indexes for DKD severity

The DKD group was merged into non-DKD (NDKD, $n = 30$) and DKD ($n = 96$) groups. ROC curves were plotted to explore the clinical predictive value of bone metabolism markers for DKD (Figure 5).

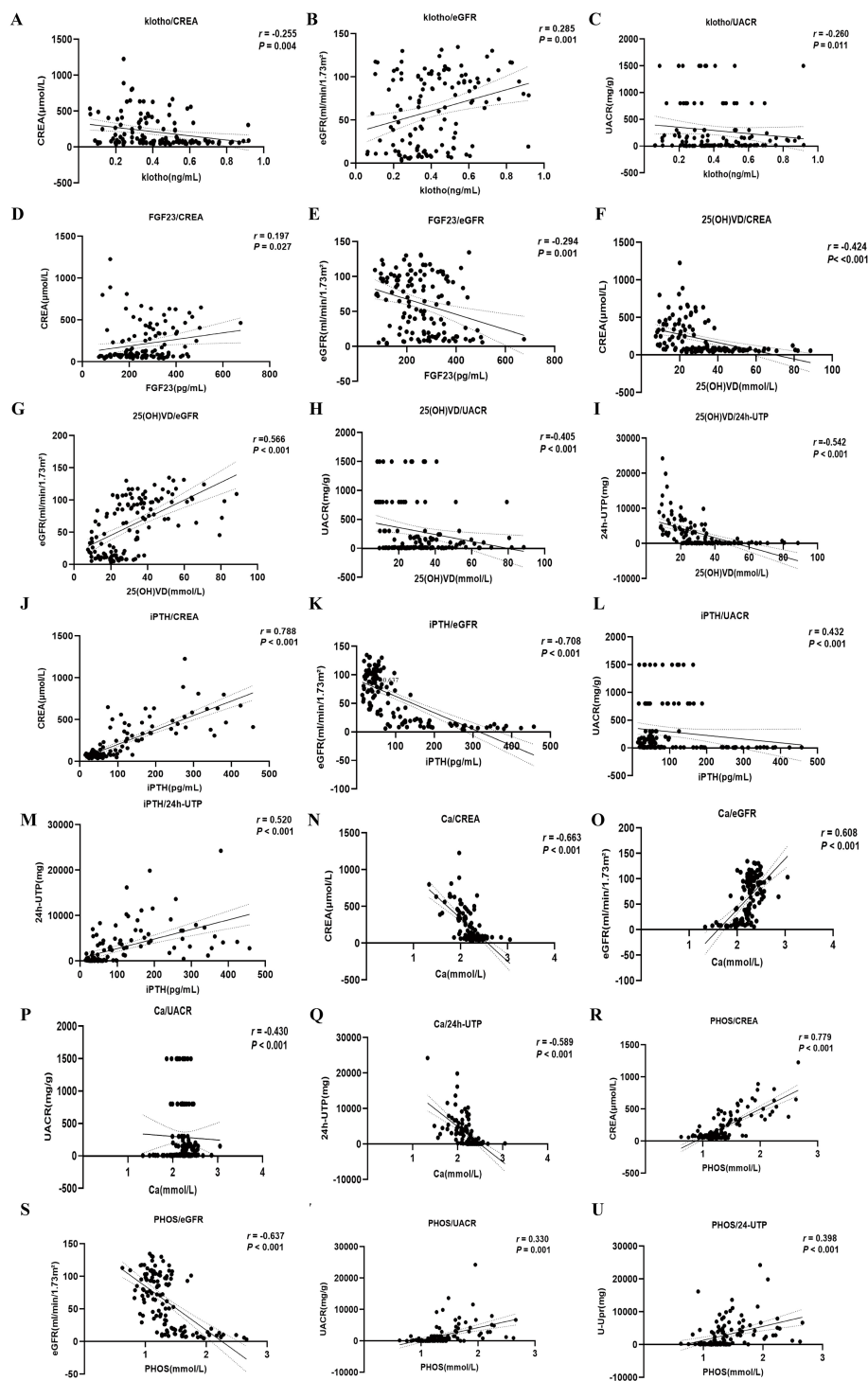


FIGURE 4
Correlation analysis of bone metabolism-related indexes and clinical biomarkers. (A–C) serum Klotho. (D, E) serum FGF23. (F–I) serum 25(OH)D3. (J–M) serum iPTH. (N–Q) serum Ca. (R–U) serum PHOS.

TABLE 2 Correlation analysis between bone metabolism markers and clinical indicators.

Variables	klotho		FGF23		25 (OH)D3		iPTH		Ca		PHOS	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age	-0.016	0.862	0.010	0.914	0.048	0.591	0.039	0.666	-0.035	0.697	-0.213	0.017
Gender	-0.018	0.838	-0.116	0.197	0.096	0.287	-0.245	0.006	0.258	0.003	-0.109	0.226
BMI(kg/m ²)	0.026	0.775	-0.055	0.538	-0.122	0.174	0.159	0.076	-0.063	0.480	0.127	0.156
SBP(mmHg)	-0.038	0.675	0.224	0.012	-0.308	<0.001	0.247	0.005	-0.172	0.054	0.242	0.006
DBP(mmHg)	0.058	0.516	0.075	0.401	-0.032	0.721	-0.100	0.263	0.128	0.155	0.020	0.826
FPG(mmol/L)	0.129	0.151	-0.045	0.616	0.083	0.357	-0.266	0.003	0.198	0.026	-0.331	<0.001
TG(mmol/L))	-0.064	0.483	-0.067	0.463	-0.110	0.228	-0.177	0.053	0.178	0.051	-0.088	0.339
LDL-C(mmol/L)	0.029	0.757	-0.136	0.143	-0.046	0.619	-0.134	0.148	0.158	0.087	-0.083	0.373
HDL-C(mmol/L)	0.027	0.765	-0.046	0.615	0.03	0.747	-0.033	0.716	0.16	0.079	0.011	0.904
TP(g/L)	0.107	0.234	-0.186	0.038	0.362	<0.001	-0.387	<0.001	0.673	<0.001	-0.287	0.001
ALP(U/L)	-0.09	0.317	0.117	0.193	-0.178	0.046	0.082	0.359	0.024	0.792	0.067	0.457
UA(μmol/L)	-0.102	0.258	0.181	0.043	-0.329	<0.001	0.328	<0.001	-0.315	<0.001	0.364	<0.001
Scr(μmol/L)	-0.255	0.004	0.197	0.027	-0.424	<0.001	0.788	<0.001	-0.663	<0.001	0.779	<0.001
eGFR(ml/min/1.73m ²)	0.285	0.001	-0.294	0.001	0.566	<0.001	-0.708	<0.001	0.608	<0.001	-0.637	<0.001
UACR(mg/g)	-0.260	0.011	0.144	0.165	-0.405	<0.001	0.432	<0.001	-0.430	<0.001	0.330	0.001
24h-UTP(mg)	-0.170	0.062	0.111	0.225	-0.542	<0.001	0.520	<0.001	-0.589	<0.001	0.398	<0.001

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, Fasting Plasma Glucose; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TP, total protein; ALP, alkaline phosphatase; UA, serum uric acid; Scr, serum Sctinine; eGFR, estimated glomerular filtration rate; UACR, urine albumin to Sctinine ratio; 24h-UTP, 24-hour Urine Total Protein. Bold values indicate $P<0.05$.

Klotho, FGF23, 25(OH)D3, iPTH, Ca and PHOS all showed predictive value for DKD in T2DM patients, with AUCs greater than 0.7 for all except FGF23 and PHOS (Figure 5A). The optimal cutoffs and corresponding sensitivity and specificity for each marker are shown in Table 3. The combined AUC for the six bone metabolism markers predicting DKD was 0.846 ($P<0.001$) (Figure 5B). DKD stages IV and V were combined into the MAP group based on proteinuria levels. ROC curves for the MP group ($n=31$) and MAP group ($n=65$) were plotted to analyze the AUC for predicting MP and MAP in DKD patients. The AUC for predicting MP and MAP with bone metabolism markers was 0.951 ($P<0.001$) (Figure 5C). Among these, 25(OH)D3, iPTH, Ca and PHOS had AUCs greater than 0.85. The optimal cutoffs and corresponding sensitivity and specificity for each marker are detailed in Table 3. The combined AUC for predicting DKD was 0.951 ($P<0.001$) (Figure 5D).

3.6 Simple linear regression analysis of eGFR with clinical and bone metabolism indicators

The eGFR was used as the dependent variable, with Age, Gender, BMI, SBP, DBP, FPG, TP, TG, LDL-C, HDL-C, ALP, UA, Scr, klotho, FGF23, 25(OH)D3, iPTH, Ca and PHOS as

independent variables (Table 4). Simple linear regression analysis showed that eGFR was positively correlated with FPG, TP, Klotho, 25(OH)D3, and Ca, and negatively correlated with SBP, UA, Scr, UACR, FGF23, iPTH and PHOS ($P<0.05$).

3.7 Multinomial logistic regression analysis of disease severity and bone metabolism markers in DKD patients

The multinomial logistic regression analysis was performed with DKD severity as the dependent variable (DKD III=3, DKD IV=4, DKD V=5), and SBP, FPG, ALP, TP, klotho, FGF23, 25(OH) D3, iPTH, Ca and PHOS as independent variables. It is aimed to assess the trends of bone metabolism markers across different DKD severity groups. In comparison to the T2DM group, klotho levels were significantly lower in patients with DKD IV and V group. Even after adjusting for different models, particularly Model 2 and Model 3, the reduction in klotho levels remained strongly linked to DKD progression, signifying that as DKD worsens, klotho levels notably decrease. Similarly, FGF23 levels in the DKD V group were significantly elevated compared to the T2DM group, with $P < 0.05$ across all models (Model 1, Model 2, and Model 3), underscoring the marked rise in FGF23 levels in advanced DKD.

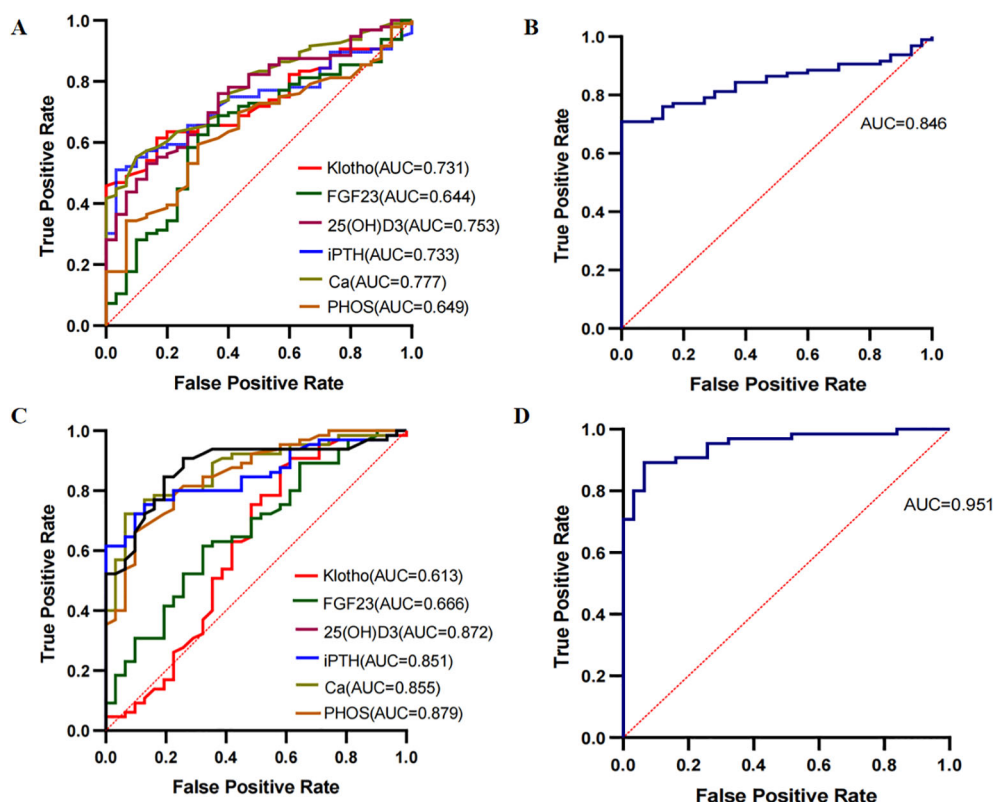


FIGURE 5

ROC curves for predicting DKD using bone metabolism markers. (A, B) ROC curves for klotho, FGF23, 25(OH)D3, iPTH, Ca and PHOS predicting NDKD and DKD groups. (C, D) ROC curves for klotho, FGF23, 25(OH)D3, iPTH, Ca and PHOS predicting MP and MAP groups.

In the DKD IV group, the levels of 25(OH)D3 were significantly lower than those in the T2DM group in Model 1 and Model 2 ($P < 0.05$), suggesting that 25(OH)D3 levels decrease significantly relative to the control group as DKD progresses to stage IV, while DKD V did not show statistical significance. When comparing to DKD s III group, iPTH levels in DKD V group were significantly elevated across all models ($P < 0.05$), showing that iPTH levels increase as DKD progresses. Ca levels were significantly reduced in DKD IV and V group when compared to both the T2DM group and DKD III group, highlighting a strong negative correlation between Ca levels and DKD severity ($P < 0.05$). PHOS levels were significantly higher in DKD III and V group compared to the T2DM group and were also elevated in DKD IV and V group when compared to DKD III group ($P < 0.05$). Notably, these bone metabolism markers varied in their timing of changes, with PHOS showing significant differences as early as DKD III group, while FGF23 levels only differed significantly in DKD V group, suggesting that disturbances in bone metabolism play a significant role in the pathological progression of DKD (Table 5).

4 Discussion

This study examined changes in bone metabolism markers in patients with T2DM and DKD, focusing on the association of klotho, FGF23, 25(OH)D3, iPTH, Ca and PHOS levels with the

severity of DKD. Furthermore, this study also assesses their predictive value for the progression of DKD. It analyzes the significance of bone metabolism markers both separately and in combination for the DKD and NDKD groups, as well as for DKD patients with microproteinuria and massive proteinuria. The results indicate that as DKD advances from stage III to V, klotho, calcium, and 25(OH)D3 levels show a significant decline, whereas FGF23, iPTH, and PHOS levels experience a marked increase. Multiple regression analyses further confirmed that these bone metabolism markers are closely related to DKD progression, suggesting their potential clinical utility in the diagnosis and prognosis of DKD.

4.1 Relationship between bone metabolism dysregulation and DKD progression

The communication between bone and kidney is a complex process involving mutual regulation among different molecules (Figure 6). FGF23 is a phosphate-regulating hormone primarily secreted by bone cells. As a crucial molecule in the communication between bone and kidney, FGF23 collaborates with active vitamin D and intact parathyroid hormone to regulate calcium and phosphate homeostasis (18), and are involved in the pathological processes of insulin resistance, inflammation, fibrosis, and podocyte injury in DKD (8, 28–30). FGF23 levels increase significantly with DKD progression, and elevated FGF23 levels during kidney function

TABLE 3 Predictive value of bone metabolism markers for DKD.

	Variables	AUC	Cut-off	Sensitivity	Specificity
NDKD group VS DKD group	klotho	0.731	< 0.331	0.458	1.000
	FGF23	0.644	> 236.6	0.625	0.700
	25(OH)D3	0.753	< 26.62	0.531	0.867
	iPTH	0.733	> 78.78	0.510	0.967
	Ca	0.777	< 2.215	0.552	0.900
	PHOS	0.649	>1.245	0.594	0.700
MP group VS MAP group	klotho	0.613	< 0.525	0.877	0.419
	FGF23	0.666	> 267.2	0.615	0.677
	25(OH)D3	0.872	< 26.16	0.723	0.936
	iPTH	0.851	> 69.35	0.723	0.903
	Ca	0.855	< 2.205	0.662	0.903
	PHOS	0.879	>1.210	0.846	0.806

decline are a compensatory response to phosphate retention, consistent with its known role in CKD (31). Chronic low-grade inflammation is a major pathogenic mechanism of DKD (32), and the inflammatory response can further increase the expression of FGF23 (33). In addition, excessive elevation of FGF23 may also lead

to complications such as left ventricular hypertrophy and cardiovascular events (34). Thus, FGF23 serves not only as a marker of bone metabolism dysregulation but also as a potential predictor of cardiovascular risk. Our study found that FGF23 is positively correlated with SBP, further suggesting that elevated FGF23 levels may be associated with increased blood pressure and worsening kidney function.

TABLE 4 Linear regression analysis of independent correlated factors.

Index	B (95%CI)	t	P
Age	-0.461(-1.104,0.182)	-1.419	0.158
Gender(Male=1,Female=2)			0.068
BMI	0.103(-1.885,2.091)	0.103	0.918
SBP	-0.698(-1.056,-0.34)	-3.86	<0.001
DBP	0.554(-0.024,1.133)	1.896	0.060
FPG	2.073(0.767,3.38)	3.141	0.002
TP	2.596(1.831,3.36)	6.72	<0.001
TG	2.177(-1.793,6.148)	1.086	0.280
LDL-C	6.886(-0.27,14.042)	1.906	0.059
HDL-C	-4.283(-26.383,17.817)	-0.384	0.702
ALP	-0.133(-0.42,0.154)	-0.916	0.361
UA	-0.216(-0.281,-0.152)	-6.625	<0.001
Scr	-0.148(-0.168,-0.128)	-14.561	<0.001
UACR	-0.046(-0.056,-0.036)	-8.974	<0.001
klotho	61.165(24.65,97.68)	3.315	0.001
FGF23	-0.109(-0.172,-0.046)	-3.427	0.001
25(OH)D3	1.37(1.015,1.724)	7.653	<0.001
iPTH	-0.289(-0.34,-0.237)	-11.163	<0.001
Ca	99.713(76.539,122.886)	8.517	<0.001
PHOS	-67.388(-81.9,-52.877)	-9.191	<0.001

Klotho is a single-pass transmembrane protein predominantly expressed in the kidneys and parathyroid glands (19, 35). Apart from acting as a co-ligand for FGF23 to regulate PHOS, Ca, 1,25 (OH)2D3, and iPTH metabolism, klotho also protects the kidneys through various mechanisms, including antioxidant stress responses, anti-inflammatory effects, anti-fibrosis, induction of autophagy, inhibition of apoptosis and aging, and maintaining renal endothelial cell integrity (20, 36). The kidneys are the primary organ for klotho gene expression (37). Our previous studies have confirmed that in the context of DKD, renal aging is accelerated, and factors such as hyperglycemia, inflammation, oxidative stress, and hypertension can induce the downregulation of klotho expression (38). Plasma klotho levels are reduced in DKD patients, and klotho levels are negatively correlated with the annual decline rate of eGFR and the occurrence of albuminuria (39, 40). Klotho levels begin to decline early in CKD and decrease further as the disease progresses (41), and considered biological markers that aid in the early diagnosis of DKD (42). Our study observed a significant decline in klotho levels with DKD progression, with klotho levels in all DKD groups being lower than those in the T2DM group, and decreasing further from stage III to stage V. We found a significant positive correlation between klotho and eGFR, which supports its role as a marker of worsening renal function.

1,25(OH)2D3 facilitates the absorption of Ca and PHOS in the intestines and regulates Ca metabolism in bone tissue (43). 1,25 (OH)2D3 participates in renal protection in DKD through pathways such as inducing autophagy, alleviating oxidative stress, anti-inflammation, anti-fibrosis, and inhibiting RAS activation (44–47). Previous studies have found that vitamin D deficiency is more

TABLE 5 Multiple logistic regression analysis of the relationship between DKD severity and bone metabolism.

Dependent variables	Independent variables	Model 1			Model 2			Model 3		
		β	Wald	P	β	Wald	P	β	Wald	P
T2DM group*	klotho	-2.113	2.216	0.137	-2.104	2.079	0.149	-2.807	3.119	0.077
DKD III group	FGF23	0.001	0.206	0.650	0.003	0.727	0.394	0.004	1.196	0.274
	25(OH)D3	-0.021	1.32	0.251	-0.025	1.818	0.178	-0.029	2.05	0.152
	iPTH	-0.016	1.246	0.264	-0.024	2.366	0.124	-0.024	1.915	0.166
	Ca	-0.202	0.013	0.911	0.179	0.009	0.925	0.712	0.076	0.783
	PHOS	-3.800	5.299	0.021	-4.462	6.139	0.013	-4.815	6.586	0.010
T2DM group*	klotho	-4.053	4.506	0.034	-4.105	4.558	0.033	-4.082	4.157	0.041
DKD IV group	FGF23	0.004	1.240	0.265	0.005	1.6	0.206	0.005	1.352	0.245
	25(OH)D3	-0.068	4.915	0.027	-0.066	4.509	0.034	-0.062	3.443	0.064
	iPTH	0.003	0.061	0.804	0.002	0.026	0.871	0.006	0.149	0.700
	Ca	-8.247	7.299	0.007	-8.821	7.342	0.007	-6.590	2.736	0.098
	PHOS	2.606	1.685	0.194	2.936	1.751	0.186	2.880	1.541	0.214
T2DM group*	klotho	-12.68	3.963	0.047	-17.052	3.962	0.047	-21.163	3.119	0.077
DKD V group	FGF23	0.018	5.005	0.025	0.021	4.97	0.026	0.024	4.088	0.043
	25(OH)D3	0.113	1.771	0.183	0.139	2.003	0.157	0.247	2.153	0.142
	iPTH	0.035	3.214	0.073	0.044	3.034	0.082	0.060	3.263	0.071
	Ca	-19.48	7.213	0.007	-21.886	6.091	0.014	-20.49	4.77	0.029
	PHOS	7.146	5.460	0.019	7.893	5.785	0.016	12.796	4.057	0.044
DKD III group*	klotho	-1.940	1.074	0.300	-2.001	1.087	0.297	-1.275	0.374	0.541
DKD IV group	FGF23	0.003	0.587	0.444	0.002	0.335	0.563	0.001	0.068	0.795
	25(OH)D3	-0.047	2.415	0.120	-0.041	1.753	0.185	-0.034	0.981	0.322
	iPTH	0.019	1.420	0.233	0.027	2.212	0.137	0.029	2.366	0.124
	Ca	-8.045	6.922	0.009	-9.00	7.351	0.007	-7.302	3.498	0.061
	PHOS	6.406	8.818	0.003	7.398	9.458	0.002	7.695	9.081	0.003
DKD III group*	klotho	-10.567	2.765	0.096	-14.949	3.048	0.081	-18.355	2.341	0.126
DKD V group	FGF23	0.017	4.290	0.038	0.018	3.747	0.053	0.020	2.912	0.088
	25(OH)D3	0.134	2.495	0.114	0.165	2.799	0.094	0.276	2.679	0.102
	iPTH	0.051	5.688	0.017	0.068	6.198	0.013	0.084	5.616	0.018
	Ca	-19.278	7.064	0.008	-22.065	6.158	0.013	-21.202	5.125	0.024
	PHOS	10.945	11.987	0.001	12.356	13.059	0.000	17.611	7.463	0.006

*reference group.
Model 1, not adjusted; Model 2, adjusted Model 1+SBP and FPG; Model 3, adjusted Model 2+ALP and TP. Bold values indicate P<0.05.

pronounced in diabetic patients compared to healthy controls and is more likely to lead to diabetic microvascular complications (48–51). Vitamin D deficiency is a significant risk factor for DKD compared to diabetic patients without renal impairment (52–54). A retrospective cohort study involving 161 patients diagnosed with DKD via biopsy further confirmed that patients with lower serum 25(OH)D3 levels had more severe glomerular lesions and higher

scores of tubular atrophy and interstitial fibrosis (55). Our study also observed a progressive decline in 25(OH)D3 levels with DKD progression, particularly marked in DKD stages IV and V. According to the diagnostic criteria for vitamin D deficiency (<75 nmol/L) set by the Italian Society of Clinical Endocrinology and the American Association of Clinical Endocrinologists (56), vitamin D deficiency was already present in stage III DKD patients. As the

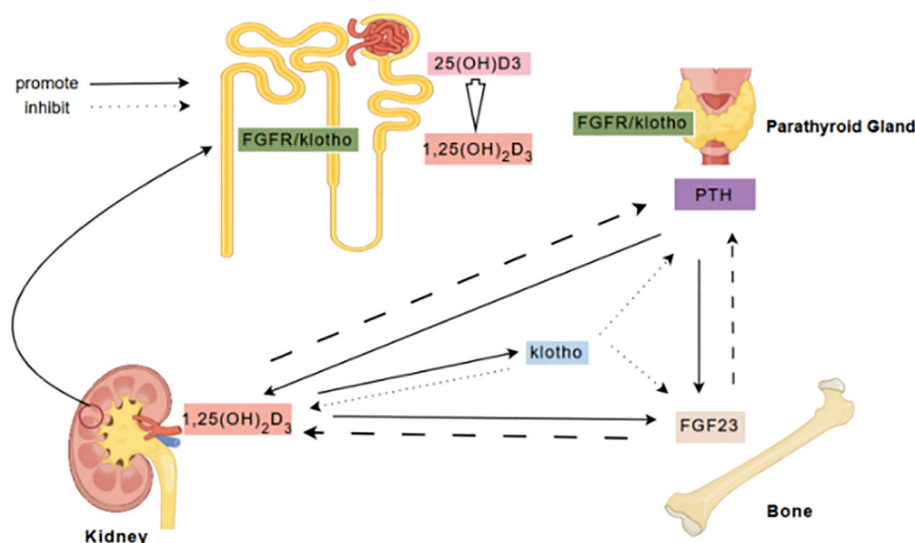


FIGURE 6

FGF23/Klotho Axis in Bone-Kidney Communication. FGF23, secreted by bones, primarily binds to the Klotho/FGFR complex in the kidneys and parathyroid glands to inhibit the secretion of 1,25(OH)₂D₃ and PTH, and regulate renal phosphate excretion. Klotho, acting as a co-receptor for FGF23, modulates phosphate metabolism by suppressing 1,25(OH)₂D₃ synthesis and promoting phosphate excretion. In the kidneys, 25(OH)D₃ is converted to 1,25(OH)₂D₃, which enhances intestinal calcium absorption and inhibits PTH secretion through negative feedback. PTH, secreted by the parathyroid glands, acts on bones and kidneys to stimulate bone resorption and renal calcium reabsorption. These factors maintain calcium-phosphate balance and bone mineral metabolism through complex feedback and feedforward mechanisms.

main circulating form of vitamin D, a decrease in 25(OH)D₃ levels may reflect a decline in renal synthetic function. Low levels of 25(OH)D₃ were significantly negatively correlated with several adverse clinical biomarkers, further supporting its protective role in DKD.

iPTH, as a key regulator of calcium-phosphate metabolism, directly affects blood calcium levels and enhances calcium absorption by influencing 1,25(OH)₂D₃ conversion. Our study found that iPTH levels significantly increase with DKD progression, particularly in stage V. Elevated iPTH often reflects the presence of CKD-MBD, which may be more common in DKD patients (57). Increased iPTH could be a compensatory response to hypocalcemia and hyperphosphatemia, but may also lead to excessive bone turnover and cardiovascular calcification (58).

The changes in serum Ca and PHOS levels also exhibited significant trends in this study. As DKD progresses from stage III to stage V, Ca levels gradually decrease while serum PHOS levels gradually increase, particularly in stage V of DKD. Hypocalcemia and hyperphosphatemia may result from hyperparathyroidism, vitamin D deficiency, and disturbances in calcium-phosphorus metabolism. Changes in serum Ca and PHOS can also feedback-regulate the levels of FGF23, klotho, 1,25(OH)₂D₃, and iPTH (59). 1,25(OH)₂D₃, FGF23, and klotho may also interactively participate in the pathogenesis of DKD. Macrophage infiltration is a significant pathological feature of DKD and is closely related to the degree of kidney injury and interstitial fibrosis (60). FGF23 promotes the polarization of macrophages to the pro-inflammatory phenotype (M1), and klotho expression is significantly upregulated in M1 macrophages. In contrast, 1,25(OH)₂D₃ can stimulate M2 macrophages to increase Arg-1 expression, inhibiting FGF23-

induced TNF- α upregulation, and participating in anti-inflammatory and anti-fibrotic responses. The balance of opposing effects between vitamin D and FGF23 may influence the trends of inflammation and fibrosis in DKD (61). Zou XR et al. discovered that Shenyan granules can modulate vitamin D levels and improve kidney injury and calcium-phosphorus metabolism abnormalities in DKD mice by intervening in the synthesis of renal 24-hydroxylase and 1 α -hydroxylase through the klotho/FGF23/Egr1 signaling pathway (62).

4.2 Clinical significance of bone metabolism markers

The results suggest that bone metabolism markers play a crucial role in DKD progression and may have significant clinical predictive value. ROC curve analysis shows that klotho, FGF23, 25(OH)D₃, iPTH, Ca and PHOS each have predictive value for DKD occurrence, with potential applications in DKD diagnosis. The combined predictive model achieved an AUC of 0.846, highlighting the potential of these markers for improving prediction accuracy. Additionally, the AUC values for 25(OH)D₃, iPTH, Ca and PHOS in predicting DKD severity were >0.85, showing high sensitivity and specificity. The combined model reached an AUC of 0.951, confirming its potential clinical utility. Previous studies have suggested that bone metabolism is closely related to women, but it is also a significant health issue for men (63). The gender differences in bone metabolism warrant further investigation.

4.3 Clinical applications and future research directions

These findings provide a valuable reference for managing bone metabolism in DKD patients. Monitoring klotho, FGF23, 25(OH)D3, iPTH, Ca and PHOS levels is crucial for early detection of DKD progression and assessing disease severity. The combined use of these markers could improve diagnostic accuracy and support personalized treatment. Future research should further explore the mechanisms of bone metabolism markers in DKD, especially their association with cardiovascular complications. Additionally, the impact of interventions targeting these markers on DKD progression needs further investigation. Such research could lead to new treatment strategies to improve DKD patient outcomes.

5 Limitations

This study has limitations including a small sample size, cross-sectional design restricting causal inference, insufficient consideration of all potential confounders, single-point measurement of bone metabolism markers, limited external validity, and lack of in-depth mechanistic studies. The study explored only certain bone metabolism indicators and did not include imaging assessments like bone density. Future research should expand the scope, validate results in larger and more diverse populations, and comprehensively evaluate the role of bone metabolism dysregulation in DKD progression.

6 Conclusion

The study indicates that changes in Klotho, FGF23, 25(OH)D3, iPTH, and Ca levels are closely related to DKD progression. These markers are significant for DKD diagnosis and prognosis and may serve as potential therapeutic targets. Further research should continue to explore the mechanisms and clinical applications of these markers to improve treatment outcomes and quality of life for DKD patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of Dongzhimen Hospital, Beijing University of Chinese Medicine (2022DZMEC-062-03). The studies were conducted in accordance with the local legislation and

institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YK: Writing – original draft, Writing – review & editing. QJ: Writing – original draft, Writing – review & editing. MZ: Writing – original draft, Writing – review & editing. ZL: Writing – review & editing. HZ: Writing – original draft, Writing – review & editing. DL: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. WL: Writing – original draft, Writing – review & editing. YW: Writing – original draft, Writing – review & editing. JL: Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Nonlinear relationship between serum Klotho and chronic kidney disease in US adults with metabolic syndrome

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Background: Current evidence regarding the effects of serum Klotho among patients with metabolic syndrome (MetS) is scarce. This study explored the relationship between serum Klotho levels and the odds of chronic kidney disease (CKD) in middle-aged and older populations with MetS.

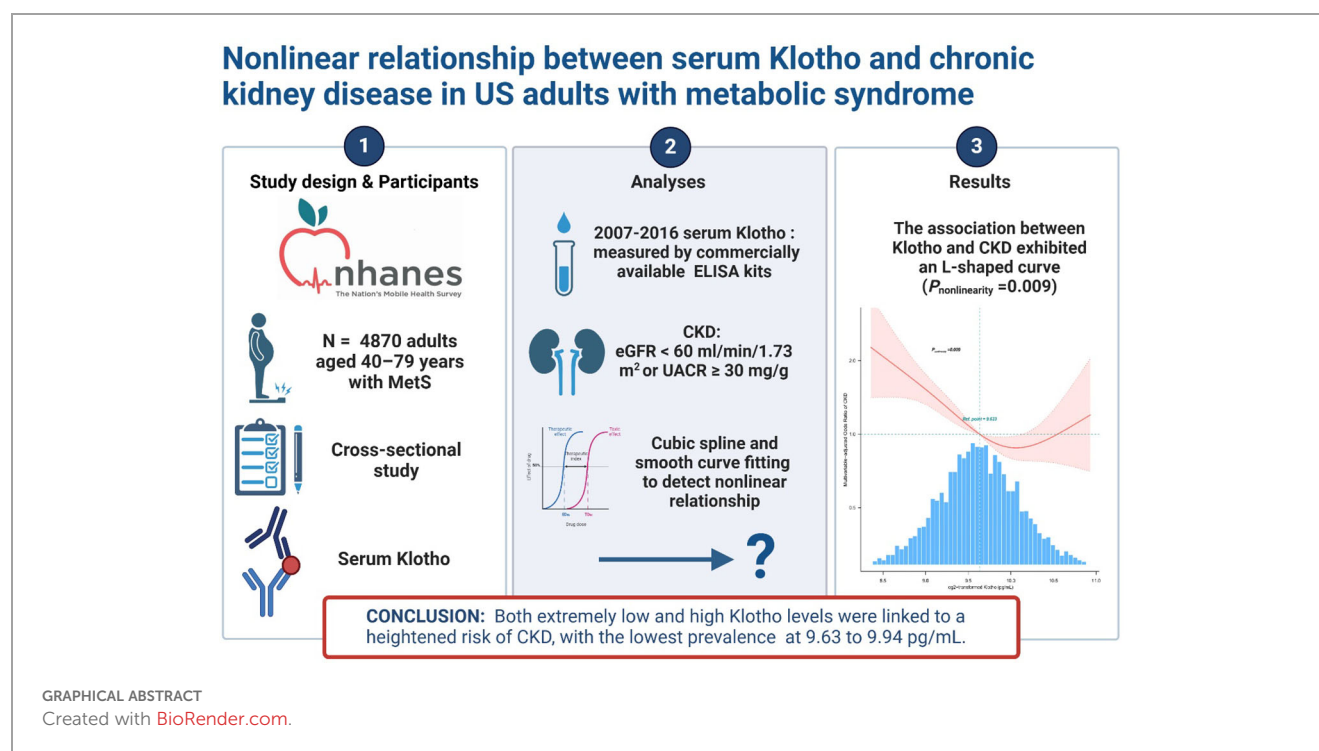
Materials and methods: This cross-sectional study analyzed data from 4870 adults aged 40–79 years who participated in the National Health and Nutrition Survey (NHANES) from 2007 to 2016. CKD was identified at urinary albumin to creatinine ratio (UACR) of 30 mg/g or higher and/or an estimated glomerular filtration rate (eGFR) below 60 mL/min/1.73 m². Measurement of serum Klotho concentration was determined via enzyme-linked immunosorbent assay (ELISA) and subsequently divided into four quartiles (Q1–Q4). The NHANES criteria were followed in calculating the sampling weights. Multivariable logistic regression models were employed to assess the correlation between Klotho and CKD, while generalized linear models with cubic spline functions and smooth curve fitting were utilized to detect any nonlinear relationship. Additionally, subgroup analysis and a range of sensitivity analyses were conducted.

Results: Results showed that a nonlinear L-shaped relationship existed between serum Klotho levels and CKD risk, with the lowest prevalence observed at 9.63–9.94 pg/mL Klotho concentrations. With a two-segment linear regression model, an inflection point of 9.88 pg/mL was noted. Hypertension status was identified as an interaction mediator ($P_{\text{interaction}} = 0.006$). Sensitivity analysis showed stable results.

Conclusions: A nonlinear L-shaped relationship exists between serum Klotho levels and risks of CKD among middle-aged and older adults with MetS, with the lowest prevalence observed at 9.63 to 9.94 pg/mL Klotho concentrations. Our findings, if replicated, underscore the need to estimate the optimal serum Klotho concentrations and the consequential inverse relationship, thus implying the potential of Klotho as both a serum biomarker and a possible preventive or therapeutic intervention.

KEYWORDS

α -Klotho, chronic kidney disease, metabolic syndrome, NHANES, L-shaped



1 Introduction

Chronic kidney disease (CKD) poses a significant healthcare burden, with its insidious onset often leading to a lack of awareness, as evidenced by its high prevalence. In the United States, the prevalence of CKD in adults had been relatively stable at just under 15% for the last 15 years, possibly due to the increasing population of older individuals and high prevalence of diabetes, hypertension, obesity, cardiovascular disease, and other conditions (1, 2). CKD can progress to end-stage renal disease (ESRD), followed by complications such as cardiovascular disease, leading to increased infection and mortality.

Metabolic syndrome (MetS) represents a cluster of metabolic dysregulations that include hypertension, hyperlipidemia, central obesity, insulin resistance, hyperglycemia, and diabetes (3). MetS is intricately linked to the development, progression, and adverse outcomes of CKD, including poor renal and cardiovascular prognoses. MetS induces renal injury through oxidative stress, inflammation, hemodynamic changes, lipotoxicity, and insulin resistance (4, 5). These interconnected mechanisms amplify kidney damage and cardiovascular risks, including glomerular hypertrophy, tubulointerstitial fibrosis, microvascular remodeling, and podocyte damage (6). MetS increases CKD risk by 1.82-fold (7, 8), with 34.8–58.4% of CKD patients having MetS (9). CKD patients with concomitant MetS face higher risks of end-stage renal disease, mortality, and cardiovascular events (5, 6). Despite this clear association, there are no specific guidelines for managing kidney risk in MetS patients, underscoring the urgent need for further research to improve early prevention and outcomes in MetS patients with renal complications. MetS causes renal damage through mechanisms like oxidative stress, chronic inflammation,

perirenal adipose tissue (PRAT) compression, and the overactivation of the renin-angiotensin-aldosterone system (RAAS), mineralocorticoid receptors (MR), and sympathetic nervous system (SNS), leading to renal hemodynamic changes, systemic insulin resistance, and lipotoxicity, which ultimately result in kidney damage and cardiovascular diseases (5). In obese patients, visceral adipose tissue exhibits elevated levels of angiotensinogen, increased leptin secretion, and upregulated MR expression also contribute to the development and progression of hypertension (6, 10). These changes collectively cause imbalances in arterial dilation and constriction, increase intrarenal pressure, and lead to glomerular damage, thereby elevating the risk of CKD (5). CKD and MetS are closely linked to several age-related diseases like cardiovascular disease, diabetes, and cancer. CKD significantly elevates cardiovascular risk, while MetS is a crucial predictor of type 2 diabetes and cardiovascular events (6, 10). Studies indicate that CKD and MetS can increase cancer risks through chronic inflammation, oxidative stress, and metabolic dysregulation (11). These conditions often coexist in elderly populations, creating complex multimorbidity that challenges clinical management. Hence, understanding their interconnections is crucial for developing effective prevention and treatment strategies for the aging population. Therefore, it is essential to identify additional effective biomarkers that could be detected in the early stages of CKD to indicate its course, thus tracking the disease's progression and preventing unfavorable outcomes.

Klotho, also known as α -Klotho, was fortuitously identified in 1997 by Kuro-o et al. (12) as a gene associated with decreased life expectancy. Transmembrane protein Klotho is primarily expressed in the distal convoluted tubules (13). The extracellular domain of Klotho is released into the bloodstream after being cleaved by a protease or by

alternative splicing. This circulating Klotho functions as a hormone that exerts pleiotropic effects including antioxidative stress, antisenescence, and antiapoptotic effects (14). Klotho is pertinent to various age-related disorders, including cardiovascular disease (15), chronic kidney disease (16), diabetes (17, 18), and cancer (19).

In recent years, there has been extensive research on the relationship between soluble Klotho and renal function in patients with CKD. A reno-protective activity of Klotho has been reported in various animal models of acute kidney injury (AKI) (20) and CKD (21, 22). To date, Klotho levels (serum and urine) have been positively correlated to eGFR in adults with CKD (23). Restoration of Klotho attenuates vascular calcification associated with CKD (24). Of note, the associations between Klotho and CKD risk among general populations are controversial. A cohort study involving patients with CKD (stages 1–5) revealed an independent relationship between serum Klotho levels and the combined outcome of doubling serum creatinine, kidney failure, or death after adjusting for age, blood pressure, eGFR, diabetes, albuminuria, and parathyroid hormone (PTH) (25). By contrast, Seiler et al. investigated the Klotho level in 321 patients with stage 2–4 CKD, in which the Klotho level did not significantly differ based on the CKD stage, and was not associated with eGFR according to Spearman correlation analysis (26).

Previous studies did not focus on the association of Klotho and CKD per se in patients with MetS. Given these knowledge gaps, we leveraged baseline cross-sectional data obtained from National Health and Nutrition Examination Survey (NHANES), to validate the association between Klotho and CKD in patients with MetS and the optimal levels of Klotho, thus providing more precise information regarding Klotho for kidney health and healthy aging.

2 Materials and methods

2.1 Data sources

The National Center for Health Statistics (NCHS) conducts the National Health and Nutrition Examination Survey (NHANES), a multistage probability sample of the civilian, noninstitutionalized US population (27). NHANES gathers a range of health-related information, such as demographic traits, physical examination results, lab findings, and dietary behavior. All participants provided written informed consent, and the NHANES study protocols received approval from the National Center for Health Statistics (NCHS) Research Ethics Review Board. Detailed information can be accessed at www.cdc.gov/nchs/nhanes/.

2.2 Study design and population

Data from five successive NHANES cycles conducted between 2007 and 2016 were combined for this cross-sectional analysis. In the NHANES investigation, serum Klotho levels were only assessed in adults between the ages of 40 and 79 (the youngest participant in

this sample was 40 years old) (28). Among the 50,588 participants aged 40–79 years, the exclusion criteria were as follows: (a) participants with unavailable data on serum Klotho ($n = 36,824$); (b) participants without MetS ($n = 7810$); (c) participants without CKD-related data ($n = 72$); and (d) incomplete covariates ($n = 1012$). Ultimately, 4870 eligible participants were included in the final analysis, as shown in Figure 1.

2.3 Determination of serum Klotho levels

Serum samples collected from NHANES 2007–2016 participants aged 40–79 years, who consented to the use of their samples for future research, were analyzed with a commercially available ELISA kit (Immuno-Biological Laboratories Co., Ltd., Fujioka, Japan) (29). All samples were kept at -80°C prior to experiments. Duplicate analyzes of samples were conducted in accordance with the manufacturer's protocol, and all the results were scrutinized against the laboratory's predefined criteria for acceptability before being finalized for reporting. Samples with duplicate results surpassing a 10% discrepancy were identified for reanalysis. If a quality control sample's value deviated beyond 2 standard deviations from the expected value, the entire analytical run was rejected, necessitating a repetition of sample analyzes (30). A more detailed description of the laboratory methodology can be found at https://wwwn.cdc.gov/Nchs/Nhanes/2015-2016/SSKL_I.htm.

2.4 Definition of MetS

The definition of Metabolic Syndrome (MetS) followed the revised National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP) III criteria, requiring the presence of three or more of the following components (3): (1) elevated waist circumference (WC) defined as a waist circumference of 102 cm for men and 88 cm for women; (2) elevated blood pressure defined as a blood pressure of 130/85 mmHg or medication treatment of previously diagnosed hypertension; (3) reduced high-density lipoprotein cholesterol (HDL-C) defined as an HDL level of <40 mg/dL for men and <50 mg/dL for women or medication treatment for reduced HDL-C; (4) elevated triglyceride (TG) defined as TG level ≥ 160 mg/dL or medication treatment for elevated TG; and (5) elevated fasting glucose defined as a fasting glucose level of ≥ 126 mg/dL or medication treatment for elevated glucose and previously diagnosed type 2 diabetes.

2.5 Assessment of CKD

The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration

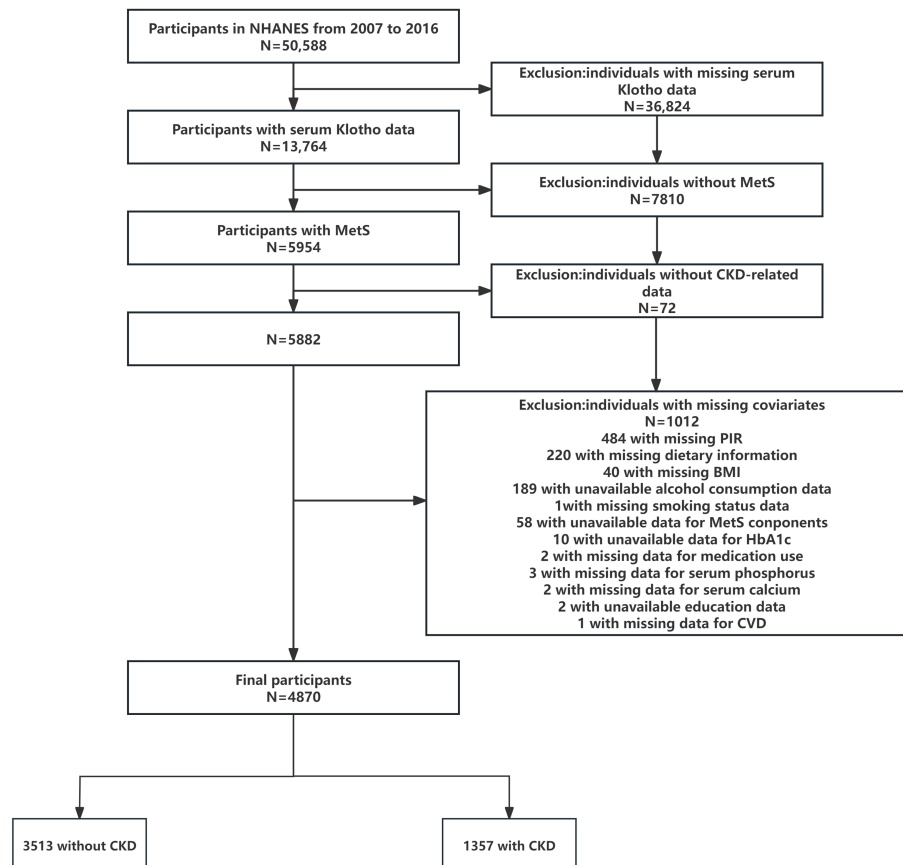


FIGURE 1

Flow diagram of the screening and enrollment of study participants. MetS, metabolic syndrome; CKD, chronic kidney disease; PIR, family poverty income ratio; BMI, body mass index; CVD, cardiovascular disease.

(CKD-EPI) equation (31), which takes into account sex, age, and serum creatinine (Scr) levels. The formula used for calculation is:

$$eGFR = 141 \times \min(Scr/\kappa, 1)^{\alpha} \times \max(Scr/\kappa, 1)^{-1.209} \times 0.993^{Age} \times 1.018[\text{if female}] \times 1.159[\text{if black}]$$

where *Scr* represents serum creatinine, $\kappa = 0.7$ (females) or 0.9 (males), α is -0.329 for females and -0.411 for males, min indicates the minimum of *Scr*/ κ or 1, and max indicates the maximum of *Scr*/ κ or 1 (31).

Urine samples from NHANES participants were collected at a standardized mobile examination center (MEC). A fluorescent immunoassay was utilized to quantify the albumin content of the urine. The Jaffé rate reaction, which forms a red creatinine-picric acid complex when creatinine and picric acid react in an alkaline solution, is used in the urine creatinine analysis (32). A modified Jaffé kinetic technique and solid-phase fluorescence immunoassay were used to measure the levels of creatinine and albumin in the urine. The urinary albumin-to-creatinine ratio (UACR) was calculated by dividing the urinary albumin concentration in milligrams by the urinary creatinine concentration in grams. An $eGFR < 60$ mL/min/1.73 m² and/or a $UACR \geq 30$ mg/g were used to diagnose CKD.

2.6 Assessment of covariates

Standardized questionnaires obtained information on sociodemographic characteristics; smoking status, alcohol consumption; physical activity; dietary intake; presence of hypertension, hyperlipidemia, CVD and diabetes; medication use; and biochemical profiles. The participants were divided into groups according to self-reported races and ethnicities: non-Hispanic white, non-Hispanic black, Mexican-American, and other groups (including multiracial participants because NHANES did not provide a detailed list of all races and ethnicities in this category). The family poverty income ratio (PIR) was categorized as ≤ 1.0 , 1–3, or > 3.0 . Educational background was categorized as below high school (≤ 9 th grade or 9–11th grade including 12th grade without diploma), high school or equivalent, and above high school (some college or associate's degree or college graduate or above).

Prior to the interview at the mobile examination center (MEC), participants underwent a dietary recall interview to gather their 24-hour nutritional data, encompassing total dietary intake of calories, protein, carbohydrates, fat, and fiber. Body mass index (BMI), waist circumference (WC), and various biochemical indicators were derived from medical evaluations and laboratory tests conducted

at the MEC. BMI was divided into three categories with cutoff values of 25 and 30 kg/m², corresponding to “normal<25, overweight 25–30, or obesity ≥30”, respectively.

In terms of smoking status, individuals who reported smoking fewer than 100 cigarettes throughout their lifetime were categorized as never smokers. Those who had smoked more than 100 cigarettes and were currently smoking were identified as current smokers, whereas former smokers were individuals who had smoked over 100 cigarettes but had stopped smoking (33).

The criteria used to diagnose alcohol consumption and status were as follows: current heavy drinking, defined as consuming a minimum of 4 drinks per day for men, 3 drinks per day for women, or engaging in binge drinking on 5 or more days per month. Current moderate drinking was characterized by consuming at least 3 drinks per day for men, 2 drinks per day for women, or binge drinking on 2 or more days per month. Individuals who did not meet these criteria were classified as current light drinkers. Additionally, individuals were categorized as never drinkers if they had consumed fewer than 12 drinks in their lifetime, and as former drinkers if they had consumed 12 or more drinks in a year but did not drink in the last year, or if they did not drink in the last year but had consumed 12 or more drinks in their lifetime (33).

Histories of medical conditions were self-reported. CVD was determined by a combination of self-reported physician diagnoses and standardized medical status questionnaires completed during individual interviews. DM was defined as (1) doctor diagnosed diabetes; (2) glycohemoglobin >6.5%; (3) fasting glucose ≥7.0 mmol/L; (4) random blood glucose ≥11.1 mmol/L; (5) 2-h oral glucose tolerance test blood glucose ≥11.1 mmol/L; and (6) use of diabetes medication or insulin (34). Glycosylated hemoglobin A1c (HbA1c) measurements were performed by the Diabetes Laboratory at the University of Minnesota using a Tosoh A1c 2.2 Plus Glycohemoglobin Analyzers (Tosoh Medics, Inc., San Francisco, CA). All blood pressure readings were obtained during a single visit. An average of up to three consecutive systolic and diastolic blood pressure readings were used. Hypertension was diagnosed if the participant meets any of the following: (1) systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg was found greater than or equal to three times during the physical examination; (2) previously diagnosed with hypertension; and (3) on medication for hypertension (35). In the meantime, if any of the three criteria were met by the participants, hyperlipidemia was diagnosed: triglycerides (TG) ≥ 1.69 mmol/L, total cholesterol (TC) ≥ 5.17 mmol/L, or low-density lipoprotein (LDL) ≥ 3.36 mmol/L; or high-density lipoprotein (HDL) < 1.03 mmol/L (male)/1.29 mmol/L (female); or were prescribed lipid-lowering drugs (36). Data concerning medications consumed in the last 30 days were gathered by skilled professionals who matched the products supplied by participants with a database containing information on drugs and dietary supplements.

In addition, strict laboratory analyzes were performed, including the assessment of serum calcium and serum phosphorus at baseline. The full measurement technique for these variables is available at <https://www.cdc.gov/nchs/nhanes/>. Details

of the laboratory analyzes are documented in the NHANES Laboratory Medical Technologists Procedures Manual.

2.7 Statistical analysis

The survey procedure was adapted to the complex survey design and appropriate sample weights were applied for this random subsample to be representative of the civilian population of the United States, as recommended by the National Center for Health Statistics (NCHS). Normally distributed variables were presented as mean ± standard deviations (SDs), while skewed variables were presented as median (interquartile range). Categorical variables were presented in the form of numbers and percentages. To test differences across quartiles of serum Klotho levels, one-way analysis of variance (ANOVA) and Kruskal-Wallis tests were conducted for continuous variables with normal and skewed distributions, respectively. Pearson's chi-square tests were performed for categorical variables. Serum Klotho concentrations were log base 2 transformed due to its skewed distribution, and it is also rational to explain that the change of the dependent variable is caused by per doubling of the exposure.

Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated to analyze the association between Klotho and CKD using survey-weighted logistic regression models. Participants were divided into four groups based on log2-transformed Klotho quartile amounts: Q1 (7.24–9.32 pg/mL; n = 1218), Q2 (9.33–9.63 pg/mL; n = 1217), Q3 (9.64–9.94 pg/mL; n = 1217), Q4 (9.95–12.30 pg/mL; n = 1218), and the lowest quartile served as the reference group. We filter covariates based on the following four criteria: (1) demographic data; (2) factors affecting CKD and Klotho levels reported in the literature (18, 25, 28, 29, 37); (3) changes in regression coefficients of the basic model exceeding 10% (38); and (4) our clinical experience. As an effort for reducing methodological bias, we also used the directed acyclic graph (DAG) approach. The DAG provides a graphical representation of the variables related to the exposure and the outcome and is helpful in identifying potential sources of bias. We used the DAGitty software, version 3.1, to make our DAG, presented in [Supplementary Figure S1](#), suggesting that minimal sufficient adjustment sets for estimating the total effect of serum Klotho on CKD: components of MetS and the number of Mets components, lifestyle factors (waist circumference, alcohol drinking, physical activity, and smoking), medication use, pre-existing diseases (CVD, diabetes, hyperlipidemia, hypertension), serum phosphorus and calcium, and socialdemographics (age, sex, race, PIR, and education).

Unadjusted, minimally adjusted, and fully adjusted findings were presented in line with the STROBE guidelines: Model 1, a non-adjusted crude model. Model 2: adjusted for social demographic characteristics (age, sex, and race). Model 3: adjusted for Model 2 plus covariates based on factors that changed the matched OR by at least 10% when it was added to this model, including HbA1c, CVD, anti-hypertensive medication use, and lipid-lowering medication use. Model 4: adjusted for Model 3 plus the covariate P<0.1 in univariate model, comorbidities, and other variables reported in the

previous literature, including education, PIR, waist circumference, smoking status, alcohol drinking status, protein intake, fiber intake, fat intake, carbohydrate, diabetes, hypertension, hyperlipidemia, anti-diabetic medication use, physical activity, serum calcium, serum phosphorus, components of MetS, and the number of MetS components. We used Klotho as both continuous and categorical variables and calculated the P_{trend} used to verify the result of Klotho as a continuous variable.

To validate the correlation between Klotho and CKD and to explore the possibility of a nonlinear relationship between Klotho and CKD, a weighted generalized additive model (GAM) was also used to visually assess the relationship between the two (39), which was selected for its flexibility in modeling nonlinear relationships without assuming a specific functional form. This approach is particularly suitable for large datasets like NHANES, as it can detect complex patterns while maintaining statistical power. For nonlinear correlations, a segmented regression model was established to calculate the relationship between Klotho and CKD using a smoothing plot. Log-likelihood ratio test comparing one-line (non-segmented) model with segmented regression (also known as piecewise regression) model was also applied to assess the robustness of the results. When the relationship between Klotho and CKD was apparent in the smoothed curve, the method automatically calculated the inflection point by maximizing the model likelihood.

To ascertain if the association between serum Klotho protein and CKD incidence was consistent across several subgroups and to identify potential effect modifiers, we performed interaction and stratified analyzes by age (40–60 years; 61–79 years), sex (male; female), BMI (<25; 25–30; >30), diabetes status (no; pre diabetes; yes), hypertension status (no; yes), smoking status (never; former; now), race (white; black; Mexican American; other) and five MetS components (yes; no). In each subgroup, we examined the association between Klotho concentration and the odds of CKD. Subgroup analyzes were performed using stratified linear regression models, while modifications and interactions among subgroups were examined through likelihood ratio tests.

To assess the robustness of our findings, we also performed several sensitivity analyzes. First, calculations were repeated with the Modification of Diet in Renal Disease study (MDRD) eGFR equation (40). Second, the International Diabetes Federation (IDF) 2009 was used as the diagnostic criteria for MetS to see if the results were consistent (41). Third, the parameter estimates may be biased as a result of the analysis of the missing data of 1,012 participants. Subsequently, we implemented multiple imputation to maintain the participants in the analysis. Fourth, to yield comparable groups across different Klotho levels, we performed propensity score matching (PSM). Propensity scores were calculated with a logistic regression where Klotho levels (divided by median) was the dependent variable. The aforementioned factors were chosen as covariates in order to produce the propensity score. Individuals in the two groups were matched 1:1 (42). The estimated propensity scores were then utilized as weighting factors. Pairwise algorithmic (PA) (43), standardized mortality ratio weight (SMRW) (44) models were used to generate a weighted cohort to adjust the baseline confounders, thus reflecting

more truly the independent association between Klotho levels and CKD. We also explored the potential for unmeasured confounding between Klotho and CKD by calculating E-values (45).

Given that the sample size was determined exclusively using the provided data, no prior statistical power assessments were carried out (46). All data analysis and visualization were conducted using R software (version 4.3.1 <http://www.R-project.org>, The R Foundation), EmpowerStats software (<http://www.empowerstats.com>, X&Y solutions, Inc., Boston, MA, USA) and the Free Statistics software (version 1.9.1; Beijing FreeClinical Medical Technology Co., Ltd, Beijing, China). $P < 0.05$ (two-sided) was considered statistically significant.

3 Results

3.1 Baseline characteristics of participants

Clinical and biochemical characteristics of participants according to the Klotho quartiles are summarized in Table 1. A total of 4870 participants selected from NHANES 2007–2016 were involved in our study, representing approximately 38,872,250 US adults aged 40–79 years. Based on the weighted analyzes, 51.6% were females, with an average age of 57.7 ± 0.2 years. The median (interquartile range, IQR) of serum Klotho was 782.9 (641.3, 968.2) pg/mL. A total of 1357 participants (22.4%) were classified as having CKD. The mean baseline eGFR was 84.5 ± 0.3 ml/min/1.73 m². The prevalence of CKD was higher among participants with lower Klotho levels (Q1: 420[28.1%]) than among participants with higher Klotho levels (Q3: 292[18.7%]). Across quartiles of Klotho, differences with statistical significance were observed regarding age; race; drinking status; smoking status; BMI; eGFR; CVD; CKD; daily energy, carbohydrate, fiber, fat, and protein intake level (all $P < 0.05$).

In our study, participants with higher Klotho levels were likely to be female and had hypertension. Serum Klotho levels across CKD stages are shown in Figure 2. Significant differences were observed among the five groups ($P < 0.001$), and the median value of Klotho in the CKD stage 4 was the lowest.

3.2 Association between serum Klotho levels and the risk of CKD

The association between serum Klotho levels and the prevalence of CKD is detailed in Figure 3. Our results suggested that higher Klotho levels were significantly associated with decreased risk of CKD both in the crude model [odds ratio (OR), 0.65; 95% CI, 0.53–0.78] and the minimally adjusted Model 2 (OR, 0.70; 95% CI, 0.58–0.85). In the fully adjusted Model 4, the negative association between Klotho and CKD remained stable (OR, 0.65; 95% CI, 0.53–0.79), indicating that when log2-transformed Klotho concentrations increased one unit, the odds of CKD were 35% lower.

Similar findings were observed when Klotho was included in the regression model as a categorical variable, divided into four

TABLE 1 Basic characteristics of NHANES participants (N = 4870).

	Total	Klotho levels quartiles (pg/mL)				P Value
Characteristics*	(n = 4870)	Q1(n = 1218)	Q2(n = 1217)	Q3(n = 1217)	Q4(n = 1217)	
Age, years [†]	57.7±0.2	59.0±0.4	57.4±0.4	57.6±0.4	56.9±0.4	0.001
Female, n (%) [‡]	2584(51.6)	627(52.3)	605(48.4)	660(51.4)	692(54.6)	0.220
Education, n (%)						0.473
Below high school	1411(17.3)	359(17.0)	347(16.9)	363(18.0)	342(17.5)	
High school	1195(25.6)	329(28.1)	278(23.4)	312(26.6)	276(24.3)	
Above high school	2264(57.1)	530(55.0)	592(59.7)	542(55.5)	600(58.2)	
PIR, n (%)						0.607
≤ 1.0	1014(12.0)	255(11.5)	246(11.0)	254(12.5)	259(13.2)	
1–3	2144(36.7)	557(37.6)	527(38.5)	542(36.0)	518(34.5)	
> 3.0	1712(51.3)	406(50.9)	444(50.5)	421(51.4)	441(52.4)	
Race, n (%)						< 0.001
White	2295(75.8)	601(76.8)	605(77.8)	596(76.7)	493(71.7)	
Black	855(7.9)	222(8.4)	188(6.6)	173(6.1)	272(10.9)	
Mexican American	863(7.0)	201(6.0)	208(6.4)	230(7.7)	224(8.1)	
Other	857(9.2)	194(8.9)	216(9.2)	218(9.5)	229(9.3)	
Drinking status, n (%)						0.010
Former	1250(22.7)	330(23.4)	297(20.9)	306(21.6)	317(25.2)	
Never	774(11.3)	165(9.8)	190(11.1)	200(11.7)	219(13.0)	
Mild	1573(37.6)	371(35.2)	403(38.8)	407(38.3)	392(38.1)	
Moderate	587(14.1)	147(15.5)	145(12.4)	136(14.2)	159(14.5)	
Heavy	686(14.3)	205(16.1)	182(16.9)	168(14.3)	131(9.3)	
Smoking status, n (%)						0.056
Former	1604(34.0)	425(35.3)	420(35.0)	405(34.7)	354(30.8)	
Never	2353(47.8)	528(44.7)	565(45.3)	594(48.8)	666(52.9)	
Now	913(18.2)	265(20.0)	232(19.7)	218(16.5)	198(16.3)	
Physical activity, n (%)						0.120
Inactive	1845(42.1)	428(42.9)	503(45.6)	465(40.9)	449(38.6)	
Active	3025(57.9)	790(57.1)	714(54.4)	752(59.1)	769(61.4)	
Physical examination						
BMI, kg/m ²	32.9±6.3	32.5±6.1	32.9±5.9	33.2±6.5	33.1±6.7	0.022
WC, cm	111.0±0.3	110.3±0.4	111.3±0.4	111.5±0.5	110.8±0.7	0.205
Laboratory data						
eGFR, ml/min/1.73 m ²	84.5±0.3	80.0±0.7	85.0±0.6	85.7±0.6	87.7±0.6	<0.001
UACR, mg/g [§]	9.0 [5.5, 21.3]	9.0 [5.4, 23.2]	9.0 [5.5, 21.4]	8.5 [5.5, 19.6]	9.5 [5.6, 21.8]	0.259
HbA1c, %	6.2±1.2	6.0±1.0	6.1±1.1	6.1±1.1	6.4±1.6	< 0.001
Phosphorus, mmol/L	1.2±0.2	1.2±0.0	1.2±0.0	1.2±0.0	1.2±0.0	0.420
Calcium, mmol/L	2.4±0.0	2.3±0.0	2.4±0.0	2.3±0.0	2.4±0.0	0.108

(Continued)

TABLE 1 Continued

	Total	Klotho levels quartiles (pg/mL)				P Value
Dietary intake						
Energy, kcal/d	2079.0±19.6	2014.4±35.2	2157.7±34.9	2079.0±42.2	2058.0±32.6	0.033
Carbohydrate, g/d	244.1±2.5	233.9±4.1	250.7±4.5	244.6±5.3	247.0±4.4	0.022
Dietary fiber, g/d	16.6±0.2	15.8±0.4	17.5±0.4	16.6±0.4	16.5±0.4	0.043
Fat intake, g/d	82.5±1.1	78.8±1.8	85.8±1.8	83.5±2.0	81.7±1.7	0.038
Protein intake, g/d	81.2±0.8	77.0±1.4	84.4±1.6	81.1±1.6	82.1±1.5	0.002
Medication use, n (%)						
Glucose-lowering drugs	1520(25.5)	412(26.7)	359(25.4)	365(23.8)	384(26.1)	0.642
Antihypertensive drugs	3252(63.3)	865(67.8)	808(62.5)	783(59.5)	796(63.5)	0.068
Lipid-lowering drugs	2049(39.9)	566(43.6)	515(40.6)	499(38.0)	469(37.3)	0.286
Comorbidities, n (%)						
Hypertension	3552(69.8)	921(72.4)	889(68.7)	864(68.2)	878(70.1)	0.316
Diabetes	2177(37.0)	542(36.0)	504(34.9)	529(36.1)	602(41.5)	0.172
CVD	957(16.9)	300(20.5)	227(14.8)	222(16.7)	208(15.8)	0.026
Hyperlipidemia	4566(93.9)	1151(94.6)	1144(94.7)	1148(93.7)	1123(92.6)	0.391
Events, n (%)						
CKD	1357(22.4)	420(28.1)	336(21.9)	292(18.7)	309(20.8)	< 0.001

*All estimates accounted for complex survey designs.†Normally distributed continuous variables are described as means ± SEs.‡Categorical variables are presented as numbers (percentages).
§Continuous variables without a normal distribution are described as medians (interquartile ranges).
OR, odds ratio; CI, confidence interval; BMI, body mass index; WC, waist circumference; PIR, family poverty income ratio; eGFR, estimated glomerular filtration rate; UACR, urine albumin-creatinine ratio; HbA1c, glycosylated hemoglobin A1c; HDL, high-density lipoprotein; CVD, cardiovascular disease; CKD, chronic kidney disease; SE, standard error.

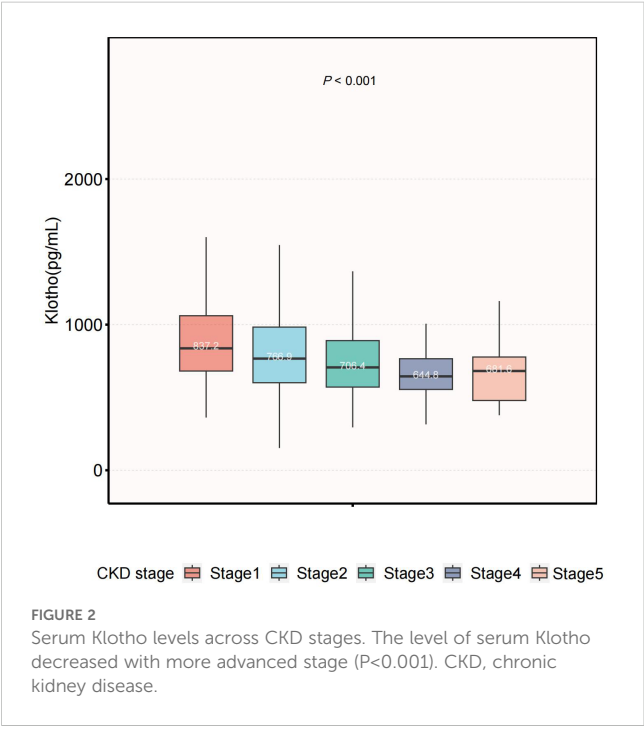
quartiles, with the lowest log2-transformed Klotho level (first quartile) as the reference. In fully adjusted Model 4, the decreases in serum Klotho levels for the second quartile, third quartile, and

fourth quartile were 20% (95% CI, 0.63–1.02), 40% (95% CI, 0.47–0.78), and 34% (95% CI, 0.51–0.87), respectively.

By utilizing a two-piecewise logistic regression model within a generalized additive model (GAM), the inflection point at 9.88 pg/mL was identified. Accordingly, the association between Klotho and CKD exhibited an L-shaped curve ($P_{\text{nonlinearity}}=0.009$) in spline curve fitting (Figure 4A). In the threshold analysis, the OR of developing CKD was 0.57 (95%CI, 0.47–0.70) in participants with a Klotho level of 9.88 pg/mL (Table 2), which means that the risk of CKD is reduced by 43% with every one unit increase in serum Klotho concentration. Serum Klotho levels were not associated with CKD when Klotho was >9.88 pg/mL (OR,1.11; 95% CI, 0.77–1.60), indicating that the risk of CKD no longer decreased with increasing serum Klotho concentrations.

3.3 Subgroup analysis

Stratified and interaction analyses were performed to investigate potential modifications in the relationship between serum Klotho protein levels and the prevalence of CKD across various subgroups (Figure 5). After adjusting for potential confounders, consistent findings were noted across strata defined by age, sex, race, PIR, educational attainment, physical activity, smoking habits, alcohol consumption, components of MetS, BMI, and diabetes status. Additionally, hypertension status produced an intriguing



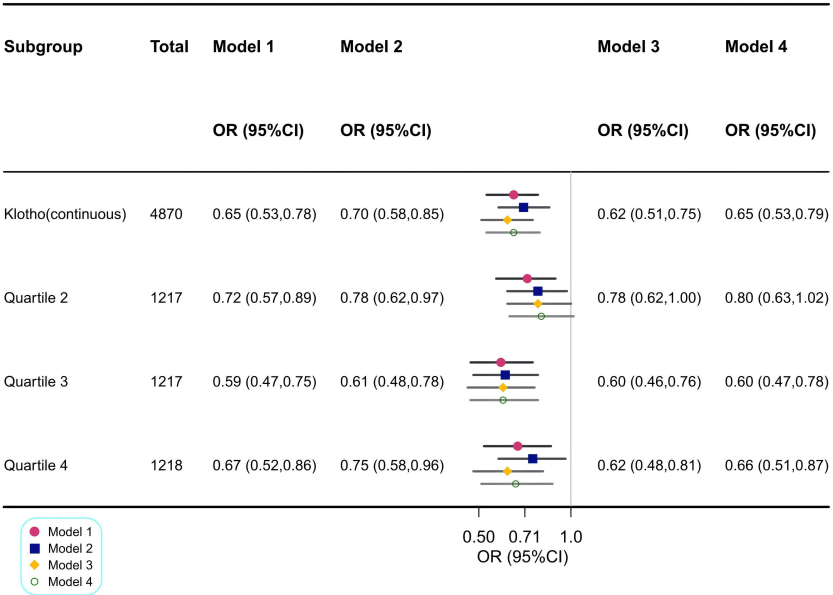


FIGURE 3
Association between log2-transformed Klotho and CKD. Model 1: Crude model. Model 2: Adjusted for age, sex and race. Model 3: Adjusted for Model 2 + HbA1c, CVD, anti-hypertensive medication use and lipid-lowering medication use. Model 4: Adjusted for Model 3 + education, PIR, waist circumference, smoking status, alcohol drinking status, protein intake, fiber intake, fat intake, carbohydrate, diabetes, hypertension, hyperlipidemia, anti-diabetic medication use, physical activity, serum calcium, serum phosphorus, components of MetS and the number of MetS components. OR, odds ratio; CI, confidence interval; CKD, chronic kidney disease; HbA1c, glycosylated hemoglobin A1c; CVD, cardiovascular disease; PIR, family poverty income ratio; MetS, metabolic syndrome; FBG, fasting blood glucose.

significant interaction whereby in patients with hypertension, every 1-pg/mL increase in log2 Klotho was associated with a 43% decrease in CKD risk, while in individuals with no hypertension, every 1-pg/mL increase in log2 Klotho was associated with a 9% increase in CKD risk ($P_{\text{interaction}}=0.006$) (Figure 4B).

3.4 Sensitivity analysis

To support our conclusions, we conducted sensitivity analyzes. The results supported the main results described above. Largely similar results were seen in sensitivity analyzes when the MDRD equation was used instead to define CKD (Supplementary Table S1). In fully adjusted model, the association was negative between Klotho and CKD (OR,0.74; 95% CI, 0.61–0.90). In addition, when MetS was redefined by IDF2009, after the adjustment of all covariates in Model 4, the association between Klotho and CKD remained (OR, 0.62; 95% CI, 0.51–0.77) (Supplementary Table S2). Multiple imputation analysis yielded consistent results (Supplementary Table S3). The results remained stable even after accounting for possible confounders using PSM and controlling for propensity score, pairwise algorithmic, overlap weight, and doubly robust analysis. The ORs were 0.64–0.76, and *P* values were all<0.001(Supplementary Figure S2). For the current study, the OR at serum Klotho concentrations for risks of CKD was 0.65 with a 95% CI of 0.53-0.79 (shown in Figure 2). The E-value for this point estimate is 2.45. Thus, following the suggested language of VanderWeele and Ding (47), the correlation between serum Klotho

and the risk of CKD was found to be robust, unless the OR of the risk of CKD of an unmeasured confounder was >2.45.

3.5 Summary of results regarding Klotho concentration and CKD risk

Our analysis reveals a significant L-shaped relationship between serum Klotho levels and CKD risk in individuals with metabolic syndrome. A specific Klotho concentration range (9.63 to 9.94 pg/mL) is linked to the lowest prevalence of CKD. Notably, a critical inflection point at 9.88 pg/mL demonstrates that lower serum Klotho levels are associated with increased CKD risk, especially in hypertensive patients. This threshold suggests that maintaining Klotho levels within this optimal range may be essential for reducing CKD risk in individuals with MetS. Our findings highlight Klotho’s potential clinical significance as a biomarker for kidney health and intervention strategies.

4 Discussion

In this nationally representative cross-sectional study involving 4,870 patients with metabolic syndrome (MetS) from the 2007–2016 NHANES, we found a significant independent negative association between serum Klotho levels and the risk of chronic kidney disease (CKD), indicating a 35% reduction in risk. Our findings revealed a dose-response relationship described by an L-

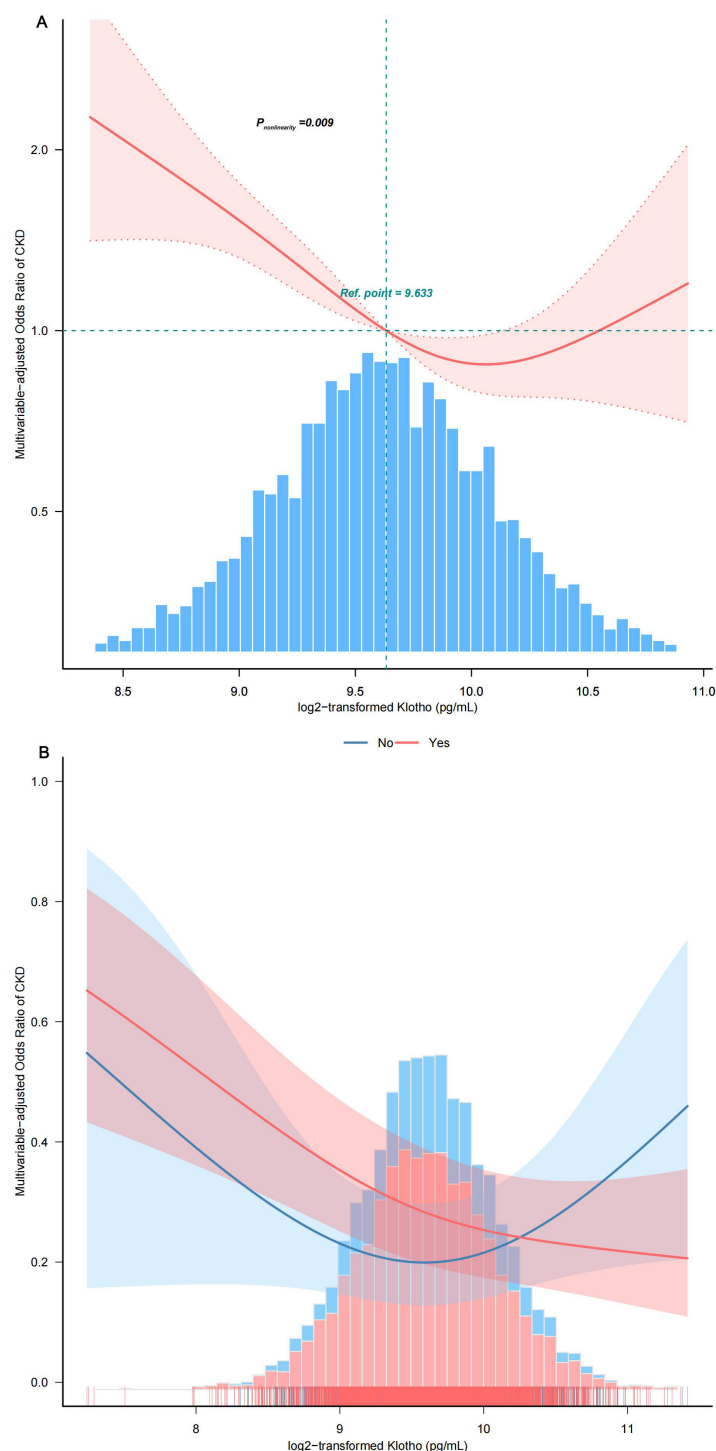


FIGURE 4

Smooth curve fitting. (A) Association between log2-transformed Klotho and the incidence of CKD. (B) Association between Klotho log2 transform and the incidence of CKD stratified by hypertension status. The solid and dashed lines represent the odds ratios and their corresponding 95% confidence intervals. Only 99% of the data is displayed after adjusting variables in Model 4. PIR, family poverty income ratio; HbA1c, glycosylated hemoglobin A1c; CVD, cardiovascular disease; MetS, metabolic syndrome; FBG, fasting blood glucose.

shaped curve ($P_{\text{nonlinearity}} = 0.009$), suggesting that the association is most pronounced at Klotho levels below 9.88 pg/mL. This threshold underscores the importance of maintaining Klotho levels within this optimal range to mitigate CKD risk, particularly in hypertensive patients ($P_{\text{interaction}} = 0.006$).

Our results are consistent with previous studies indicating that systemic Klotho levels decrease in CKD patients and decline further as estimated glomerular filtration rate (eGFR) declines. For example, Drew et al. identified an independent association between higher soluble Klotho levels and a reduced risk of kidney

TABLE 2 Threshold effect analysis of Klotho on CKD using the two-piecewise logistic regression model.

Models	CKD _§	
	OR (95%CI)	P value
Model 1*		
One line effect	0.69 (0.60, 0.80)	<0.001
Model 2†		
Turning point(K)	9.88	
<9.88 slope1	0.57 (0.47, 0.70)	<0.001
>9.88 slope2	1.11 (0.77, 1.60)	0.586
slope2-1	1.94 (1.19, 3.14)	0.008
Model fit value at K	-1.19 (-1.30, -1.08)	
Log-likelihood ratio test‡	–	0.008

*Model 1: linear analysis. †Model 2: nonlinear analysis. ‡Log-likelihood ratio test: P value<0.05 means Model 2 is significantly different from Model 1, which indicates a nonlinear relationship. Only 99% of the data is displayed. §Adjusted for variables in Model 4. CKD, chronic kidney disease; OR, odds ratio; CI, confidence interval.

function decline in individuals without albuminuria (25). However, their study’s small sample size limits its generalizability. Similarly, Zhang et al. reported a positive relationship between Klotho levels and eGFR and a negative association with advanced CKD stages (37), but did not specifically analyze individuals with MetS, who are particularly vulnerable to renal abnormalities.

Some observational studies yield inconsistent results. Seiler et al. (26) and Bob et al. (48) reported no significant association between Klotho levels and kidney function, with variations attributed to differences in study populations, CKD stages, and methodologies. Discrepancies may stem from complex factors such as oxidative stress, inflammation, medications (49, 50), and disease progression that influence Klotho levels. The fluctuating nature of Klotho concentrations, especially in advanced CKD stages and during dialysis (13, 51, 52), further complicates these observations.

Our study provides new insights into the nonlinear relationship between Klotho levels and CKD risk, with a critical inflection point at 9.88 pg/mL. This finding implies that Klotho’s renoprotective effects are most pronounced at higher levels, whereas CKD risk sharply increases below this threshold. The mechanisms underlying this relationship may involve Klotho’s roles in regulating mineral metabolism (53), inhibiting fibrosis (54), and modulating oxidative stress (21) and inflammation (54). Additionally, Klotho deficiency has been associated with the activation of pro-inflammatory and pro-fibrotic pathways (55, 56), which are critical drivers of CKD progression. This identified inflection point may represent a threshold where these detrimental pathways predominate, leading to rapid deterioration in kidney function. At optimal levels, Klotho may effectively maintain these homeostatic processes, but below a critical threshold, protective mechanisms may be impaired, resulting in increased CKD risk. These findings emphasize the importance of Klotho management in clinical practice, particularly for patients with MetS, who are at heightened risk for CKD (3).

The interaction with hypertension further complicates Klotho’s role in kidney function. Our results suggest that Klotho deficiency may create a vicious cycle with hypertension, potentially increasing CKD risk through mechanisms such as impaired endothelial function (24, 57) and activation of inflammatory pathways (55, 56).

The identified threshold of 9.88 pg/mL serves as a potential clinical target for Klotho management. Clinicians should consider monitoring Klotho levels as an early indicator of kidney health, particularly in patients with MetS and hypertension. Our study has several strengths, including a large sample size that ensures robust statistical power, representation of the general U.S. population, which enhances external validity, and rigorous adherence to standardized data collection methods to minimize non-sampling and measurement errors according to stringent NHANES criteria. Additionally, complex sampling weights and sample design were applied to achieve a nationally representative sample estimate.

While our study presents compelling evidence, it is essential to acknowledge its limitations. First, the cross-sectional design of our study precludes establishing causality between CKD and MetS. While a strong correlation is evident, the directionality of the association and potential confounding factors remain unclear. This design does not capture temporal changes in metabolic parameters, which could oversimplify the CKD-MetS relationship. Reverse causality is also a possibility, as lifestyle modifications in CKD patients might influence metabolic parameters. Future research should focus on longitudinal studies and randomized controlled trials to enhance causal inference.

Second, serum creatinine and albuminuria were assessed only once, limiting our ability to evaluate dynamic changes in serum Klotho concentrations. While baseline measurements are informative, fluctuations due to various influences may affect the validity of our findings. Consequently, our results should be considered preliminary, emphasizing the need for longitudinal studies with multiple assessments to further investigate Klotho’s role in renal function.

Third, our findings are based on middle-aged and older adults with MetS in the U.S., which may restrict generalizability. Additionally, though the ELISA kit used for Klotho measurement underwent stringent quality control (46), precision concerns persist. Nonetheless, our large sample size and advanced statistical methodologies help mitigate the effects of batch-to-batch variation. Future studies should utilize multiple measurement techniques for Klotho to improve accuracy.

Lastly, unmeasured confounding variables could impact the relationship between α -Klotho and CKD. We were unable to account for serum fibroblast growth factor 23 (FGF23), which plays a critical role in phosphate homeostasis and kidney function (58, 59). Further multicenter studies are necessary to clarify this relationship. While we adjusted for related minerals in our regression model, other factors such as nutritional status (28) and oxidative stress biomarkers (55) may still influence associations. The observed odds ratio of 0.65 for incident CKD could be impacted by an unmeasured confounder with a risk ratio exceeding 1.79. However, it is improbable that such confounding would negate the effect of serum Klotho levels, as this ratio exceeds those for established CKD risk factors. Given the consistent findings

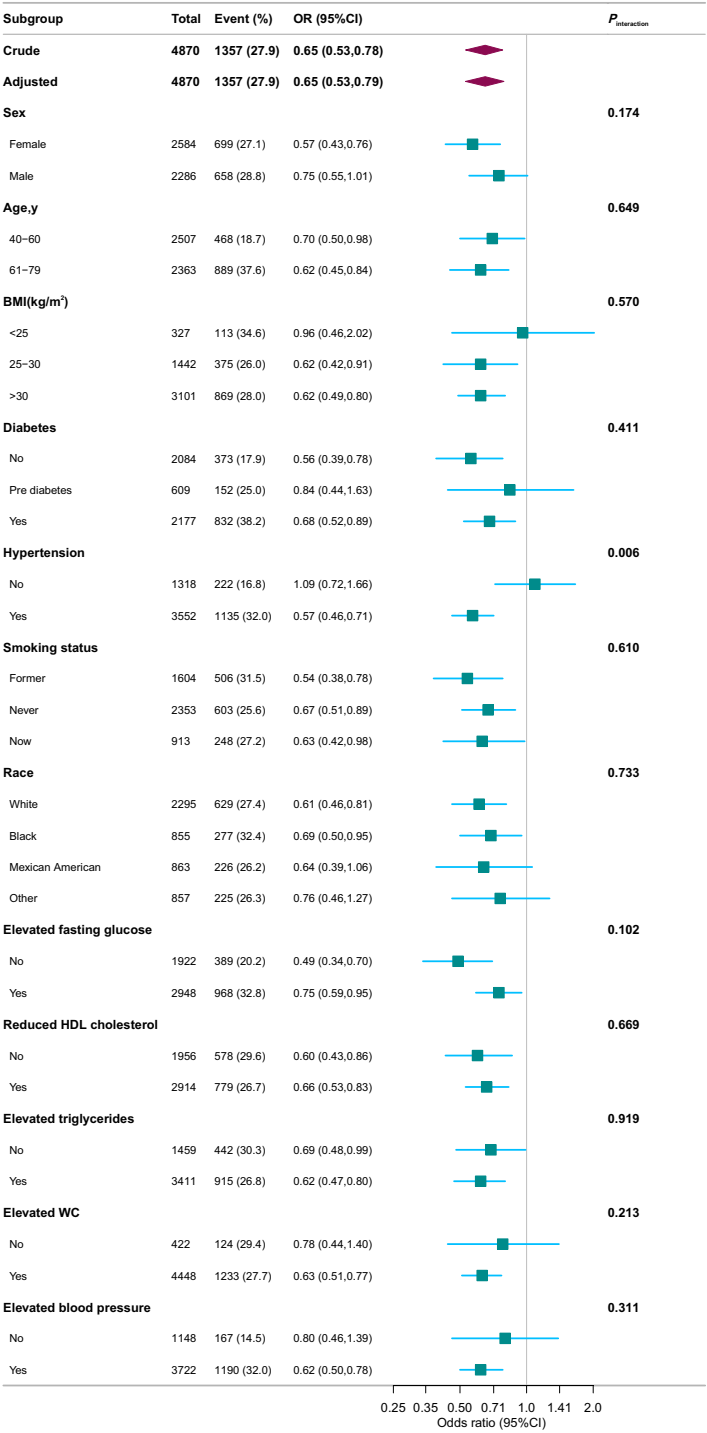


FIGURE 5
Associations between serum Klotho and CKD in different subgroups. Except for the stratification component itself, each stratification factor was adjusted for variables in Model 4. OR, odds ratio; CI, confidence interval; PIR, family poverty income ratio; HbA1c, glycosylated hemoglobin A1c; CVD, cardiovascular disease; HDL, high-density lipoprotein; WC, waist circumference.

across various models, the observed relationships are unlikely to be attributable to random chance.

Future studies should prioritize longitudinal research to establish causal links between Klotho levels and CKD progression, investigate the molecular mechanisms behind the observed L-shaped

relationship, and develop targeted interventions to maintain Klotho levels within the optimal range, particularly for patients with MetS and hypertension. Additionally, exploring the impact of Klotho management on CKD progression in diverse populations could enhance our understanding of its clinical implications.

5 Conclusion

An L-shaped association was found between serum Klotho levels and the risk of CKD in patients with MetS, with an inflection point at 9.88 pg/mL. The findings from our study consistently indicated that both extremely low and high Klotho levels were linked to a heightened risk of CKD. This underscores the significance of regulating serum Klotho levels within an optimal range to prevent CKD in middle-aged and older individuals with MetS.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: <https://wwwn.cdc.gov/nchs/nhanes>.

Ethics statement

The studies involving humans were approved by The National Center for Health Statistics Research Ethics Review Board (NCHS ERB). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XL: Conceptualization, Project administration, Writing – original draft, Writing – review & editing. LY: Conceptualization, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2024.1409560/full#supplementary-material>.

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The role of vitamin D in slipped capital femoral epiphysis in children and adolescents: a retrospective case-control study

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Objective: To explore the correlation between vitamin D levels, related endocrine/metabolic factors, and the risk of slipped capital femoral epiphysis (SCFE) in children and adolescents, and to assess whether vitamin D levels are associated with SCFE severity.

Methods: A retrospective case-control study was conducted from March 2014 to October 2023 in Shengjing hospital. Patients diagnosed with SCFE were categorized as the SCFE group. The control group consisted of healthy children matched by gender, age, weight, height, body mass index (BMI), and date of blood tests at a 1:2 ratio from the pediatric developmental clinic. The analysis included relevant laboratory tests such as 25-hydroxyvitamin D (25(OH)D), hemoglobin (Hb), serum alkaline phosphatase (ALP), calcium (Ca), phosphorus (P), and magnesium (Mg), etc. Univariate and multivariate conditional logistic regression analyses were conducted to identify factors associated with SCFE, with a particular focus on the correlation between 25(OH)D levels and the risk of SCFE. The study also explored whether these factors were correlated with SCFE severity, determined by measuring the slip angle and displacement on the anteroposterior pelvic or frog-leg lateral views.

Results: One hundred and twenty subjects were finally included, with 40 SCFE patients (36 males, 4 females) and 80 controls (72 males, 8 females). There were no significant differences in gender, age, weight, height, BMI, Hb, albumin (ALB), creatinine (Cr), free triiodothyronine (FT3), thyroid stimulating hormone (TSH), Ca, and P ($P > 0.05$). Significant differences were found in 25(OH)D, ALP, free thyroxine (FT4), and Mg ($P < 0.05$). The SCFE group had lower 25(OH)D and ALP levels but higher FT4 and Mg. Univariate analysis showed that 25(OH)D, FT4, and ALP were associated with SCFE, but multivariate analysis indicated only 25(OH)D had a significant correlation ($P < 0.05$). 25(OH)D levels were not linked to SCFE severity ($P > 0.05$).

Conclusions: The results of this study indicate that a higher level of vitamin D is associated with a decreased risk of SCFE, suggesting potential benefits of vitamin D sufficiency. However, no correlation was observed between 25(OH)D levels

and the severity of SCFE. Serum FT4 and ALP also seem to have some association with SCFE, but the clinical significance is unproven. Future multi-center studies in various regions are necessary to further validate the protective role of vitamin D against SCFE.

KEYWORDS

slipped capital femoral epiphysis (SCFE), severity, hormone, adolescent, 25 hydroxyvitamin D

1 Introduction

SCFE mainly affects the hips of elder children and adolescents. It manifests as the relative displacement of the capital epiphysis of the femoral head in relation to the proximal femoral metaphysis. This displacement takes place when acute or chronic shear force works on a pathological physis.

The majority of SCFE occurred in children aging 10–14 years, especially those that are overweight or obese (1, 2). There is a slight male dominance. The incidence of SCFE varied slightly by region. In Sweden, the average annual incidence from 2007 to 2013 was 0.44 per 100,000 girls and 0.57 per 100,000 boys aging 9–15 years (1). In South Korea, SCFE showed an overall increasing trend in incidence, rising from 0.96 per 100,000 in 2009 to 2.05 per 100,000 in 2019 (3). Most SCFE patients presented with long-lasting dull pain around the hip and limping without specific traumatic history (4). Acute hip pain, inability to bear weight and limited range of motion may present when there is an acute aggravation of the slip. SCFE may lead to complications such as avascular necrosis of the femoral head, growth arrest and chondrolysis (4). Therefore, it is of great significance to explore the factors associated with the risk of SCFE, aiming to prevent its progression and improve the prognosis.

The etiology of SCFE includes biomechanical, histochemical and endocrinologic factors such as obesity and endocrine abnormality (5, 6). Obesity may promote skeletal maturation and increase the mechanical load on the physis to trigger the slip. Additionally, it disrupts normal metabolism such as serum leptin and promotes early puberty, which may contribute to the incidence of SCFE (6). Endocrine diseases, such as hyper- and hypothyroidism, hypogonadism, and hypopituitarism could also increase the risk of SCFE by directly or indirectly influencing hormone levels that are relevant to physis development (7). SCFE also demonstrated seasonal variation with incidence peaking in late summer or early autumn (8, 9). This

seasonal change was more prominent in northern regions and less evident in areas with minimal fluctuations in annual average temperature (8, 9). This variation was related to the latitude, skin color, sunlight exposure, seasonal variation of growth and intensity of growth plates and potentially the seasonal synthesis of VD (8, 9).

Vitamin D (VD) plays a crucial role in maintaining the homeostasis of the human body, particularly within the musculoskeletal system (10). The classical role of VD is to regulate calcium and phosphate metabolism and stimulate protein expression in the intestinal wall to enhance calcium absorption. VD is essential for optimizing bone health in childhood (11). Recent clinical studies have reported a high prevalence of VD deficiency in cases of fractures (12) and idiopathic scoliosis (13) among children and adolescents. Additionally, animal studies have demonstrated that VD plays a regulatory role in cartilage development, including the growth plate (14–16). In patients with SCFE, the growth plate was thicker with disrupted extracellular matrix and disarranged physis (17), which is somehow similar to that of vitamin D deficiency (16, 17). Therefore, it is reasonable to hypothesize that VD may play a role in the biological or pathological development of the proximal femoral growth plate. In recent years, increasing researches have explored the relationship between VD and SCFE (18–22). Most children with SCFE have insufficient or deficient levels of VD, suggesting that VD deficiency may be one of the risk factors for SCFE (18, 19, 21, 22). One study found that obese children with SCFE have a higher rate of VD deficiency compared to non-obese controls (22), while another study suggested that low VD level is uncorrelated to the development of SCFE in obese children (20). Therefore, the independent effect of VD on SCFE remains controversial.

Based on a retrospective case-control design, this study aimed at investigating the independent role of VD level in SCFE. Some other relevant factors that may be associated with the incidence and severity of SCFE were also included and analyzed.

2 Methods

This retrospective case-control study (1:2 pair-wise matching) was conducted in Shengjing hospital from March 2014 to October 2023, with the approval of the ethics committee (No.2024PS214K).

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; ALB, Albumin; ALP, Alkaline phosphatase; BMI, Body mass index; Ca, Serum calcium ions; Cr, Creatinine; EDA, Epiphysis-diaphyseal angle; FT3, Free triiodothyronine; FT4, Free thyroxine; Hb, Hemoglobin; HAS, Head-shaft angle; Mg, Serum magnesium ions; P, Serum phosphorus ions; SCFE, Slipped capital femoral epiphysis; TSH, Thyroid stimulating hormone; VD, Vitamin D.

2.1 Subjects

Medical data of all SCFE patients from the Department of Pediatric Orthopedics were collected. The inclusion criteria were: (1) the patients are younger than 16 years old; (2) the patients were diagnosed SCFE when other musculoskeletal diseases had been ruled out; (3) the patients received biochemical tests including regular blood test, serum 25(OH)D level and bone-related nutrients measurement; (4) the patients received relevant hormones measurements.

Data of the control group was collected from the Department of Developmental Pediatrics. They were matched with the case group in terms of gender, age, BMI, and date of blood tests. All the subjects were free of any diagnosed diseases, but in order to match with the SCFE group, some participants were overweight or obese. All the subjects selected received the same tests as the SCFE patients.

The exclusion criteria were: (1) VD supplementation in six months before the blood test; (2) presence of other systems diseases that may affect physeal health or VD metabolism such as hypothyroidism and precocious puberty; (3) long-term use of medications that may affect physeal health or VD metabolism such as anticonvulsive drugs; (4) lack of complete medical record.

2.2 Demographic and laboratory data

General information of participants included gender, age, height and weight, BMI ($\text{BMI} = \text{mass}(\text{kg})/\text{height}(\text{m})^2$) and date of blood test.

The sample for regular blood test was whole blood, while serum was used for all other tests. The test results were provided by the Department of Clinical Laboratory at Shengjing Hospital. Levels of Hb and serum ALB were included to reflect overall nutritional status. Serum Cr was included as an indicator of renal function. Levels of serum Ca, P, Mg and ALP were measured to evaluate bone metabolism. FT3, FT4 and TSH were measured to identify patients with thyroid dysfunction. Sex-related hormones, growth hormone and adrenal gland hormones were tested to assist in the diagnosis of abnormal gonadal and adrenal development.

The levels of serum 25(OH)D were measured by electrochemiluminescence immunoassay. Based on the results, VD status was classified into three levels: sufficiency ($\geq 30 \text{ ng/mL}$), insufficiency ($21\text{--}29 \text{ ng/mL}$), and deficiency ($< 20 \text{ ng/mL}$) (23).

2.3 Assessment of SCFE

The severity of SCFE was determined by measuring the slip angle and displacement in the anteroposterior pelvis and the frog-leg lateral views. Currently, the generally accepted assessments were proposed by Wilson and Southwick. Wilson et al. (24) described an assessment method based on the proportion of epiphyseal displacement on the femoral neck on the frog-leg lateral view (Figure 1A). Southwick et al. (25, 26) measured the epiphysis-diaphyseal angle (EDA) on the frog-leg lateral view (Figure 1B) and the proximal femoral head-shaft angle (HSA) on the anteroposterior view (Figure 1C). HSA can only be used in unilateral SCFE, as the severity of SCFE is defined according to the difference in HSA between the two sides.

A senior pediatric orthopedist (>20-year experience in pediatric orthopedics), a junior pediatric orthopedist (>10-year experience in pediatric orthopedics) and an orthopedic resident (<5-year experience in pediatric orthopedics) performed the measurements independently, and the mean of the three results was calculated to reduce observer bias. Discrepant results (where the proportion of epiphyseal displacement on the femoral neck differed by more than 5% or the EDA and HSA values differed by more than 5 degrees) were finally confirmed by another senior pediatric orthopedist.

Severity of SCFE were assessed using all the three methods, except for bilateral cases that cannot be assessed with HSA. The criteria are as follows:

1. Mild:
 - proportion of epiphyseal displacement $< 1/3$
 - $\text{EDA} < 30^\circ$
 - Difference in bilateral HSAs $< 30^\circ$
2. Moderate:
 - Proportion of epiphyseal displacement between $1/3$ and $1/2$
 - EDA between 30° and 50°

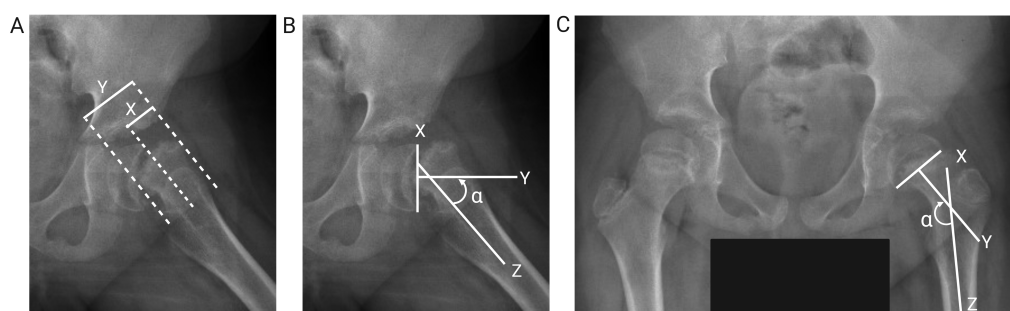


FIGURE 1

Diagram of measurement assessment criteria. (A) Epiphyseal displacement: the proportion of epiphyseal displacement (X) relative to the width of the femoral neck (Y) in the frog-leg lateral view. (B) HSA: the angle (α) between the mid-perpendicular line (Y) drawn through the line connecting the medial and lateral sides of the femoral epiphyseal plate (X) and the axis of the femoral shaft (Z) in the frog-leg lateral view. (C) EDA: the angle (α) between the mid-perpendicular line (Y) drawn through the line connecting the medial and lateral sides of the femoral epiphyseal plate (X) and the axis of the femoral shaft (Z) in the anteroposterior view.

- Difference in bilateral HSAs between 30° and 60°
3. Severe:
- Proportion of epiphyseal displacement > 1/2
 - EDA > 50°
 - Difference in bilateral HSAs > 60°

2.4 Statistical analysis

Data were analyzed using SPSS software version 26.0 (SPSS Inc., Chicago, Illinois). All variables were tested for normal distribution. Normally distributed data were expressed as mean ± standard deviation, and comparisons between groups were performed using the *t* test. Data that did not follow a normal distribution were presented using the median (Q1, Q3), and comparisons between groups were performed using the non-parametric test. Data with inter-group differences were further evaluated for their potential association with SCFE using univariate and multivariate conditional logistic regression analyses. Finally, both linear (Pearson correlation) and nonlinear models were employed to assess the relationship between the levels of these factors and the severity of SCFE. *P*-values <0.05 were considered statistically significant.

3 Results

3.1 General data

Finally, this study enrolled a total of 40 patients (36 males, 4 females) with SCFE. The mean age of the patients was 10.6 ± 2.3 years, and their BMI was 26.5 ± 4.0 kg/m². Thirty-six cases of SCFE were unilateral (refer to [Table 1](#) for basic information), while four cases were bilateral (refer to [Table 2](#) for basic information). Accordantly, 80 healthy controls (72 males, 8 females) were matched. The mean age of the control group was 10.8 ± 1.9 years, with a mean BMI of 26.2 ± 3.6 kg/m².

3.2 Association between laboratory data and SCFE

Data of bodyweight, Hb, ALB, Cr, P, Mg, 25(OH)D, FT3, FT4 and TSH were normally distributed, whereas data of age, height, BMI, ALP, and Ca followed a non-normal distribution. After comparing the two groups, statistically significant differences were observed in the levels of 25(OH)D, ALP, Mg and FT4, as shown in [Table 3](#).

Next, the univariate conditional logistic regression analysis was performed on 25(OH)D, Mg, FT4 and ALP. 25(OH)D, FT4, and ALP were associated with the occurrence of SCFE (*P*<0.05), as presented in [Table 4](#). While in the multivariate conditional logistic regression analysis, only 25(OH)D remained significantly associated (*P*<0.05), as shown in [Table 5](#). The odds ratio (OR) for 25(OH)D was 0.779 (95% confidence interval, CI 0.610-0.995), indicating that a higher level of 25(OH)D were associated with a reduced risk of SCFE.

TABLE 1 Basic information of unilateral SCFEs.

	Males (n=32)	Females (n=4)	Total (n=36)
25(OH)D			
Sufficient	1 (2.8%)	0 (0.0%)	1 (2.8%)
Insufficient	30 (83.3%)	3 (8.3%)	33 (91.6%)
Deficient	1 (2.8%)	1 (2.8%)	2 (5.6%)
Side			
Left	13 (36.1%)	2 (5.6%)	15 (41.7%)
Right	19 (52.7%)	2 (5.6%)	21 (58.3%)
Type			
Stable	27 (75.0%)	3 (8.3%)	30 (83.3%)
Unstable	5 (13.9%)	1 (2.8%)	6 (16.7%)
Severity of the slip displacement (proportion)			
Mild	15 (41.6%)	2 (5.6%)	17 (47.2%)
Moderate	8 (22.2%)	2 (5.6%)	10 (27.8%)
Sever	9 (25.0%)	0 (0.0%)	9 (25.0%)
Severity of the EDA			
Mild	1 (2.8%)	0 (0.0%)	1 (2.8%)
Moderate	16 (44.4%)	1 (2.8%)	17 (47.2%)
Sever	15 (41.7%)	3 (8.3%)	18 (50.0%)
Severity of the difference in bilateral HSAs			
Mild	25 (69.4%)	2 (5.6%)	27 (75.0%)
Moderate	7 (19.4%)	1 (2.8%)	8 (22.2%)
Sever	0 (0.0%)	1 (2.8%)	1 (2.8%)

Bolded values indicate statistical significance (*P* < 0.05).

3.3 Association between 25(OH)D levels and the severity of SCFE

We further investigated the relationship between the level of 25 (OH)D and the severity of SCFE. For bilateral SCFEs, the more severe side was used to represent its severity.

Linear (Pearson correlation) and nonlinear models including the logarithmic, inverse, quadratic, cubic, compound, power, S curve, growth, exponential and logistic models were all not suitable for describing the relationship between the level of 25 (OH)D and the severity of SCFE (all *P* > 0.05), as shown in [Table 6](#) and [Figure 2](#).

4 Discussion

SCFE is a multi-factorial disease that is associated with personal characteristics, behaviors and environment. This study was designed to reduce the influence of confounding factors by matching variables including gender, age, weight, height, BMI,

TABLE 2 Basic information of bilateral SCFEs.

	Right condition (n=4)	Left condition (n=4)	Total (n=8)
Type			
Stable	3 (37.5%)	2 (25.0%)	5 (62.5%)
Unstable	1 (12.5%)	2 (25.0%)	3 (37.5%)
Severity of the slip displacement (proportion)			
Mild	4 (50.0%)	2 (25.0%)	6 (75.0%)
Moderate	0 (0.0%)	1 (12.5%)	1 (12.5%)
Sever	0 (0.0%)	1 (12.5%)	1 (12.5%)
Severity of the EDA			
Mild	2 (25.0%)	2 (25.0%)	4 (50.0%)
Moderate	1 (12.5%)	0 (0.0%)	1 (12.5%)
Sever	1 (12.5%)	2 (25.0%)	3 (37.5%)

All four cases of bilateral SCFE were male, with one VD deficiency and three VD insufficiency. Bolded values indicate statistical significance ($P < 0.05$).

and the date of blood tests. The influence of endocrine abnormalities was also eliminated. Our study showed significant differences in 25(OH)D, ALP, FT4, and Mg ($P < 0.05$) between SCFE patients and the control group. Further univariate conditional logistic regression revealed that 25(OH)D, ALP, and FT4 were associated with the risk of SCFE ($P < 0.05$), while Mg was not ($P > 0.05$). Multivariate conditional logistic regression analysis demonstrated that only 25(OH)D was significant ($P < 0.05$). Univariate conditional logistic regression initially identified variables associated with SCFE risk, while multivariate conditional logistic regression, controlling for other factors, indicated that the optimal level of VD might be a protective factor against SCFE with the OR of 0.779. As the level of 25(OH)D increased, the risk of SCFE decreased ($P < 0.05$).

This conclusion was consistent with the findings of several other studies (18, 19, 22). Madhuri et al. (18) first suggested that SCFE was not only related to high BMI but also to VD deficiency. Judd et al. (19) observed that most children diagnosed with SCFE were VD deficient. They also noted that in children with VD deficiency who underwent *in situ* fixation with cannulated screws, there was a varying degree of delay in the time of the growth plate closure. Natalie et al. (22) found that VD deficiency was a risk factor for not only the incidence of SCFE but also post-slip osteonecrosis.

Our study found that serum 25(OH)D level was not correlated with the severity of SCFE ($P > 0.05$), which was consistent with the findings of Elbeshry et al. (21), but contrary to those of Judd et al (19). Elbeshry et al. (21) found no significant relationship between VD levels and the lateral Southwick angle in the frog-leg view in 39 children with SCFE. In contrast, Judd et al. (19) conducted a retrospective cohort study on 27 children with SCFE and found that low VD levels were associated with greater slip severity reflected by the posterior sloping angle and prolonged time to physeal fusion. Both these two studies used a single method to judge

TABLE 3 Comparison between the SCFE group and the control group.

	SCFE (n=40)	Control (n=80)	P
Age (years old)	11.000 (10.000, 12.000)	11.000 (10.000, 12.000)	0.257
Height (cm)	159.000 (147.000, 164.000)	153.000 (144.500, 160.000)	0.262
Weight (kg)	62.213 ± 14.803	60.269 ± 11.640	0.434
BMI (kg/m ²)	26.0 00 (25.000, 27.400)	26.400 (24.910, 27.930)	0.641
25(OH)D (ng/mL)	19.027 ± 6.374	23.109 ± 5.489	<0.001
Hb (g/L)	136.00 ± 8.231	137.410 ± 8.500	0.423
ALB (g/L)	45.540 ± 3.438	45.763 ± 2.189	0.725
ALP (U/L)	200.000 (169.000, 258.000)	278.000 (238.000, 318.000)	0.001
Ca (mmol/L)	2.430 (2.340, 2.530)	2.370 (2.300, 2.430)	0.405
Cr (μmol/L)	41.773 ± 8.458	44.284 ± 8.621	0.182
P (mmol/L)	1.682 ± 0.140	1.647 ± 0.123	0.166
Mg (mmol/L)	0.920 ± 0.057	0.885 ± 0.101	0.030
FT3 (pmol/L)	5.420 ± 0.894	5.768 ± 0.701	0.074
FT4 (pmol/L)	14.697 ± 2.328	13.061 ± 1.297	0.001
TSH (μIU/mol)	2.251 ± 1.060	2.566 ± 1.110	0.226

Normally distributed data were expressed as mean ± standard deviation. Data that did not follow a normal distribution were presented using median (Q1, Q3). Bolded values indicate statistical significance ($P < 0.05$).

the severity of SCFE. While in this study, we used three different methods to assess the severity of SCFE, providing more solid evidence that there was no significant relationship between the VD level and the severity of SCFE.

Seasonal variation in VD levels among children and adolescents was also observed in this study. In both the SCFE group and the control group, higher VD levels were observed in the summer (SCFE group average: 22.53 ng/ml, control group average: 27.34 ng/ml) and autumn (SCFE: 21.64 ng/ml, control: 22.67 ng/ml) compared to spring (SCFE: 15.40 ng/ml, control: 21.32 ng/ml)

TABLE 4 Univariate conditional logistic regression analysis.

	B	SE	Z	OR	P	95%CI
25 (OH) D	-0.238	0.065	13.403	0.788	<0.001	0.694-0.895
FT4	0.503	0.207	5.912	1.653	0.015	1.102-2.497
ALP	-0.017	0.005	13.584	0.983	<0.001	0.974-0.992
Mg	5.623	3.071	3.353	276.593	0.067	0.673-113639.960

B, coefficients; SE, standard error; Z, Wald, $Z = B/SE$; OR, Odds Ratio. Bolded values indicate statistical significance ($P < 0.05$).

TABLE 5 Multivariate conditional logistic regression analysis.

	B	SE	Z	OR	P	95%CI
25 (OH) D	-0.250	0.125	4.002	0.779	0.045	0.610-0.995
FT4	0.188	0.344	0.296	1.206	0.586	0.614-2.369
ALP	-0.012	0.009	1.751	0.988	0.186	0.970-1.006

B, coefficients; SE, standard error; Z, Wald, Z, B/SE; OR, Odds Ratio.
Bolded values indicate statistical significance ($P < 0.05$).

and winter (SCFE: 15.60 ng/ml, control: 22.05 ng/ml). Similar trend had also been observed in pediatric patients with upper limb fractures (12). However, the seasonal variation in the incidence or severity of SCFE was not observed in this study, possibly due to the limited size of the sample.

Although there is a lack of mechanical research on how VD directly affects SCFE, some animal studies have provided indirect evidence about their relationship. The pathology of SCFE is characterized by irregular cluster arrangement of chondrocytes in the thickened growth plate with an altered ratio between the thickness of the resting zone and the proliferating, maturation, hypertrophic and degenerating zones (17, 27). A comparison of the proximal tibial growth plate in VD-deficient mice and the growth plate of SCFE showed similar structural disorganization, including changes in chondrocyte arrangement and irregular widening of the proliferative and hypertrophic zones (16, 17). In porcine animal experiments, it was found that the VD-deficient group showed lower tolerance to shear stress and strain compared to the VD-normal group (14). Other murine experiments confirmed that VD supplementation had a favorable impact on the development of articular cartilage thickness, joint lubrication, and extracellular matrix fiber deposition (15). These implies that VD might influence the incidence of SCFE by working on the development and function of the physis. However, possibly due to the difficulties

in establishing an animal model of SCFE, the confirmation of this hypothesis still requires further efforts.

Ca, P, and Mg are all important ions that are involved bone growth and metabolism, and their serum levels generally fluctuate within a normal reference range (28). In this study, only one child with SCFE and three children in the control group had slightly lower serum Ca levels, and two children with SCFE had slightly elevated serum P levels. Those slight variations are believed to be natural among the population. This suggested that the metabolism of Ca and P are not disturbed in SCFE cases. Although there was a significant difference in serum Mg levels between the two groups ($P < 0.05$), subsequent univariate logistic regression analysis showed no association between Mg levels and the risk of SCFE ($P > 0.05$). Therefore, it was believed that serum levels of Ca, P, and Mg had no significant impact on the risk of SCFE.

In addition to 25(OH)D, FT4 and ALP were found to be associated with the risk of SCFE. The univariate logistic regress analysis showed that the OR of FT4 was 1.653 ($P = 0.015$), and the OR of ALP was 0.983 ($P < 0.001$), which indicated that FT4 level is positively correlated with the risk of SCFE, while the ALP level is negatively correlated. The clinical significance of this result, however, remains unclear. Imbalance of thyroid hormones, sex hormones, growth hormones, and adrenal hormones may be associated with the risk and development of SCFE (29), among which hypothyroidism is the most common (30). The thyroid hormones may be regulated by gender, age, BMI and puberty (31, 32). Hypothyroidism generally presents with low levels of thyroxine (T4) and triiodothyronine (T3), and a high level of TSH. T4 includes both the bound and the free forms (FT4). Although the concentration of FT4 in circulation is low, it is of high diagnostic value and is widely used as an indicator of T4 level in clinical practice. The average FT4 level in SCFE patients (14.697 ± 2.328 pmol/L) was about 1.6 pmol/L higher than in the control group

TABLE 6 Correlation analysis between 25(OH)D level and SCFE severity.

Model		The Severity of SCFE		
		Slip Displacement	EDA	Difference in Bilateral HSAs
P	Linear/Pearson	0.736	0.688	0.873
	Logarithmic	0.815	0.933	0.890
	Inverse	0.862	0.816	0.952
	Quadratic	0.617	0.193	0.977
	Cubic	0.374	0.256	0.900
	Compound	0.571	0.720	0.690
	Power	0.629	0.980	0.680
	S curve	0.681	0.758	0.723
	Growth	0.571	0.720	0.690
	Exponential	0.571	0.720	0.690
	Logistic	0.571	0.720	0.690

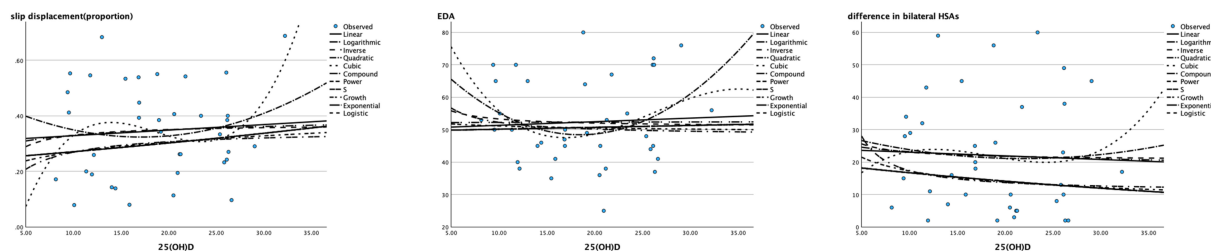


FIGURE 2

Scatter plot of 25(OH)D (X axis) and severity of SCFE (Y axis) The X-axis represents the 25 (OH) value of children with SCFE, and the Y-axis represents their severity (slip displacement described by proportion, EDA and the difference in bilateral HSAs). None of the models in the diagram could match their correlation.

(13.061 ± 1.297 pmol/L). But according to an survey of 1279 Chinese healthy children (33), only one 11-year-old male SCFE patient in this study had an FT4 level (9.97 pmol/L) below the normal range, while the rest were all within the normal range (12.65–23.16 pmol/L). Elevated FT4 levels with normal TSH are rare in children and are typically associated with TSH suppression and hyperthyroidism (34), but none of the SCFE cases in this study met the clinical criteria of hyperthyroidism. The elevation in FT4 levels might be related to SCFE, but its clinical significance is still unclear. A larger sample is needed to confirm the clinical significance of this phenomenon. Meanwhile, current evidence is still insufficient to clarify its exact mechanism.

We also observed the decreased serum ALP level in SCFE patients compared to controls, and further analysis indicated a negative relation between serum ALP level and SCFE. Most of the serum ALP in children comes from the bone, and ALP level in children during their active growth periods can be 1.5–2.5 times that of adults (35). Mice with non-specific ALP knockout exhibited insufficient dephosphorylation of matrix phosphoproteins, delayed or blocked bone mineralization, disrupted epiphyseal development, and reduced number of hypertrophic chondrocytes (36). This was consistent with the microscopic changes in SCFE observed by Tresoldi (17). Decreased ALP level may imply decreased activity of the physeal chondrocytes, or disrupted physeal development that predispose the subject to capital epiphysis displacement. However, there are no basic studies directly investigating the relationship between ALP and SCFE. Furthermore, many factors can affect serum ALP levels, such as age, gender, overall developmental status and hepatobiliary and hematological diseases (37). Further statistical analysis that can eliminate those confounding factors may be necessary to demonstrate the role of ALP in SCFE.

This study has several limitations (1): Although this study has a relatively large sample size among studies on SCFE, the number of cases is limited and all the cases come from one research center (2); Despite being matching by age, gender, weight, and height, and excluding six SCFE patients diagnosed with hypothyroidism, it was still challenging to control for all variables such as the degree of gonadal development. Some matching was based on BMI, resulting in higher average weight and BMI in the control group compared to

the normal population. Further prospective, multi-center studies are needed to confirm the protective role of VD in SCFE. Such studies would include more SCFE cases from different latitude and more healthy controls to match, enhancing the reliability of the conclusion and ensuring their applicability across diverse populations and clinical settings.

5 Conclusion

In conclusion, this study found that vitamin D deficiency is more severe in patients with SCFE, indicating that lower serum 25 (OH)D level may be correlated with increased occurrence of SCFE. It supports a potential protective role of vitamin D against SCFE, though no association between the severity of vitamin D deficiency and the severity of SCFE was found. Serum FT4 and ALP also seems to be correlated with the incidence of SCFE, but the clinical significance of this finding remains to be investigated. Future multi-center studies including centers of different latitude are necessary to further validate the role of vitamin D in SCFE.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was approved by the Ethics Committee at Shengjing Hospital of China Medical University, with approval number No.2024PS214K. Written informed consent was obtained from individual or guardian participants. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

PN: Data curation, Investigation, Methodology, Software, Formal analysis, Writing – original draft, Writing – review & editing. SL: Data curation, Investigation, Methodology, Software, Formal analysis, Writing – original draft, Writing – review & editing. HG: Formal analysis, Investigation, Supervision, Writing – review & editing. TL: Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A novel LC-MS/MS analysis of vitamin D metabolites in mice serum and hair: impact of diet and light exposure

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Introduction: Numerous physiological systems, such as the functioning of the immune system, bone health, and the regulation of expression of genes, depend critically on vitamin D. Considering the significance of vitamin D for health, it is critical to understand how it is metabolized and the factors that affect its levels.

Methods: The objective of this study was to develop and validate an LC-MS/MS method to examine the effects of light exposure and dietary vitamin D consumption on the levels of vitamin D and its metabolites in a mouse model under consistent growth conditions throughout the year. Serum and hair samples from mice were analyzed under various experimental conditions for vitamin D and its metabolites using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The experimental conditions included a vitamin D-deficient diet, a vitamin D-standard diet, and changes in ambient light exposure ranging from complete darkness to a regular light-dark cycle.

Results: Mice fed a standard vitamin D diet and exposed to a regular light-dark cycle exhibited significantly higher levels of 25OHD₃ in both serum and hair, indicating the synergistic effect of dietary vitamin D intake and light exposure. Mice fed a standard vitamin D diet but kept in continuous darkness showed moderately elevated 25OHD₃ levels, demonstrating the efficacy of dietary vitamin D in maintaining adequate levels despite the absence of light. Conversely, mice fed a vitamin D-deficient diet and housed in darkness displayed 25OHD₃ levels below the limit of quantification, highlighting the combined detrimental effects of dietary deficiency and lack of light exposure.

Discussion: This study provides valuable insights into the complex interplay between dietary vitamin D intake, light exposure, and the regulation of vitamin D metabolism in mice. Moreover, our results underscore the potential implications for human health, suggesting the importance of adequate vitamin

D intake and sunlight exposure in maintaining optimal vitamin D levels. Further research in this area has the potential to unveil additional factors influencing vitamin D metabolism, offering valuable insights into strategies for optimizing vitamin D levels in both animal models and human subjects.

KEYWORDS

LC-MS, vitamin D metabolites, mice serum, mice hair, 25OHD 25-hydroxyvitamin D

1 Introduction

Vitamin D is a vital, fat-soluble nutrient which plays a pivotal part in maintaining calcium and phosphate balance within the body (1, 2). This balance of calcium and phosphate is crucial for healthy bone development and mineralization (3, 4). The calcium and phosphorus balance also influence the conditions such as hypertension, chronic kidney disease, psoriasis, cancer, and several autoimmune diseases (5–9). Beyond its primary functions, it significantly influences various physiological processes, including the immune system's regulation, cellular growth, and differentiation (10–13). In addition to its nutritional role, vitamin D acts as a steroid hormone integral to numerous biological functions across the cardiovascular, nervous, skeletal, and reproductive systems (14–16).

Vitamin D exists in two primary forms within the human body: vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol), with D₃ recognized for its paramount biological importance (17, 18). After being exposed to ultraviolet B radiation (UVB) from the sun, 7-dehydrocholesterol is converted in the skin to vitamin D₃ (19). Vitamin D₃ is also obtained from dietary sources. Vitamin D₃ requires activation through two hydroxylation steps (20). It is first converted to 25-hydroxyvitamin D₃ (25OHD₃) in the liver (21), and then to 1 α 25(OH)₂D₃, the active form, in the kidneys (22). The regulation of this pathway is tightly controlled by feedback mechanisms involving calcium and phosphate homeostasis, as well as parathyroid hormone (PTH) levels. Dysregulation of these processes can lead to endocrine disorders such as rickets, osteomalacia, and vitamin D-dependent hypercalcemia (23–27). Our study's analytical method enables precise quantification of vitamin D metabolites, including epimers, which are emerging as critical indicators of endocrine health. This capability offers insights into vitamin D metabolism in physiological and pathological states, advancing our understanding of endocrine dysfunctions.

Unlike D₃, vitamin D₂ is exclusively obtained through the diet and metabolized similarly to yield its active forms and respective epimers (28, 29). The most potent form, 1 α 25(OH)₂D₃, exerts its effects through genomic and non-genomic pathways, largely due to its affinity for vitamin D receptors (VDRs), including both the nuclear and membrane-bound receptors (22, 30). Interestingly, vitamin D epimers also contribute to physiological health, affecting bone mineral density and growth (31, 32). Notably, these

epimers have been observed at elevated levels in mice receiving dietary vitamin D₃ supplementation (29).

In humans, newborns and children exhibit elevated concentrations of vitamin D epimers reaching up to 60% till reaching the age of one year (33). There is a range of 0 to 45% for the ratio of 3-epi-25OHD₃ to 25OHD₃. Notably, the concentration of 3-epi-25OHD₃ increases linearly with vitamin D₃ medication (29, 34). According to a research, certain pregnant women and babies have higher than average concentrations of 3-epi-25OHD₃, and the reason for these higher-than-average concentrations throughout infancy is probably early embryogenesis (31, 35).

Vitamin D's role as an endocrine regulator extends beyond calcium and phosphate homeostasis. Its active form, 1 α 25(OH)₂D₃, binds to VDRs to modulate the transcription of genes involved in bone metabolism, immune responses, and cell proliferation (11, 36, 37). Non-genomic actions, mediated by membrane-bound VDRs, rapidly activate signaling cascades such as MAPK and PI3K/AKT, influencing cellular responses (38, 39). Dysregulation in these pathways contributes to endocrine disorders such as rickets, osteoporosis, and autoimmune diseases, underlining the clinical importance of accurately quantifying vitamin D and its metabolites (40–42).

Given how important vitamin D is to the body, several analytical techniques have been developed for investigating the metabolites of vitamin D in samples from both humans and animals. The low concentration of vitamin D and metabolites in biological matrices, together with the availability of metabolites that are structurally identical, provide significant analytical problems for this research (43–45). The most effective of these approaches is the LC-MS/MS method, overcoming the limitations associated with immunoassay techniques while offering high sensitivity and specificity (46). Its short run time, ability to separate interfering co-eluting epimers and isobar effectively, and use of an internal standard that allows for the evaluation of instrumental and matrix-related effects are among its few noteworthy benefits (47–51).

Animal models, particularly rodents, have been instrumental in elucidating the metabolic pathways of vitamin D and the physiological consequences of its deficiency or excess (52, 53). However, fewer studies have comprehensively analyzed the effects of dietary variation and environmental factors, such as light exposure, on vitamin D metabolism in these models (54, 55). In addition to serum, hair has emerged as a valuable matrix for vitamin

D metabolite analysis due to its unique properties. Hair offers several advantages as a non-invasive, integrative biomarker that reflects long-term exposure to nutrients and environmental factors, making it suitable for assessing vitamin D accumulation over extended periods. Unlike serum, which provides a snapshot of current vitamin D status, hair accumulates metabolites over time, allowing for retrospective evaluation of vitamin D levels. Studies suggest that vitamin D and its metabolites are deposited in hair, providing a more stable record of nutritional status that is less affected by short-term fluctuations. Thus, analyzing vitamin D in hair complements serum analysis and provides a holistic view of vitamin D dynamics in response to dietary and environmental changes (49, 56–58).

In this study, our primary objective was to enhance and validate an LC-MS/MS methodology for the comprehensive analysis of vitamin D and its metabolites in both mouse hair and serum samples. Additionally, our study aimed to elucidate the impact of varying vitamin D dietary routines and exposure to light on the concentrations of vitamin D and its metabolites within the mouse population. The study by Zgaga et al. (59) was the first to extract and quantify 25OHD₃ from human hair, highlighting hair's potential for tracking vitamin D levels. Our research extends this work by focusing on mouse hair and serum under varied growth conditions. Notably, external factors such as diet and light exposure may influence vitamin D levels differently across species, emphasizing the need to understand these distinctions. This is essential for translating findings from animal models to potential human applications. To the best of our knowledge, no prior study has developed a method for the quantification of vitamin D in mouse hair and effect of different growth conditions highlighting the innovative aspect of our approach. Building upon our prior investigations into vitamin D analysis in mice serum (29), we have expanded our research to incorporate mice hair sample analysis and to investigate the effect of different growth and diet conditions. This comprehensive study aims to elucidate the relationship between vitamin D and its metabolites concentration in mice serum and hair and to examine the impact of different diet and light exposure conditions on these analytes, providing valuable insights into the dynamics of vitamin D metabolism in mouse population.

2 Materials and methods

2.1 Standards and reagents

A range of standards and reagents is employed in this study to ensure accurate and precise analysis. Vitamin D₃, vitamin D₂, 25OHD₃, 25OHD₂, 3-epi-25OHD₃, 3-epi-25OHD₂, 1 α 25(OH)₂D₂, and 1 α 25(OH)₂D₃, 7 α C4 and Internal standard (ISTD) [25-hydroxyvitamin-D₃ (6,19,19-d₃)] were utilized as standards in our experiment. LC-MS grade Water, methanol, acetonitrile, ethyl acetate, formic acid, ammonium formate, and ammonium hydroxide were used for the preparation of the solutions during the study. LABCO LLC in Dubai, UAE, supplied all of these chemicals, which were purchased from Sigma Aldrich. Isopropanol, hexane, and dichloromethane sourced from Emirates

Scientific & Technical Supplies LLC in Dubai, UAE, were also integral components of our experimental setup. These meticulously selected standards and reagents were crucial for achieving reliable and reproducible results throughout our study.

2.2 Growth condition

The Ethics Committee of United Arab Emirates University (UAEU) granted approval for the study (Ref # ERA_2017_5684). The mice used in this study were of the C57BL/6J strain, which is widely recognized for its genetic predisposition to lower bone density. This strain's skeletal phenotype is well-documented, characterized by reduced bone formation and altered bone turnover dynamics (60, 61). Mice were kept in the animal facility of the UAEU Faculty of Medicine and Health Sciences in a specialized pathogen-free (SPF) environment. Upon weaning at 3 weeks old, a cohort of 40 mice was carefully allocated to three distinct groups, each characterized by unique dietary compositions and light exposure routines. The first group, designated as SDL, consisted of 15 mice and received a standard-vitamin D diet while being exposed to alternating 12-hour cycles of UV light and darkness. The second group, labelled as SDD (control group), also included 15 mice and was provided with a standard-vitamin D diet (Standard AIN-93G Rodent Diet with 1000 IU VD₃, D10012Gi, Research Diet) but was housed in continuous darkness. Lastly, the third experimental group, DDD, comprised 10 mice and was fed a diet deficient in vitamin D (AIN-93G Growing Rodent Diet with 25 IU VD₃/kg of diet, D17053003i, Research Diet), also maintained in darkness. Each group was housed in separate cages to ensure consistency in exposure conditions. Mice were housed in temperature-controlled rooms maintained at 22 $\pm$ 2°C, with humidity levels of 50 $\pm$ 5%. For groups exposed to UV light, the wavelength was set at 280–320 nm, which effectively supports the cutaneous synthesis of vitamin D₃. Cages were kept separate to ensure controlled light exposure and dietary conditions. These conditions were rigorously maintained over a period of 12 months. A 12-month study duration was chosen to capture long-term endocrine adaptations, as vitamin D metabolism and deposition, particularly in hair, are influenced by cumulative dietary and environmental exposures. This duration aligns with previous research indicating that hormonal adaptations in rodents occur over extended periods, reflecting chronic physiological changes. Sample sizes were determined based on power analyses, ensuring sufficient statistical power (>80%) to detect significant differences in vitamin D metabolite concentrations between groups and time points. Figure 1 below provides a schematic representation of the experimental setup, including the C57BL/6J mice strain, dietary and light exposure conditions, sample collection, and subsequent LC-MS/MS analysis.

2.3 Anesthesia protocol

In our study, the mice were anesthetized using diethyl ether at a concentration of 1.9%, which was vaporized in a sealed chamber. Specifically, 80 microliters of diethyl ether per liter of container volume were used to create the anesthetic atmosphere. The mice were placed in

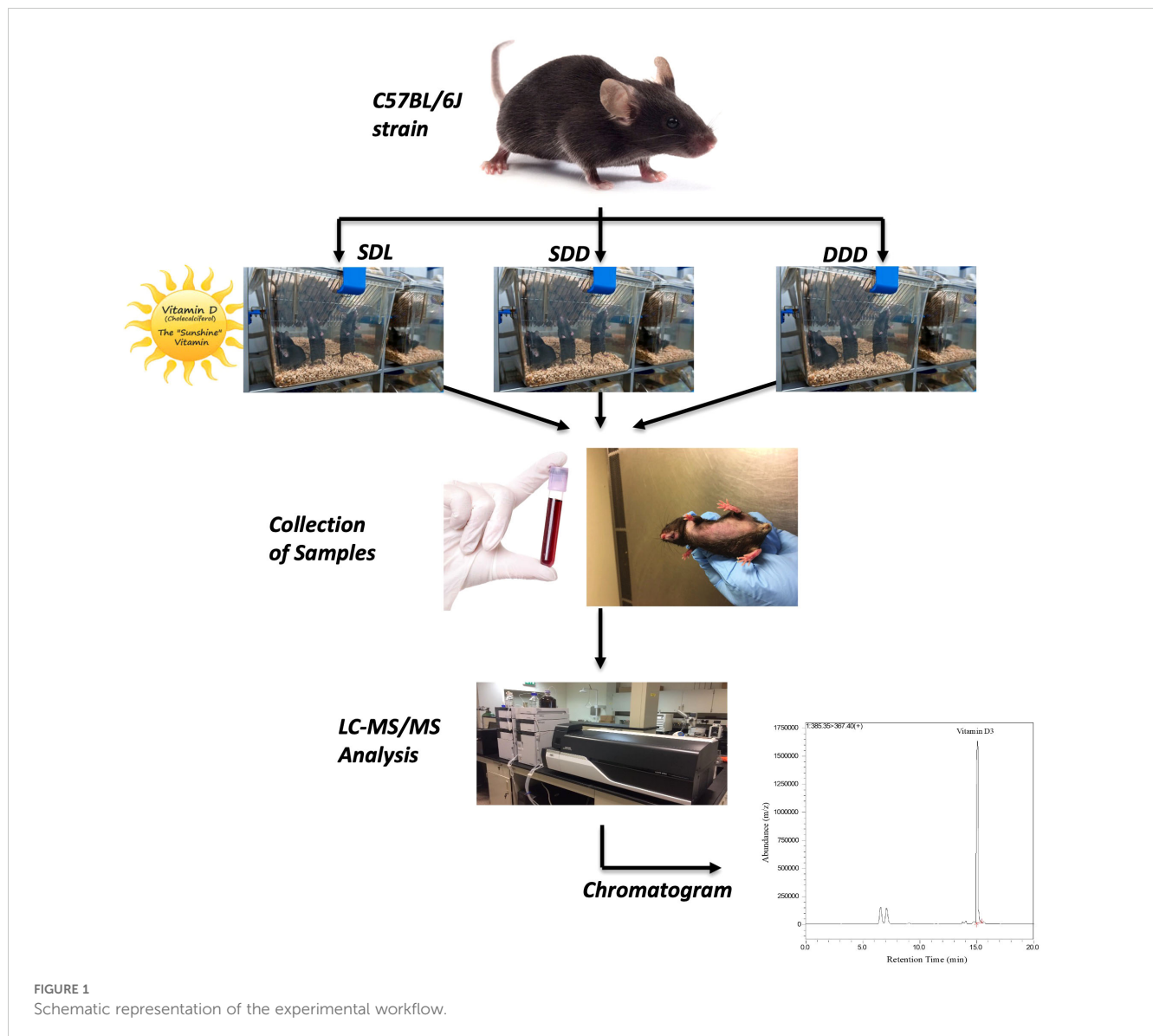


FIGURE 1
Schematic representation of the experimental workflow.

the chamber, and the induction of anesthesia was monitored by observing the loss of the righting reflex and the absence of response to tactile stimuli, indicating adequate anesthesia. The duration of exposure was carefully controlled to ensure humane treatment and avoid prolonged exposure. All procedures were performed in compliance with ethical guidelines and institutional protocols to ensure the welfare of the animals (62, 63).

2.4 Hair sample collection & pre-treatment

Hair samples were collected to analyze vitamin D metabolite deposition. To minimize variability arising from the hair growth cycle, the growth cycle was synchronized by shaving hair at the onset of the study. Samples were collected after six months (6-M) and 12 months (12-M) of regrowth, ensuring consistency across the experimental groups. Hair samples were consistently obtained from the dorsal region of the mice due to its uniform growth characteristics

and reduced susceptibility to external contaminants. Each hair sample was carefully shaved, weighed, and placed in labelled plastic bags, maintaining clear identification and stored at 4°C to prevent degradation. In preparation for analysis, collected hair samples corresponding were subjected to pre-treatment procedures. This involved washing the hair samples with a 1:1 methanol/water mixture to remove sweat, sebum, and contaminants. The washed hair samples were then dried and pulverized into a fine powder using a mini ball mill. A precise amount of 30 mg of grounded hair was weighed and transferred to labelled borosilicate culture tubes for analysis.

Additionally, blank hair samples were included in the analysis to serve as controls. These blank hair samples were obtained from the DDD group of mice, which were maintained on a vitamin D-deficient diet in dark. These samples underwent a rigorous selection process to ensure their cleanliness and absence of interfering substances. Selected hair samples were subjected to an LC-MS/MS analysis for this purpose, and no interference peaks were seen

during the retention time (RT) of any vitamin D metabolites. After selection, the calibration curves and quality controls were prepared using one of the cleanest blank samples.

2.5 Hair sample extraction

For the extraction of vitamin D and its metabolites from the hair samples, a systematic approach was followed. Standards and the internal standard [25-hydroxyvitamin-D₃ (6,19,19-d₃)] were introduced to the hair samples that were being investigated, calibrants, and quality controls. After they had been sonicated in a 50:50 v/v combination of methanol and water, the top layer of these samples was collected by centrifugation at 4,000 rpm for 10 minutes. The obtained layer was dried and filtered using nitrogen gas before being reconstituted in a solution of methanol and water (50:50 v/v). subsequently, the LCMS/MS system was equipped with the reconstituted samples to begin the analysis. The blank samples were prepared as per the given extraction procedure without the addition of analyte. These samples were confirmed to contain no interference peaks at the RT of analyte, ensuring their suitability as blanks for the analysis.

2.6 Serum sample collection and pre-treatment

To investigate the presence of vitamin D and its metabolites in serum, blood samples were taken from each of the three experimental mouse groups (DDD, SDD, and SDL) at two time points: 6 months and 12 months. To reduce circadian variability, blood collection was performed consistently in the morning, between 8:00 and 10:00 AM. Each mouse was carefully anesthetized to minimize stress and discomfort. Approximately 300 µL of blood was drawn from each mouse using BD vacutainers (part no. 367957) to ensure uniformity and reliability of the samples. The blood samples were collected, centrifuged at 1300 rpm for 15 minutes at 4°C, and then kept at -80°C. Stability tests confirmed that vitamin D metabolites remained stable for up to 12 months under these storage conditions, with less than 10% variation in measured concentrations. During thawing and preparation, light exposure was minimized to prevent degradation. Upon collection, the mouse serum samples underwent a series of preparatory steps to ensure optimal conditions for subsequent analysis. Each serum sample (0.100 mL) was allowed to thaw at room temperature for 15 minutes to prevent thermal shock and maintain sample integrity. After thawing, the samples were vortexed thoroughly to ensure homogeneity and minimize any potential for sample stratification. Except for the blank, 50 µL of a working internal standard solution containing 25-hydroxyvitamin-D₃-(6,19,19-d₃) was carefully added to all calibration standards, quality controls, and serum samples in order to aid accurate measurement. To ensure precise calibration of the analytical instrument and to simulate the matrix effect, calibration standards and quality controls samples were carefully prepared in blank serum.

2.7 Extraction of serum sample

Following pre-treatment, the serum samples underwent extraction to isolate vitamin D and its metabolites for subsequent analysis. Liquid-liquid extraction was performed using a well-established protocol with a mixture of hexane: ethyl acetate (9:1; v/v). This extraction method ensured efficient extraction of all analytes from the serum matrix while minimizing interference from other components. The extracted mixture was vigorously vortexed for 10 minutes to ensure thorough mixing of the solvent and sample components. Subsequently, the mixture underwent centrifugation at 1320 rpm for 10 minutes to separate the organic layer containing the analytes of interest from the aqueous layer. Pasteur pipettes were used to gently decant the translucent organic layer of the supernatant after centrifugation and transport it to other test tubes. To maximize extraction efficiency, the remaining lower layer underwent three additional extraction cycles using the same protocol. All extracted samples were then pooled together and dried under N₂ gas in a sample concentrator to remove residual solvent. The dried residue was reconstituted in a carefully prepared mixture of 100 µL LC-MS/MS-grade methanol and water (75:25, v/v) to ensure optimal conditions for subsequent analysis.

2.8 LC-MS/MS system

The analytical system employed for this study included a tandem mass spectrometer, the model 8060 from Shimadzu, Japan, integrated with a Nexera ultra-high-performance liquid chromatography (UHPLC) setup, Nexera X2 series, also from Shimadzu, Japan. The UHPLC system was equipped with essential components including a pump, an auto-sampler, a column oven, and a degasser, enhancing the sensitivity of our analysis using columns that were both narrow bore and packed with small particles, capable of withstanding high pressures. For ionization, the mass spectrometer operated in the positive electrospray ionization (ESI) mode. Data acquisition and analysis were conducted using Shimadzu's Lab-Solutions software, ensuring precise data handling. We optimized the mass spectrometer's operational settings such as nebulizer, drying and heating gas flows, interface, and block temperatures to maximize the detection and quantification accuracy for vitamin D metabolites. Specific conditions were set as follows: nebulizing gas at 2 L/min, drying gas at 8 L/min, and heating gas also at 8 L/min, with interface and block temperatures maintained at 300°C and 400°C, respectively.

To prevent any degradation of samples by light exposure, sample handling and LC-MS/MS injections were performed under ambient lighting conditions. Analyte stability was assessed under various conditions, including room temperature, freeze-thaw cycles, and prolonged storage at -80°C. All metabolites demonstrated stability, with less than 10% variation in measured concentrations. Metabolite separation was achieved using an Ascentis Express F5 column, measuring 150 mm by 2.1 mm and with a 2.7 µm particle size, maintained at 40°C complemented by a pre-column guard. The injection volume for all samples was set at 10 µL. A methanol-water solution (50:50, v/v) was employed to

rinse the injector needle between runs, mitigating carryover and contamination risks. The mobile phase consisted of 5 mM ammonium formate in water (Phase A) and methanol (Phase B), each containing 5 mM ammonium formate, propelled at a flow rate of 0.5 mL/min via a binary gradient pump system. The gradient program was meticulously set to begin with 25% Phase A and 75% Phase B for the first 11 minutes, transitioning to 100% Phase B from 11 to 15 minutes, maintained at this composition till the 16th minute, and then returning to the initial conditions of 25% A and 75% B from 16.1 to 20 minutes to ensure optimal separation.

In the analysis of vitamin D and its metabolites, an electrospray ionization (ESI) source served as the primary method for ionization and protonation, yielding molecular ions of the form $[M + H]^+$. To ensure maximum sensitivity and accuracy in quantification, optimization of multiple reaction monitoring (MRM) parameters was conducted. The optimization protocol aimed to identify the most sensitive MRM transition for each analyte, focusing on precursor and product ions that exhibited the highest sensitivity. Our method involved the use of three MRM transitions for each analyte, comprising one quantifier ion and two qualifier ions. To

ensure optimal data acquisition, we set the target cycle time at 0.5 seconds, allowing for efficient sampling while maintaining sensitivity. Additionally, a retention-time window of 90 seconds and an interscan delay of 3 milliseconds were implemented to accurately capture the analytes of interest.

Through rigorous experimentation and data analysis, the most sensitive precursor and product ions were selected for LC-MS/MS analysis, enhancing the detection capabilities of the instrument. Interestingly, chromatographic separation facilitated by the LC system allowed for clear differentiation between closely related compounds such as 25OHD₃, 3-epi-25OHD₃, and 7 α C₄, despite their identical ionization precursor and product ions, as well as collision energies. Similarly, despite having the identical precursor, product, and collision energies, differentiation between 25OHD₂ and 3-epi-25OHD₂ was accomplished based on retention times. **Table 1** provides a detailed presentation of the chosen MRM transitions and the associated parameters, such as precursor/product ions, retention times, and collision energy.

The validation process for the current study followed strict guidelines set forth by the US Food and Drug Admin.

TABLE 1 MRM parameters for vitamin D and its metabolites.

No.	Analytes	Retention Time (min)	Precursor Q1 (m/z)	Product Q3 (m/z)	Collision Energy (eV)
1	Vitamin D ₃	15.212	385.0	367.0	-13
				259.0	-16
				91.0	-56
2	Vitamin D ₂	15.180	397.1	379.4	-17
				69.0	-19
3	25OHD ₃	6.991	383.2	365.3	-15
				107.1	-30
4	25OHD ₂	7.809	395.1	377.3	-17
				81.1	-38
5	3-epi-25OHD ₃	7.701	383.2	365.3	-15
				107.1	-30
6	3-epi-25OHD ₂	8.401	395.1	377.1	-17
				81.1	-38
7	1 α 25(OH) ₂ D ₃	3.799	399.1	381.3	-14
8	1 α 25(OH) ₂ D ₂	3.989	411.1	135.3	-13
				133.1	-12
9	7 α C ₄	14.501	401.5	383.25	-16
				97.1	-29
				91.2	-23
10	ISTD [25 hydroxyvitamin-D ₃ (6,19,19-d ₃)]	7.010	386.3	368.2	-15
				257.2	-183
				95.2	-35

2.9 Method validation

The validation process for the current study followed strict guidelines set forth by the US Food and Drug Administration (FDA) to ensure the reliability and accuracy of the analytical method (64). Optimized Multiple Reaction Monitoring (MRM) parameters were precisely established for the analysis of vitamin D and its metabolites using LC-MS/MS. The MRM mode enabled the tracking of protonated molecules ($[M+H]^+$), precursor ions, and diagnostic product ions, ensuring specificity and sensitivity in compound detection. Chromatographic separation allowed differentiation of closely related compounds, such as 25OHD₃ and 3- ϵ -25OHD₃, based on their retention times. Given the presence of other steroid hormones in serum and hair samples, matrix effects were evaluated using post-extraction spiked samples. Ion suppression/enhancement was quantified by comparing responses from spiked extracts to those from neat solutions. No significant interference was observed for the quantification of vitamin D metabolites. To further enhance the rigor of the validation process, stability studies were conducted to evaluate the resilience of vitamin D metabolites under physiological conditions. Stability was assessed under ambient lighting, at room temperature for up to 24 hours, across three freeze-thaw cycles, and during long-term storage at -80°C. The metabolites demonstrated excellent stability, with less than 10% variation in concentrations under all tested conditions, ensuring reliable quantification. Calibration curves were prepared in blank matrices to simulate the physiological environment, minimizing the impact of other hormones.

To evaluate linearity, specificity, and accuracy, a series of quality control samples quality control low (QCL), quality control medium (QCM), and quality control high (QCH) were prepared at different concentrations covering the entire analytical range. These samples were subjected to LC-MS/MS analysis alongside calibration curves to assess the method's ability to accurately quantify vitamin D metabolites. Analyzing the signal-to-noise (S/N) ratio and comparing it to the lowest concentration of the analyte allowed for the determination of the LOD. To do this, the analyte concentration had to be gradually lowered until a response three times the background level was seen. The blank serum was used as the matrix for the calibration curve and quality controls (QCs), which were made by combining albumin serum with phosphate-buffered saline. The linearity of the calibration curves was assessed using regression analysis, ensuring that the method provided accurate measurements across the specified concentration range. By computing the % CV and % Accuracy based on the examination of quality control samples, precision and accuracy were evaluated. % CV stands for coefficient of variation, and it was computed as $\frac{\text{Standard Deviation}}{\text{Mean}} \times 100$. The quality control samples' % accuracy was determined by taking the $\left[\frac{\text{Mean value}}{\text{Nominal value}} \times 100 \right]$ formula. These parameters provided insights into the method's reproducibility and reliability in quantifying vitamin D metabolites in mice serum and hair samples. Recovery experiments were conducted to evaluate the method's efficiency in extracting vitamin D metabolites from serum and hair samples. Serum and hair samples were spiked with known quantities of vitamin D metabolites, followed by extraction and the validated LC-MS/MS technique was then used

for analysis. The formula for calculating the absolute percentage recovery is $\left[\frac{\text{Mean unextracted QC value}}{\text{Mean extracted QC value}} \times 100 \right]$, yielding a measure of the method's extraction efficiency and accuracy.

The serum and tissue samples used in this study were part of a broader research project that investigated multiple aspects of vitamin D metabolism, including its interactions with dietary and environmental factors, as detailed in our prior publications (65–67). These studies focused on the interplay between vitamin D metabolism, gut microbiota, and metabolic pathways, utilizing the available biological materials comprehensively. As a result, the remaining samples were fully utilized for complementary analyses, and no further biological materials remain for additional investigations. Consequently, while we acknowledge the importance of measuring parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and the expression of enzymes such as CYP27B1 and CYP24A1 to provide a more complete understanding of the vitamin D endocrine axis, it was not feasible to perform these analyses within the scope of this study due to sample unavailability. Despite these limitations, the current work provides valuable insights into the impact of dietary vitamin D and light exposure on the metabolite profile of vitamin D in serum and hair, laying the groundwork for future studies.

2.10 Statistical analysis:

A statistical analysis was conducted to compare the levels of vitamin D metabolites across the three experimental groups (SDL, SDD, and DDD) and over the two time points (6 months and 12 months). One-way ANOVA was performed to compare the means of vitamin D metabolites levels between the groups at both time points. The results were considered statistically significant when $p < 0.05$. *Post-hoc* analysis using Tukey's HSD test was applied to further assess differences between groups. Additionally, effect size calculations were included using Cohen's d to assess the magnitude of differences between the SDL and DDD groups, and partial eta-squared values were calculated to determine the strength of the ANOVA effects. Paired t -tests were used to evaluate the change in vitamin D metabolites levels within each group from 6 months to 12 months.

3 Results & discussion

3.1 Hair validation results

Validation of the LC-MS/MS method for analyzing vitamin D and its metabolites in hair samples involved a comprehensive assessment of precision, accuracy, linearity, and recovery values for multiple analytes. LC-MS/MS offered exceptional sensitivity, allowing for precise identification of analytes based on retention time on the LC column and characteristic fragmentation reactions. During the validation process, we carefully examined the retention times and signal intensities of the vitamin D metabolites and internal standard (ISTD). This ensured a strong correlation between the concentration of each metabolite and its signal strength, allowing for accurate quantification across different

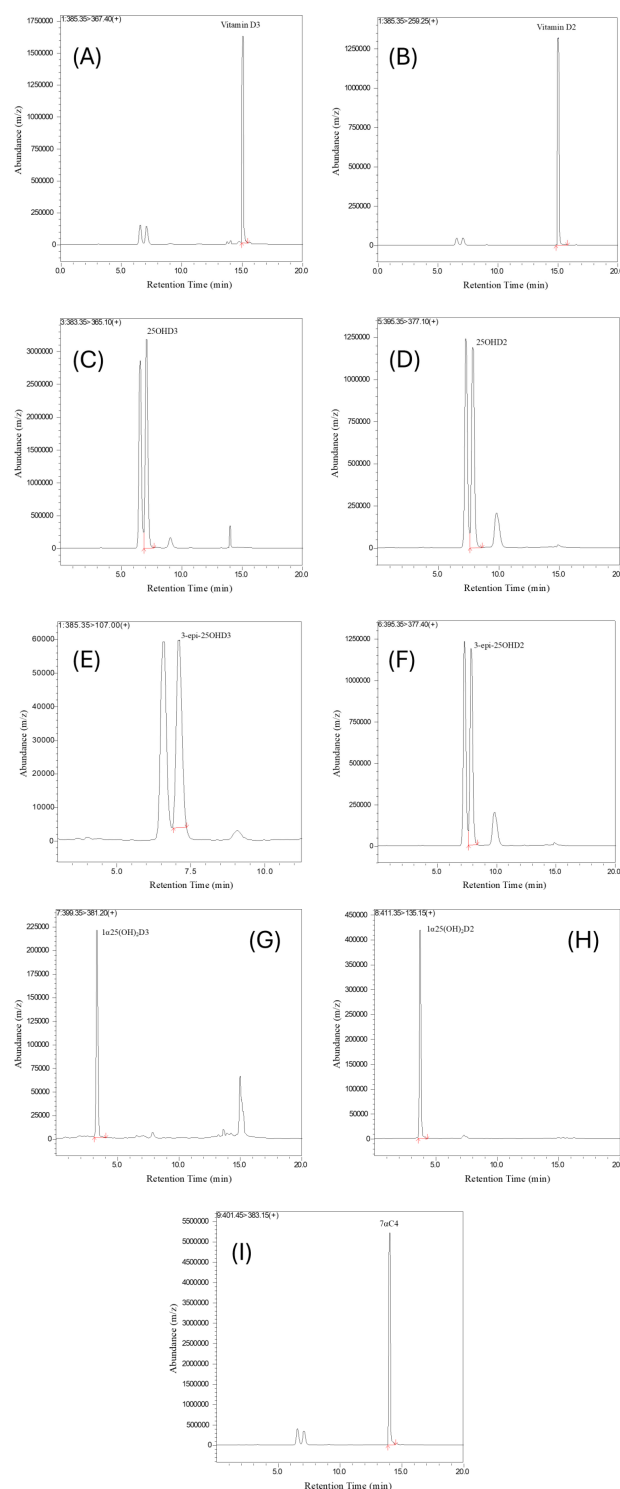


FIGURE 2

Chromatograms of Internal standard and Vitamin D metabolites (A) Vitamin D₃ (B) Vitamin D₂ (C) 25OHD₃ (D) 25OHD₂ (E) 3-epi-25OHD₃ (F) 3-epi-25OHD₂ (G) 1 α 25(OH)₂D₃ (H) 1 α 25(OH)₂D₂ (I) 7 α C₄ in spiked hair samples.

concentration levels. Figure 2 provides visual representations of Vitamin D metabolites in spiked hair samples along with ISTD under optimal conditions.

The chromatograms in Figure 2 were obtained by spiking 240 pg/mg (HQC) of standards in blank hair samples. The

chromatograms show well-defined and sharp peaks at their specific retention time for each vitamin D metabolites, highlighting the method's precision and ability to accurately measure the target compounds. The clear and separate peaks are crucial for accurate quantification, ensuring that each vitamin D

TABLE 2 Method validation results for intraday/interday precision, accuracy, and recovery.

Analytes	QC's (pg/mg)	Intraday		Interday		% Recovery
		%CV	%Accuracy	%CV	%Accuracy	
Vitamin D ₃	14	14.90	106.1	14.82	109.8	79
	90	7.99	97.4	7.95	99.4	77
	240	6.20	99.9	8.39	100.2	88
Vitamin D ₂	14	13.21	105.1	12.9	103.2	88
	90	9.81	98.5	7.1	99.8	89
	240	5.64	99.4	6.2	101.3	92
25OHD ₃	14	13.22	99.9	13.4	102.1	86
	90	9.90	95.3	8.7	99.8	89
	240	11.00	99.7	9.3	98.7	94
25OHD ₂	14	14.78	107.9	11.2	101.8	82
	90	8.01	99.4	7.3	98.9	90
	240	5.21	99.9	4.3	97.8	80
1 α 25(OH) ₂ D ₃	14	12.81	107.2	14.7	104.5	75
	90	7.21	102.3	10.9	102.6	96
	240	3.20	100.1	8.9	99.9	78
1 α 25(OH) ₂ D ₂	14	13.78	98.9	10.2	101.5	76
	90	5.92	99.6	5.7	93.1	87
	240	2.10	89.6	3.2	91.3	89
3-epi-25OHD ₃	14	12.77	98.3	13.3	106.2	78
	90	13.20	102.6	9.1	95.3	89
	240	14.01	91.9	10.6	108.1	97
3-epi-25OHD ₂	14	13.24	99.3	12.3	101.2	92
	90	5.01	98.7	6.1	97.8	94
	240	7.99	101.2	7.2	96.9	97
7 α C ₄	14	14.36	97.2	12.7	103.2	75
	90	7.22	91.7	5.8	97.1	79
	240	2.78	89.3	3.7	93.5	72

metabolite is accurately identified and measured. Additionally, **Figure 2** demonstrates the method's selectivity by distinguishing target analytes from potential interferences in the sample matrix. These peaks in (**Figures 2A–I**) reinforce the method's reliability and suitability for precise quantification of vitamin D metabolites in mouse serum and hair samples. These chromatographic results demonstrate the method's effectiveness thereby enhancing the reliability of the analytical approach for vitamin D analysis.

The method validation encompassed key vitamin D compounds including vitamin D₃, vitamin D₂, 25OHD₂, 25OHD₃, 1 α 25(OH)₂D₂, 1 α 25(OH)₂D₃, 3-epi-25OHD₃, and 3-epi-25OHD₂. Recovery rates, as well as intra- and inter-day precision and accuracy values, were

carefully evaluated and presented in a **Table 2**. All analytes had a lower limit of detection (LOD) of 5 pg/mg, and all vitamin D metabolites had a linear range of 5 pg/mg to 300 pg/mg. The FDA guidelines' criterion for linearity were met when the linear regression (R^2) values for the calibration curve were equal to or higher than 0.986.

The method's effectiveness was further demonstrated through the identification of precursor and product ion pairs, alongside optimized collision energies used for the multiple reaction monitoring (MRM) method. The LC retention time and MRM parameters for the analysis of 25OHD₃ in hair samples were aligned with the validated procedure, underscoring the method's robustness and reliability.

3.2 Hair sample analysis

In this experimental study, we provide the measurement of vitamin D metabolites in the three groups of mice: the group fed a diet deficient in vitamin D and kept in the dark (DDD), the group given a diet rich in vitamin D and kept in the dark (SDD), and the group exposed to light in addition to the regular diet of vitamin D (SDL). We conducted sample collection and analysis at two intervals, specifically at the 6th month and 12th month marks, spanning over the course of a year. Our objective was to assess the impact of both diet and exposure of radiation on the concentration of Vitamin D and its metabolites. The analysis of mouse hair samples revealed that only 25OHD₃ was found among the tested forms of vitamin D when samples were subjected to the above-mentioned validated procedure. This finding likely reflects the higher physiological stability and abundance of 25OHD₃ compared to other metabolites. The incorporation of metabolites into hair may also vary based on solubility and deposition efficiency, with 25OHD₃ being preferentially accumulated. The levels of 25OHD₃ varied among the different groups of mice (SDL, SDD, and DDD) and over the 6-month and 12-month periods. Specifically, at the 6-month mark, the average 25OHD₃ levels were 17.7 ng/mg for SDL (N=15), 16 ng/mg for SDD (N=15), and below limit of quantification (BLQ) for DDD population of mice (N=10). Similarly, at the 12-month mark, the average 25OHD₃ levels increased to 18.3 ng/mg for SDL, 17.7 ng/mg for SDD, and remained undetectable for DDD.

The results indicated a statistically significant difference in 25OHD₃ levels between the SDL and DDD groups at both time points ($p < 0.05$). However, no significant difference was observed between the SDL and SDD groups, suggesting that light exposure plays a crucial role in maintaining higher 25OHD₃ levels when dietary vitamin D is sufficient. Cohen's d for differences between the SDL and DDD groups was calculated as 2.1 at 6 months and 2.3 at 12 months, indicating large effect sizes. Partial eta-squared values

from the one-way ANOVA tests were 0.68 and 0.71 at the 6-month and 12-month intervals, respectively, reinforcing the strong statistical significance of these differences. *Post-hoc* analysis using Tukey's HSD test further confirmed that the DDD group had significantly lower 25OHD₃ levels compared to both the SDL and SDD groups at each time point ($p < 0.01$). Additionally, a paired t -test was conducted to evaluate the increase in 25OHD₃ levels within each group over time, revealing a significant increase from 6 months to 12 months for both the SDL and SDD groups ($p < 0.05$), whereas the DDD group remained below the limit of quantification throughout the study period.

The findings suggest that the concentrations of 25OHD₃ in mouse hair samples vary based on dietary and light exposure conditions, with the SDL group of mice model consistently exhibiting the highest levels over the duration of the study. **Figure 3** illustrates the observed variations in 25OHD₃ concentrations among the SDL, SDD, and DDD groups at both the 6-month and 12-month time points. The detection of 25OHD₃ in hair highlights its potential as a long-term biomarker for vitamin D exposure, integrating both dietary and environmental sources. The significant differences between the SDL and DDD groups underscore the critical role of light exposure in maintaining adequate vitamin D levels, even when dietary sources are absent. The lack of significant differences between SDL and SDD groups suggests that dietary intake alone may be sufficient to maintain similar 25OHD₃ levels as light exposure under certain conditions. These results support the notion that hair-based vitamin D analysis can serve as a complementary tool to serum measurements, especially in studies of chronic exposure or deficiency. These findings also highlight the role of light exposure in sustaining higher vitamin D levels, even when dietary intake is sufficient, and emphasize the biological significance of these results. While the assay's capability to quantify up to eight forms of vitamin D is promising, further advancements in instrument sensitivity and sample pre-concentration techniques could potentially expand the

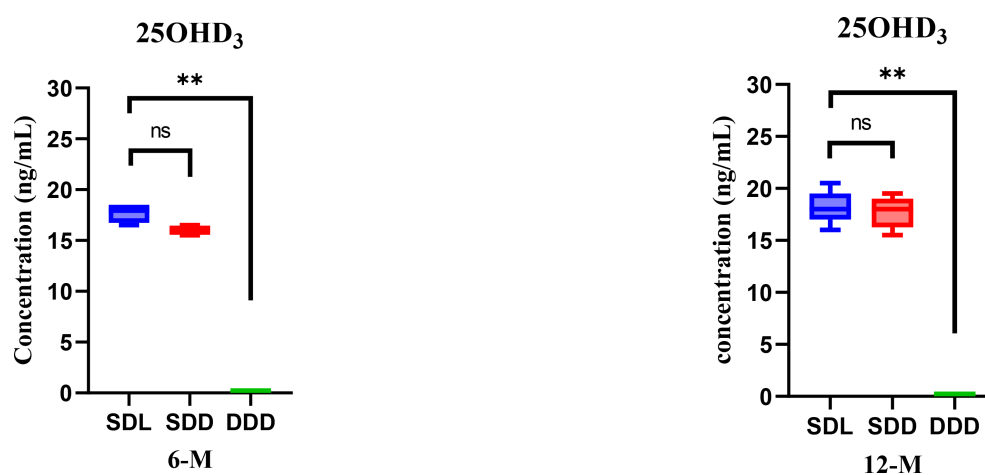


FIGURE 3

Comparison of 25OHD₃ levels among SDL (N=15), SDD (N=15), and DDD (N=10) groups of mice hair at 6th and 12th month interval. Asterisk (**) represent significant differences between SDL and DDD groups. The error bars represent standard deviations. The differences between the results were considered significant if $p < 0.05$, refuting the null hypothesis ($p < 0.05$). "ns" means no significant difference.

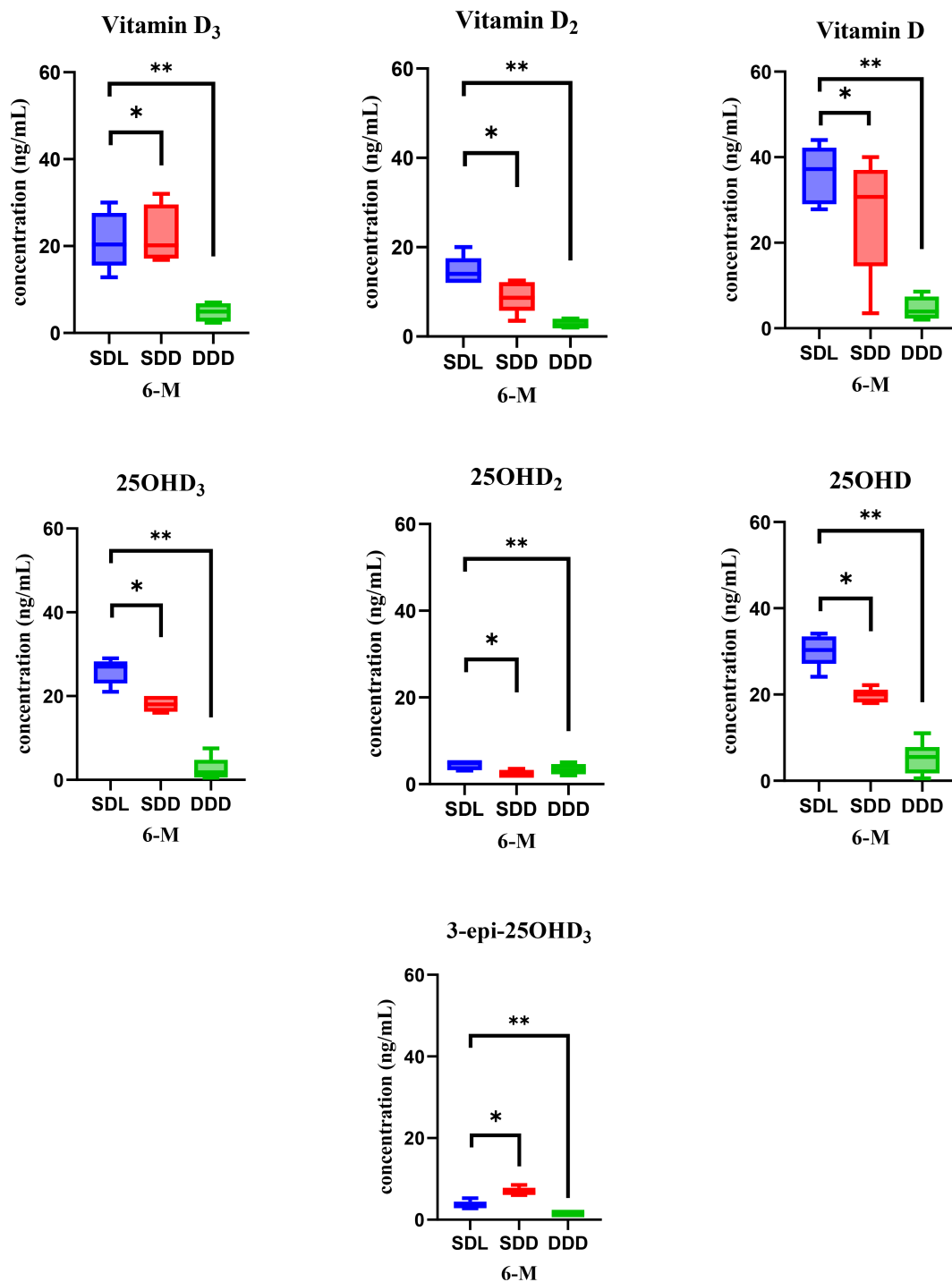


FIGURE 4

Representation of vitamin D metabolite's concentrations in mouse serum of three study groups (SDL (N=15), SDD (N=15), and DDD (N=10)) at the 6-month mark. Asterisk (* & **) represent significant differences between SDL, SDD and DDD groups. The error bars represent standard deviations. The differences between the results were considered significant if $p < 0.05$, refuting the null hypothesis ($p < 0.05$). Vitamin D: 20–50 ng/mL (normal), <20 ng/mL (deficient). 25OHD: 15–40 ng/mL (normal), <15 ng/mL (deficient).

scope of analytes quantified. The hair-based assay, focusing primarily on 25OHD₃, presents a cost-effective and non-invasive alternative for long-term monitoring of vitamin D levels, complementing routine clinical tests and overcoming logistical challenges associated with traditional blood-based assays.

3.3 Serum sample analysis

The validation results for analysis of Vitamin D and its metabolites in serum of mice has been already reported in our previous study (29). In this study, we aimed to further explain

the variations in the concentration of vitamin D and its metabolites over the course of a year. Serum samples were collected twice annually, at the 6-month and 12-month marks, enabling a thorough analysis of changes in vitamin D levels. This analysis was conducted

under consistent experimental and growth conditions for mice model throughout the year. At the 6-month analysis period, the concentrations of vitamin D and its metabolites were determined in the serum samples collected from the three study

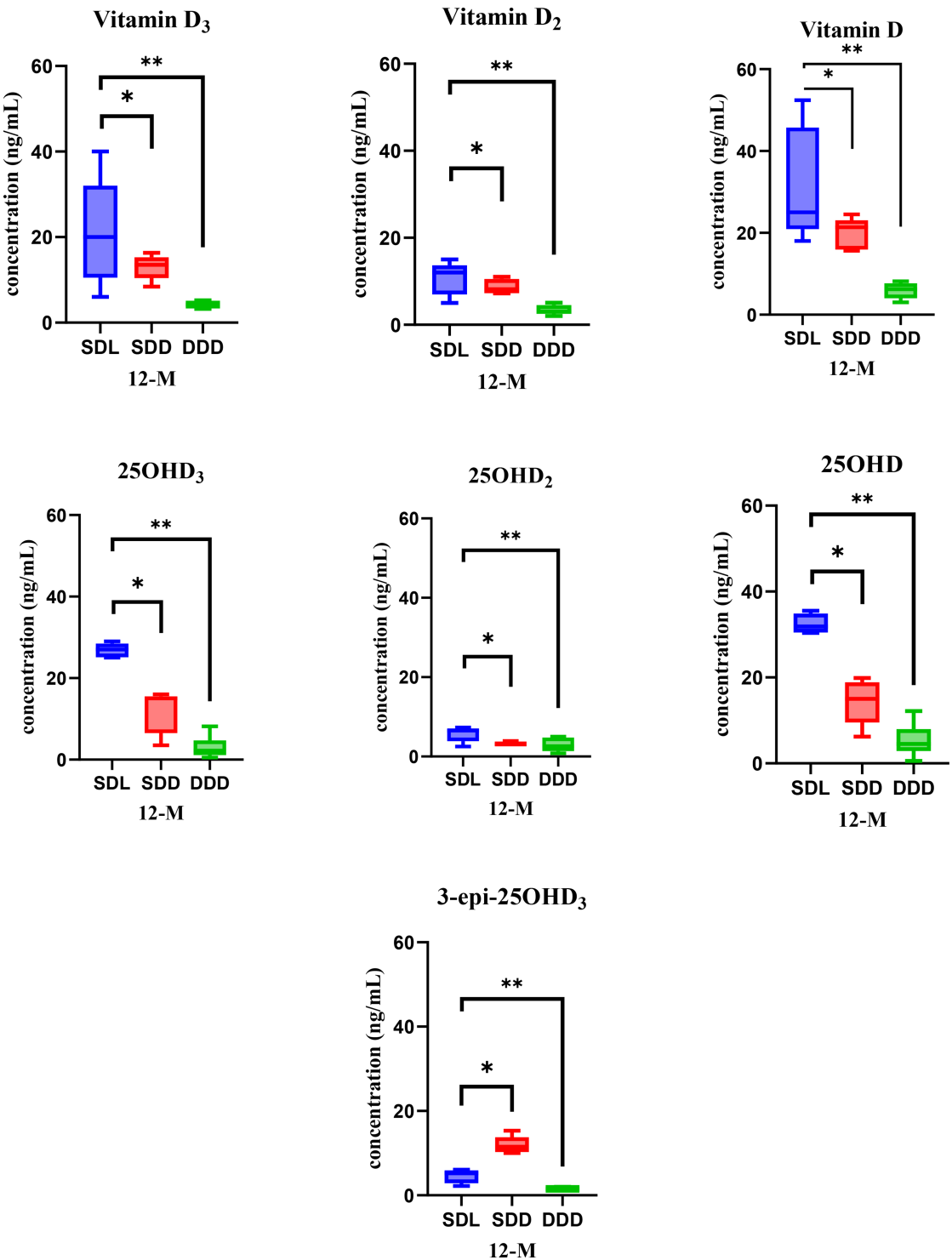


FIGURE 5 Bar and whisker plots showcasing vitamin D metabolite's concentrations in mice serum of three study groups (SDL (N=15), SDD (N=15), and DDD (N=10)) at the 12-month mark. Asterisk (* & **) represent significant differences between SDL, SDD and DDD groups. The error bars represent standard deviations. The differences between the results were considered significant if $p < 0.05$, refuting the null hypothesis ($p < 0.05$). Vitamin D: 20–50 ng/mL (normal), <20 ng/mL (deficient). 25OHD: 15–40 ng/mL (normal), <15 ng/mL (deficient).

groups of mice: SDL (N=15), SDD (N=15), DDD (N=10) as shown in **Figure 4**. Statistical analysis (ANOVA) indicated significant differences between the SDL and DDD groups for both vitamin D and 25OHD levels ($p < 0.01$). Additionally, a significant difference was observed between the SDL and SDD groups for 25OHD concentrations ($p < 0.05$).

The analysis revealed notable variations in the concentrations of the metabolites among the groups. Specifically, the SDL group exhibited the highest average concentration of vitamin D (35.9 ng/ml), followed by the SDD group (26.8 ng/ml), and notably lower levels were observed in the DDD group (4.8 ng/ml). Similarly, for the 25OHD metabolite of vitamin D, the SDL group displayed the highest average concentration (30.3 ng/ml), followed by the SDD group (19.7 ng/ml), with the DDD group showing the lowest levels (5 ng/ml). The bar and whisker plots in **Figure 4** illustrate the concentrations of all analyzed vitamin D metabolites in the serum samples of mice at the 6-month mark. The error bars represent the standard deviation, indicating the variability in the concentrations within each group. As depicted in the **Figure 4**, the SDL group consistently exhibited higher concentrations of vitamin D and its metabolites compared to the other groups, while the DDD group consistently showed the lowest concentrations across all metabolites.

When analyzing the serum samples at the 12th month of the study year, the concentration of 25OHD exhibited the highest levels across all samples, followed by vitamin D and 25OHD₃. **Figure 5** presents the bar and whisker plots of all analyzed metabolites in mice serum at the 12th month analysis, with error bars indicating the standard deviation. ANOVA results at this time point also indicated significant differences between SDL and DDD groups for both vitamin D and 25OHD ($p < 0.01$), while SDL showed higher levels than SDD for 25OHD ($p < 0.05$). The data reveal distinct concentration levels for each metabolite across the three experimental groups. Specifically, the SDL group demonstrates the highest concentrations for most metabolites, reflecting the influence of standard vitamin D intake and UV light exposure. Conversely, the DDD group exhibits significantly lower concentrations, indicating the impact of a vitamin D-deficient diet and absence of light exposure. These findings underscore the critical role of dietary vitamin D intake and light exposure in modulating vitamin D metabolite concentrations in the serum over time. Additionally, the variations observed among the experimental groups highlight the importance of environmental factors in shaping vitamin D metabolism in mice models.

Vitamin D metabolites, particularly 25OHD and its active form 1 α 25(OH)₂D₃, play critical roles in regulating endocrine functions. The observed variations in serum metabolite concentrations among the groups are reflective of the regulatory influence of vitamin D on calcium and phosphorus homeostasis, primarily mediated through the suppression of parathyroid hormone (PTH) secretion. The high concentrations of 25OHD observed in the SDL group may enhance PTH suppression, thereby maintaining calcium-phosphorus balance more effectively than the SDD or DDD groups. Conversely, the DDD group's low serum concentrations likely reflect a deficiency-driven feedback loop, with reduced 25OHD levels failing to adequately regulate PTH and downstream metabolic pathways. These findings highlight the interplay between vitamin D

status and systemic endocrine adaptation, emphasizing the importance of both dietary intake and light exposure in maintaining endocrine health (22, 25, 36, 68).

3.4 Statistical analysis

A comprehensive statistical analysis was conducted to evaluate differences in vitamin D and its metabolites among the three groups (SDL, SDD, and DDD) and across two time points (6 months and 12 months). The results indicated statistically significant differences in the concentrations of vitamin D and its metabolites between the SDL and DDD groups at both time points ($p < 0.01$). Significant differences were also observed between the SDL and SDD groups, particularly for 25OHD levels ($p < 0.05$), underscoring the role of UV light exposure in enhancing vitamin D metabolite levels. *Post-hoc* analysis confirmed that the DDD group consistently had significantly lower concentrations of vitamin D and its metabolites compared to both the SDL and SDD groups ($p < 0.01$).

To ensure sufficient statistical power, a *post-hoc* power analysis was performed based on the observed effect sizes for vitamin D metabolite concentrations across groups and time points. This analysis confirmed that the sample sizes (SDL: N=15, SDD: N=15, DDD: N=10) provided >80% power ($\alpha = 0.05$) to detect significant differences. Effect sizes were calculated using Cohen's *d* for pairwise comparisons and partial eta squared (η^2) for ANOVA results to quantify the magnitude of observed differences. Paired *t*-tests were conducted to analyze temporal changes in metabolite levels within each group from 6 months to 12 months. The results revealed significant increases in both vitamin D and 25OHD levels in the SDL and SDD groups ($p < 0.05$), while the DDD group showed no significant changes, reflecting a persistent deficiency due to the absence of dietary supplementation and light exposure.

3.5 Correlation analysis between hair and serum samples

To evaluate the consistency of vitamin D levels between hair and serum, a correlation analysis was conducted on the concentrations of 25OHD₃ in both matrices across the SDL, SDD, and DDD groups at 6 and 12 months. Our findings demonstrated a moderate positive correlation ($r = 0.56$, $p < 0.05$) between hair and serum 25OHD₃ concentrations, suggesting that hair vitamin D levels reliably reflect serum levels over time. This correlation was most pronounced in the SDL group, which received UV light exposure and a standard vitamin D diet. Notably, the DDD group, which was vitamin D-deficient, consistently exhibited the lowest 25OHD₃ levels in both hair and serum, reinforcing the reliability of hair samples for identifying deficiencies. The moderate correlation indicates that while hair captures long-term vitamin D status, serum levels may respond more dynamically to short-term dietary and environmental variations. This consistency between hair and serum vitamin D measurements supports the validity of hair as a viable biomarker for vitamin D status, particularly in studies aiming to monitor long-term exposure.

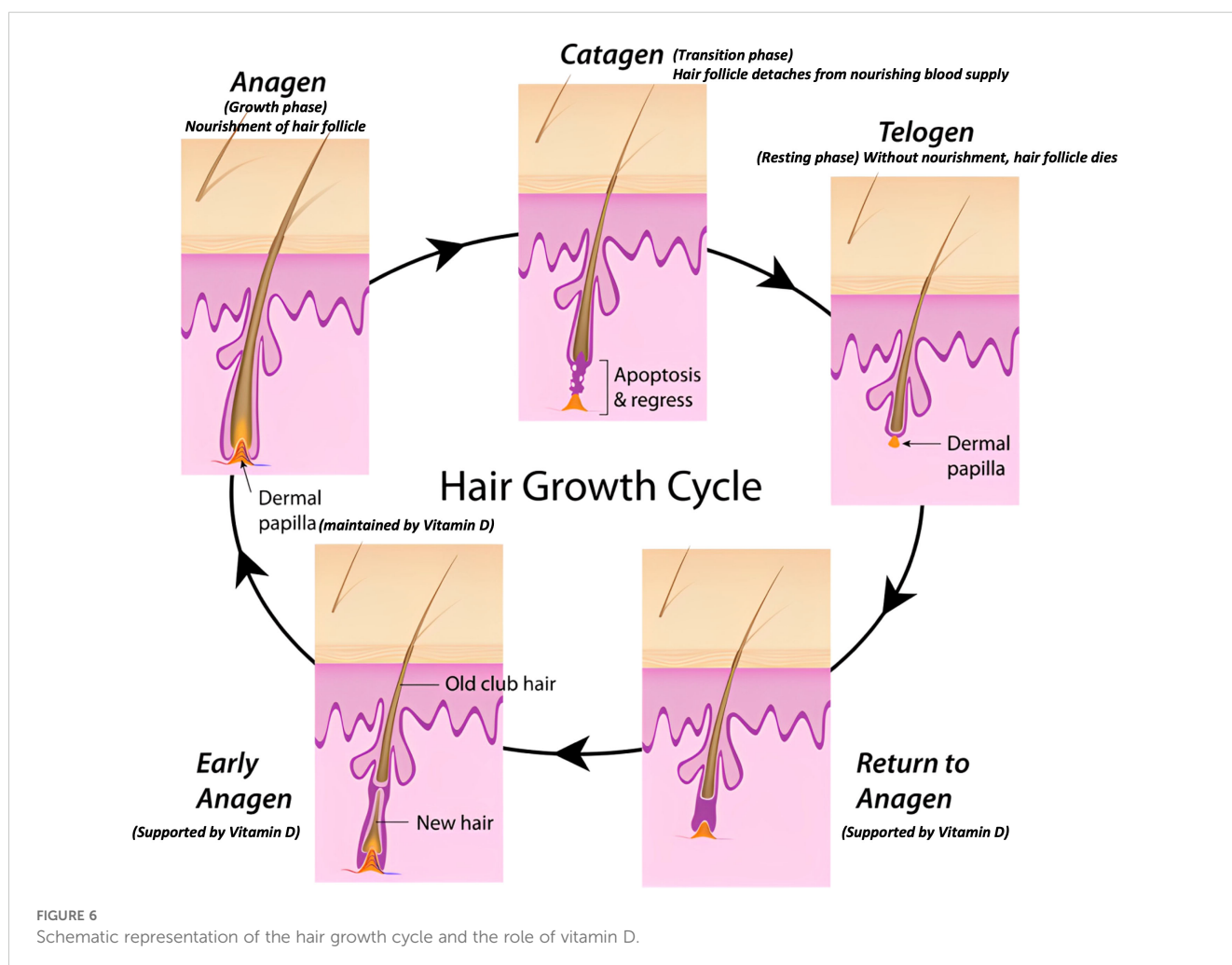
4 Discussion

The analysis of serum samples revealed substantial differences in the concentrations of vitamin D and its metabolites among the various experimental groups. This comparison of vitamin D metabolite concentrations allowed us to discern the impact of experimental conditions on serum vitamin D dynamics over time. The graphical representation in the [Figures 4, 5](#) illustrates the levels of vitamin D metabolites in mouse serum after 6 months (6-M) and 12 months (12-M) period provides insights into these variations. These plots give a visual comparison of concentrations among different experimental groups. Each bar represents the concentration of a specific vitamin D metabolite, with error bars indicating the standard deviation. Significant differences observed between the SDL, SDD and DDD groups of mice across various parameters, including vitamin D and its metabolites. $1\alpha25(\text{OH})_2\text{D}_3$, $1\alpha25(\text{OH})_2\text{D}_2$ and $1\alpha25(\text{OH})_2\text{D}$ were below limit of quantification (BLQ) and is not detected in the samples. These variations are attributed to differences in dietary intake and exposure to light, both of which significantly influence the levels of vitamin D metabolites in mice serum.

Comparing the SDL and DDD groups, it was evident that both diet and light exerted considerable effects on the concentrations of

vitamin D metabolites in the blood. This observation underscores the importance of environmental factors in modulating vitamin D metabolism and maintaining optimal levels of its metabolites in serum. In particular, a significant difference in the concentrations of 25OHD_3 metabolite was observed between the SDL and SDD groups, attributed to the influence of UV light. These findings suggest that UV light exposure enhances the production of vitamin D epimers in serum, either by stimulating their synthesis or by promoting their metabolic efficiency. The notable increase in Vitamin D concentrations in the SDL group, compared to the DDD and SDD groups, further supports the beneficial effects of a standard vitamin D intake coupled with UV light exposure on the levels of vitamin D metabolites. Furthermore, levels of vitamin D metabolites in serum were markedly low in the DDD group, indicative of a vitamin D-deficient diet and lack of light exposure. Conversely, both SDD and SDL groups exhibited substantially higher concentrations of these metabolites, underscoring the synergistic effect of dietary vitamin D intake and UV light exposure on maintaining optimal serum levels.

Hair grows in cycles comprising anagen (active growth), catagen (transition), and telogen (resting) phases, which influence metabolite incorporation into the hair shaft ([Figure 6](#)). During the anagen phase, systemic metabolites are actively deposited into



growing hair, whereas metabolite deposition decreases significantly during the catagen and telogen phases as the follicle becomes metabolically inactive. Consequently, variability in the proportion of hair follicles in different phases at the time of collection can affect the metabolite levels observed (58, 69, 70). In this study, to mitigate the impact of hair growth cycle variability, we coordinated the growth cycle by shaving hair at the study's onset and collected samples after six months of regrowth for the first time point and at 12 months for the second. This approach ensured that hair samples represented cumulative vitamin D metabolite deposition during stable growth phases, minimizing the influence of transient growth cycle variability. Hair composition, growth characteristics, and environmental exposure vary across different body regions, potentially affecting metabolite deposition and measurement. For example, dorsal hair is less exposed to contaminants such as grooming residues compared to ventral or tail hair and typically exhibits more uniform growth patterns. Additionally, differences in lipid content and structural properties across regions can alter the incorporation and retention of metabolites (70–72). This standardization minimized variability and enhanced reproducibility across experimental groups.

The interpretation of these findings is further contextualized by the characteristics of the C57BL/6J mouse strain used in this study. This strain is widely recognized for its genetic predisposition to lower bone density, attributed to reduced bone formation rates and altered bone turnover dynamics. Histomorphometric studies have demonstrated that C57BL/6J mice exhibit impaired bone formation, characterized by reduced trabecular thickness and bone volume. These skeletal characteristics provide a valuable model for understanding genetic influences on vitamin D metabolism and bone health. The observed differences in vitamin D metabolite concentrations in the SDL and DDD groups align with the strain's phenotype, emphasizing the critical role of dietary vitamin D intake and light exposure in mitigating the effects of lower bone density in this genetic model (60, 61).

The application of LC-MS/MS in this study highlights its utility as a powerful tool for exploring nutrigenomic aspects of hair biology. By precisely quantifying vitamin D metabolites in serum and hair samples, this methodology enables the investigation of how systemic and local vitamin D metabolism influences genetic and epigenetic regulation in hair follicles (57, 73, 74). Vitamin D status, for instance, could affect the expression of genes involved in keratinocyte differentiation, stem cell activation, and immune modulation, all of which are crucial for maintaining hair follicle health and promoting the hair growth cycle (5, 73, 75). Future applications of this technique could include correlating metabolite profiles with gene expression data to uncover molecular pathways through which vitamin D exerts its effects on hair follicle biology. The observed differences in vitamin D metabolite profiles among experimental groups provide a foundation for exploring their potential roles in gene regulation. Vitamin D metabolites, particularly $1\alpha,25(\text{OH})_2\text{D}$, interact with the vitamin D receptor (VDR) to modulate the transcription of target genes. This interaction has been implicated in processes such as keratinocyte differentiation, immune response, and oxidative stress management within the hair follicle microenvironment. Elevated levels of $1\alpha,25$

$(\text{OH})_2\text{D}$ in groups exposed to standard vitamin D diets and regular light cycles suggest enhanced VDR activity, which may positively influence gene expression patterns critical for anagen initiation and maintenance. These mechanisms provide a molecular framework linking vitamin D metabolism to nutrient-gene interactions in hair follicle development.

Vitamin D plays a pivotal role in regulating gene expression in hair follicles by binding to VDR, which subsequently interacts with vitamin D response elements (VDREs) in target genes. This interaction influences the transcriptional regulation of keratinocyte differentiation, essential for hair follicle integrity and normal hair growth cycle progression. Specifically, vitamin D metabolites may modulate the expression of genes regulating cell proliferation, differentiation, and apoptosis within the hair follicle matrix. These findings align with previous studies suggesting that vitamin D deficiency disrupts hair follicle cycling, potentially contributing to alopecia. The LC-MS/MS results presented here provide a platform for further exploration of these transcriptional dynamics under varying vitamin D statuses. The interplay between nutrient availability and gene expression in the hair follicle is a critical aspect of hair biology. Vitamin D metabolites are known to influence nutrient-gene interactions, particularly in regulating genes associated with the anagen, catagen, and telogen phases of the hair growth cycle. For instance, alterations in vitamin D levels can modulate β -catenin signaling pathways, which are crucial for the activation of hair follicle stem cells during anagen. Additionally, nutrient deficiencies may exacerbate telogen effluvium, a condition characterized by premature hair follicle dormancy. These insights underscore the significance of maintaining optimal vitamin D levels to support gene regulatory networks that govern hair growth and cycling. Our findings demonstrate that supplementation with vitamin D or exposure to UV radiation leads to a significant increase in circulating vitamin D metabolite concentrations. However, the mechanisms driving vitamin D epimerization and its differential regulation in cellular contexts remain poorly understood, warranting further investigation. It is essential to acknowledge the inherent physiological differences between mice and humans in vitamin D metabolism. While our results provide valuable insights into dietary intake and light exposure effects in mice, extrapolating these findings to humans requires cautious interpretation and further study. Human metabolism involves a combination of sunlight exposure and dietary intake for vitamin D synthesis, unlike the nocturnal dietary intake predominant in mice. These distinctions should inform future research to clarify the broader implications of these findings for human health (37, 58, 76–78).

Emerging evidence suggests that vitamin D status can influence epigenetic modifications, with profound implications for hair follicle biology. Vitamin D metabolites, particularly $1\alpha,25(\text{OH})_2\text{D}$, have been shown to modulate DNA methylation patterns and histone modifications in keratinocytes. These epigenetic changes regulate the expression of genes critical for the hair growth cycle, such as those involved in stem cell activation, apoptosis, and immune response. Alterations in vitamin D-mediated epigenetic regulation may also contribute to conditions such as alopecia areata, characterized by dysregulation of immune-related genes. While our study did not directly assess epigenetic modifications, the findings

provide a biochemical basis for future research into how vitamin D metabolites shape the epigenetic landscape of hair follicles. Incorporating analyses of methylation and histone modifications in future studies could yield valuable insights into the mechanisms linking vitamin D metabolism to nutrient-gene interactions in hair follicle development. The findings of this study have significant implications for understanding nutrient-gene interactions in hair follicle development. Vitamin D metabolites not only serve as markers of systemic nutrient status but also act as key regulators of hair follicle function through transcriptional and epigenetic mechanisms. By elucidating the relationships between metabolite profiles, gene expression, and hair follicle biology, this research contributes to a growing body of evidence supporting vitamin D's role in maintaining hair health. Integrating LC-MS/MS data with transcriptomic and epigenomic analyses in future studies could provide deeper insights into how nutrient availability modulates gene regulatory networks in hair follicle biology. This integrative approach will enhance our understanding of the molecular underpinnings of hair growth and its response to environmental and dietary factors (74, 79, 80).

5 Conclusions

In this study, we successfully developed and validated a sensitive LC-MS/MS assay for the analysis of vitamin D and its metabolites in both mouse hair and serum samples. Unlike previous assays, our method is simple, precise, accurate, and it offers sensitivity, and improved precision, without the need for extensive sample pre-treatment and purification. Through the analysis of hair and serum samples collected over a year, we investigated the variations in vitamin D metabolite concentrations under different experimental conditions. Our results indicate that administering a standard vitamin D diet increased the levels of vitamin D metabolites in both mouse hair and serum. Furthermore, when combined with UV irradiation, the levels of metabolites were further elevated, suggesting a synergistic effect between diet and light exposure. These findings have important endocrine implications, highlighting the role of dietary intake and light exposure in maintaining vitamin D homeostasis. The validated assay offers potential for studying vitamin D-related endocrine disorders and monitoring treatment responses. Its application to non-invasive hair analysis provides a novel tool for long-term monitoring of vitamin D status, with promising utility in both research and clinical settings.

5.1 Future perspectives

This study highlights the potential of serum and hair-based assays for assessing vitamin D status, offering a non-invasive, sensitive approach to monitor vitamin D metabolism. Future efforts should focus on expanding the range of analytes to include additional vitamin D metabolites and refining the assay to become the gold standard for hair analysis. An important direction for future research would be to integrate analyses of key endocrine

regulators, including parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), to further elucidate the interplay between vitamin D metabolism and calcium-phosphorus homeostasis. These parameters could provide deeper mechanistic insights into the vitamin D endocrine axis and its role in systemic nutrient signaling. Additionally, exploring the expression of enzymes such as CYP27B1 and CYP24A1 in tissue samples would shed light on how vitamin D metabolites are processed in specific physiological contexts and under varying environmental conditions. Our findings also open the door to investigating the potential molecular and nutrigenomic implications of vitamin D metabolism. Future studies could leverage this assay to study how vitamin D as a micronutrient regulates gene expression in hair follicles and other tissues. Specifically, understanding the interactions between vitamin D status and nutrient-gene interactions affecting hair growth cycles, transcriptional regulation, and epigenetic modifications could provide valuable insights into vitamin D's broader physiological roles. Translational research could further explore the application of this methodology to monitor treatment responses in vitamin D-related endocrine disorders such as rickets, osteoporosis, and chronic kidney disease. These applications would contribute to a more comprehensive understanding of how vitamin D metabolism influences disease progression and management. Investigating additional factors such as age, ethnicity, gender, and seasonal variations on vitamin D levels would further enrich our knowledge of vitamin D biology and its implications for diverse populations.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The Ethics Committee of United Arab Emirates University (UAEU) granted approval for the study (Ref #C57BL/6J). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MH: Methodology, Writing – original draft, Writing – review & editing. AA: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. SE: Methodology, Writing – original draft, Writing – review & editing. IS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Association between magnesium depletion score and prevalence of hyperuricemia in American adults: a study based on NHANES 2007-2018

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Background: The Magnesium Depletion Score (MDS) is a novel indicator that integrates multiple factors to assess systemic magnesium depletion. However, its association with hyperuricemia (HUA) prevalence remains unclear. This study aims to investigate the relationship between MDS and the prevalence of HUA.

Methods: A cross-sectional analysis was conducted using data from the 2007-2018 National Health and Nutrition Examination Survey (NHANES). The MDS was derived by integrating multiple risk factors associated with magnesium depletion: the use of diuretics and proton pump inhibitors (PPI), estimated glomerular filtration rate (eGFR), and alcohol consumption exceeding recommended thresholds. Univariable and multivariable logistic regression models assessed the association between MDS and HUA. Subgroup and sensitivity analyses, including the exclusion of gout patients, further evaluated this association.

Results: Among 18,761 participants, higher MDS were associated with an increased prevalence of HUA. Multivariable logistic regression confirmed a significant positive association between MDS and HUA (OR = 1.73, 95% CI = 1.62-1.84). Restricted cubic splines (RCS) further characterized the non-linear relationship between MDS and HUA prevalence. Subgroup analysis indicated a positive association between MDS and HUA, with significant interactions observed for sex and body mass index. Sensitivity and additional analyses reinforced the robustness of these findings.

Conclusion: Among U.S. adults, higher MDS were significantly associated with an increased prevalence of HUA, suggesting that interventions targeting magnesium deficiency could be beneficial in reducing HUA prevalence within this population. However, prospective studies are needed to further confirm these findings.

KEYWORDS

magnesium depletion score, hyperuricemia, uric acid, cross-sectional study, NHANES

1 Introduction

Hyperuricemia (HUA) is characterized as a chronic metabolic disease resulting from purine metabolism disorders (1), with elevated serum uric acid (SUA) levels as its primary clinical symptom. As the condition progresses, it may develop into gout, which leads to acute arthritis and tophi. Epidemiological studies estimate that about 38 million people reside in the US exhibit elevated SUA levels (2), especially among the obese population (3), with the prevalence of HUA rising annually (4). HUA serves as the primary risk factor for gout, an inflammatory arthritis (5); prolonged HUA may result in the formation of monosodium urate crystals (6), which damage joint structures and cause severe pain, significantly impacting a patient's quality of life (7). Moreover, elevated SUA levels are linked to various diseases, including hypertension, kidney diseases, and cardiovascular diseases (8, 9). HUA has emerged as a serious public health issue, emphasizing the importance of research to evaluate factors associated with HUA.

Among the minerals found in the body, magnesium is the fourth most abundant cation and one of the important elements. It is essential for several physiological processes (10), such as energy metabolism, protein synthesis, and membrane integrity, among others (11, 12). A magnesium shortage can lead to alterations in biochemical pathways and is prevalent among obese patients (13), potentially increasing the risk of heart disease, diabetes, and other chronic conditions (14, 15). Current literature primarily focuses on serum or dietary magnesium, yet it does not adequately address the role of systemic magnesium deficiency in metabolic disorders, such as HUA. The conventional reliance on serum magnesium levels as an indicator of magnesium status may be insufficient, as these levels can remain within the normal range even during chronic deficiency, limiting their reliability in reflecting overall magnesium status. The Magnesium Deficiency Score (MDS) was initially proposed by Fan et al. in 2021 as a systematic scoring tool for assessing magnesium deficiency (16). A higher MDS indicates a greater degree of magnesium depletion. MDS aims to identify individuals with magnesium deficiency and encourage them to increase magnesium intake, thereby reducing systemic inflammation and maintaining stability in various biochemical and metabolic processes (17, 18). A significant relationship was found in a cross-sectional investigation among elevated MDS and a greater incidence of metabolic syndrome (19). The potential mechanisms linking MDS with HUA may involve inflammation and oxidative stress (20, 21). Additionally, low magnesium levels may affect DNA modification and repair, where damaged DNA and the ultimate breakdown of purine nucleotides can lead to increased uric acid production (22). However, studies are still lacking on the relationship between MDS and HUA. This study aims to address

this gap by investigating the relationship between MDS and HUA in a nationally representative sample of U.S. adults, hypothesizing that a higher MDS is associated with an increased risk of HUA. Understanding this association may inform potential prevention strategies, underscoring the importance of magnesium status in metabolic health management.

2 Methods

2.1 Survey description

The National Health and Nutrition Examination Survey (NHANES), which is intended to assess the health and nutritional status of the American population, provided all information about participants for this research. This poll is cross-sectional and nationally representative. The Ethics Assessment Committee approved the study endeavor, and each participant provided signed informed permission.

2.2 Study population

Data for this study were collected from the NHANES between 2007 and 2018. Inclusion criteria included: (1) subjects aged 20 years or older and not pregnant at the time of examination; (2) participants with complete data on SUA concentrations and gout; (3) participants with comprehensive data necessary for assessing the MDS, including data on serum creatinine concentration, alcohol consumption, proton pump inhibitor (PPI) usage, and diuretic medication.

2.3 Diagnosis of HUA

SUA data for participants were obtained from the NHANES database. Blood samples from participants were collected, processed, and subsequently refrigerated or frozen in accordance with NHANES protocols. These samples were analyzed in laboratories designated by NHANES. SUA concentrations were measured using a standardized colorimetric method. HUA was defined as SUA levels above 7 mg/dL in males and 6 mg/dL in females. These thresholds, recommended by major clinical guidelines, are widely used in epidemiological studies to identify individuals at higher risk for HUA-related conditions (23, 24).

2.4 Calculation of MDS

The MDS is a scoring system developed based on previous studies to assess systemic magnesium depletion by integrating multiple risk factors associated with magnesium deficiency, including the use of diuretics and proton pump inhibitors (PPI), estimated glomerular filtration rate (eGFR), and alcohol intake exceeding recommended limits. MDS has shown predictive validity in identifying magnesium-related health risks, such as

Abbreviations: BMI, Body Mass Index; CAD, Coronary Artery Disease; CI, Confidence Interval; CRP, C-Reactive Protein; eGFR, estimated glomerular filtration rate; HUA, Hyperuricemia; IL-6, Interleukin-6; MDS, Magnesium Depletion Score; NHANES, National Health and Nutrition Examination Survey; OR, Odds Ratio; PIR, Poverty Income Ratio; Q, Quartiles; SCR, Serum Creatinine; SUA, Serum Uric Acid; TNF- α , Tumor Necrosis Factor-alpha.

metabolic syndrome and systemic inflammation (16, 19). The score is calculated based on the following components: (1) one point for current diuretic use; (2) one point for current PPI use; (3) one point if eGFR is between 60 and 90 mL/min/1.73 m² and two points if eGFR is below 60 mL/min/1.73 m²; (4) one point for excessive alcohol intake, defined as more than two drinks per day for male and more than one drink per day for female. eGFR is calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula. MDS range from 0 to 5, categorizing individuals into six groups: MDS = 0, 1, 2, 3, 4, and 5.

2.5 Covariates

The study's main variables are chronic comorbidities and demographic traits. Age, race, ethnic origin, a person's body mass index (BMI), poor index ratio (PIR), education level, smoking and drink alcohol, diabetes, hypertension, hypercholesterolemia and cardiovascular disease (CAD) are some of these factors. PIR is divided into three groups based on the poverty threshold: < 1, 1 - 3, and ≥ 3. It is computed by dividing the family's earnings by this criterion. Individuals who have smoked in excess of 100 cigarettes in all of their lives are classified as smokers based on their answers to the questionnaire. Drinking status is defined as the consumption of at least 12 alcoholic beverages in a year. A history of CAD, heart failure that is congestive, or angina pectoris, or chest pain, is among the self-reported data from the questionnaire that is used to diagnose coronary heart disease. Other chronic comorbidities, including diabetes, hypertension and hypercholesterolemia, are identified through physician diagnoses or self-reports in the questionnaire.

2.6 Statistical analysis

In this study, data from the NHANES database, spanning six cycles from 2007 to 2018, were utilized. Following a filtering of the data according to participant inclusion criteria, the remaining participants were classified for statistical analysis according to their HUA status. Data that was categorical were reported as proportions, while continuous variables were shown as means ± deviations in standard form. Univariable and multivariable regression logistic analyses were carried out, yielding ratios of odds (OR) and 95% confidence intervals (CI), in order to investigate the association between the MDS and HUA. To explore the relationship between different MDS and HUA, and to illustrate the dose-response interaction, we used restricted cubic splines (RCS). The RCS model included three knots placed at the 10th, 50th, and 90th percentiles of the MDS distribution to capture potential non-linear relationships between the MDS and HUA. Subgroup analyses were conducted to assess the relationship between MDS and HUA across variables such as age, sex, smoking status, and the presence of various chronic illnesses. Sensitivity analyses were performed separately by excluding participants with gout and excluding participants with missing values for covariates. Three models were tested: Model 1 included

no covariates; Model 2 adjusted for age, sex, and race; and Model 3 included additional adjustments for BMI, educational level, PIR, smoking, drink alcohol, hypertension, hypercholesterolemia, CAD and diabetes. All statistical analyses were performed using R (version 4.2.3), with a p-value < 0.05 considered indicative of statistical significance.

3 Results

3.1 Characteristics of study population

Data were taken from the NHANES registry for 59842 participants between 2007 and 2018. The screening process is shown in Figure 1, with a final inclusion of 18,761 participants. Supplementary Table 1 presents the demographic characteristics of the excluded population. Table 1 displays initial traits based on the existence of HUA, whereas Supplementary Table 2 displays weighted baseline features. Participants with HUA were generally older, male, and had higher BMI and education levels compared to those without the condition. Additionally, they exhibited higher SUA and serum creatinine (SCR) levels, coupled with lower eGFR. Additionally, there was a higher incidence of smoking in this group, as well as a higher chance of developing diseases including gout, diabetes, CAD, hypercholesterolemia and hypertension.

3.2 Association between MDS and HUA

Table 2 displays the results of the logistic regression study evaluating the interaction among MDS and HUA. Univariate logistic regression analysis demonstrated a positive association between MDS and HUA (OR=1.85, 95% CI=1.75-1.95).

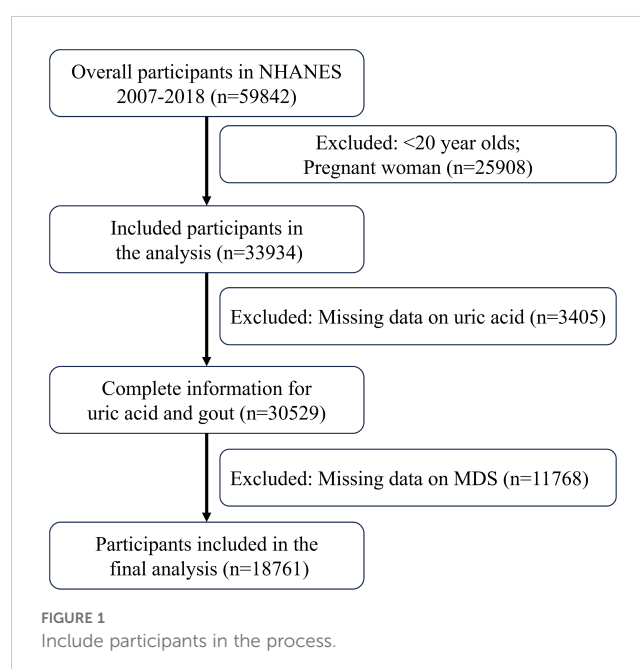


TABLE 1 Baseline characteristics of the study population.

Characteristic	Overall	Non-hyperuricemia	hyperuricemia	P-value
n	18761	15277	3484	
Age (%)				<0.001
<50	10666 (56.9)	9043 (59.2)	1623 (46.6)	
>50	8095 (43.1)	6234 (40.8)	1861 (53.4)	
Sex (%)				<0.001
Female	8685 (46.3)	7356 (48.2)	1329 (38.1)	
Male	10076 (53.7)	7921 (51.8)	2155 (61.9)	
Race (%)				<0.001
Mexican American	2768 (14.8)	2387 (15.6)	381 (10.9)	
Non-Hispanic black	3758 (20.0)	2900 (19.0)	858 (24.6)	
Non-Hispanic white	8330 (44.4)	6744 (44.1)	1586 (45.5)	
Others	3905 (20.8)	3246 (21.2)	659 (18.9)	
BMI (%)				<0.001
Underweight	271 (1.5)	259 (1.7)	12 (0.3)	
Normal	5173 (27.8)	4708 (31.0)	465 (13.5)	
Overweight	6249 (33.5)	5206 (34.3)	1043 (30.2)	
Obese	6934 (37.2)	5006 (33.0)	1928 (55.9)	
Education level (%)				0.004
Under high school	3613 (19.3)	2966 (19.4)	647 (18.6)	
High school or equivalent	4145 (22.1)	3296 (21.6)	849 (24.4)	
Above high school	10994 (58.6)	9007 (59.0)	1987 (57.0)	
No record	9 (0.0)	8 (0.1)	1 (0.0)	
PIR (%)				0.108
<1	3049 (17.8)	2507 (18.0)	542 (17.1)	
1-3	6866 (40.1)	5542 (39.8)	1324 (41.8)	
>3	7194 (42.0)	5889 (42.3)	1305 (41.2)	
Smoke (%)				<0.001
No	9583 (51.1)	7939 (52.0)	1644 (47.2)	
Yes	9164 (48.8)	7327 (48.0)	1837 (52.7)	
No record	14 (0.1)	11 (0.1)	3 (0.1)	
Drink alcohol (%)				0.31
No	1590 (8.5)	1317 (8.6)	273 (7.8)	
Yes	17164 (91.5)	13954 (91.3)	3210 (92.1)	
No record	7 (0.0)	6 (0.0)	1 (0.0)	
Hypertension (%)				<0.001
No	12507 (66.7)	10853 (71.0)	1654 (47.5)	
Yes	6235 (33.2)	4408 (28.9)	1827 (52.4)	

(Continued)

TABLE 1 Continued

Characteristic	Overall	Non-hyperuricemia	hyperuricemia	P-value
Hypertension (%)				<0.001
No record	19 (0.1)	16 (0.1)	3 (0.1)	
Hypercholesterolemia (%)				<0.001
No	10662 (63.5)	8896 (65.2)	1766 (55.8)	
Yes	6042 (36.0)	4671 (34.3)	1371 (43.3)	
No record	99 (0.6)	70 (0.5)	29 (0.9)	
Diabetes (%)				<0.001
No	16402 (87.4)	13535 (88.6)	2867 (82.3)	
Yes	1931 (10.3)	1430 (9.4)	501 (14.4)	
No record	428 (2.3)	312 (2.0)	116 (3.3)	
CAD (%)				<0.001
No	17689 (94.3)	14561 (95.3)	3128 (89.8)	
Yes	1072 (5.7)	716 (4.7)	356 (10.2)	
Hyperuricemia (%)				<0.001
No	15277 (81.4)	15277 (100.0)	0 (0.0)	
Yes	3484 (18.6)	0 (0.0)	3484 (100.0)	
Gout (%)				<0.001
No	17919 (95.6)	14829 (97.1)	3090 (88.8)	
Yes	828 (4.4)	439 (2.9)	389 (11.2)	
MDS (mean (SD))	1.19 (0.92)	1.08 (0.84)	1.68 (1.10)	<0.001
MDS (%)				
0	4019 (21.4)	3573 (23.4)	446 (12.8)	<0.001
1	9163 (48.8)	7906 (51.8)	1257 (36.1)	
2	3895 (20.8)	2901 (19.0)	994 (28.5)	
3	1311 (7.0)	745 (4.9)	566 (16.2)	
4	348 (1.9)	146 (1.0)	202 (5.8)	
5	25 (0.1)	6 (0.0)	19 (0.5)	
eGFR (mean (SD)) (ml/min/1.73m ²)	92.95 (21.62)	95.42 (20.17)	82.11 (24.26)	<0.001
SCR (mean (SD)) (mg/dL)	0.89 (0.36)	0.86 (0.34)	1.03 (0.38)	<0.001
SUA (mean (SD)) (mg/dL)	5.52 (1.43)	5.04 (1.04)	7.60 (0.99)	<0.001

Mean ± SD for continuous variables, % for categorical variables. BMI, Body Mass Index; CAD, Coronary Artery Disease; MDS, Magnesium Depletion Score; PIR, Poverty Income Ratio; SCR, Serum Creatinine; SUA, Serum Uric Acid.

Multivariable adjustments, incorporating demographic and chronic disease covariates, sustained this association (OR=1.73,95% CI=1.62-1.84). When MDS was changed from a continuous to a categorical variable, high levels of MDS significantly increased the prevalence of HUA compared with low levels of MDS. RCS analysis further identified a nonlinear positive correlation between MDS and HUA prevalence, as shown in [Figure 2](#). The prevalence of HUA gradually increased with increasing levels of MDS. The above results suggest that MDS maintains a robust positive association with the prevalence of HUA.

3.3 Subgroup evaluation from MDS with HUA

Subgroup analyses, stratified by age, sex, BMI, smoke, CAD, hypertension, diabetes, and hypercholesterolemia, were carried out to investigate any connections between MDS and HUA. [Figure 3](#) shows that a positive connection among MDS and HUA was found across certain subgroups after correcting for all variables. Interaction tests indicated potential influences on the correlation associated with sex and BMI. This positive correlation was

TABLE 2 The relationship between MDS and Hyperuricemia.

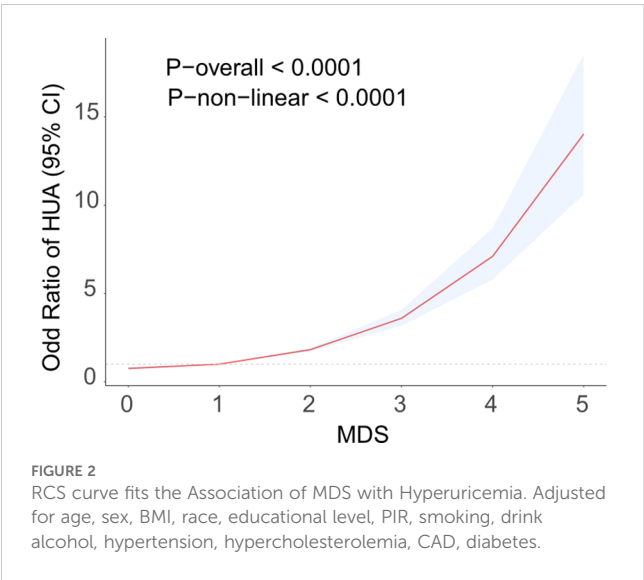
		Model 1 OR (95%CI) P-value	Model 2 OR (95%CI) P-value	Model 3 OR (95%CI) P-value
Hyperuricemia	MDS	1.85 (1.75, 1.95) <0.001	1.93 (1.82, 2.05) <0.001	1.73 (1.62, 1.84) <0.001
	0	[Reference]	[Reference]	[Reference]
	1	1.29 (1.10, 1.50) 0.002	1.36 (1.17, 1.58) <0.001	1.27 (1.06, 1.52) 0.011
	2	2.62 (2.25, 3.05) <0.001	2.50 (2.04, 3.07) <0.001	2.48 (2.06, 2.99) <0.001
	3	5.78 (4.73, 7.06) <0.001	6.77 (5.48, 8.36) <0.001	4.77 (3.73, 6.09) <0.001
	4	10.0 (7.48, 13.50) <0.001	12.7 (9.17, 17.60) <0.001	8.10 (5.75, 11.40) <0.001
	5	21.4 (6.90, 66.30) <0.001	24.0 (8.11, 71.20) <0.001	10.70 (2.91, 39.1) <0.001
	P for trend	<0.001	<0.001	<0.001

MDS, Magnesium Depletion Score; OR, Odds Ratio; CI, Confidence Interval; Model 1: No covariates adjusted; Model 2: Adjusted for age, sex, and race; Model 3: Adjusted for age, sex, BMI, race, educational level, PIR, smoking, drink alcohol, hypertension, hypercholesterolemia, CAD, diabetes.

particularly pronounced among females and participants with a lower BMI.

3.4 Sensitivity analysis

To rigorously evaluate the robustness of our findings, we performed a sensitivity analysis. Excluding 842 individuals with a gout diagnosis yielded a remaining sample of 17,919 participants. In this adjusted cohort, Model 3 logistic regression identified a significant positive association between MDS and HUA (Supplementary Table 3). Additionally, we excluded participants with missing covariate data, resulting in 16,216 participants available for further sensitivity analysis. The sensitivity analysis outcomes corroborated the primary study results, underscoring the robustness of the observed associations (Supplementary Table 4).



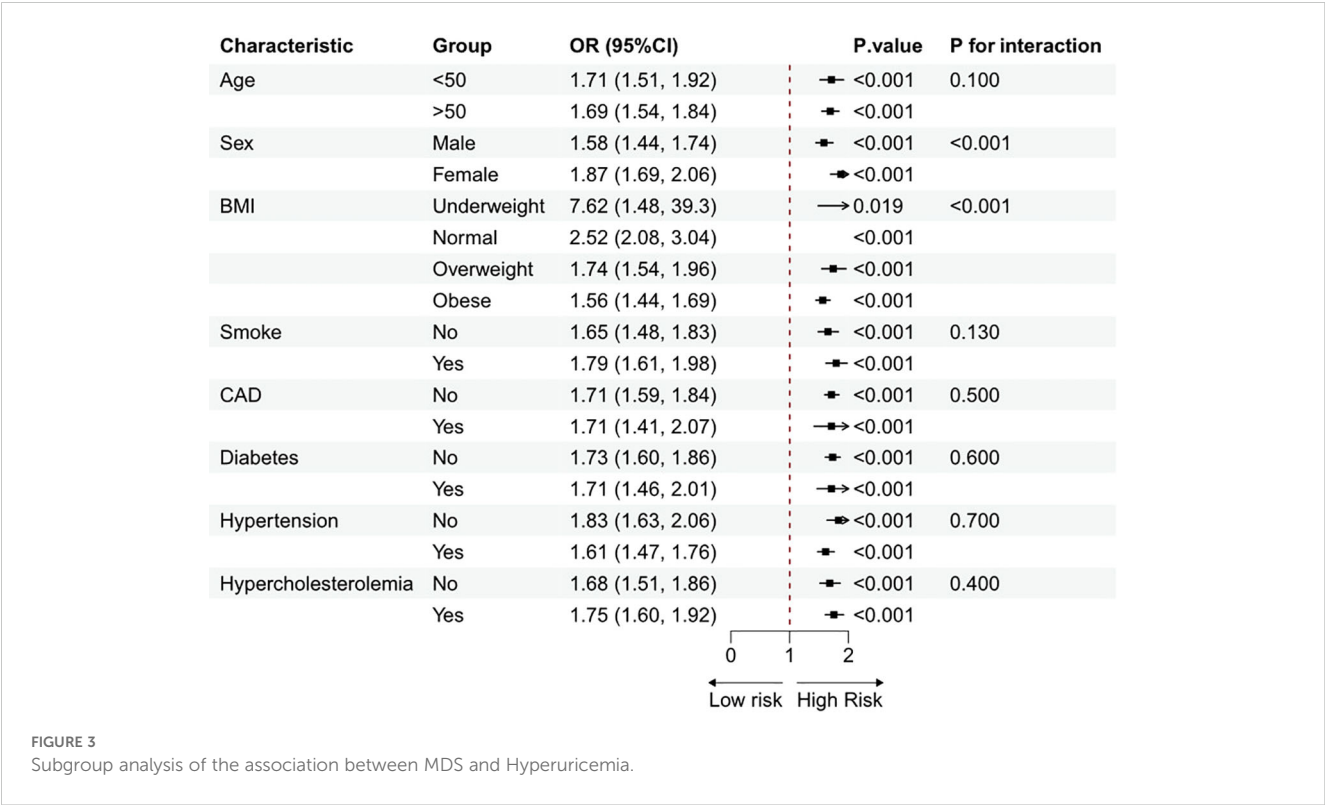
3.5 Additional analysis

Linear regression was employed in the additional analysis to investigate the relationship between MDS and SUA levels (Supplementary Table 5). The results indicated a stable positive correlation between MDS and SUA levels, whether MDS was considered a continuous or categorical variable. The RCS curve further demonstrated a nonlinear positive correlation between MDS and SUA levels (Supplementary Figure 1). These findings align with our analysis, enhancing the reliability of the relationship between MDS and HUA in this study.

4 Discussion

Using data from NHANES from 2007 to 2018, we investigated the relationship between MDS and HUA among adults in the United States. Our study found that participants with HUA were typically older, male, had a higher BMI, and also had higher MDS. A positive association between MDS and HUA prevalence was analyzed by logistic regression and was consistently confirmed in subgroup analyses and sensitivity tests. The above results suggest that MDS may be a potential indicator for assessing the prevalence of HUA.

Increasing evidence suggests that in addition to traditional factors such as genetics, high-purine diet, obesity, and metabolic syndrome, mineral intake also influences the risk of HUA (25, 26). Several studies indicate a correlation between serum magnesium levels and HUA (27, 28). Serum magnesium is the most commonly used method to assess magnesium status in clinical practice. Lower serum magnesium levels reflect higher MDS. Regarding the relationship between magnesium levels and HUA, existing studies have primarily focused on the effects of serum magnesium levels or dietary magnesium intake. For example, in India's south, a study of 94 individuals with retinopathy caused by diabetes discovered an negative correlation between the patients' blood magnesium concentration and urinary acid levels (29). Similarly, serum



magnesium and HUA prevalence in males over 40 were shown to be inversely related in an observational study with 2,904 respondents. The study showed that, compared to the lowest quartile, the highest quartile had a 41% lower prevalence of HUA (OR = 0.59, 95% CI = 0.41-0.84) (28). However, because blood magnesium levels can stay within the usual range even during chronic insufficiency, they do not accurately reflect the underlying condition of the whole body magnesium (30, 31). Recent focus has shifted to dietary magnesium intake as a more accurate measure of magnesium status. A cross-sectional investigation of 5168 individuals over forty years of age old conducted in China found an adverse connection between HUA and magnesium from diet consumption (32). An further descriptive research comprising 26796 adult Americans found a correlation between a higher magnesium consumption and a lower incidence of HUA (29). Another way to measure magnesium status is by urine, which requires complicated 24-hour specimen gathering and is easily altered by food, diuretics, and renal function (30, 33, 34). The magnesium endurance test is complicated and challenging to extensively execute since it requires collecting 24-hour samples of urine, giving venous magnesium, and then collecting another 24-hour urinary sample. This test is frequently thought to be the best way to assess body magnesium levels (35). In contrast, this study selected MDS as a tool for systematically evaluating magnesium deficiency, as MDS provides a more comprehensive assessment of magnesium depletion by incorporating multiple factors, such as renal function and diuretic use, which addresses the limitations of traditional magnesium assessments (16). Based on population background information, men generally exhibit a higher prevalence of HUA, likely due to the protective effect of estrogen

on uric acid metabolism in women (36). Additionally, men tend to consume alcohol and high-purine foods more frequently, both of which contribute to increased uric acid production (37). Conversely, patients with chronic diseases sometimes show lower uric acid levels, potentially due to medications like benzbromarone and allopurinol that promote uric acid excretion (38). The results of this study indicate a stable positive correlation between MDS and HUA, based on a broader MDS assessment, suggesting that MDS may better capture the role of magnesium in HUA risk than serum magnesium levels alone.

The potential mechanisms linking magnesium depletion and HUA may involve inflammation, oxidative stress, and metabolic disorders. Numerous studies have reported a positive correlation between HUA and inflammatory biomarkers, including CRP, TNF- α and N-methyl-D-aspartate, suggesting that uric acid may play a role in inflammation and subsequent inflammation-related diseases (39–41). Combined testing of serum uric acid and blood inflammatory markers may enhance the diagnostic accuracy for HUA (42, 43). Mechanistic studies have indicated a negative correlation between magnesium intake and CRP levels (44–48). Excessive serum uric acid crystallization can induce inflammation in joints and surrounding tissues (49), with patients experiencing uric acid crystals often showing markedly elevated serum CRP concentrations during acute episodes (50). Magnesium is essential for DNA folding and functional activity (22), with its role in DNA stability being concentration-dependent; low concentrations can lead to DNA deficiency and instability (51). Low magnesium levels may impact oxidative stress, subsequent oxidative DNA modifications, and DNA repair (52–54), potentially resulting in

significant DNA damage and the release of purine nucleotides, which eventually degrade to produce uric acid (55). Given the association between HUA and oxidative stress and inflammatory cascades, the anti-inflammatory and antioxidant properties of magnesium may help mitigate these effects, providing biological plausibility for the positive correlation between MDS and HUA.

Our study has notable strengths, including its novelty and methodological rigor. It is the first to leverage a nationally representative sample to evaluate the association between HUA risk and the MDS. The findings demonstrate a consistent positive association between MDS and HUA, indicating that this relationship is unlikely due to random variation. Rigorous adjustments were made for demographic and chronic disease confounders, and stratified subgroup analyses were conducted to examine the MDS-HUA association across diverse demographic groups, highlighting the potential need for more targeted HUA prevention strategies. However, our study has several limitations. Firstly, the cross-sectional design of this study inherently limits causal inference; thus, future research incorporating *in vivo* or *in vitro* experimental validation is essential to substantiate these associations and clarify underlying mechanisms. Secondly, despite adjusting for a comprehensive set of covariates to assess the relationship between the MDS and HUA, unmeasured confounding variables may still have influenced the results, contributing to residual confounding. Finally, as a retrospective analysis, this study is susceptible to data collection biases, underscoring the need for prospective studies to confirm and reinforce the robustness of these findings.

5 Conclusions

Among U.S. adults, higher MDS were significantly associated with an increased prevalence of HUA, suggesting that interventions targeting magnesium deficiency could be beneficial in reducing HUA prevalence within this population. However, prospective studies are needed to further confirm these findings.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: <https://www.cdc.gov/nchs/nhanes/nhanes>.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/participants or patients/participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

YX: Data curation, Investigation, Resources, Software, Writing – original draft, Writing – review & editing. YM: Investigation, Validation, Writing – original draft, Writing – review & editing. PW: Investigation, Validation, Writing – original draft, Writing – review & editing. KW: Investigation, Writing – review & editing. TC: Project administration, Writing – review & editing. ZC: Data curation, Writing – review & editing. HL: Data curation, Writing – review & editing. HY: Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing. ZJ: Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2025.1438639/full#supplementary-material>

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Adults with Phenylketonuria have suboptimal bone mineral density apart from vitamin D and calcium sufficiency

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Introduction: Improvement of early diagnosis and treatment in patients with Phenylketonuria (PKU) allowed for healthy survival into adulthood of these patients, but non-neurological health impairments of unknown etiology emerged. One of these is impaired bone health that manifests in adolescence and adulthood, potentially depending not only on treatment adherence but also on additional lifestyle factors and nutrition.

Methods: Eighteen adults with PKU (18.3–51.6 years, ♀ n = 11) and 19 age- and gender-matched controls (18.3–54.9 years, ♀ n = 10) participated in the study. Bone metabolism-related parameters (BMRPs) in plasma, serum, and urine were analyzed. Dietary habits and lifestyle factors were obtained from questionnaires; a 6-min walk test and a dual X-ray absorptiometry measurement at two sites were performed. Phenylalanine (Phe) serum concentrations from the 5 years prior to study participation were collected from the patients' charts.

Results: Patients had reduced bone mineral density (BMD) T-score in hips (-0.67 ± 1.05) and lumbar spine (-0.71 ± 1.11 , both $p = 0.018$). Most BMRPs in plasma, serum, and urine, as well as markers of oxidative stress did not differ from healthy controls. Whereas 89% of adults with PKU were vitamin D-sufficient, only 68% of controls reached vitamin D sufficiency. 25-Hydroxy vitamin D concentration was significantly higher in adults with PKU than that in controls [33.1 ng/mL (26.2–40.3) vs. 23.4 ng/mL (17.2–24.9); $p < 0.001$], whereas parathyroid hormone concentrations showed no difference [PKU: 27.6 pg/mL (19.6–42.8) vs. Co: 36.1 pg/mL (29.2–40.8); $p = 0.089$]. Neither Phe blood concentration nor dietary habits or lifestyle factors were associated with BMD in regression analysis.

Conclusion: Neither dietary habits nor lifestyle factors showed an association with BMD in this group of adults with PKU, whereas BMD was reduced.

KEYWORDS

aromatic amino-acids, bone turnover, dietary compliance, calciotropic hormones, oxidative stress, dietary habits

1 Introduction

Phenylketonuria (PKU; OMIM (Online Mendelian Inheritance in Man) #261600) is an inborn error of the phenylalanine hydroxylase with an incidence of 19.3/100,000 newborns in 2020 in Germany (1, 2). The reduced activity of the enzyme results in the accumulation of Phenylalanine (Phe) and its metabolites in blood and brain, leading to irreversible intellectual disability, motor, and developmental deficits, if left untreated (1). Diagnosis via newborn screenings and subsequent early start of treatment with reduction of Phe intake from natural protein and supplementation of amino acids (AA) can prevent the development of most symptoms (1, 3). Additionally, tetrahydrobiopterin (BH₄), the co-factor of phenylalanine hydroxylase, is available as therapeutic agent for patients if they respond to the treatment. This allows them to either increase their intake of natural protein by $\geq 100\%$ and/or achieve better metabolic control (1). A strict dietary regime is important during maturation of the brain; thereafter, higher blood Phe concentrations can be tolerated (1). In children and adolescents with PKU, quality of life is significantly impaired by their reduced enjoyment of food, in part due to AA supplements (4). As brain development progresses in adolescents, diet restrictions are relaxed and higher Phe concentrations are tolerable (1). Due to the impairment of quality of life observed in adolescents, a less strict diet is usually followed in adolescents and adults with PKU. To reduce the intake of natural proteins, adults tend to adopt a vegan-like diet, with optional vitamin- and mineral-enriched AA supplement consumption, depending on activity level of the phenylalanine hydroxylase (5). The leading goal in therapy of patients with PKU is the prevention of neurocognitive deficits (1); however, other health impairments of unknown etiology occur (6). One of these health impairments is reduced bone mineral density (BMD) of patients with PKU (6). In pediatric patients with PKU, a low BMD Z-score was observed in 28%–45% of patients, even without family history of osteoporosis (7, 8). In adults with PKU, BMD Z-score below -2 in lumbar spine, femoral neck, total proximal femur, or total body was observed in 1.6%–5.5% of patients, whereas overall mean BMD Z-scores were significantly lower compared to reference, without higher proportion of fractures (9). Factors for impaired bone health could be dietary compliance as high blood Phe is associated with reduced bone quality and higher spontaneous osteoclastogenesis (10, 11), whereas the intake of AA supplements might affect bone mineralization negatively due to

high renal acid load (12–16). Energy and protein intake play a crucial role in bone formation. In fact, 21% of total body bone area could be explained by protein intake in children and adolescents (17–19). However, the impact of protein intake on bone formation is lower in adults (18). Additionally, physical activity influences not only bone mass accrual but also maintenance even in patients with low BMD (17, 19). Furthermore, various influencing factors on BMD have been studied in the general population throughout the last years (17, 19). One of these is educational attainment which is associated with higher BMD, although this may be mediated partially by heightened coffee intake and reduced consumption of processed meats (20). Because bone can be influenced by various factors, we analyzed involvement of lifestyle factors and dietary compliance in adult patients with PKU and healthy controls.

2 Materials and methods

2.1 Subjects

Patients were recruited during routine check-up at the outpatient clinic in the Children's Hospital in Bochum. As 32%–77% of adults are lost to follow-up of care in specific centers for PKU care (21), additional recruitment by word of mouth, advertisement in prints for interest groups, and posts on social media were used to reach patients outside of specific clinical care centers. Patients were asked to bring a friend, partner, or sibling as control. Missing controls were recruited by word of mouth. Nineteen age- and sex-matched controls (Table 1) were included into the study on the same day as the patients with PKU.

Classical PKU was defined as either being treated with AA supplements and/or BH₄ supplementation or untreated Phe levels $> 600 \mu\text{mol/L}$. Patients with hyperphenylalaninemia do not require treatment to maintain blood Phe levels $< 600 \mu\text{mol/L}$ (1). Patients with PKU and hyperphenylalaninemia were invited to participate, as all patients did either exceed $600 \mu\text{mol/L}$ of Phe or were treated with AA supplements, we can classify all included patients with PKU as patients with classical PKU.

A written informed consent was given by all participants. The study was approved by the Ethics Committee of the Ruhr-University Bochum (No. 20-7008). The number of eligible data of the parameters varied due to missing or small sample volumes in patients and controls.

TABLE 1 Characteristics of the participating patients with Phenylketonuria (PKU) and controls (Co).

Parameter	PKU	Co	p	Effect size
n	18	19	–	–
Age (years)	37.9 (23.6–45.0)	38.3 (23.8–46.5)	0.869	–0.03 [⊖]
Female, n (%)	11 (61%)	10 (53%)	0.603	–
Height (cm)	167.3 ± 9.3	176.4 ± 9.7	0.006	–0.95[~]
Weight (kg)	70.1 ± 17.3	76.4 ± 14.5	0.235	–0.40 [~]
BMI (kg/m ²)	23.6 (22.1–26.8)	24.5 (21.4–26.3)	0.916	–0.02 [⊖]
Smoking habits, n (%)				
Never	10 (56%)	11 (58%)	0.708 ^a	–
Not any more	2 (11%)	4 (21%)		
Sometimes	3 (17%)	1 (5%)		
Daily	3 (17%)	3 (16%)		
Heavy smoking according to RKI [26]	2 (11%)	2 (11%)	1.000 ^a	–
Pack-years	0.0 (0.0–2.5)	0.0 (0.0–10.0)	0.711	–0.06 [⊖]
Not fit 6MWT, n (%)	6 (33%)	3 (16%)	0.269 ^a	–
ΔDistance 6MWT	16.3 (–48.0–70.7)	50.3 (3.0–129.0)	0.134	–0.25 [⊖]
SES	6.7 ± 2.2	8.6 ± 2.6	0.021	–0.79[~]
WISH	60.6 ± 16.4	59.4 ± 18.3	0.846	0.07 [~]
Protein-rich foods (% [#])	8.6 (3.5–15.0)	32.3 (21.3–48.8)	< 0.001	–0.58[⊖]
Women who were pregnant before, n (%)	3 (27%)	5 (50%)	0.387 ^a	–
Phe during last 5 years (μmol/L)	591 ± 340	–	–	–
Patients with mean blood Phe > 600 μmol/L, n (%)	7 (47%)	–	–	–

^aFisher’s exact test; [~]Cohen’s d; [⊖]Pearson’s r; [#]ratio of protein- to carbohydrate-rich food items. 6MWT, 6-min walk test; ΔDistance 6MWT, difference between walked distance and predicted distance 6MWT (m); BMI, body mass index; RKI, Robert Koch Institute; SES, socioeconomic status; WISH, World Index for Sustainability and Health; Phe, Phenylalanine. Data are reported as median (25th–75th percentile) (non-normal distribution) or as mean ± standard deviation (normal distribution). Significant results are marked bold.

2.2 Anthropometric data, lifestyle, and nutrition

Dietary habits were recorded by a validated self-administered food frequency questionnaire on 53 food and drink items previously used in the German Health Interview and Examination Survey for Adults 1 (DEGS1) (22, 23) and extended by current use of AA supplementation in patients with PKU. Dietary quality was calculated by the World Index for Sustainability and Health (WISH) using the calculated daily intake of the 53 items of the food frequency questionnaire (24, 25). For further analysis of food intake, healthy and unhealthy subsections were calculated. Healthy subsection included vegetables, fruits, nuts, fish, poultry, whole grains, fish, milk and dairy products, and eggs, whereas unhealthy subsection included red meat, saturated fats, sugar sweetened beverages, sugary and salty snacks, and spreads (24). As mentioned in the introduction, adult patients with PKU tend toward a vegan diet, excluding most protein-rich foods and substitute these with carbohydrate-rich foods, which, in turn, might be disadvantageous for bone health. Therefore, food items were categorized into protein-rich (milk and dairy products, eggs, white and red meats, fish, and legumes) and carbohydrate-rich

(fruit juices, fruits, vegetables, grains, potatoes, rice, sweets, and snacks) items, and proportions of these were calculated. As fats were only estimated from butter and oil usage, proportions of these were not calculated.

Dietary compliance of patients with PKU was recorded from blood Phe concentrations during the last 5 years prior to study participation. As all participants were adults at time of study participation, the target Phe levels during the 5 years before recruitment were 120–600 μmol/L, according to the European guidelines (1). For evaluation, the mean blood Phe concentration during the last 5 years was calculated, and patients were grouped into good compliance when their mean blood Phe concentration during the 5 years stayed below 600 μmol/L or poorly compliant, when their mean blood Phe concentration during the 5 years was above 600 μmol/L.

Lifestyle factors such as highest level of educational, socio-economic factors, smoking habits, current medication, and physical activity were examined by self-administered questionnaire previously used in DEGS1 (23). Fractures during the last 12 months prior to study participation were recorded by self-administered questionnaire and examination by the responsible physician. In female participants, the number of pregnancies and

births, the use of hormonal contraceptives, the status of menopause (defined as no menstruation in the prior 12 months), and hysterectomy or ovariectomy were recorded. Smoking habits were recorded as previous and current smoking, with additional classification of heavy smokers as defined by Robert Koch Institute (RKI) (26), and pack-years were calculated (27). Socioeconomic status (SES) was calculated on the basis of the work by Lampert et al. (2013) (28), including highest education and employment status of the main earner among the household members. Here, household income was not included in the SES; therefore, the lowest SES possible was 2 points, with the highest points being 14. A 6-min walk test (6MWT) was used for further examination of physical fitness. Participants were instructed to walk along a corridor of 36 m for 6 min as quickly as possible, whereas the distance was recorded by study personnel. Participants were defined as physically active if they achieved > 96% of the predicted distance in the 6MWT calculated by considering age and gender of participants (29, 30).

BMD from dual X-ray absorptiometry (DXA) was either acquired from records of patients with PKU or measured wherever indicated, expressed as T- and in Z-scores at the left proximal femur (hip) and at the lumbar spine (L1-L4; LS). DXA device used was Hologic Delphi C (Hologic Medicor GmbH, Berlin, Germany), each standardized for gender, weight, and ethnicity by the provider.

2.3 Sampling and biochemical analyses

Unfasted venous blood was drawn in ethylenediaminetetraacetic acid (EDTA) monovettes and monovettes for serum collection (Kabe, Nümbrecht-Elsenroth, Germany). EDTA blood was centrifuged at 3,000 rpm at 4°C for 10 min. Serum was incubated at least 30 min at room temperature to clot, centrifuged at 3,000 rpm at 4°C for 10 min, and the plasma and serum were stored at −80°C until analysis. Spot urine samples were collected and stored at −80°C until further analysis. In plasma, serum, and urine bone metabolism-related parameters (BMRPs), markers of oxidative stress and anti-oxidative vitamins were analyzed. Intact parathyroid hormone (PTH), 25-hydroxy vitamin D3 (25-OH D), cross-linked C-telopeptide of type I collagen (CTX), deoxypyridinoline (DPD), pyridinoline (Pyr), insulin-like growth factor 1 (IGF1), total alkaline phosphatase (ALP), and osteocalcin (OCN), as well as anorganic phosphate, calcium, and creatinine in urine and serum and were analyzed at the MVZ Dr. Eberhard & Partner GbR Dortmund, Germany.

Analytical assays for anorganic phosphate in serum and urine, calcium in serum and urine, ALP, creatinine in serum and urine, and C-reactive protein (CRP) were performed on an automated clinical chemistry module cobas c701 (Roche Diagnostics GmbH, Mannheim, Germany). 25-OH D, IGF1, CTX, and PTH were analyzed via ECLIA technology on an automated cobas 8000 e801 analytical unit (Roche Diagnostics, Mannheim, Germany). OCN was analyzed on the fully automated immunodiagnostic Liaison XL platform (DiaSorin, Saluggia, Italy) using CLIA technology. Here, OCN assay detects both the intact OCN (1–49) and the N-terminal midfragment (1–43). Intra-assay and inter-assay coefficients of

variation (CVs) were, respectively, the following: 0.6% and 0.7% for phosphate in serum, 0.6% and 1.1% for calcium in serum, 0.7% and 0.8% for creatinine in serum, 0.4% and 1.1% for ALP, 2.6% and 3.5% for 25-OH D, 1.2% and 1.5% for CTX, 0.8% and 1.4% for IGF-1, 0.9% and 1.0% for PTH, 1.4% and 2.0% for OCN, 0.6% and 0.8% for CRP, 1.2% and 1.9% for urinary calcium, 0.9% and 2.4% for urinary creatinine, and 0.9% and 1.2% for urinary anorganic phosphate. Analysis of Pyr and DPD was performed on a modular Ultra high pressure liquid chromatography (UHPLC) system with fluorescence detector (Waters Acquity H-Class system, equipped with binary solvent manager, autosampler with flow through needle and column manager, all from Waters GmbH, Eschborn, Germany) using an Acquity UPLC HSS T3 Column (1.8 μ m, 2.1 $\times$ 100 mm, Waters GmbH, Eschborn, Germany). Separation was achieved using a gradient of mobile phase A (0.2% heptafluorobutyric acid in water) and mobile phase B (acetonitrile) at a flow rate of 0.5 mL/min. Excitation wavelength was 290 nm, and emission wavelength was 395 nm. After hydrolysis of 250 μ L of the sample material with 250 μ L of 25% hydrochloric acid and 50 μ L internal standard (#48004) at 100°C for at least 12 h, extraction was achieved by addition of 2.5 mL of extraction buffer (#48005, both, Chromsystems Instruments & Chemicals GmbH, Gräfelfing/Munich, Germany). The sample clean-up columns (#48008) were primarily conditioned with 2.0 mL of wash buffer (#68007). Subsequently, SPE-Columns were loaded with the hydrolyzed sample material and washed three times with 2.5 mL of wash buffer. Elution of the analytes was achieved using 1 mL of elution buffer (#68010, all Chromsystems Instruments & Chemicals GmbH, Gräfelfing/Munich, Germany). The eluate (5 μ L) was injected into the HPLC system. The calibration standard (#48003) and the two control levels (#0046 and #0047, all Chromsystems Instruments & Chemicals GmbH, Gräfelfing/Munich, Germany) were prepared analogous to the patient sample material for daily calibration and quality control. Intra-assay and inter-assay CVs were, respectively, 1.1% and 2.9% for low levels (315 μ g/L) and 1.5% and 3.3% for high levels (911 μ g/L) of Pyr, as well as 1.6% and 3.5% for low levels (63 μ g/L) and 1.5% and 3.2% for high levels (167 μ g/L) of DPD. Urinary analytes calcium, Pyr, and DPD were normalized to creatinine excretion. In serum, osteoprotegerin (OPG) and tartrate-resistant acid phosphatase 5b (TRAP) were measured by using commercially available ELISA kits (OPG: Abcam Limited, Cambridge, UK; TRAP: BoneTRAP® Immunodiagnostic Systems, Bordon Colliery, UK) according to the manufacturer's specifications.

25-OH D was used for evaluation of vitamin D sufficiency, as suggested by the Institute of Medicine (IOM) (31). To evaluate bone health, bone pathology harbinger (BPH) score was calculated as suggested by Schündeln et al. (32). For this score, one point was given if the levels of PTH, OCN, ALP, or DPD were outside the normal range, knee pain on exercise or spontaneous back pain occurred, and if BMD Z-score in any area was below −2. The BPH was calculated if at least five of these items were available, and points were summed up and divided by available items to achieve a score between 0 and 1.

Total oxidative status was measured as total lipid peroxides using a commercially available photometric test system (Immuchrom GmbH, Heppenheim, Germany) according to the

manufacturer's instructions. Intra-assay and inter-assay CVs were 3.6% each for total lipid peroxides. Additionally, vitamin E (Vit E) and vitamin C (Vit C) were measured. Analysis of Vit E was performed on a modular UHPLC system with dual wavelength UV-VIS detector (LC-20ADXR pump, DGU-20A3R degassing unit, SIL-20ACXR autosampler, CTO-20AC column oven, SPD-20AV UV-VIS detector, and CBM-20A communications bus module, all from Shimadzu Deutschland GmbH, Duisburg, Germany) using the Conformité Européenne - In vitro diagnostics (CE-IVD) validated Chromsystems One Step Vitamin A and E in Serum/Plasma – UHPLC analysis kit (#34900/UHPLC, Chromsystems Instruments & Chemicals GmbH, Gräfelfing/Munich, Germany). Mobile phase and UHPLC column are part of the analysis kit. UV detection was performed at 295 nm. Serum (100 μ L) was vortexed with 200 μ L of the pre-mixed solution (#34966, Chromsystems Instruments & Chemicals GmbH, Gräfelfing/Munich, Germany) for 30 s and centrifuged for 10 min at 14,000 U/min. The supernatant (5 μ L) was injected into the HPLC system. Intra-assay and inter-assay CVs were, respectively, 2.3% and 4.6% for low levels (9 mg/L) of Vit E and 1.0% and 4.7% for high levels (19 mg/L) of Vit E. Analysis of Vit C was performed on a Waters Alliance e2695 HPLC system with dual wavelength Waters 2489 UV-VIS detector (Waters GmbH, Eschborn, Germany) using the CE-IVD-validated Chromsystems Vitamin C in Plasma/Serum – HPLC analysis kit (#65065, Chromsystems Instruments & Chemicals GmbH, Gräfelfing/Munich, Germany). Mobile phase and HPLC column are part of the analysis kit. UV detection was performed at 245 nm. For sample preparation, 100 μ L of serum and 100 μ L of Internal Standard (#65044, Chromsystems Instruments & Chemicals GmbH, Gräfelfing/Munich, Germany) were vortexed for 30 s and centrifuged for 5 min at 14,000 U/min. The supernatant (20 μ L) was injected into the HPLC system. The kit calibration standard (#65003) and the two control levels (#0074, both Chromsystems Instruments & Chemicals GmbH, Gräfelfing/Munich, Germany) were prepared analogous to the patient sample material for daily calibration and quality control. Intra-assay and inter-assay CVs were, respectively, 2.1% and 3.5% for low levels (6 mg/L) and 3.2% and 2.5% for high levels (21 mg/L) of Vit C.

2.4 Statistical analyses

The statistical software package IBM® SPSS® Statistics for Windows, version 29.0 (IBM Corp., Armonk, NY, USA), was used for the statistical analyses. Descriptive data were analyzed by the Chi-squared test or, where applicable, by Fisher's exact test. Shapiro–Wilk test was used to evaluate for normal distribution in small groups. The QQ plots were used to test for normal distribution in groups larger than $n = 10$. Normally distributed data were analyzed using parametric tests. Between-group comparisons of normally distributed data were analyzed with Student's t-test; for comparison to general population, one sample t-test was used (9, 33). For normally distributed data, effect size was calculated using Cohen's d for groups larger than 20 and Hedges g for groups smaller than 20 observations. Non-normally distributed data were analyzed using non-parametric tests (Mann–Whitney

test). Non-normally distributed data effect sizes were calculated by Pearson's r. Values of $p < 0.05$ were considered significant. Linear regression analysis with heteroscedasticity consistent co-variance matrix (HC3) was applied to evaluate associations between potential influencing factors and BMD. Normally distributed data are presented as mean $\pm$ standard deviation (SD), non-normally distributed data as median (25th–75th interquartile range). Results from DXA scans, height, and weight were compared to population by one-sample t-test.

3 Results

3.1 Study population characteristics

Eighteen adult patients with PKU and 19 age- and gender-matched controls participated in the study (PKU: 18.3–51.6 years; Co: 18.3–54.9 years; [Table 1](#)). Neither smoking habits nor body mass index (BMI), current education, or fitness according to 6MWT differed between healthy controls and patients with PKU ([Table 1](#)). Although, patients with PKU were significantly shorter than controls ($p = 0.006$ for both sexes, [Table 1](#); female PKU: 161.9 ± 6.8 cm vs. Co: 169.5 ± 5.5 cm, $p = 0.012$, Hedges' $g = -1.17$; male PKU: 175.7 ± 5.6 cm vs. Co: 184.0 ± 7.4 cm, $p = 0.028$, Hedges' $g = -1.17$), patients with PKU were of normal height for the general population and weight did not differ between groups [[Table 1](#), [Supplementary Table S1](#) (33)]. Controls had a significantly higher SES than patients with PKU ($p = 0.021$, [Table 1](#)). One control subject reported a bone fracture within the 12 months prior to participating in the study; none of the patients with PKU reported any fractures during the past 12 months. Nutrition, as evaluated by WISH, did not differ between patients with PKU and controls, whereas patients with PKU consumed significantly smaller proportions of protein-rich foods and significantly higher proportions of carbohydrate-rich foods, compared to controls. While four control subjects were menopausal, no patient was (data not shown).

Blood Phe concentrations during the 5 years prior to study participation were available from 15 patients with PKU who had a mean blood Phe concentration of $591 \mu\text{mol/L}$ ($\pm 340 \mu\text{mol/L}$; min. $170 \mu\text{mol/L}$; max. $1,500 \mu\text{mol/L}$) during this time period ([Table 1](#)).

3.2 Bone metabolism-related parameters and parameters of oxidative stress in patients with PKU and controls

Neither the bone formation markers ALP, IGF1, and OCN nor the bone resorption markers Pyr, DPD, and CTX or BPH differed between the groups of PKU and Co ([Figure 1](#)). Only 25-OH D was significantly higher in patients with PKU [33.1 ng/mL (26.2–40.3)] than in controls [23.4 ng/mL (17.2–24.9); $p < 0.001$; $r = -0.53$], without a significant difference between the groups in PTH plasma concentration [PKU: 27.6 pg/mL (19.6–42.8) vs. Co: 36.16 pg/mL (29.2–40.8); $p = 0.089$; $r = -0.29$]. Although, no significant difference in vitamin D sufficiency can be reported ($p = 0.114$), 89% of the participants with PKU were vitamin D-sufficient,

whereas 68% of controls were sufficient in vitamin D (data not shown). One participant in each group had 25-OH D serum concentration above 50 ng/mL but below 100 ng/mL. Parameters of oxidative stress, Vit C, and Vit E did not differ between patients with PKU and controls (Supplementary Table S2).

BMRP did not differ between patients with mean Phe below 600 $\mu\text{mol/L}$ during the 5 years prior to study participation (good compliance) and those with mean Phe above 600 $\mu\text{mol/L}$ or no measurements of Phe (poor compliance, Supplementary Table S3). Only Vit C was higher in patients with good compliance (12.3 ± 2.7 mg/L) compared to those with poor compliance (8.7 ± 3.4 mg/L, $p = 0.031$, Hedges' $g = 1.10$), whereas CRP tended to be lower in patients with good compliance [0.06 mg/dL (0.04 – 0.17)] compared to those with poor compliance [0.21 mg/dL (0.09 – 0.45), $p = 0.083$, $r = -0.41$].

3.3 Bone mineral density in patients with PKU

DXA measurements were only available for patients with PKU. Out of the 18 patients, 17 had calculated T-scores for hips and LS, whereas Z-scores were available for all 18 patients. T-scores and Z-scores are depicted in Table 2. T-scores were significantly lower than zero, whereas Z-scores were only marginally reduced.

Eight patients (47.1%) with PKU had a T-score in the hip below the margin of -1 standard deviations and, therefore, met the definition of osteopenia, whereas seven (41.2%) had a T-score below -1 in LS. No significant difference was observed in T-scores or Z-scores of patients with mean Phe below 600 $\mu\text{mol/L}$ and those exceeding the 600 $\mu\text{mol/L}$ in the 5 years prior to study participation in hips (good compliance vs. poor compliance: T-

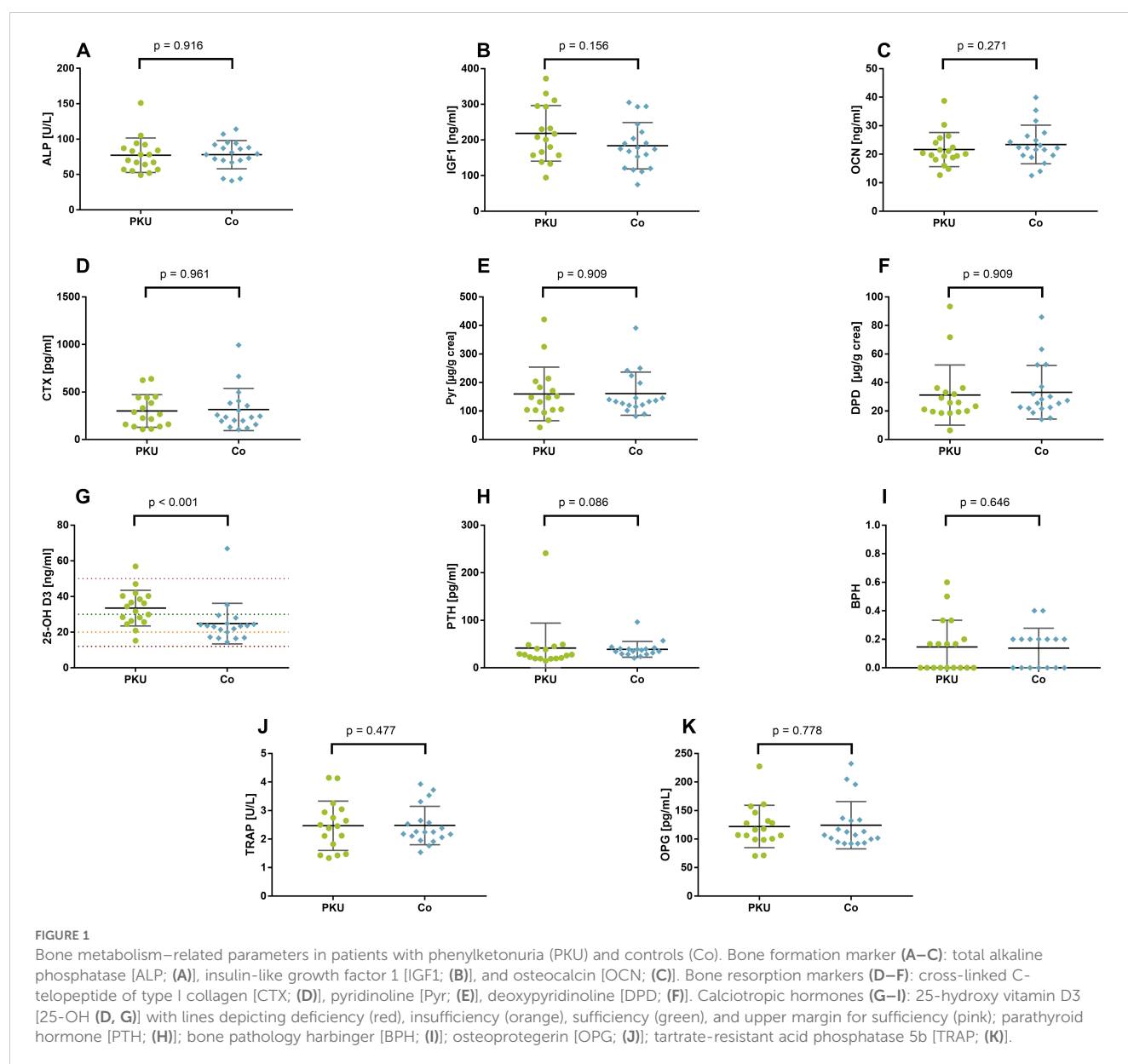


TABLE 2 Results from dual X-ray absorptiometry scans of patients with Phenylketonuria (PKU) in hips and lumbar spine (LS) compared to healthy 30-year-old reference population (T-score).

		PKU	p	Effect size
Hip	T-score	-0.668 ± 1.05	0.018	-0.61[†]
	Z-score	-0.419 ± 1.13	0.133	-0.36 [†]
LS	T-score	-0.712 ± 1.11	0.018	-0.61[†]
	Z-score	-0.517 ± 1.16	0.075	-0.43 [†]

[†]Hedges' g. Data are reported mean ± standard deviation (normal distribution). Significant results are marked bold.

score: -0.53 ± 1.22 vs. -0.79 ± 0.93 , $p = 0.613$; Z-score: -0.39 ± 1.21 vs. -0.45 ± 1.12 , $p = 0.918$, **Supplementary Table S4**) and LS (good compliance vs. poor compliance: T-score: -0.56 ± 1.18 vs. -0.84 ± 1.10 , $p = 0.618$; Z-score: -0.40 ± 1.21 vs. -0.61 ± 1.17 , $p = 0.714$, **Supplementary Table S4**).

3.4 Diet compliance and dietary behavior of patients with PKU in association with their bone health

Information on AA supplementation was missing from four patients with PKU, 13 patients with PKU took vitamin- and mineral-enriched AA supplements, whereas one patient did not consume AA supplements. Out of the 13 patients consuming AA supplements, 46.1% ($n = 6$) took supplements with glycomacropeptides (GMPs) (data not shown). Additionally, one patient was treated with BH₄ and AA supplementation, and no patient was treated with Pegvaliase. The patients taking GMP were significantly older than those taking AA [GMP: 45.1 (42.5–48.0) years vs. AA: 23.8 (19.7–25.2) years; $p = 0.002$; $r = -0.79$].

During the last 5 years prior to study participation, three patients with PKU did not evaluate their blood Phe concentration. Out of the remaining 15 patients with PKU who had measured their blood Phe concentration, eight patients had mean blood Phe concentration below 600 μmol/L during the 5 years prior to study participation and were, therefore, classified as well compliant to treatment. Seven patients had mean blood Phe above

600 μmol/L during the 5 years prior to study participation and were, therefore, poorly compliant; as guidelines suggest, blood Phe concentrations in adults should stay below 600 μmol/L (1). The three participants who during the prior 5 years never evaluated their blood Phe concentrations were also grouped as poorly compliant.

In addition, to dietary compliance the patients with PKU also differed in some lifestyle characteristics. Although patients with good compliance had a significantly higher SES (good compliance: 8.3 ± 1.2 vs. poor compliance: 5.4 ± 2.1 , $p = 0.003$, Hedges' $g = 1.57$), they also smoked significantly less [pack-years: good compliance: 0 years (0–0) vs. poor compliance: 1.5 (0–4.5), $p = 0.034$, $r = -0.55$; **Table 3**; **Supplementary Table S3**] and walked further than expected during the 6MWT [good compliance: 55.9 m (10.1–173.3) vs. poor compliance: -9.9 m (-139.4 – 37.4), $p = 0.027$, $r = -0.52$]. No difference was observed in age, BMI, or gender distribution, as well as WISH score (**Table 3**).

Patients with PKU and low hip BMD had a significantly lower WISH ($p = 0.010$; **Table 3**) than patients with BMD above -1 . Here, patients with PKU and low hip BMD reached significantly less points in the unhealthy food section of WISH than patients with BMD above -1 (below -1 : 7.5 ± 6.6 vs. above -1 : 18.3 ± 6.4 ; $p = 0.005$; Hedges' $g = 1.58$) and tended to reach lower points in healthy food section of WISH (below -1 : 42.1 ± 9.2 vs. above -1 : 50.6 ± 7.7 ; $p = 0.065$; Hedges' $g = 0.95$). Additionally, patients with BMD below -1 tended to consume higher proportion of protein-rich foods ($p = 0.063$) than patients with BMD above -1 (**Table 3**), whereas none of these differences were observed in LS patients with high vs. low LS BMD. Patients with a LS BMD above -1 were significantly less often active during their everyday life, as suggested by results of the 6MWT. None of the other basic parameters were different between patients with PKU with high versus low LS BMD (**Table 3**; **Supplementary Table S4**).

3.5 Association of physiological, lifestyle, and disease parameters with bone mineral density in patients with PKU

In the regression analysis, only body weight was positively associated with hip and LS BMD, whereas neither lifestyle factors

TABLE 3 Comparison of characteristics of the participating patients with Phenylketonuria with bone mineral density (BMD) T-score above -1 and below -1 in either hips or lumbar spine (LS).

Parameter	Hip BMD T-score		p	Effect size	LS BMD T-score		p	Effect size
	Over -1	Below -1			Over -1	Below -1		
n	9	8	–	–	10	7	–	–
WISH	68.9 ± 11.8	49.5 ± 14.0	0.010	1.42[†]	59.3 ± 19.4	59.1 ± 11.8	0.984	0.01 [†]
Protein-rich foods (%#)	7 ± 5	17 ± 13	0.063	-0.95 [†]	11 ± 11	13 ± 12	0.766	-0.14 [†]
Phe during last 5 years (μmol/L)	543 ± 233	674 ± 531	0.526	-0.34 [†]	672 ± 363	386 ± 255	0.180	0.79 [†]
Ratio of all Phe measurements over 600 μmol/L	80 (10–100)	100 (0–100)	0.837	-0.07 [⊖]	90 (15–100)	67 (0–100)	0.875	-0.04 [⊖]

#percentage from sum of carbohydrate-rich and protein-rich foods; [†]Hedges' g; [⊖]Pearsons r; 6MWT, 6-min walk test; ΔDistance 6MWT, difference between walked distance vs. predicted distance 6MWT (m); BMI, body mass index; SES, socioeconomic status; Phe, Phenylalanine; WISH, World Index for Sustainability and Health. Data are reported as median (25th–75th percentile) (non-normal distribution) or as mean ± standard deviation (normal distribution). Significant results are marked bold.

nor mean Phe during the last 5 years were associated with BMD (Table 4).

4 Discussion

As the main symptom of PKU, neurologic damages, is minimized by treatment and patients are living a longer and healthier life, comorbidities have started to become prevalent. One of these comorbidities frequently described is a reduced BMD. About 40%–60% of patients with PKU are affected by low bone health, already showing first signs in childhood and adolescence (34, 35). The aim of our study was to determine the influence of nutrition, midterm dietary compliance, and lifestyle factors on bone health in patients with PKU. Whereas 47.1% of the participating patients with PKU met the criteria for osteopenia based on DXA assessment at the left hip, no differences in BMRP in patients with osteopenia and no-osteopenia or controls were detectable. A recent meta-analysis found similar results with BMD in patients with PKU being significantly lower than in the general population and 42% of patients being classified as low BMD (Z -score ≤ -1) (36). As previously described (37, 38), patients with PKU tend toward better 25-OH vitamin D status than the general population, which was also observed in this cohort. Patients with PKU and controls were included on the same day; therefore, seasonal differences in vitamin D status are unlikely to have led to higher 25-OH D concentration in patients with PKU. A higher vitamin D ingestion from AA supplements is probably the cause for the higher 25-OH D serum concentration (39). Nagasaka et al. reported higher bone resorption in patients with PKU and lower 25-OH D than controls (40). Compared to that study, patients in this study were older and had higher 25-OH D, which may explain why no differences in bone resorption were observed. However, BMD-results were comparable to the study by Nagasaka et al. (40). Whereas total body BMD was significantly reduced in Greek children and adolescents with PKU and also in LS BMD of children and adolescents with mild hyperphenylalaninemia, serum calcium (Ca), protein, and ALP were not different in these patients (8). Modan-Moses et al. observed BMD Z -scores in adult patients slightly lower than in these patients (34). In 183 patients with PKU from eight different metabolic centers in Europe, significantly reduced BMD Z -scores in LS, femoral neck, and total proximal femur were observed (9). These results were included into a recent meta-analysis that found significantly reduced LS BMD Z -scores in the pooled 304 adolescent and adult patients with PKU out of the 10 included studies (36). Patients in our study had comparably lower BMD Z -scores in LS and hip to the patients in the meta-analysis, although results missed significance, despite relatively normal BMRPs, probably due to the small sample size and the large range of measurements. Whereas skeletal changes that affect BMD take some time to become obvious, BMRPs represent actual changes in the activity and regulation of osteoblasts and osteoclasts *in situ* (41). A direct correlation of BMRPs and BMD is not guaranteed (41). Whereas some studies found relationships of some BMRPs and BMD in parts of the participants, others did not find any relationship of BMD and BMRPs (41–43).

However, even subclinical changes in BMRP can add up to changes in BMD over time (44–46). Therefore, lower BMD readings in the study participants could be a result of accumulated shortcomings in bone mass formation over several years before study participation. Several studies found reduced BMD or bone quality in pediatric and adolescent patients (35, 47–49), with low serum concentrations of bone-specific ALP, OCN, and Ca and lower urinary creatinine than controls and reduced bone turnover (45, 48). Tanaka et al., however, reported improvements in bone quality in participating children and adolescents with PKU, following only 34 days of Ca supplementation (47). The recommended initial treatment for low BMD in adolescents with PKU consists out of optimization of diet and physical activity, followed by further analysis of other factors and treatments in case of persistent low BMD measures (1). The exact onset of impaired skeletal mineralization in patients with PKU is unclear and further research is needed to evaluate the onset of bone mass reduction in patients with PKU to start treatment as early as possible.

As in the general population (50), there was a trend toward better BMD with higher BMI in the present study, whereas Doulgeraki et al. did not find differences in BMI between patients with PKU and controls or correlation between BMI and BMD (8). As BMD accrual depends on muscle-bone interaction, reduced muscle mass in favor of higher fat mass could have led to reduced bone mass accrual in the patients with PKU. Choukair et al. observed significantly reduced volumetric total BMD in the radius in a group of 56 adolescent and adult patients with PKU. This finding was associated with reduced grip strength, but the association was weaker than in controls. Therefore, the authors concluded that, in addition to a reduced muscle size, the adaptation of bones to loading is reduced in patients with PKU (51). In the present study, patients with PKU had similar everyday activity compared to controls, but a weaker performance in the 6MWT. Others observed a significantly reduced BMD and maximal capacity in incremental workload in patients with PKU who discontinued AA supplements compared to healthy controls, whereas patients who adhered to the dietary treatment only showed a slightly reduced maximal capacity (11). Furthermore, lower lean body mass Z -scores than expected were observed in adolescents and young adults with PKU who had blood Phe over 600 $\mu\text{mol/L}$ (52). Reduced muscle accrual during adolescents and early adulthood could have led to reduced bone mass accrual in the patients with PKU, but we are lacking data on lean body mass and 6MWT might not have been sensitive enough to detect differences in this cohort. As in adolescents and young adults with high blood Phe concentrations, lean body mass of adolescents with inherited AA metabolism disorders other than PKU also was significantly smaller than in controls (53). In a small study population ($n = 15$), men with PKU had low-normal lean body mass, whereas women with PKU had significantly higher body fat mass than men (54). In a larger study population, patients with non-classical PKU ($n = 38$) not only showed a significantly higher proportion of body fat than patients with classical PKU ($n = 58$) but also were taller than these, which was predicted by Phe concentration in serum (14). Reduced protein consumption could lead to the observed changes in body composition. Therefore, Evans et al. identified a sweet spot of 12%–

TABLE 4 Regression analysis on influencing factors bone mineral density (BMD) in hip and lumbar spine (LS).

Dependent variable	Block	Variables	B	Standard error ^a	95% CI		p
					Lower limit	Upper limit	
Hip BMD T-score	Physiological	Age (years)	−0.035	0.029	−0.097	0.026	0.238
		Height (cm)	−0.056	0.044	−0.151	0.039	0.225
		Weight (kg)	0.049	0.009	0.029	0.069	<0.001
	Lifestyle	WISH	0.046	0.025	−0.009	0.101	0.092
		SES	−0.071	0.128	−0.352	0.210	0.590
		Pack-years	0.090	0.163	−0.268	0.447	0.593
		ΔDistance 6MWT	0.006	0.005	−0.005	0.017	0.274
	Disease	Mean Phenylalanine last 5 years (μmol/L)	0.000	0.001	−0.003	0.002	0.748
Hip BMD Z-score	Physiological	Age (years)	−0.029	0.027	−0.087	0.029	0.301
		Height (cm)	−0.068	0.038	−0.149	0.012	0.090
		Weight (kg)	0.054	0.010	0.033	0.075	<0.001
	Lifestyle	WISH	0.050	0.026	−0.006	0.107	0.077
		SES	−0.155	0.158	−0.498	0.188	0.345
		Pack-years	0.074	0.188	−0.335	0.483	0.701
		ΔDistance 6MWT	0.004	0.005	−0.006	0.014	0.393
	Disease	Mean Phenylalanine last 5 years (μmol/L)	0.000	0.002	−0.004	0.003	0.862
LS BMD T-score	Physiological	Age (years)	−0.023	0.036	−0.101	0.056	0.546
		Height (cm)	−0.047	0.049	−0.153	0.059	0.353
		Weight (kg)	0.036	0.013	0.007	0.064	0.019
	Lifestyle	WISH	0.014	0.046	−0.088	0.115	0.774
		SES	0.045	0.219	−0.436	0.526	0.842
		Pack-years	0.017	0.183	−0.386	0.420	0.929
		ΔDistance 6MWT	00.000	0.007	−0.015	0.015	0.999
	Disease	Mean Phenylalanine last 5 years (μmol/L)	0.000	0.001	−0.001	0.002	0.550
LS BMD Z-score	Physiological	Age	−0.005	0.031	−0.071	0.062	0.878
		Height (cm)	−0.042	0.038	−0.124	0.040	0.293
		Weight (kg)	0.037	0.013	0.009	0.065	0.012
	Lifestyle	WISH	0.017	0.047	−0.085	0.118	0.729
		SES	0.060	0.194	−0.362	0.482	0.761
		Pack-years	0.043	0.220	−0.435	0.522	0.847
		ΔDistance 6MWT	0.001	0.007	−0.014	0.016	0.929
	Disease	Mean Phenylalanine last 5 years (μmol/L)	0.001	0.001	−0.001	0.003	0.550

ΔDistance 6MWT, difference between walked distance vs. predicted distance 6MWT (m); 6MWT, 6-min walk test. Significant results are marked bold. ^aCorrected by the HC3 method.

18% of energy from all protein sources combined to ensure healthy growth and reduced body fat mass of children with PKU (55). As the diet of patients with PKU excludes most animal foods such as meat, dairy products, and fish due to their high protein content, one could argue that the diet is comparable to that of vegans. A meta-analysis of studies with vegan children reported lower bone mineral content (BMC), smaller height, and lower protein intake, although recommended ranges for body height, calcium, and protein intake were still attained (56). The amount of protein consumption is discussed to influence the development of osteoporosis and its progression. Current literature on the consumption of milk and dairy products suggests that a higher intake is beneficial, whereas the consumption of plant proteins seems not to be detrimental as long as an adequate amount of micronutrients such as Ca and vitamin D are consumed (57). Furthermore, Kędzia et al. discussed a benefit for BMD in case of carbohydrate reduction in favor of protein consumption especially in women (57). As patients with PKU need to reduce their intake of natural protein, they consumed a higher proportion of carbohydrate-rich foods in this study population. Inappropriate protein supply by reduced supplement intake in some patients could, therefore, account for some reduction in BMD, as previously reported in 43 patients with PKU (58), as well as in patients with inherited AA metabolism disorders other than PKU (53). As we were unable to calculate the exact uptake of protein from natural protein and supplements, we cannot conclude if reduced protein supply at some point during the patient's life has negatively affected BMD, even though the amount of natural protein rich foods consumed by the patient was lower than in controls.

Overall, we did not observe differences in nutrition between controls and adult patients with PKU in WISH, but major food groups like milk and meats were frequently substituted by patients with PKU, which did not influence WISH. We observed higher WISH in patients without osteopenia than in patients with osteopenia. As WISH can be split into an unhealthy subsection and a healthy subsection (24), we applied these subsections to analyze differences in nutrition of patients with and without osteopenia. Patients with osteopenia consumed higher amounts of foods in the unhealthy subsection of WISH (red meat, saturated fats, sugar sweetened beverages, sugary and salty snacks, and spreads) than patients with higher BMD. A negative association of BMD or BMC and unhealthy food patterns has been described previously (59). As high salt intake could negatively impact calcium metabolism, salty snacks are advised to be consumed in low amounts (19). In a large cohort of older adults ($n = 4,028$), adherence to a food pattern with higher amounts of fruits, vegetables, and dairy products was associated with a lower risk of osteoporotic fractures, whereas adherence to a food pattern defined by sweets, animal fat, and low meat was associated with a higher hazard for these (60). Additionally, a 2021 meta-analysis found a significant inverse association between the intake of sugar-sweetened beverages and BMD, especially in adults younger than 30 years old (61).

Previously, it was suggested that elevated Phe concentrations could influence bone health negatively (10, 52, 62, 63). However,

there was no correlation of mean blood Phe in the year prior to study participation and BMD in two studies on patients with PKU (34, 64). Similarly, we did not observe differences in mean Phe in the 5 years prior to participation in patients with PKU with osteopenia and those without osteopenia or significant result in the regression analysis. Coakley et al. found no significant association between dietary Phe:tyrosin ratio, but a significant influence of compliance to diet and BMD Z-score (49). As almost 70% of these study participants were underage, a stricter dietary regime and a broader deviation of dietary regimes can be expected than in participants of the present study. Furthermore, a significantly higher current Phe serum concentration was observed in patients with PKU than in patients with untreated hyperphenylalaninemia, without a difference in BMD between these groups (14). Roato et al. observed a reduced phalangeal quantitative ultrasound (a parameter for bone quality) in patients with Phe blood concentration over 600 $\mu\text{mol/L}$ (10). However, these patients were also younger than the participants of this study, especially those with low Phe concentration (10).

Next to blood Phe influencing bone mass, medical foods might influence bone mass as well. Acid load on kidneys is discussed to have a negative effect on BMD in patients with PKU, and, previously, a very high acidity of low Phe preparations was a problem in treatment (16, 65). Up until today, higher renal acid load is observed in patients with PKU consuming AA supplements, compared to patients consuming GMP (16). After lifelong AA consumption, a switch to GMP resulted in lower urinary Ca and magnesium excretion (16). Additionally, male patients with PKU consumed higher amounts of AA and showed higher renal Ca excretion and simultaneously lower BMD Z-scores than female patients with PKU (54). Therefore, higher renal loss of Ca is discussed to result in reduced BMD in patients consuming AA mixtures (16, 66). Because only few patients, who were significantly older than the patients taking AA, were consuming GMP in this study, BMRPs were not compared between these groups.

4.1 Limitations

Despite intensive efforts to recruit, the number of participating patients was quite small, making comparisons between subgroups impossible. As loss to follow-up in adults with PKU is a large issue (21), not only in Germany (67), recruitment in additional specialized clinics would be needed for further analyses. Additionally, it was not possible to obtain DXA scans for healthy controls; thus, the results were solely compared to a reference population. We calculated a BPH to account for this problem, classifying participants by BMRP within and out of reference margins. Moreover, as this was a retrospective cross-sectional study, we cannot determine whether the reduced BMD observed in the patients with PKU persisted throughout their lives or developed only recently. Furthermore, Phe blood concentrations in the 5 years prior to study participation were only available for some of the study participants, probably because some participants were no longer in regular care of a specialized center. Regrettably,

we do not have data on early childhood treatment, information on genetic mutation or time of diagnosis. Because we have data on Phe concentration or treatment of 17 patients with PKU, all of these can be classified as having classical PKU. Additionally, only usual foods could be included into the calculation of the WISH score (24, 25); therefore, a potential influence of different AA supplements or specialized food could not be considered in the evaluation of the usual diet. Because adult patients with PKU tend to eat a vegan-like diet with a wider variety of natural foods than children (1, 5), we did not observe a difference between healthy adults and patients with PKU in regards of the WISH score.

5 Conclusion and perspective

In summary, almost half of the participating adults with PKU had reduced BMD in the left hip on DXA examination, but no differences in BMRP or lifestyle factors were seen in patients with osteopenia, without osteopenia or in controls. In addition, dietary adherence in the 5 years prior to study participation was not associated with observed BMD. Further studies are needed to assess the first occurrence of bone loss in patients with PKU and to optimize treatment options. Further improvements in AA supplementation could lead to better outcomes, like it has been observed with some GMP products. In addition, other mediating factors such as the gut microbiome, low muscle mass, or low-grade inflammation could influence the formation and resorption of bone mass and require future research.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Ruhr-University Bochum. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

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Author contributions

BH: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. AS: Methodology, Writing – review & editing. CG: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. TL: Resources, Supervision, Writing – review & editing. KS: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2025.1488215/full#supplementary-material>

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The inflection point: α -Klotho levels and the risk of all-cause mortality

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Purpose: The controversial nature of the association between α -Klotho and mortality risk in the general population warrants further investigation. This study aims to examine the correlation between circulating α -Klotho levels and the risk of all-cause mortality

Methods: A sample size of 13,748 individuals from the NAHNES 2005–2016 cycles was included in this study. The effect of different α -Klotho levels (divided into quartiles) on survival was assessed using Kaplan-Meier (KM) curves. Cox proportional hazards models were used to analyze the linear relationship between log α -Klotho and the risk of all-cause mortality. Restricted cubic spline Cox proportional hazards regression model was used to analyze the non-linear relationship between log α -Klotho and risk of all-cause mortality. Threshold effect analysis was performed to determine the most favorable inflection point for log α -Klotho. Stratification and sensitivity analyses were performed to assess the robustness of the results.

Results: A total of 1,569 deaths were reported during the median follow-up period of 5.33 years (2.83–7.83 years). Among the log α -Klotho quartile groups, quartile 1 had the highest mortality rate compared to quartiles 2, 3, and 4. Multifactorial Cox regression analysis revealed a weak association between log α -Klotho and a 44% reduction in the risk of all-cause mortality ($p=0.0473$). We also found a U-shaped non-linear association between log α -Klotho and risk of all-cause mortality, with an optimal inflection point identified at 2.89 pg/mL. The stability of the U-shaped association between log α -Klotho and mortality risk was observed in various stratification and sensitivity analyses.

Conclusion: This study identified a U-shaped association between circulating α -Klotho levels and risk of all-cause mortality, with a notable inflection point at 2.89 pg/mL. Further investigation is warranted to fully elucidate the potential mechanisms underlying the association between α -Klotho and risk of all-cause mortality in the broader population.

KEYWORDS

α -Klotho, all-cause mortality, NHANES, U-shaped relationship, inflection point

Introduction

In 1997, Kuro-o (1) and his research group demonstrated that the deletion of the Klotho gene resulted in accelerated aging in mice. It is hypothesized that this gene may have anti-ageing properties. Reduced levels of α -Klotho have been linked to an increased risk of developing a number of diseases. In the context of cardiovascular disease, low levels of Klotho have been associated with a number of adverse outcomes, including myocardial ischemia, cardiac hypertrophy, sinus node dysfunction and sudden cardiac death (2–4). Furthermore, in relation to nephropathic diseases, reduced Klotho has been associated with increased adverse clinical outcomes in patients with chronic kidney disease (CKD), including elevated creatinine levels, CKD progression and CKD-mineral bone disease (5–7). In tumors, Klotho expression is reduced in the majority of tumor samples, and low Klotho expression in tumors is associated with poorer overall survival (8).

Although α -Klotho has been shown to have anti-aging and cardio-protective effects on cardiac and renal function, its association with all-cause mortality has shown different patterns in different populations and disease contexts. Some studies have found that lower α -Klotho levels are associated with a higher risk of mortality in older populations and in patients with CKD (6, 9). However, other studies have observed a U-shaped relationship between α -Klotho levels and the risk of death in CKD populations, where both low and high levels of α -Klotho are associated with a higher risk of death (10). In addition, other studies have failed to find a significant association between α -Klotho levels and mortality risk (11, 12). These contradictory results suggest that the relationship between α -Klotho and mortality risk may be influenced by a variety of factors, and further studies could help to clarify its mechanism of action in the general population.

Given the controversy surrounding the relationship between Klotho and mortality risk, the objective of this study was to re-examine the potential association between Klotho and mortality risk using National Health and Nutrition Examination Survey (NHANES) data.

Methods

The NHANES is an ongoing research initiative designed to collect demographic data on the nutritional and health status of adults and children in the United States. In order to achieve its stated objectives, the survey employs a stratified, multistage probability sampling design, with the aim of obtaining a sample that accurately represents the US population. The data collection process comprises face-to-face structured interviews conducted in participants' homes, health screenings conducted at mobile screening centers, and laboratory analysis of biospecimens. The NHANES program has obtained ethical approval from the National Center for Health Statistics Ethics Review Board (<https://www.cdc.gov/nchs/nhanes/irba98.htm>). This study is in accordance with the Strengthening

the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for reporting cross-sectional studies (13).

This study employed a series of continuous data from 2007 to 2016, which included the variables α -Klotho and survival. Furthermore, a number of additional variables were incorporated, including fundamental demographic data (e.g., age, gender, race, education, and economic status), biochemical assessments (e.g., fasting blood glucose (FBG), hemoglobin A1C (HBA1C), total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL), serum uric acid (SUA), estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio (UACR)). The following variables were also considered: body mass index (BMI), medical history including hypertension, diabetes mellitus, cardiovascular disease (CVD), and chronic kidney disease (CKD), as well as smoking and drinking habits. Adults who participated in NHANES between 2007 and 2016 were included in this study (48,711 individuals). Specific inclusion and exclusion criteria are listed below:

Inclusion criteria: 1. age $\geq$ 18 years. 2. complete data on α -Klotho levels were provided. 3. provide complete survival data.

Exclusion criteria: 1. participants lacking α -Klotho data (34,947). 2. participants lacking survival data (16 individuals). 3. individuals who did not meet the age requirement.

Ultimately, a total of 13,748 participants met the inclusion criteria and were included in the analysis.

Measurement of α -Klotho

Frozen serum samples were stored at -80°C for a period spanning from 2007 to 2016. α -Klotho levels were assayed by the NHANES team between 2019 and 2020. The specific steps are as follows:

1. Reagents and antibodies: commercial enzyme-linked immunosorbent assay (ELISA) kits manufactured by IBL International (Japan) were used for the assay.
2. Detection steps: Serum samples were divided into two parts and measured separately. Two independent measurements were made for each sample and the average value was taken as the final result. If the difference between the two measurements exceeds 10%, the assay is repeated. The value of the quality control sample needs to be within two standard deviations of the specified value, and if it does not meet the requirements, the same re-measurement is required.
3. Sensitivity and reference range: the sensitivity of the ELISA kit was 4.33 pg/mL. a reference range was established by 114 healthy donor samples, ranging from 285.8 to 1,638.6 pg/mL, with a mean value of 698.0 pg/mL (14).
4. Data processing: log10 transformation was performed in this study in order to make the data conform to normal distribution.

Outcome variables

The NHANES dataset was matched to the National Death Index records to determine the survival of the participants. International Classification of Diseases-Tenth Revision was used to determine the cause of death. Follow-up for each participant began at the time of the NHANES baseline interview and continued until death or the last follow-up visit, which occurred on 31 December 2019.

Covariates

The NHANES database provides a comprehensive range of data from 2007 to 2016, encompassing questionnaires, physical measurements, laboratory measurements, and dietary information. In the context of this study, demographic data, including gender, age, race, educational attainment, and economic status (as indicated by the poverty income ratio (PIR)), was gathered through the administration of questionnaires. Furthermore, blood biomarkers, including FBG, HBA1C, SUA, TC, TG, HDL, and LDL, were quantified. Urine markers, specifically urinary creatinine and urinary albumin, were collected for the purpose of facilitating the calculation of the urinary albumin to creatinine ratio (UACR). Moreover, the estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI formula (15). Body measurements, including weight and height, were employed to ascertain the body mass index (BMI).

The past medical history was determined through a combination of physical examinations, self-reporting, and an analysis of the prescription medications that the participants were taking. In summary, hypertension was defined as three blood pressure measurements (systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg) or a diagnosis of hypertension by a medical professional or the use of antihypertensive medication. A diagnosis of diabetes mellitus was defined as having been informed by a medical practitioner that the individual in question has diabetes mellitus or is taking medication to control their blood glucose levels. The presence of CVD was determined based on the participant's history of congestive heart failure, coronary heart disease, or angina/angina pectoris. CKD was defined as an eGFR of less than 60 ml/min/1.73 m² and a UACR of 30 mg/g or greater (16).

The questionnaire was used to determine the definitions of smoking and alcohol consumption. Individuals who smoked more than 100 cigarettes during their lifetime were classified as smokers. On the other hand, those who consumed more than 12 drinks within a year were classified as alcohol drinkers.

Statistical analysis

Dummy variables were set to indicate missing covariate values to avoid losing sample size. Continuous variables were tested for normality using the Lilliefors test. The continuous variables in this study (including age, PIR, α -Klotho, FBG, HBA1C, SUA, TC, TG, HDL, LDL, UACR, eGFR, and BMI) were not normally distributed. Therefore, these variables were expressed using medians

(interquartile ranges). Comparisons between groups were made using the Kruskal-Wallis rank sum test. Categorical variables were expressed as percentages (%), and comparisons between groups were made using the Fisher exact probability test. Kaplan-Meier (KM) curves were used to demonstrate participant survival between log α -Klotho four groups, and survival was compared between groups using Cox regression analysis.

Three models were fitted for this study, with Model I adjusted for age (continuous), gender (male/female), race (Non-Hispanic white, Non-Hispanic black, Mexican American, and Other), education (junior high school education or below, high school education and college education or above) and PIR (continuous) were adjusted. Model II was further adjusted for FBG, HBA1C, SUA, TG, TC, HDL, LDL, UACR, eGFR, BMI, smoking and drinking habits, and disease history (hypertension, diabetes, CVD, and CKD). Model III was further adjusted for a set of dummy variables. The above adjustments for potential confounders were made based on existing literature (6, 9–12, 17) and clinical observations.

To evaluate the nonlinear association between log α -Klotho and the risk of death, we employed a restricted cubic spline (RCS) Cox proportional hazards regression model. Initially, a Cox model incorporating RCS was fitted to capture potential nonlinear relationships. Nonlinearity was assessed by comparing the RCS model to a traditional linear Cox model using a likelihood ratio test, with a significant p-value ($p < 0.05$) indicating deviation from linearity. Upon confirming nonlinearity, a threshold effects analysis was conducted to identify the optimal inflection point of log α -Klotho that best delineates the risk of death. Furthermore, three models were constructed to account for potential confounders: Model I adjusted for demographic variables, Model II further incorporated biochemical indicators and lifestyle factors, and Model III included additional dummy variables. To ensure robustness and reproducibility, sensitivity analyses were performed, and the consistency of the inflection points across different models was verified.

We conducted multiple sensitivity analyses to ensure the accuracy and consistency of our findings. Firstly, we excluded participants who had passed away within two years of follow-up to prevent any potential bias related to reverse causality. Secondly, we created subgroups based on different follow-up periods. Lastly, we stratified the analyses based on participants' demographic characteristics such as age and sex, as well as their disease history, including hypertension, diabetes, CVD, and CKD.

The software tools used for all the above analyses were R (version 4.3.1) and Empower Stats (version 4.2). A two-sided $P < 0.05$ was considered statistically significant.

Results

Demographic and clinical baseline characteristics of the study population

The study included a total of 13,748 participants, of whom 1,569 died. The participants were followed for 5.33 years, with a range of

2.83 to 7.83 years. Those who died were mainly older and more likely to be male. They also had lower levels of α -Klotho and higher levels of FBG, HBA1C, SUA, TG, LDL and UACR compared to their living counterparts. In addition, the deceased participants had higher prevalence probabilities of hypertension, diabetes, CVD and CKD, and were more likely to smoke tobacco and consume alcohol (Table 1).

Examination of the log α -Klotho quartile groups showed that individuals in quartile 1 (2.18-2.81 pg/mL) had an older age and higher levels of FBG, HBA1C, SUA, TG, UACR and UACR. There was also an increased incidence of hypertension, diabetes, CVD and CKD, as well as higher proportions of smoking and alcohol consumption (Table 2).

Association of log α -Klotho with all-cause mortality in NHANES 2006-2017

KM curves were utilized to illustrate the correlation between pairwise comparisons of log α -Klotho and all-cause mortality. It was observed that individuals falling within the Quartile 1 range of log α -Klotho exhibited the lowest survival rate ($P<0.05$)

(Figure 1A). Similar outcomes were observed in participants with a follow-up period of 5-10 years (Figure 1C), indicating a potential association between lower α -Klotho levels and decreased mortality risk. However, similar results were not observed in participants with follow-up periods of less than 5 years and greater than 10 years (Figures 1B, D). Subsequently, univariate Cox regression analysis was conducted to examine the variables associated with participants' susceptibility to all-cause mortality (Supplementary Table 2). Our study shows a weak association between log α -Klotho and all-cause mortality.

To further investigate the independent role of log α -Klotho in the risk of all-cause mortality, multifactor Cox regression analysis was conducted, as presented in Supplementary Table 3. Controlling for various confounders and dummy variables (Supplementary Table 1), the risk of all-cause mortality was significantly reduced by 44% for each 1 pg/mL increase in log α -Klotho. Additionally, the investigation involved the examination of the correlation between log α -Klotho and the risk of mortality by dividing log α -Klotho into quartiles. The results revealed that only in quartile 1 (2.18-2.81 pg/mL) was log α -Klotho weakly linked to a reduced risk of all-cause mortality. Conversely, log α -Klotho in quartile 2 (2.82-2.90 pg/mL), quartile 3 (2.91-2.99 pg/mL), and quartile 4 (3.0-3.70 pg/mL) did

TABLE 1 Baseline characteristics of 2007-2016 NHANES enrolled study subjects grouped according to survival status.

Mortality	No	Yes	P-value
N	12179	1569	
Age (years)	56.00 (48.00-65.00)	68.00 (60.00-74.00)	<0.001
Sex [N (%)]			<0.001
Female	6445 (52.92%)	646 (41.17%)	
Male	5734 (47.08%)	923 (58.83%)	
Ethnic [N (%)]			<0.001
Non-Hispanic white	2025 (16.63%)	160 (10.20%)	
Non-Hispanic black	2372 (19.48%)	354 (22.56%)	
Mexican American	5068 (41.61%)	849 (54.11%)	
Other	2714 (22.28%)	206 (13.13%)	
Education [N (%)]			<0.001
Junior high school education or below	3301 (27.12%)	586 (37.42%)	
High school education	2668 (21.92%)	379 (24.20%)	
College education or above	6205 (50.97%)	601 (38.38%)	
PIR (%)	2.31 (1.16-4.54)	1.52 (0.98-2.88)	<0.001
α -Klotho (pg/mL)	806.70 (659.80-997.60)	761.50(602.60-957.00)	<0.001
Log α -Klotho (pg/mL)	2.91 (2.82-3.00)	2.88 (2.78-2.98)	<0.001
FBG (mmol/L)	5.72 (5.27-6.38)	6.05 (5.44-7.22)	<0.001
HBA1C (%)	5.70 (5.40-6.00)	5.80 (5.40-6.50)	<0.001
SUA (μ mol/L)	321.20(267.70-380.70)	345.00(285.50-416.40)	<0.001
TG(mmol/L)	1.23 (0.86-1.79)	1.32 (0.93-1.91)	0.040

(Continued)

TABLE 1 Continued

Mortality	No	Yes	P-value
TC(mmol/L)	5.12 (4.42-5.82)	4.78 (4.06-5.64)	<0.001
HDL(mmol/L)	1.32 (1.09-1.60)	1.24 (1.01-1.55)	<0.001
LDL(mmol/L)	3.00 (2.43-3.65)	2.71 (2.02-3.44)	<0.001
UACR (mg/g)	7.31 (4.81-13.79)	13.11 (6.67-45.00)	<0.001
eGFR(mL/min/1.73m ²)	90.50 (76.18-102.18)	76.09 (58.86-91.64)	<0.001
BMI (kg/m ²)	28.70 (25.20-33.10)	28.80 (24.83-33.45)	0.630
Follow-up time (years)	8.00 (5.50-10.46)	5.33 (2.83-7.83)	<0.001
Smoking [N (%)]			<0.001
No	6533 (53.67%)	532 (33.93%)	
Yes	5640 (46.33%)	1036 (66.07%)	
Drinking [N (%)]			0.421
No	1664 (14.75%)	204 (13.96%)	
Yes	9614 (85.25%)	1257 (86.04%)	
Hypertension [N (%)]			<0.001
No	5952 (48.88%)	409 (26.07%)	
Yes	6226 (51.12%)	1160 (73.93%)	
Diabetes mellitus[N (%)]			<0.001
No	9380 (77.07%)	930 (59.31%)	
Yes	2791 (22.93%)	638 (40.69%)	
CVD[N (%)]			<0.001
No	10822 (88.87%)	1019 (64.95%)	
Yes	1355 (11.13%)	550 (35.05%)	
CKD[N (%)]			<0.001
No	10016 (82.70%)	826 (54.02%)	
Yes	2095 (17.30%)	703 (45.98%)	
Cause of death[N (%)]			
Accidents (unintentional injuries)	-	32 (2.04%)	
All other causes (residual)	-	419 (26.70%)	
Alzheimer's disease	-	31 (1.98%)	
Cerebrovascular diseases	-	92 (5.86%)	
Chronic lower respiratory diseases	-	78 (4.97%)	
Diabetes mellitus	-	64 (4.08%)	
Diseases of heart	-	353 (22.50%)	
Influenza and pneumonia	-	34 (2.17%)	
Malignant neoplasms	-	443 (28.23%)	
Nephritis, nephrotic syndrome and nephrosis	-	23 (1.47%)	

Continuous variables are expressed using medians (Q1-Q3) and categorical variables are expressed using percentages.

PIR, poverty income ratio; FBG, fasting blood glucose; HbA1C, hemoglobin A1C; SUA, serum uric acid; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; UACR, urinary albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; BMI, body mass index; CVD, cardiovascular disease; CKD, chronic kidney disease.

TABLE 2 Baseline characteristics of study participants included in NHANES 2007-2016 grouped according to α -Klotho quartiles.

Log α -Klotho quartile (pg/mL)	Quartile 1 2.18-2.81	Quartile 2 2.82-2.90	Quartile 3 2.91-2.99	Quartile 4 3.0-3.70	P-value
N	3437	3436	3438	3437	
Age	60.00 (50.00-68.00)	58.00 (49.00-66.00)	57.00 (48.00-66.00)	56.00 (47.00-64.00)	<0.001
Sex					<0.001
Female	1664 (48.41%)	1659 (48.28%)	1811 (52.68%)	1957 (56.94%)	
Male	1773 (51.59%)	1777 (51.72%)	1627 (47.32%)	1480 (43.06%)	
Ethnic [N (%)]					<0.001
Non-Hispanic white	556 (16.18%)	538 (15.66%)	560 (16.29%)	531 (15.45%)	
Non-Hispanic black	698 (20.31%)	568 (16.53%)	585 (17.02%)	875 (25.46%)	
Mexican American	1554 (45.21%)	1590 (46.27%)	1503 (43.72%)	1270 (36.95%)	
Other	629 (18.30%)	740 (21.54%)	790 (22.98%)	761 (22.14%)	
Education [N (%)]					0.001
Junior high school education or below	1009 (29.39%)	967 (28.15%)	951 (27.69%)	960 (27.93%)	
High school education	827 (24.09%)	745 (21.69%)	771 (22.45%)	704 (20.48%)	
College education or above	1597 (46.52%)	1723 (50.16%)	1713 (49.87%)	1773 (51.59%)	
PIR (%)	2.11 (1.11-4.19)	2.24 (1.16-4.43)	2.27 (1.16-4.46)	2.22 (1.13-4.37)	0.048
FBG (mmol/L)	5.77 (5.27-6.38)	5.72 (5.33-6.44)	5.72 (5.33-6.38)	5.77 (5.33-6.61)	<0.001
HBA1C (%)	5.70 (5.40-6.10)	5.70 (5.40-6.00)	5.60 (5.40-6.00)	5.70 (5.40-6.10)	<0.001
SUA (μ mol/L)	333.10 (279.60-398.50)	327.10 (273.60-386.60)	321.20 (267.70-374.70)	303.30 (249.80-362.80)	<0.001
TG (mmol/L)	1.30 (0.91-1.91)	1.28 (0.91-1.86)	1.24 (0.87-1.78)	1.15 (0.81-1.69)	<0.001
TC (mmol/L)	5.07 (4.32-5.87)	5.09 (4.40-5.79)	5.07 (4.42-5.79)	5.07 (4.40-5.77)	0.920
HDL (mmol/L)	1.29 (1.06-1.60)	1.29 (1.06-1.58)	1.29 (1.06-1.60)	1.32 (1.09-1.63)	0.011
LDL (mmol/L)	2.92 (2.30-3.60)	3.00 (2.43-3.64)	3.00 (2.43-3.65)	2.97 (2.40-3.60)	0.031
UACR (mg/g)	7.89 (4.90-16.94)	7.67 (5.00-15.19)	7.40 (4.77-14.27)	7.74 (5.08-15.26)	<0.001
eGFR(mL/min/1.73m ²)	84.96 (68.27-98.65)	88.65 (73.37-100.48)	90.01 (75.92-101.82)	92.29 (79.08-104.09)	<0.001
BMI (kg/m ²)	28.90 (25.50-33.20)	28.80 (25.22-33.18)	28.60 (25.10-33.00)	28.60 (24.73-33.20)	0.499
Follow-up time (years)	7.42 (4.67-10.25)	7.67 (5.17-10.33)	7.83 (5.42-10.33)	7.92 (5.42-10.25)	<0.001
Smoking [N (%)]					<0.001
No	1572 (45.79%)	1698 (49.43%)	1829 (53.22%)	1966 (57.22%)	
Yes	1861 (54.21%)	1737 (50.57%)	1608 (46.78%)	1470 (42.78%)	
Drinking [N (%)]					<0.001
No	399 (12.40%)	469 (14.72%)	454 (14.31%)	546 (17.27%)	
Now	2820 (87.60%)	2718 (85.28%)	2718 (85.69%)	2615 (82.73%)	
Hypertension [N (%)]					<0.001
No	1433 (41.69%)	1605 (46.71%)	1669 (48.56%)	1654 (48.12%)	
Yes	2004 (58.31%)	1831 (53.29%)	1768 (51.44%)	1783 (51.88%)	
Diabetes mellitus[N (%)]					0.016
No	2536 (73.83%)	2621 (76.32%)	2617 (76.12%)	2536 (73.89%)	
Yes	899 (26.17%)	813 (23.68%)	821 (23.88%)	896 (26.11%)	

(Continued)

TABLE 2 Continued

Log α -Klotho quartile (pg/mL)	Quartile 1 2.18-2.81	Quartile 2 2.82-2.90	Quartile 3 2.91-2.99	Quartile 4 3.0-3.70	P-value
CVD [N (%)]					<0.001
No	2840 (82.65%)	2965 (86.29%)	2994 (87.09%)	3042 (88.53%)	
Yes	596 (17.35%)	471 (13.71%)	444 (12.91%)	394 (11.47%)	
CKD [N (%)]					<0.001
No	2494 (73.18%)	2711 (79.43%)	2826 (82.95%)	2811 (82.39%)	
Yes	914 (26.82%)	702 (20.57%)	581 (17.05%)	601 (17.61%)	
Mortality [N (%)]					<0.001
No	2932 (85.31%)	3071 (89.38%)	3090 (89.88%)	3086 (89.79%)	
Yes	505 (14.69%)	365 (10.62%)	348 (10.12%)	351 (10.21%)	
Cause of death [N (%)]					0.482
Accidents (unintentional injuries)	8 (1.58%)	11 (3.01%)	8 (2.30%)	5 (1.42%)	
All other causes (residual)	133 (26.34%)	91 (24.93%)	92 (26.44%)	103 (29.34%)	
Alzheimer's disease	7 (1.39%)	10 (2.74%)	4 (1.15%)	10 (2.85%)	
Cerebrovascular diseases	33 (6.53%)	15 (4.11%)	25 (7.18%)	19 (5.41%)	
Chronic lower respiratory diseases	25 (4.95%)	23 (6.30%)	17 (4.89%)	13 (3.70%)	
Diabetes mellitus	17 (3.37%)	15 (4.11%)	13 (3.74%)	19 (5.41%)	
Diseases of heart	121 (23.96%)	91 (24.93%)	75 (21.55%)	66 (18.80%)	
Influenza and pneumonia	12 (2.38%)	5 (1.37%)	7 (2.01%)	10 (2.85%)	
Malignant neoplasms	139 (27.52%)	101 (27.67%)	104 (29.89%)	99 (28.21%)	
Nephritis, nephrotic syndrome, and nephrosis	10 (1.98%)	3 (0.82%)	3 (0.86%)	7 (1.99%)	

Continuous variables are expressed using medians (Q1-Q3) and categorical variables are expressed using percentages.
PIR, poverty income ratio; FBG, fasting blood glucose; HbA1C, hemoglobin A1c; SUA, serum uric acid; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; UACR, urinary albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; BMI, body mass index; CVD, cardiovascular disease; CKD, chronic kidney disease.

not exhibit any association with a decreased risk of all-cause mortality. Furthermore, we conducted a comprehensive examination of the correlation between log α -Klotho and the probability of mortality over different follow-up periods. Our findings indicate that log α -Klotho was not significantly associated with all-cause mortality within follow-up periods of <5 years, 5-10 years, and >10 years.

Log α -Klotho showed U-shaped non-linear relationship with risk of all-cause mortality

The present study examined the association between log α -Klotho and the risk of all-cause mortality, taking into account various confounding factors. Our findings revealed a non-linear relationship, characterized by a U-shaped pattern, between log α -Klotho and the risk of all-cause mortality, as depicted in [Figures 2A–D](#). Furthermore, through threshold effect analysis, we

identified the optimal inflection point, as presented in [Table 3](#). Specifically, when log α -Klotho levels were below 2.89 pg/mL, the risk of all-cause mortality decreased by 86%. Conversely, log α -Klotho levels exceeding 2.89 pg/mL were associated with a 2.15-fold increase in the risk of all-cause mortality.

In addition, a sensitivity analysis was conducted to exclude participants who died within two years of follow-up to limit the effect of reverse causality. Notably, the U-shaped association between log α -Klotho and all-cause mortality remained consistent, as depicted in [Supplementary Figure 1](#). Additionally, participants were categorized based on the duration of follow-up, revealing that the U-shaped relationship between log α -Klotho and all-cause mortality remained unchanged across follow-up periods of less than five years, 5-10 years, and exceeding ten years, as illustrated in [Figure 3](#). After stratifying the data by age, sex, and medical history, including hypertension, diabetes, CVD, and CKD, a U-shaped correlation between log α -Klotho and mortality risk was still observed ([Supplementary Figures 2, 3](#)). Nevertheless, the

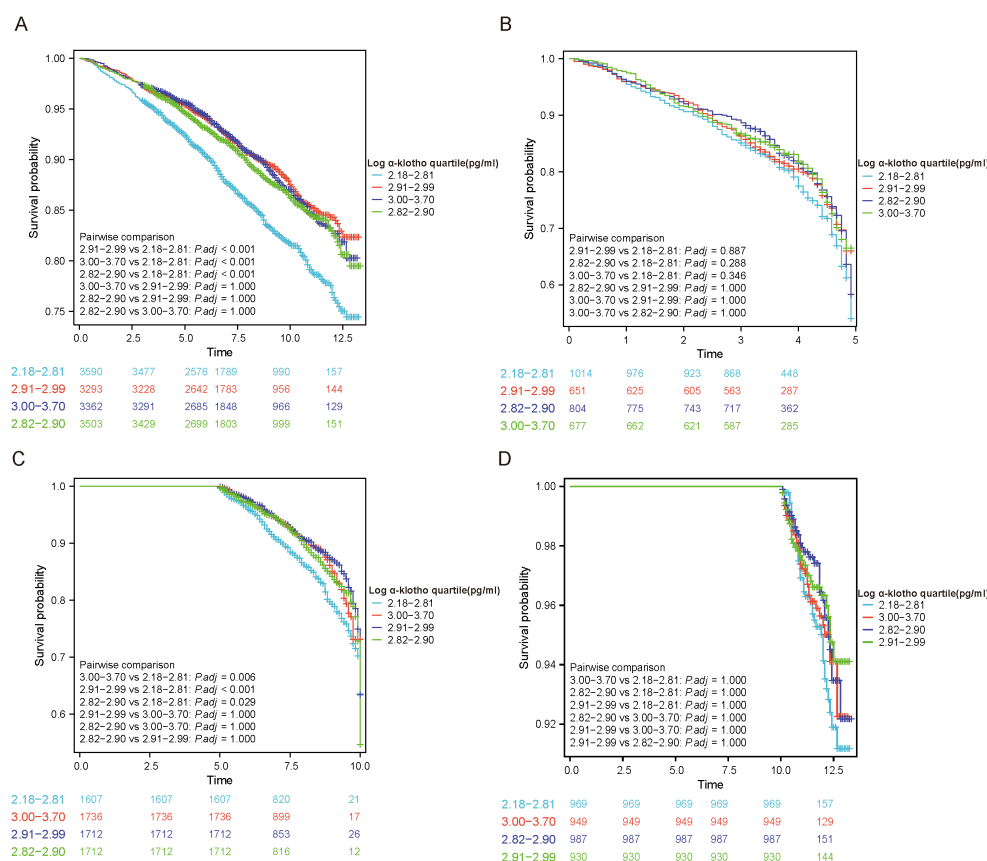


FIGURE 1

KM curves to analyze outcomes of included study subjects. (A) Analysis based on α -Klotho quartiles; (B-D) Analysis based on duration of follow-up (<5, 5-10 and >10 years).

statistical significance of this U-shaped relationship was not consistently observed in certain subgroups, primarily due to the constraints imposed by the limited sample size. Moreover, [Supplementary Table 4](#) presents the inflection points for the various stratifications.

Discussion

The aim of our study was to examine the association between log α -Klotho levels and the likelihood of mortality. In a retrospective analysis, we observed a modest association between log α -Klotho and a reduced risk of mortality, particularly in individuals in the first quartile. In addition, we examined the dose-response relationship between log α -Klotho levels and mortality risk and found a U-shaped association while controlling for various confounders. We also identified the optimal inflection point for log α -Klotho at 2.89 pg/mL. Finally, we demonstrated the stability of our findings in various stratification and sensitivity analyses.

There is currently no precise value for the normal range of α -Klotho. Yamazaki Y (18) measured circulating serum α -Klotho levels by ELISA. His (18) study found that normal serum α -Klotho levels in adults ranged from 239-1266 pg/mL (mean $\pm$ standard

deviation 562 ± 146 pg/mL). Later, Drew-DA (19) also used an ELISA to measure circulating serum α -Klotho levels. His (19) study found a median α -Klotho level of 631 pg/mL (25th-75th percentile 477-817 pg/mL). However, the specificity of the ELISA assay may be lower than that of immunoprecipitant immunoblotting assays (20). The reference range for α -Klotho in the NHANES database is 285.8-1638.6 pg/mL, which is in general agreement with the results of the above study. In addition, there is still a lack of a robust and credible assay for circulating α -Klotho, and scientists have expressed concern about the accuracy of commercially available kits (21). The advent of new antibodies (22), branched polypeptides (23) and other assays is expected to provide more accurate results.

Klotho is an anti-aging protein, and transgenic mice that overexpress α -Klotho have a 30% longer lifespan (1). Klotho has organ-protective effects. Klotho deficiency makes the kidneys more susceptible to acute injury, delays renal regeneration, and promotes renal fibrosis. In addition to direct renal effects, Klotho deficiency triggers and exacerbates disorders of mineral metabolism, secondary hyperparathyroidism, vascular calcification, and cardiac hypertrophy and fibrosis (24). Moreover, Klotho expression is significantly decreased in malignant tumors, and low Klotho levels are an independent poor prognostic factor for cancer-specific and progression-free survival (25). Klotho deficiency has been associated with several neurological disorders, including

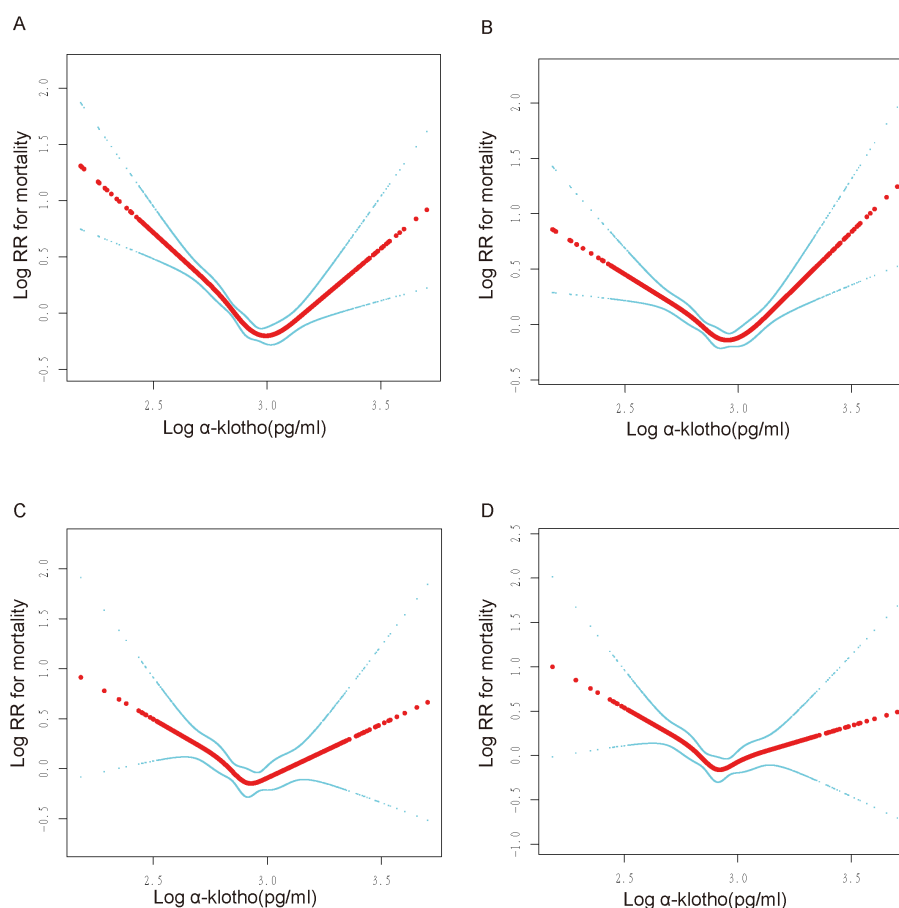


FIGURE 2

U-shaped non-linear relationship between log α -Klotho and mortality. RR: relative risk. (A) No variables were adjusted. (B) Adjusted for age, sex, ethnic, poverty income ratio, education. (C) Adjusted for Model 1+fasting blood glucose, hemoglobin A1c, serum uric acid, triglyceride, total cholesterol, high-density lipoprotein, low-density lipoprotein, urinary albumin-to-creatinine ratio, estimated glomerular filtration rate, body mass index, smoking, drinking, hypertension, diabetes, cardiovascular disease, chronic kidney disease. (D) Adjusted for Model 2+poverty income ratio dummy variable, fasting blood glucose dummy variable, hemoglobin A1c dummy variable, triglyceride dummy variable, high-density lipoprotein dummy variable, low-density lipoprotein dummy variable, urinary albumin-to-creatinine ratio dummy variable, estimated glomerular filtration rate dummy variable, body mass index dummy variable, hypertension dummy variable, diabetes mellitus dummy variable, cardiovascular disease dummy variable, chronic kidney disease dummy variable, smoking dummy variable, drinking dummy variable.

multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease (26). It has been found that Alzheimer's disease patients have lower Klotho concentrations in their cerebrospinal fluid than healthy individuals (27). Klotho-overexpressing mice have 1.76 times higher axon density than wild-type mice, which suggests a substantial beneficial role of Klotho proteins in myelin sheath regeneration (28). Klotho also enhances cognitive performance in mammals (29). In summary, low levels of Klotho are not beneficial for physical health. In fact, in our study, participants with low levels of α -Klotho had a higher prevalence of hypertension, diabetes, CVD and CKD, as well as mortality.

Several previous studies have examined the relationship between Klotho and mortality risk. However, the results of these studies are contradictory. Plasma Klotho is an independent predictor of all-cause mortality in the elderly (9). During hemodialysis, Klotho < 280 pg/mL will significantly increase the

incidence of combined cardiovascular time and cardiovascular death (30). In addition, low concentrations of Klotho predicted CVD death in non-calcified and mildly calcified hemodialysis patients. In CKD patients, the low Klotho concentration group had a reduced probability of survival and an increased risk of all-cause mortality than the high Klotho concentration group (31). However, it has also been noted that serum α -Klotho is not associated with a higher risk of mortality (11, 12). A study by Brandenburg Vincent M et al. also noted that Klotho does not increase the predictive power of cardiovascular and mortality risk assessment in patients with normal renal function (32). In the present study, we revealed a U-shaped association between α -Klotho levels and mortality risk in the general population. In addition, consistent associations between α -Klotho and mortality risk were observed in different subsets of the population regardless of sex, age, and presence of hypertension, diabetes, CKD, or CVD. This finding may shed light on the inconsistent findings of previous

TABLE 3 Two-piecewise Cox proportional risk regression analysis the effect of all-cause mortality on in NHANES 2007-2016.

Outcome:	All-cause mortality		
	HR (95%CI)	P Value	P non-linear value (P for log-likelihood ratio test)
Crude			
Log α -Klotho < 3 pg/mL	0.13 (0.09, 0.21)	<0.0001	<0.001
Log α -Klotho > 3 pg/mL	5.56 (2.35, 13.14)	<0.0001	
Model 1			
Log α -Klotho< 2.99 pg/mL	0.27 (0.17, 0.42)	<0.0001	<0.001
Log α -Klotho < 2.99 pg/mL	8.02 (3.41, 18.88)	<0.0001	
Model 2			
Log α -Klotho < 2.96 pg/mL	0.23 (0.10, 0.50)	0.0003	0.002
Log α -Klotho < 2.96 pg/mL	3.55 (1.04, 12.20)	0.0439	
Model 3			
Log α -Klotho < 2.89 pg/mL	0.14 (0.05, 0.38)	0.0001	0.002
Log α -Klotho < 2.89 pg/mL	2.15 (0.81, 5.72)	0.1252	
Follow-up time < 5 years			
Log α -Klotho < 2.86 pg/mL	0.04 (0.01, 0.17)	<0.0001	<0.001
Log α -Klotho < 2.86 pg/mL	4.76 (1.44, 15.77)	0.0106	
Follow-up time 5-10 years			
Log α -Klotho < 3.16 pg/mL	0.34 (0.14, 0.85)	0.0207	0.002
Log α -Klotho < 3.16 pg/mL	1427.72 (21.73, 93818.47)	0.0007	
Follow-up time > 5 years			
Log α -Klotho < 2.98 pg/mL	0.20 (0.01, 5.86)	0.3532	0.093
Log α -Klotho < 2.98 pg/mL	89.79 (0.80, 10130.14)	0.0621	

Model 1: Adjusted for age, sex, ethnic, poverty income ratio, education.
Model 2: Adjusted for Model 1+fasting blood glucose, hemoglobin A1c, serum uric acid, triglyceride, total cholesterol, high-density lipoprotein, low-density lipoprotein, urinary albumin-to-creatinine ratio, estimated glomerular filtration rate, body mass index, smoking, drinking, hypertension, diabetes, cardiovascular disease, chronic kidney disease.
Model 3: Adjusted for Model 2+poverty income ratio dummy variable, fasting blood glucose dummy variable, hemoglobin A1c dummy variable, triglyceride dummy variable, high-density lipoprotein dummy variable, low-density lipoprotein dummy variable, urinary albumin-to-creatinine ratio dummy variable, estimated glomerular filtration rate dummy variable, body mass index dummy variable, hypertension dummy variable, diabetes mellitus dummy variable, cardiovascular disease dummy variable, chronic kidney disease dummy variable, smoking dummy variable, drinking dummy variable.

studies regarding the relationship between Klotho and mortality risk.

Several studies have explained the Klotho association with mortality risk. More than ten single nucleotide polymorphisms (SNPs) have been identified in the human Klotho gene, and numerous studies have been conducted to assess the association between allelic variation in the Klotho gene and the etiology of aging-related diseases (33–35). Gang Jee Ko’s (36) study found that in the promoter region of Klotho, G395A, the A allele carrier status is a factor affecting the survival rate of hemodialysis patients, and the survival rate of the GA+AA group was lower than that of the GG group. A study by Serafi Cambray (37) also identified three SNPs in Klotho: rs562020 carrying the most common allele (G), a rare allele (C) at rs2283368, and a pureblood at rs2320762 rare allele (G), which could help in the prediction of non-cardiovascular mortality in CKD. In addition, the involvement of low concentrations of Klotho in the progression of various diseases also explains the relationship between Klotho and mortality.

Numerous scientists have worked to investigate the underlying mechanisms of how low Klotho levels increase the risk of death, and Stephanie S. Fischer (38) and colleagues have proposed that Klotho deficiency leads to severe disturbances in mineral and electrolyte metabolism, as well as lowered blood pressure, in mice. In addition, knockdown of the Klotho gene can promote neuronal cell death by regulating oxidative stress (39). Low Klotho levels have also been associated with accelerated aging and premature death by affecting the immune system (40). Despite the existence of a large body of basic research, there is no consensus in the scientific community on how low Klotho levels contribute to premature death.

Klotho overexpression has been demonstrated to attenuate the progression of various diseases. Specifically, it has been found to mitigate the physiologic compensatory hypertrophy of the kidney following nephrectomy and has shown efficacy in reducing disease progression (41). Additionally, Klotho overexpression has been observed to hinder cell proliferation and promote apoptosis in A549 cells, suggesting its potential as a tumor suppressor (42). Moreover, the inclusion of Klotho has been acknowledged as a potential therapeutic approach for conditions such as multiple sclerosis, Duchenne muscular dystrophy, cardiovascular disease, and osteoarthritis, and neuronal damage (43–47). Certain drugs have demonstrated the ability to enhance Klotho levels. For instance, Astragaloside IV has been found to elevate Klotho expression, ameliorate renal function, and mitigate podocyte apoptosis and injury in mice with diabetic nephropathy (48). Simvastatin alleviated cognitive dysfunction in rats and found a significant increase in klotho-positive cells in the hippocampus of simvastatin-treated rats (49). Rosiglitazone inhibits melanoma resistance by increasing serum levels of klotho and decreasing levels of Wnt5A in the blood and tumor microenvironment of mice (50). Consequently, the elevation of Klotho levels through external means presents a novel approach for alleviating symptoms and treating various diseases.

The U-shaped relationship between log α -Klotho levels and all-cause mortality observed in our study suggests that both low and high levels of α -Klotho may be detrimental to health, with

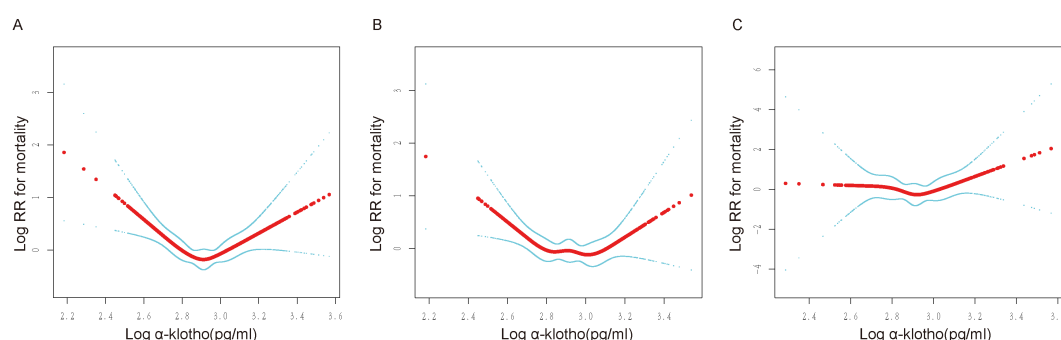


FIGURE 3

Non-linear relationship between log α -Klotho and mortality at different follow-up durations. RR: relative risk. (A) Association of log α -Klotho with mortality in less than 5 years, (B) Association of log α -Klotho with mortality over 5–10 years, (C) Association of log α -Klotho with mortality in >10 years. Adjusted for age, sex, ethnic, poverty income ratio, education, fasting blood glucose, hemoglobin A1c, serum uric acid, triglyceride, total cholesterol, high-density lipoprotein, low-density lipoprotein, urinary albumin-to-creatinine ratio, estimated glomerular filtration rate, body mass index, smoking, drinking, hypertension, diabetes, cardiovascular disease, chronic kidney disease, poverty income ratio dummy variable, fasting blood glucose dummy variable, hemoglobin A1c dummy variable, triglyceride dummy variable, high-density lipoprotein dummy variable, low-density lipoprotein dummy variable, urinary albumin-to-creatinine ratio dummy variable, estimated glomerular filtration rate dummy variable, body mass index dummy variable, hypertension dummy variable, diabetes mellitus dummy variable, cardiovascular disease dummy variable, chronic kidney disease dummy variable, smoking dummy variable, drinking dummy variable.

intermediate levels being optimal. Several potential mechanisms may explain this phenomenon:

1. Regulation of Physiological Homeostasis:

Low levels of α -Klotho: α -Klotho is well-established for its antioxidant, anti-inflammatory, and anti-aging properties (1, 51). Reduced levels of α -Klotho may result in the loss of these protective effects, increasing the risk of various conditions such as cardiovascular disease, chronic kidney disease, and neurodegenerative disorders.

Excessive levels of α -Klotho: While moderate levels of α -Klotho are beneficial, excessive concentrations may disrupt mineral metabolism and calcium-phosphorus homeostasis, potentially leading to vascular calcification and other metabolic abnormalities (52). Furthermore, supraphysiological levels of α -Klotho may interfere with the feedback mechanisms of endocrine pathways, resulting in adverse effects.

2. Feedback Regulatory Mechanisms:

α -Klotho interacts with the FGF23/FGFR signaling pathway, which plays a key role in phosphate and vitamin D metabolism (52). Deviations from optimal α -Klotho levels may disrupt this pathway, leading to dysregulation of phosphate homeostasis. This imbalance can result in complications such as cardiovascular and renal dysfunction.

3. Genetic and Epigenetic Factors:

Genetic variations in the Klotho gene, such as single nucleotide polymorphisms (SNPs), can influence the expression and function of α -Klotho (33–36). These genetic differences may result in varying individual responses to α -Klotho levels, contributing to the observed U-shaped relationship. Additionally, epigenetic modifications that affect Klotho gene expression may further complicate this association.

4. Disease State Regulation:

α -Klotho levels undergo significant changes in various disease states, such as chronic kidney disease and heart failure (31, 32). These fluctuations may reflect either compensatory mechanisms or pathological processes, which could differentially affect mortality risk depending on the α -Klotho concentration.

5. External Factors and Interventions:

External factors, including pharmacological agents that influence α -Klotho levels (e.g., astragaloside IV, simvastatin, rosiglitazone) (48–50), may cause fluctuations in α -Klotho concentrations outside the optimal range. Such deviations may inadvertently increase the risk of adverse outcomes, including mortality, particularly at high α -Klotho levels.

6. Cellular and Molecular Effects:

At optimal levels, α -Klotho promotes cellular homeostasis, enhances cell survival, and inhibits apoptosis (42, 48). However, excessive α -Klotho levels may induce cellular stress, autophagy, or apoptosis, increasing the risk of tissue damage and adverse health outcomes.

These potential mechanisms collectively highlight the complex and context-dependent role of α -Klotho in human health, providing a theoretical framework for understanding its dual biological effects and implications for disease and mortality.

The present study offers several novel contributions. First, it leverages NHANES data from a nationally representative sample of 13,748 participants, significantly enhancing the external validity of the findings. Second, it is the first to confirm, within a general population, the U-shaped nonlinear relationship between serum α -Klotho levels and all-cause mortality, identifying a specific inflection point at 2.89 pg/mL. This finding sheds light on previously conflicting results and provides a clearer interpretation of the association. Furthermore, the robustness of this relationship was verified through multiple adjustments and sensitivity analyses, thereby reinforcing the study's credibility. Lastly, this research delves into the bidirectional biological effects of α -Klotho at different concentrations, offering a valuable theoretical foundation for future basic research and clinical applications. Collectively, these contributions advance the understanding of α -Klotho in aging and disease while providing new perspectives for clinical risk assessment and the development of targeted interventions.

It is crucial to acknowledge certain limitations inherent in this observational study, which prevented us from establishing a causal relationship between α -Klotho and susceptibility to all-cause mortality. In addition, not all blood samples were collected on the same day and were not consistent. In addition, this blood sample was stored at -80°C without timely testing, which may pose a potential risk of protein degradation. Therefore, better study designs are needed in the future to demonstrate the relationship between α -Klotho and mortality risk. Furthermore, our exclusive reliance on a single baseline measurement of α -Klotho concentration has limited our ability to assess the influence of time-dependent fluctuations in α -Klotho on the risk of all-cause mortality. Next, the limited sample size for each specific cause of mortality has hindered our ability to examine the relationship between α -Klotho and mortality risk across different causes. It is crucial to acknowledge that residual and unidentified confounding factors may persist despite our efforts to minimize them. A substantial proportion of the sample had to be excluded due to the absence of data, consequently constraining the study's capacity to accurately depict the US population.

Conclusion

In a nationally representative U.S. population, we found a U-shaped association between circulating α -Klotho levels and risk of all-cause mortality, with a specific inflection point at 2.89 pg/mL. The results of different levels and sensitivity analyses supported this association. Future studies need to further explore the specific mechanisms of the effect of α -Klotho on mortality risk at different levels.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author/s.

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JS: Data curation, Investigation, Writing – original draft. HL: Data curation, Methodology, Software, Writing – review & editing. XF: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2025.1405003/full#supplementary-material>

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Advancing patient evidence in XLH (APEX): rationale and design of a real-world XLH global data unification program

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X-linked hypophosphatemia (XLH) is a rare, genetic, progressive, lifelong disorder caused by pathogenic variants in the *phosphate-regulating endopeptidase homolog, X-linked (PHEX)* gene, resulting in excess fibroblast growth factor 23 (FGF23) and consequent renal phosphate wasting. Chronic hypophosphatemia leads to deficits of the musculoskeletal system affecting bone, muscle, joint, and dental health. XLH treatments include oral phosphate and active vitamin D—which are associated with a burdensome dosing regimen, gastrointestinal disturbances, hyperparathyroidism, and nephrocalcinosis—or burosumab, a fully human anti-FGF23 antibody. Randomized clinical trials (RCTs) demonstrated burosumab to be well tolerated and efficacious in improving serum phosphate, rickets, bone turnover, and patient-reported outcomes. However, there are limited data on the natural history of XLH or real-world comparisons of the safety, effectiveness, and long-term outcomes of XLH treatments. Advancing Patient Evidence in XLH (APEX) is a global data unification project aiming to describe the burden and lifelong progression of XLH, collect real-world data on treatment effectiveness and safety, and investigate regional differences in treatment outcomes. Participants from three observational, noninterventional, retrospective and prospective, multicenter, longitudinal (10-year) studies of patients with XLH will be included: XLH Disease Monitoring Program (NCT03651505), International XLH Registry (NCT03193476), and SUNFLOWER (NCT03745521). Data collected in the Americas, Europe, Israel, Japan, and South Korea will be processed to unify identical and similar data elements. Data unification will be an iterative process with a clinical and programming review, ensuring validity and accuracy. In this observational study, unified data involving approximately 2000 pediatric and adult participants with XLH will be analyzed to address research questions in an

exploratory manner. Long-term observational studies and patient registries provide opportunities to generate real-world data and address knowledge gaps in rare diseases. APEX aims to improve clinical decision-making and practice by bridging evidence gaps that cannot be addressed by RCTs or regional registries.

KEYWORDS

X-linked hypophosphatemia (XLH), *phosphate-regulating endopeptidase homolog X-linked (PHEX)* gene, fibroblast growth factor 23 (FGF23), musculoskeletal, rickets, osteomalacia, odontomalacia, Advancing Patient Evidence in XLH (APEX)

1 Introduction

X-linked hypophosphatemia (XLH) is a rare, progressive and lifelong disorder caused by pathogenic variants in the *phosphate-regulating endopeptidase homolog, X-linked (PHEX)* gene, resulting in excess fibroblast growth factor 23 (FGF23) and consequent renal phosphate wasting (1–3) affecting approximately 1 in 20,000 to 70,000 people globally (4–7). XLH is caused by inactivating *PHEX* variants (1, 8) and is inherited in an X-linked dominant pattern (8). *De novo* variants occur in approximately 20% to 30% of patients (9, 10). Increased levels of FGF23 cause downregulation of the sodium-dependent phosphate co-transporters NPT2a and NPT2c in proximal renal tubules leading to renal phosphate wasting, decreased 1 α -hydroxylase, and increased 24-hydroxylase activity, resulting in decreased serum 1,25-dihydroxyvitamin D levels, and decreased phosphate absorption in the intestines (1). Resultant chronic hypophosphatemia impairs bone and tooth mineralization resulting in rickets, osteomalacia, and odontomalacia (1, 8), amongst other multi-system consequences. In children, impaired and disproportionate growth with lower limb deformities are typical; delayed motor development and craniosynostosis may occur (1, 8, 11, 12). Adults with XLH may manifest fractures or pseudofractures, early onset osteoarthritis, enthesopathy, and less frequently, spinal stenosis (1, 8, 11). Hearing loss is not infrequent (1, 8). Dental abscesses are common across age groups as well as bone and joint pain, stiffness, and muscle weakness (1, 8, 11). The disorder severely impacts physical function, mobility, and health-related quality of life (QoL) (13, 14). In addition to the well characterized role of elevated FGF23 and hypophosphatemia in XLH, inactivating *PHEX* variants also modify levels of the small integrin-binding ligand, N-linked glycoproteins (SIBLING) family, such as osteopontin and the inhibitory acidic serine aspartate-rich-MEPE-associated protein (ASARM) peptides (1, 15–17). These lead to local impairment of mineralization of bone and teeth, and altered tooth morphology, contributing to osteomalacia and odontomalacia in XLH (1, 15–17). Efforts to treat XLH medically have focused primarily on modifying the influence of FGF23 on renal phosphate handling and 1,25-dihydroxyvitamin D production, or administration of conventional therapy, neither of which are likely to address aspects of the disease that are not mediated by FGF23, hypophosphatemia, or impaired vitamin D activation.

“Conventional therapy” over the past several decades has consisted of a combination of oral phosphate salts and active vitamin D analogs (8, 18). While this treatment is usually beneficial, it does not address the elevated levels of FGF23, and phosphate levels cannot be safely normalized (8, 18). Furthermore, conventional therapy does not normalize growth, nor entirely resolve bone and dental manifestations, or improve health-related QoL (13, 14, 19–21). Additionally, significant side effects, such as secondary or tertiary hyperparathyroidism, hypercalciuria, and nephrocalcinosis, may occur, and progression to chronic kidney disease has been described (18). Finally, oral phosphate supplementation is associated with poor adherence due to its unpleasant taste, the burdensome regimen of frequent dosing, and uncomfortable side effects such as diarrhea and abdominal cramping (8, 22, 23).

Burosumab is a fully human monoclonal antibody against FGF23, approved initially in 2018 for the treatment of children and adults with XLH, with varied approvals and payor coverage in children and adults across the world (24–26). Unlike conventional therapy, burosumab targets an earlier stage in the underlying pathophysiology of XLH by blocking effects of excess FGF23 and improving serum phosphate levels (24, 25, 27, 28). Clinical trials have shown burosumab to improve serum phosphate levels without hypercalciuria or elevating parathyroid hormone levels (29, 30). In children, burosumab improved rickets, and increased height and walking distance in the 6-Minute Walk Test (30). In adults, insufficiency fractures healed, walking distance in the 6-Minute Walk Test improved, and patient-reported outcomes of stiffness, pain, physical function improved (31). These benefits of burosumab treatment have been shown to be maintained long-term, for up to 144 to 184 weeks in adults (32, 33) and up to 160 weeks in children (34). Burosumab was well tolerated in clinical trials with few, if any, treatment-related serious adverse events (SAEs) (29–31, 35). Restless leg syndrome was observed as an adverse event (AE), primarily in adults (35). There were no AEs or SAEs, including deaths, that led to withdrawal of participants from the studies (29–31, 35). Neutralizing antibodies against burosumab were infrequently detected (29–31, 35, 36) and patients positive for neutralizing antibodies responded to burosumab treatment (36). To date, burosumab has not been found to

increase nephrocalcinosis, ectopic myocardial mineralization, or incidence of hyperparathyroidism. However, there are important knowledge gaps regarding the long-term effects of burosumab treatment.

One such knowledge gap is the expected inability of burosumab to treat manifestations of XLH that are mediated by factors other than FGF23, such as osteopontin and ASARM peptides. To date, the effect of burosumab is not known regarding final height, degree of improvement of lower limb deformities, craniosynostosis, or the long-term effects regarding dental complications in patients with XLH (27, 30, 37–49). However, it is important to consider that most of these patients were previously treated with conventional therapy prior to burosumab initiation, and the age of burosumab initiation varied, which may influence the cumulative development of disease-related complications. Outside of randomized clinical trials (RCTs), burosumab was able to improve or prevent the worsening of some of these complications (27, 38–49), and earlier age of initiation improved outcomes relative to later age of initiation (42, 47, 49). Additionally, patients who initiate burosumab treatment tend to be those who exhibit more severe symptomatology compared with patients who persist in treatment with conventional therapy (43, 46). Therefore, information on the effect of long-term burosumab treatment and the impact of the age of initiation will be important in understanding the role burosumab can play in treating the wider symptomatology of XLH and, in addition, will also provide insights into which disease features cannot be addressed by targeting FGF23.

Due to the rarity of XLH, and with treatment expertise limited to specialized centers, significant unmet needs exist for affected patients (8). Real-world evidence is required to address knowledge gaps and to provide further information on the natural history of XLH, disease progression and burden, as well as long-term effectiveness, safety and the overall experience with burosumab outside of clinical trial conditions (2). Patient registries have been

shown to be effective in the collection of large-scale, real-world patient data in rare disorders (50).

The Advancing Patient Evidence in XLH (APEX) is a ten-year global data unification project, combining data from three Kyowa Kirin-/Ultragenyx-sponsored regional observational studies to increase the overall size of the datasets and address a set of questions that would not be feasible or complete using the regional studies alone.

2 Methods

2.1 Regional study identification

Three Kyowa Kirin-/Ultragenyx-sponsored, regional observational studies in patients with XLH have been established and registered with ClinicalTrials.gov (Table 1). The XLH Disease Monitoring Program (XLH DMP, NCT03651505), initiated in July 2018 and with enrollment completed in December 2022, collects data in the Americas (51). The International XLH Registry (IXLHR, NCT03193476), collecting data from Europe and Israel, was initiated in September 2017 (52). The Study of Longitudinal Observation for Patients With X-linked Hypophosphatemic Rickets/Osteomalacia in Collaboration With Asian Partners (SUNFLOWER, NCT03745521), collecting data from participants in Japan and Korea, was initiated in April 2018 and with enrollment completed in April 2022 (53).

2.2 Inclusion and exclusion criteria

Inclusion and exclusion criteria for the three regional studies are described in Table 2. Briefly, patients diagnosed with XLH who provided consent and were not participating in a clinical trial at the time (or who had received prior approval to do so in the case of the XLH DMP) were eligible for participation in the individual studies

TABLE 1 Characteristics of Kyowa Kirin-/Ultragenyx-sponsored regional XLH studies.

Registry	Country	Sponsor	Study Type	Number of Patients*	Patient Age	Number of sites	Study Period
XLH DMP	US, Canada, and Latin America	Ultragenyx, Kyowa Kirin North America	Company-sponsored, prospective, multicenter observational, phase IV study for XLH	776, enrollment completed Dec 2022	All ages	35	Jul 2018–Jul 2028 (10 years)
IXLHR	Europe, Israel; 21 countries†	Kyowa Kirin International	Company-sponsored, prospective, multicenter, non-interventional observational registry	1257, as of Apr 15, 2024	All ages	121	Sep 2017–Dec 2028 (10 years)
SUNFLOWER	Japan, Korea	Kyowa Kirin Co., Ltd.	Company-sponsored, prospective, observational cohort study	226, enrollment completed Apr 2022	All ages	20	Apr 2018–Dec 2028 (10 years)

*Not all participants enrolled at the regional studies are included in the APEX study, depending on data availability and participant consent. †Countries participating in the IXLHR: Belgium, Bulgaria, Czech Republic, Denmark, France, Germany, Hungary, Ireland, Israel, Italy, Latvia, The Netherlands, Norway, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, United Kingdom. DMP, Disease Monitoring Program; IXLHR, International XLH Registry; SUNFLOWER, Study of Longitudinal Observation for Patients With X-linked Hypophosphatemic Rickets/Osteomalacia in Collaboration With Asian Partners; XLH, X-linked hypophosphatemia.

TABLE 2 Inclusion/exclusion criteria of Kyowa Kirin-sponsored regional XLH studies.

Inclusion/Exclusion	Criteria	XLH DMP	IXLHR	SUNFLOWER
Inclusion criteria	Age and sex	Patients of all ages at baseline		Patients of any age who have been clinically diagnosed with XLH
	XLH diagnosis	Clinical diagnosis of XLH based on clinical features including short stature or leg deformities AND biochemical profile consistent with XLH, OR confirmed <i>PHEX</i> mutation in patient or in family member	Diagnosis of XLH with clinical, radiological, biochemical, genetic AND/OR family mapping findings consistent with XLH	Patients have one or more of the following: a documented <i>PHEX</i> mutation, at least one family member with a documented <i>PHEX</i> mutation or a documented intact FGF23 level >30 pg/mL (cut-off value for diagnosing FGF23-related hypophosphatemia)
	Symptomatology	Not defined		Participants must also have a history or current physical signs of rickets/osteomalacia, regardless of treatment history (presence/absence of treatment or type of treatment)*
	Consent	Participants must provide written informed consent/assent		
Exclusion criteria	Consent	Patient or their legally authorized representative is not willing and able to provide informed consent after the nature of the study has been explained and prior to any study procedures	Patient or their legally designated representative does not have the cognitive capacity to provide informed consent	Not defined
	Clinical trial participation	Concurrent enrollment in a clinical trial without prior approval from sponsor	Patients who are currently participating in an interventional clinical trial	Participation in another clinical study at the time of informed consent
	Comorbidity	Serious medical or psychiatric comorbidity	Not defined	Presence of any characteristic that may make participation inappropriate or unsafe
	Life expectancy	Less than 1 year of life expectancy	Not defined	

*No inclusion/exclusion criteria based on the stage or severity of the disease were utilized by the SUNFLOWER study, to allow inclusion of patients with symptoms ranging from mild to severe. DMP, Disease Monitoring Program; FGF23, fibroblast growth factor 23; IXLHR, International XLH Registry; SUNFLOWER, Study of Longitudinal Observation for Patients With X-linked Hypophosphatemic Rickets/Osteomalacia in Collaboration With Asian Partners; XLH, X-linked hypophosphatemia.

(2, 51–54). Patients were identified and enrolled by participating clinical sites.

2.3 Study designs

Characteristics of the three regional studies are summarized in Table 1: the XLH DMP is a prospective, multicenter, longitudinal long-term outcomes program for participants with XLH (51); the IXLHR is a prospective, non-interventional observational registry (2, 52); and SUNFLOWER is a prospective, multicenter, longitudinal, long-term, observational cohort study (53, 54).

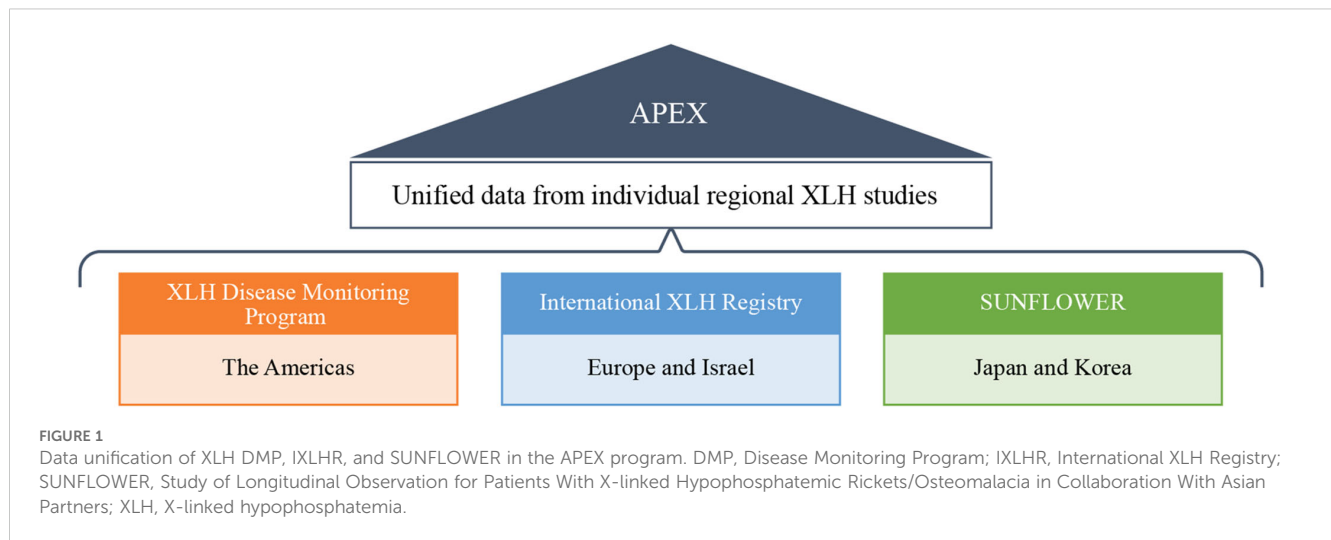
In all three studies, participants will be followed for up to ten years to capture treatment details and clinical outcome variables until withdrawal from the study or a loss to follow-up event. Participants will be treated at the discretion of the treating physician and will have access to burosumab only through

authorized prescribed (commercial) use, or via early access programs in countries where burosumab is not commercially available. Where applicable and feasible, APEX will utilize these datasets through their unification (Figure 1).

2.4 Study objectives

The overall goals of the APEX study are to:

- Describe the progressive nature of the disorder and associated burden of patients with XLH in a global real-world setting.
- Generate data on the real-world effectiveness and safety of XLH treatment options.
- Investigate regional differences in the real-world treatment of patients with XLH.



This study was planned to analyze collected information on XLH and associated clinical, patient, and disease burden data through the long-term observation of patients with XLH. APEX will enable the exploration of regional differences and generate evidence regarding the real-world effectiveness and safety of the available therapeutic options. These data will provide further context for results from RCTs to better inform clinical decision-making, and long-term consequences of XLH or its management.

2.5 Determination of sample size

Due to the rarity of XLH, statistical guidance from prior research, including the three regional studies, was limited. Sample sizes of the regional studies were determined based on feasibility and the number of potential XLH outpatients who could be enrolled in participating clinical sites (2, 54). The number of participants enrolled at the regional studies is described below; however, depending on data availability, due to the status of data processing/analysis, or whether participants in the IXLHR or SUNFLOWER studies provided additional informed consent allowing for inclusion in APEX, the number of participants in the regional studies may differ from the number available for the APEX analyses. Additional consent was not required from participants in XLH DMP. At the time of submission South American data were not available for inclusion in the APEX program but may be included at a later date.

- XLH DMP: 776 patients were enrolled.
- IXLHR: 1257 patients were enrolled as of April 15, 2024.
- SUNFLOWER: 226 patients were enrolled.

2.6 Data collection

Detailed information on the individual regional studies' methods of data collection is available (2, 51–54). In brief, where

data are available, each study collects demographic information, medical history, *PHEX* mutation, family history, medication history, disease and medication-related complications, laboratory assessments, functional assessments, and QoL assessments. When patients enroll into their respective regional study, both retrospective and baseline data are collected. Prospective data will be collected post-baseline at routine clinical visits and added periodically to the study databases (2, 51–54).

2.7 Data collection tools and data management

Regional study data are collected in purposely designed electronic case report forms (eCRF) via electronic data capture tools, and study databases are checked automatically using logical checks. The eCRF is completed and signed electronically for each individual participant and is completed only by qualified individuals trained in the completion and data verification of the eCRF information. Data are stored according to local regulations in order to maintain data protection.

2.8 Data quality assurance

To ensure the accuracy and robustness of the collected data, the individual regional studies have mechanisms in place to assess and, if necessary, verify the data to either confirm or correct the relevant data entry(s). These include the monitoring and auditing of study sites and automatic checks built into the electronic data capture tools (2, 51–54).

2.9 Data unification process

Data unification and the preparation of the data for analysis are separate processes and will be performed by Arbor Research Collaborative for Health (Ann Arbor, Michigan, United States).

The data will initially undergo a data checking process, including a check on the range of the values, the units used, internal consistency and the distribution of the data. Any data flagged as impossible will be queried and cross-checked, and either corrected or removed. Following the data check, the data will undergo unification.

Data unification is an iterative process with clinical and programming review to maintain accuracy. As part of the process, a consistent naming convention for variables will be applied across the study data for variables that are identical, such as age at study entry. The data will be brought into a consistent data structure to group the data that will hold all shared values, for example baseline or longitudinal data. Finally, the data will be processed to handle inconsistent variables. This will possibly include transforming variables, such as health-related QoL patient-reported outcome values, into indicators that identify values that pass clinically meaningful thresholds. Therefore, rather than trying to translate an entire incompatible scale, established diagnostic values that identify similar concepts across the different scales can be used. The unification process will also base unification schemes on clinical input and the available literature. For example, when unifying data on permanent tooth removal where XLH DMP excludes wisdom tooth removal but the other studies do not, we may restrict comparisons to specific age ranges where, according to clinical judgment and the literature, wisdom tooth removal is likely to be rare in order to have comparable data across the individual studies.

Once the data have been unified, they will be prepared for the analysis of specific research questions. This will include handling missing data and creating clinically based categories of continuous variables. When research questions require analyses of longitudinal data, regression-based methods that are robust to differences in data collection schedules will be utilized (55). Wherever possible the full combined dataset will be used to maximize statistical power; however, region-specific analyses of variables may not be available in all studies.

2.10 Statistical analyses

Appropriate to an observational study, the data will be analyzed in an exploratory manner. Continuous variables will be described by standard summary statistics (e.g. number of patients, mean and standard deviation or median and inter-quartile range, as appropriate). Categorical variables will be described by frequency and proportion. Regression modeling will be used to adjust for confounding variables between comparison groups, propensity score matching will enable the identification of comparison groups to estimate the impact of an intervention in the absence of a control group, and contrasting analyses will investigate regional differences. All analyses will attempt to identify evidence of potential biases, and diagnostic tools will be used to assess goodness of fit and appropriateness of models according to accepted statistical practices. Details of all analyses will be specified in the Statistical Analysis Plan (SAP), including how missing data will be handled.

2.11 Management and reporting of AEs

Details of AE reports associated with any treatment for XLH will be captured by the study entry for the affected patient and included in the APEX dataset. AEs will be coded at each clinical center and reported as per the local regulatory guidelines.

2.12 APEX oversight

A global medical committee (GMC) has been formed with two representatives from each regional study's scientific steering committee, and the members of the GMC may rotate with other representatives from their study. The GMC will provide scientific oversight of the APEX program, provide their input on the data collected and the analyses performed, and author APEX reports.

3 Discussion

Multicenter, international observational studies and patient registries can be an invaluable source of real-world data in rare disorders, providing important information that would not be possible by other means, such as RCTs. It can be challenging to derive statistically robust conclusions from RCTs in rare disorders due to the limited number of patients available for inclusion. Observational studies and patient registries, in contrast, can recruit a greater number of patients as they have fewer inclusion and exclusion criteria than RCTs and typically have a longer patient follow-up. This provides invaluable information on the natural history of a disease, treatment effectiveness, additional outcomes, and safety beyond that possible in smaller RCTs (2, 50, 56, 57). Observational studies and patient registries more closely resemble real-world clinical practice compared with RCTs, and with relatively fewer exclusion criteria, they are able to provide a more generalizable insight on the impact of certain patient characteristics on disease outcomes.

APEX is an important extension of the regional XLH studies, collecting and unifying their data to form the largest XLH patient dataset in the world to date. An advantage of creating a global dataset by pooling data and increasing the sample size is in allowing analyses to be conducted that would not normally be feasible in many rare disorders. This initiative also enables a comparison of region-specific XLH populations, treatment pathways and outcomes.

There are several areas of research in which APEX may be particularly beneficial. Firstly, there is the continuing uncertainty in the management of adult patients with XLH. Clinical practice recommendations published in 2019 recommended conventional therapy for symptomatic adults (8). However, small-scale RCTs and case studies have demonstrated benefits of burosumab treatment in adults with moderate to severe XLH (31, 32, 58, 59), but real-world evidence of treatment with burosumab is lacking in this patient group. Secondly, data from APEX may also provide invaluable information on adolescent patients with XLH transitioning into adult care, as this population can be lost to follow-up and there is

uncertainty regarding optimal management (60). The absence of data from this age group occurs due to their exclusion from the burosumab RCTs (29, 30), thus real-world evidence regarding their treatment and outcomes is a significant gap in our knowledge. The large patient numbers available to the APEX project and the ability to compare regional differences in practice may provide additional evidence that can be used to further refine treatment approaches in patients with XLH, and importantly, in patient groups for whom data are lacking. Finally, the analysis of the outcomes of long-term burosumab treatment and the impact of the age of initiation on outcomes may help to identify which disease complications cannot be prevented, improved, or resolved with burosumab treatment. This will hopefully lead to additional studies to characterize and address the unmet medical needs of patients with XLH.

3.1 Limitations

The limitations of APEX include inconsistencies of protocol (e.g. study visit requirements) and differences in data collection and reporting methods between the individual regional studies, which may limit the data that can be pooled or used for comparison. Therefore, it may not be possible to combine all datasets within APEX. Differences in the number of patients recruited regionally, may also result in imbalances in representation, thereby affecting the ability to describe clinical practice in underrepresented regions. In addition, there are inherent limitations of non-interventional observational studies. Unlike RCTs, there is often no standardized or mandated follow-up in registries, and this can be influenced by regional differences in data collection and clinical practice. This increases the likelihood of missing or incomplete data collection and is a potential source of bias. The XLH DMP study aims to improve follow-up, with specified visit schedules and measurements funded by the study. However, these may often be carried out in conjunction with the clinical care, leading to variability in timing and collection. SUNFLOWER has specified assessments to be collected either annually in pediatric participants or every two years in adults, while IXLHR has recommended clinical variables and collection schedule, but data are collected as per routine clinical practice. While for some registries there can be a lack of supervised enrollment, the enrollment of participants in these regional studies are supervised by the study investigators based on inclusion/exclusion criteria. The clinical diagnosis is based on the local practice of expert physicians, which ensures that the regional studies accurately reflect real-world practice. However, the lack of central definitions of diseases or diagnostic practices may result in regional variability in the diagnosis and reporting of clinical conditions.

4 Conclusion

Observational studies and patient registries offer valuable opportunities to generate real-world data and address knowledge

gaps in rare diseases. The APEX program will enable the global collection and analysis of data relating to the natural history, treatment, and outcomes of patients with XLH. Analyses may help address research questions that would not be feasible in the individual regional studies, as well as allow the comparison of regional practices and outcomes. It is anticipated the findings from APEX will further increase the understanding of XLH, its treatment, and help to improve clinical practice for patients with XLH.

5 Ethics and dissemination

The XLH DMP required Institutional Review Board approvals for each participating local institution. Patients who have provided informed consent, or assent where required, with informed consent by a legally authorized representative, are included in XLH DMP (51).

The IXLHR is run in accordance with the Declaration of Helsinki and received ethical, regulatory, and institutional approvals at national, regional, and site levels for each participating country. Patient data are kept in accordance with the EU General Data Protection Regulations on the processing of personal data and the protection of privacy in the electronic communication sector (2016/679/EU). Once a patient or their legal representative provides informed consent, or assent for minors aged ≥ 12 years, they are enrolled in the IXLHR (2, 52).

Ethics approval for SUNFLOWER was obtained from the Ethics Committee of Osaka University, the Ethics Committee of Kyowa Kirin Co., Ltd., and the Ethics Committee of each participating medical institution. Patients or their parents/guardians are required to give informed consent before inclusion in SUNFLOWER (53, 54).

Before inclusion in the APEX project, participants in the IXLHR and SUNFLOWER are provided with information regarding the objectives and the procedures and are required to re-consent to participate.

The datasets used and analyzed that support the findings of this manuscript are available from the corresponding author and Kyowa Kirin Co., Ltd. on reasonable request.

Ethics statement

The studies involving humans were approved by the Research Ethics Review Committee of Kyowa Kirin Co., Ltd.. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants or their legal guardians.

Author contributions

MLB: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. TOC: Conceptualization, Investigation, Writing – review & editing. SF: Conceptualization, Investigation, Writing – review & editing. DH: Conceptualization,

Investigation, Writing – review & editing. EAI: Conceptualization, Investigation, Writing – review & editing. MK: Conceptualization, Project administration, Writing – original draft, Writing – review & editing. KPM: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. KO: Conceptualization, Investigation, Writing – review & editing.

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Conflict of interest

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