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ORIGINAL ARTICLE

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Association between circulating 25-hydroxyvitamin D metabolites and periodontitis: Results from the NHANES 2009–2012 and Mendelian randomization study

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Abstract

Aim: This study sought to investigate associations of 25-hydroxyvitamin D (25(OH)D) metabolites with periodontitis and to assess causality using Mendelian randomization (MR).

Materials and Methods: This study included 7246 participants of the National Health and Nutrition Examination Survey, 2009–2012. The association of periodontitis with 25(OH)D metabolites was assessed using multivariable logistic regression analysis. Two-sample MR for 25(OH)D, 25(OH)D_3, and C3-epi-25(OH)D_3 with periodontitis (n = 17,353 cases/28,210 controls) was conducted. The principal analysis employed the inverse-variance-weighted (IVW) approach. We controlled for horizontal pleiotropy using five additional methods.

Results: Based on the observational study, each 1-point increase in standard deviation of 25(OH)D lowered the risk of periodontitis by 15% (OR = 0.85, 95% confidence interval [CI]: 0.78–0.93, p = .006) after multivariable adjustment. A similar relationship was observed between 25(OH)D_3 and periodontitis (OR = 0.88, 95% CI: 0.80–0.97, p = .031). Furthermore, a potential non-linear association was found between periodontitis and both 25(OH)D and 25(OH)D_3. However, C3-epi-25(OH)D_3 was not found to be associated with periodontitis risk. IVW-MR showed that periodontitis risk was not significantly associated with genetically increased levels of 25(OH)D (OR = 1.02, 95% CI: 0.90–1.16, p = .732), 25(OH)D_3 (OR = 1.04, 95% CI: 0.93–1.17, p = .472), or C3-epi-25(OH)D_3 (OR = 1.11, 95% CI: 0.87–1.41, p = .400). The pleiotropy-robust MR approaches yielded similar results after we had eliminated the variants with horizontal pleiotropy risk.

Conclusions: Cross-sectional observational analysis identified significant relationships between periodontitis with 25(OH)D metabolites, while findings based on MR study did not support a causal role.

KEYWORDS
25(OH)D metabolites, Mendelian randomization, NHANES, periodontitis

Wenjing Li and Qiwen Zheng should be considered the co-first authors of this article.

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Clinical Relevance

Scientific rationale for study: Previous observational studies have found that low vitamin D levels are associated with the occurrence of periodontitis. However, most of these studies only measured the 25-hydroxyvitamin D [25(OH)D] levels but did not assess the levels of its subtypes, including 25(OH)D_3 and C3-epi-25(OH)D_3, an isomer of 25(OH)D_3. It is not clear whether the associations of 25(OH)D metabolites with periodontitis are causal or not. If periodontitis is caused by vitamin D deficiency, it would be clinically relevant to prevent periodontitis in people at high risk, as vitamin D deficiency is not uncommon and can be corrected safely.

Principal findings: In this study, we used multivariable regression of observational data and two-sample Mendelian randomization analysis to investigate the relationship and potential causality between 25(OH)D metabolites, including total 25(OH)D, 25(OH)D_3, and C3-epi-25(OH)D_3, and periodontitis. There were conflicting findings for a link with periodontitis for the observational analysis of biochemically measured 25(OH)D metabolites versus the genetically predicted levels of these metabolites. The present findings do not suggest a definitive association of periodontitis risk with measured and genetically predicted levels of 25(OH)D and its metabolites.

Practical implications: This study compared observational estimates of the association between 25(OH)D metabolites and periodontitis with Mendelian randomization estimates based on genetic instruments. It might provide a whole picture of observational association and causal relationship between vitamin D status and periodontitis, which implies that the blood levels of 25(OH)D or its metabolites are not likely to be causal for the development of periodontitis.

1 | INTRODUCTION

Periodontitis is a complex disease caused by an imbalance in the interaction between oral microbes and the host inflammatory response. On account of its high prevalence rate and its potential role in the development of diabetes, cardiovascular diseases, depression, and cancer, it poses a heavy global medical burden and presents a global public health challenge (Lalla & Papapanou, 2011; Araujo et al., 2016; Disease, Injury, & Prevalence, 2017; Czesnikiewicz-Guzik et al., 2019; Peres et al., 2019; Nwizu et al., 2020; Sanz et al., 2020; Jiao et al., 2021). Vitamin D status is key to the metabolism of calcium and phosphorus. Accumulating evidence from in vitro and animal studies suggests that vitamin D might be beneficial for periodontal health via its anti-inflammatory effects and reduction in the number of harmful bacteria (Jagelaviciene et al., 2018; X. Hu et al., 2020).

A number of epidemiological studies have evaluated the association between vitamin D and periodontitis; however, the relationship remains uncertain. A large-scale cross-sectional study involving over 15,000 participants showed that lower levels of vitamin D were associated with the presence and severity of periodontitis (Ebersole et al., 2018). The findings were further confirmed by a meta-analysis (Machado et al., 2020). Additionally, a non-randomized clinical trial on 82 patients with moderate periodontitis found that vitamin D supplementation, as an adjunct to non-surgical periodontal therapy (NSPT), was beneficial for the treatment of periodontitis (Perayil et al., 2015). In contrast, Antonoglou et al. reported a case–control study on 55 chronic periodontitis patients and 30 periodontally healthy subjects, and demonstrated that there was no association between the 25-hydroxyvitamin D [25(OH)D] levels and periodontal health status (Antonoglou et al., 2015). In addition, a randomized, double-blinded, placebo-controlled trial of 360 patients with moderate or severe periodontitis after NSPT reported that using vitamin D supplementation as an NSPT adjunctive had limited clinical importance and no durable efficacy with regard to probing depth and attachment loss (Gao et al., 2020).

Existing observational studies are subject to a few limitations, such as small sample size, insufficient adjustment of some important covariates, and lack of assessment of different metabolites of total 25(OH)D. Moreover, older clinical trials reported inconsistent findings and were limited by administration of inadequate doses or inability to separate the effect of vitamin D and calcium. Genetic Mendelian randomization (MR) analysis is a tool that uses genetic data to elucidate the causal relationship between exposure and outcome (Smith & Ebrahim, 2003). Since genetic alleles are randomly assigned during meiosis and are not correlated with environmental factors, the genetic associations observed from MR analysis are less likely to be affected by confounding bias and reverse causation risk (Smith et al., 2008). Therefore, MR studies are referred to as "nature-created randomized, double-blind trials" and are considered as a complementary approach to randomized controlled trials (RCTs). Given the inconsistent findings from observational studies and the lack of strong evidence from RCTs, MR studies may be a useful supplementary tool to explore the causality between vitamin D and periodontitis.

Therefore, the present study aims to investigate the association of 25(OH)D and its metabolites 25(OH)D_3 and C3-epi-25(OH)D_3 with periodontitis based on observational data, and to assess evidence for the causal relationship between periodontitis and 25(OH)D and its metabolites using publicly available genetic data under the framework of the MR analysis.

2 | METHODS

2.1 | Overall study design

The present study was conducted in two stages, as shown 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 association of total 25(OH)D, 25(OH)D_3, and C3-epi-25(OH)D_3 with periodontitis (CDC, 2006). In stage 2, we assessed the causal effect of genetically determined levels of 25(OH)D metabolites on periodontitis by MR analysis of summary statistics data from the genome-wide association study (GWAS). Data from the largest published GWAS datasets were used, which contained the 25(OH)D levels in 443,734 participants and 25(OH)D_3 and C3-epi-25(OH)D_3 levels in 40,562 participants (Manousaki et al., 2020; Zheng et al., 2020). Further, the effect of genetic variants on periodontitis was estimated using the data for 17,353 periodontitis patients and 28,210 controls obtained from the Gene-Lifestyle Interactions in Dental Endpoints (GLIDE) consortium (Shungin et al., 2019).

2.2 | Data sources and study population

NHANES is a continuous cross-sectional series of surveys that were conducted on non-institutionalized U.S. civilians. It uses multistage probability sampling to select a nationally representative sample and assesses their health and nutritional status. The survey includes household interviews, physical examinations, and laboratory tests. It was performed by the National Center for Health Statistics of the Centers for Disease Control and Prevention (CDC). Information about the sampling method and data collection is available in a previous publication (CDC, 2006). The study received the approval of the Ethics Review Board of the National Center for Health Statistics, and all the participants provided their written informed consent.

To investigate the association between 25(OH)D metabolites in serum and periodontitis, we used publicly available NHANES survey data from the 2009/2010 and 2011/2012 cycles. Periodontal status was evaluated by dentists through a full-mouth periodontal examination, which included assessment of gingival recession and pocket depth measurements. Participants aged 30 years and older were eligible for the periodontal examination if they had one or more natural teeth and no health conditions that required antibiotic prophylaxis before periodontal probing. The current study included 9402 eligible adults for further analysis. After excluding participants without serum 25(OH)D measurements ($n = 525$) or results of periodontal examination ($n = 1631$), a total of 7246 individuals were included for further analysis. A flow-chart depicting the selection of the study participants is shown in Figure S1.

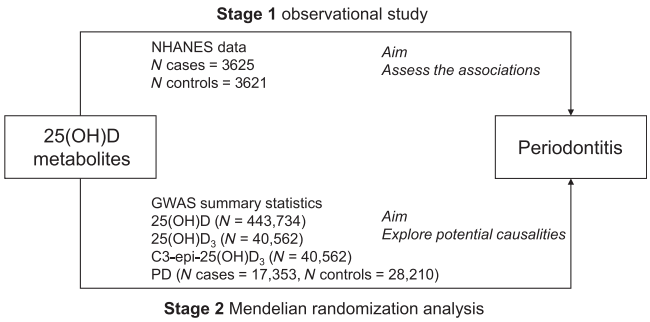

FIGURE 1 Overall study design based on observational analysis and Mendelian randomization.

2.3 | Measurement of serum 25(OH)D and its metabolites

Sample collection, transformation, storage, and analysis have been described in the laboratory procedure manual. Briefly, the serum levels of 25(OH)D metabolites were assessed with liquid chromatography–tandem mass spectrometry (LC–MS/MS) at the National Center for Environmental Health. Calibration and quality control were conducted according to the laboratory procedure manual of NHANES. For LC–MS/MS, the total 25(OH)D level (nmol/L) was calculated by adding the levels of 25(OH)D_3 and 25(OH)D_2; the level of C3-epi-25(OH)D_3 was not included in this calculation.

2.4 | Ascertainment of periodontitis

Classical diagnosis of periodontitis was conducted based on clinical parameters such as periodontal pocket probing, bleeding on probing (BOP), plaque index, and clinical attachment levels (CALs) (Eke et al., 2012). The following conditions were diagnosed as periodontitis: mild periodontitis: ≥2 interproximal sites with AL ≥3 mm and ≥2 interproximal sites with PD ≥4 mm (not on the same tooth) or one site with PD ≥5 mm; moderate periodontitis: ≥2 interproximal sites with AL ≥4 mm (not on the same tooth) or ≥2 interproximal sites with PD ≥5 mm (not on the same tooth); severe periodontitis: ≥2 interproximal sites with AL ≥6 mm (not on the same tooth) and ≥1 interproximal site with PD ≥5 mm. The others were diagnosed as "No periodontitis": no evidence of mild, moderate, or severe periodontitis.

2.5 | Assessment of covariates

Information on demographic characteristics (age, gender, ethnicity, education level, and family income), lifestyle factors (smoking, drinking, diet, and physical activity), and health conditions (hypertension, diabetes, hypercholesterolaemia, cardiovascular disease, and cancer) was obtained with the help of a questionnaire interview. Body weight and height were obtained from physical examinations and used to calculate body mass index (BMI). A non-smoker was defined as someone with a smoking history of less than 100 cigarettes per lifetime. Individuals with a smoking history of more than 100 cigarettes per lifetime

who were currently not smoking were classified as former smokers, and those who currently had a regular smoking habit were classified as current smokers. Based on alcohol use, the participants were divided into non-drinkers (0 drinks per day), moderate drinkers (<2 drinks per day for males and <1 drink per day for females), and heavy drinkers (≥2 drinks per day for males and ≥1 drink per day for females). The overall dietary quality was assessed using the Health Eating Index-2015 (HEI-2015), which was a score ranging from 0 to 100, with a higher score indicating a healthier diet (Krebs-Smith et al., 2018). Total HEI-2015 scores were computed by summing the scores for 13 components, including 9 adequacy components (total fruits, whole fruits, total vegetables, greens and beans, whole grains, dairy, total protein foods, seafood and plant proteins, and fatty acids) and 4 moderation components (refined grains, sodium, added sugars, and saturated fats). Both dietary data and food serving equivalents are needed to compute the HEI-2015 score. The dietary intake was estimated using the mean value of the two 24-h recall data. The first day's interview was conducted in person in the mobile examination centre, where the participants underwent physical examination. The second day's interview was conducted by telephone 3–10 days after the first day. The food serving equivalents were computed using the USDA Food Patterns Equivalent Database. Leisure-time physical activity was assessed based on the number of times the participants engaged in physical activities in a week and the corresponding metabolic equivalents (METs). Based on the MET values, the participants were grouped into low level (no leisure-time physical activity), moderate level (1–5 times with METs ranging from 3 to 6 or 1–3 times with METs >6), and vigorous level (>5 times with METs ranging from 3 to 6 or >3 times with METs >6). Healthy eating index was assessed based on 24-h dietary recall data from interviews. Medical conditions were confirmed if the response was "yes" to the question "Have you ever been told by a doctor or other health professional that you had hypertension, diabetes, hypercholesterolaemia, coronary heart disease, congestive heart failure, stroke, angina, heart attack, or cancer?".

2.6 | Statistical analysis

Data from 2009/2010 and 2011/2012 were combined, and 4-year sampling weights were constructed and incorporated in all the analyses based on the sampling strategy of NHANES. Rao–Scott chi-square tests and *t*-tests were applied for analysing the association of periodontitis with categorical variables and continuous variables, respectively. A multivariable logistic regression analysis was performed to ascertain the effects of serum 25(OH)D metabolites on the likelihood of prevalent periodontitis. Odds ratios and the corresponding 95% confidence intervals were calculated. The levels of 25(OH)D metabolites were categorized into four groups based on their quantiles. The values of all the metabolites were log-transformed and analysed as continuous variables based on their skewness. In the multivariable analysis, we adjusted for age, gender, and ethnicity in model 1 and for BMI, education level, family income-poverty ratio, smoking status, alcohol use, leisure-time physical activity, and healthy eating index in model 2. Further, model 3 was adjusted for conditions including hypertension, diabetes, hypercholesterolaemia, cardiovascular disease, and cancer. The non-linear association between each vitamin D metabolite and periodontitis risk was assessed using restricted cubic spline regression with three knots (25th, 50th, and 75th), with the multivariable adjustment mentioned above. All analyses were performed using R 4.1.0 (http://www.r-project.org). Two-sided levels of significance were calculated, and the significance level was set as .05.

2.7 | Mendelian randomization

2.7.1 | Basic concept of MR analysis

Since genetic variants are randomly allocated at the time of gamete formation and not correlated with environmental factors, MR analysis is less vulnerable to bias from reverse causation and confounding than traditional observational methods. Therefore, in this study, MR analysis was used to identify single nucleotide polymorphisms (SNPs) associated with 25(OH)D metabolites and the SNPs associated with periodontitis, and the SNPs identified were combined to determine the relationship between 25(OH)D metabolites and periodontitis risk. For valid causal estimates, the genetic variants used as instrumental variables (IVs) in MR analyses need to meet the following criteria (Figure 2): (1) the genetic variants need to be correlated with the levels of 25(OH)D metabolites; (2) they should not be affected by confounding factors; and (3) they should affect periodontitis only via 25(OH)D metabolite levels.

2.7.2 | Data sources

Our MR analysis used a two-sample design and used publicly available summary statistics from large-scale GWAS datasets. For total 25(OH)D, we obtained genetic data for 25(OH)D from one of the biggest GWAS meta-analyses on circulating levels of 25(OH)D deposited in the UK Biobank (which included white British participants [$N = 401{,}460$]) and combined it with a previous GWAS involving 42,274 Europeans (Manousaki et al., 2020). Measurement of 25(OH)D was performed at baseline (2006–2010) using chemiluminescence immunoassay, and the measured values were log-transformed and standardized to adjust the skewness in the distribution of 25(OH)D levels. Additionally, the season in which the data were collected and vitamin D supplementation were adjusted for, in order to identify genetic variants that were significantly correlated with circulating 25(OH)D levels. We obtained genetic estimates for 25(OH)D_3 and C3-epi-25(OH)D_3 based on GWAS data for 40,562 participants of European origin from the EPIC-InterAct study, the EPIC-Norfolk study, and the EPIC-CVD study (Zheng et al., 2020). The plasma levels of 25(OH)D metabolites were assessed with LC–MS/MS at VITAS, Oslo. Genetic variants associated with periodontitis were identified from publicly available data of the GLIDE consortium, which included a meta-analysis of seven GWAS studies on populations of European

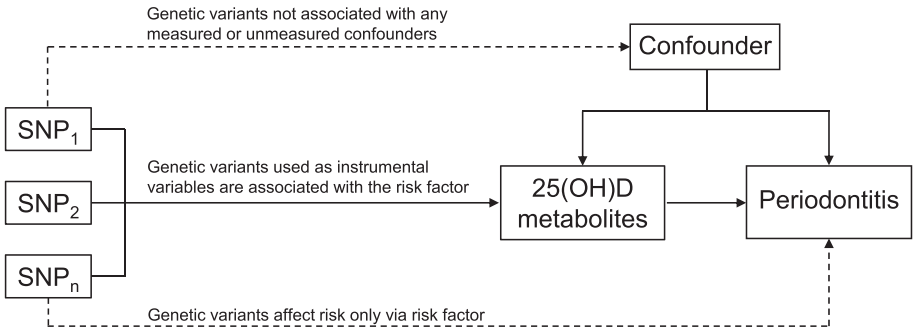

FIGURE 2 Principles of Mendelian randomization and assumptions that need to be satisfied to derive unbiased causal effect estimates.

descent (17,353 periodontitis cases and 28,210 controls) (Shungin et al., 2019). Periodontitis cases were defined according to either the CDC/AAP or the Community Periodontal Index criteria (World Health Organization, 1997; Page & Eke, 2007). Additionally, cases were also defined based on probing depth and/or number of deep periodontal pockets and self-reported clinical diagnosis of periodontitis. More details regarding the definition of periodontitis adopted in each study cohort are available in the original publications (D. Shungin et al., 2015, 2019). Further details of the GWAS studies that were included in our MR analysis can be found in Table S1.

2.7.3 | Selection of SNPs for MR analysis

SNPs (i.e., the IVs used for the analysis) associated with 25(OH)D, 25(OH)D_3, and C3-epi-25(OH)D_3 at a genome-wide significance level ($p < 5 \times 10^{-8}$) were selected for analysis. Among these SNPs, those with coefficients of linkage disequilibrium (LD) of <1% were selected based on the European 1000 Genomes dataset as the LD reference panel. In the next step, SNPs that showed genome-wide association with periodontitis at a significance level of 1.0×10^{-5} were eliminated. Also excluded were BMI-associated SNPs, as BMI has been associated with both 25(OH)D metabolite levels and periodontitis risk (Vimaleswaran et al., 2013; D. Shungin et al., 2015). A detailed flowchart of the selection process for SNPs is shown in Figure S2.

The SNPs used as IVs in this study are listed in Table S2. For 25(OH)D, we used 66 common independent SNPs that explain 3.5% of the phenotypic variation. For 25(OH)D_3, seven SNPs together explained 4.6% of the variance. For C3-epi-25(OH)D_3, three SNPs served as IVs that could explain 2.9% of the variance at the observed scale. For each instrument, all *F* statistics were >10 (range, 28.1–6310.5), with an overall *F* statistic of 225.9, 279.3, and 403.7 for 25(OH)D, 25(OH)D_3, and C3-epi-25(OH)D_3, respectively. This indicated that the causal effect estimate was less likely to be affected by weak instrument bias.

2.7.4 | MR analysis

MR analysis was performed using the R statistical software with the TwoSampleMR package (Hemani et al., 2018). We investigated the causal effect of a 1-point standard deviation (SD) increase in the log-transformed levels of 25(OH)D or 25(OH)D_3 on periodontitis predisposition using several MR approaches. We used the same method to determine the relationship between genetically predicted high C3-epi-25(OH)D_3 levels (above vs. below the low limit of quantification) with the risk of periodontitis. We performed principal analyses with inverse-variance-weighted (IVW) meta-analysis under a fixed-effects model, which combines the IV ratio estimates across the exposure-associated SNPs (Burgess et al., 2013). The robustness of the IVW results was checked with variance heterogeneity tests. The IVW method provides consistently robust causal effect estimates, provided the genetic variants meet the assumptions of an IV.

2.8 | Sensitivity analysis

We performed multiple sensitivity analyses to evaluate the extent to which directional pleiotropy might bias the MR causal estimates. First, we applied the MR-Egger method, which is based on the Instrument Strength Independent of Direct Effect assumption. In this method, the effect of SNPs on 25(OH)D metabolite levels is charted against the effect of SNPs on periodontitis, and an intercept dissimilar from that of the source is considered as proof of pleiotropic effects. This method yields bias-free estimations even if all the selected SNPs are unfounded (Bowden et al., 2015). Furthermore, we used four more MR models that are more robust under horizontal pleiotropy conditions: simple mode, weighted mode, weighted median, and penalized weighted median (Slob & Burgess, 2020). Using median values provides better robustness than using individual values, which have strong outlying causal estimates. With median-based analysis, it is possible to obtain consistent estimates of the causal effect under conditions in which at least 50% of the variants are valid IVs. Under conditions of equal weights, the simple median method is similar to the weighted median method. The penalized weighted median method is equivalent to the weighted median method when there is no causal effect heterogeneity. In the presence of directional pleiotropy, the contribution of heterogeneous SNP-specific measures to the total measure is down-weighted by a penalization parameter with the penalized weighted median method (Bowden et al., 2016). The mode-based method assumes that the most frequent causal effect is equivalent to the true casual effect. According to this assumption, the rest of

TABLE 1 Baseline characteristics of study population in NHANES 2009–2012 and prevalence of periodontitis (weighted) by characteristics

Characteristics	Overall ($n = 7246$)	Periodontitis ($n = 3625$)	Healthy controls ($n = 3621$)	*p*-Value
Age, years	51.5 ± 0.3	54.5 ± 0.4	49.3 ± 0.4	<.001
Gender				
Male	3629 (49.0)	2135 (59.0)	1494 (41.4)	<.001
Female	3617 (51.0)	1490 (41.0)	2127 (58.6)	
Ethnicity				
Non-Hispanic White	3201 (69.8)	1326 (61.5)	1875 (76.0)	<.001
Non-Hispanic Black	1500 (10.2)	867 (13.3)	633 (8.0)	
Mexican American	1024 (7.6)	668 (11.0)	356 (4.9)	
Others	1521 (12.4)	764 (14.2)	757 (11.1)	
Education				
Less than high school	1821 (18.3)	1073 (23.2)	748 (14.5)	<.001
High school or equivalent	1560 (20.7)	852 (24.1)	708 (17.9)	
College or above	3668 (61.2)	1596 (52.7)	2072 (67.6)	
Family income-to-poverty ratio				
≤1	1342 (12.3)	772 (16.1)	570 (9.6)	<.001
1–3	2740 (35.0)	1500 (41.6)	1240 (30.0)	
>3	2538 (52.7)	1010 (42.3)	1528 (60.4)	
BMI				
<25.0	1907 (27.2)	913 (25.5)	994 (28.5)	.060
25.0–30.0	2546 (36.1)	1279 (36.6)	1267 (35.7)	
≥30.0	2735 (36.7)	1410 (37.9)	1325 (35.8)	
Smoking status				
Non-smoker	3919 (54.6)	1779 (47.0)	2140 (60.3)	<.001
Former smoker	1911 (27.2)	972 (27.9)	939 (26.7)	
Current smoker	1413 (18.2)	871 (25.1)	542 (13.0)	
Alcohol consumption				
Non-drinker	2214 (26.6)	1180 (29.5)	1034 (24.3)	<.001
Moderate drinker	3963 (64.3)	1891 (59.8)	2072 (67.7)	
Heavy drinker	506 (9.1)	291 (10.7)	215 (8.0)	
Leisure-time physical activity				
Low	3938 (48.0)	2131 (55.9)	1807 (42.1)	<.001
Moderate	2101 (33.1)	937 (27.8)	1164 (27.1)	
Vigorous	1206 (18.9)	556 (16.3)	650 (20.8)	
Healthy Eating Index	55.6 ± 0.3	54.4 ± 0.3	56.4 ± 0.5	<.001
Self-reported diseases				
Diabetes	973 (9.7)	571 (12.5)	402 (7.6)	<.001
Hypertension	2771 (33.6)	1505 (38.0)	1266 (30.2)	<.001
Cardiovascular disease	721 (8.0)	403 (10.0)	218 (6.5)	<.001
Hypercholesterolemia	2630 (40.2)	1350 (43.3)	1280 (37.9)	<.001
Cancer	713 (10.6)	349 (10.5)	364 (10.7)	.854
25-Hydroxyvitamin D metabolites				
25(OH)D, nmol/L	70.7 ± 1.0	67.2 ± 1.0	73.3 ± 1.2	<.001
25(OH)D_3, nmol/L	66.8 ± 1.1	63.5 ± 1.0	69.3 ± 1.3	<.001
C3-epi-25(OH)D_3, nmol/L	4.3 ± 0.2	4.1 ± 0.1	4.4 ± 0.2	.042

Note: Data are numbers (percentages) unless otherwise indicated. All estimates accounted for complex survey designs.
Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; NHANES, National Health and Nutrition Examination Survey.

the IVs could be invalid, but they may not result in a bias in the estimated causal effect (Hartwig et al., 2017).

In the second sensitivity analysis, for the levels of total 25(OH)D and 25(OH)D_3, we limited our instrument selection to SNPs in or close to the genes (*DHCR7*, *CYP2R1*, *GC*, *CYP24A1*) directly involved in synthesizing or degrading vitamin D (Figure S3) (Manousaki et al., 2020). In the third analysis, to further reduce the risk of horizontal pleiotropy, we searched PhenoScanner to check whether these selected instruments were associated with other diseases or traits that affect periodontitis susceptibility independent of 25(OH)D metabolites (Staley et al., 2016). Lastly, we performed MR analysis using periodontitis-associated SNPs as IVs to determine whether the associations observed between periodontitis and 25(OH)D metabolite levels are a result of reverse causality.

We also performed power calculations using Brion's method (Brion et al., 2013). Based on the sample size of 45,563 individuals (17,353 cases and 28,210 controls), an alpha level of 0.05, and an assumed variance of 3.5% explained by the genetic instruments, the present study had a power of 80% for detecting an effect on periodontitis as small as OR = 1.16 per 1-SD increase in the natural log-transformed levels of 25(OH)D metabolites.

3 | RESULTS

3.1 | Population characteristics of NHANES

For the given study period (2009–2012), a total of 7246 adults from the United States with data on exposure (25(OH)D metabolites) and outcome (periodontitis) were eligible for analysis, whose main characteristics are shown in Table 1. Of these participants, 3625 (50.0%) met the diagnostic criteria for periodontitis according to CDC/AAP. Compared with the healthy controls, participants with periodontitis were more likely to be older, male, current smokers, and obese; had a higher prevalence of cardiometabolic conditions (hypertension, diabetes, hypercholesterolaemia, and cardiovascular diseases); and had lower levels of education, family income, leisure-time physical activity, and healthy eating index. In addition, the levels of total 25(OH)D, 25(OH)D_3, and C3-epi-25(OH)D_3 were significantly lower among participants with periodontitis than healthy controls.

3.2 | Association between serum 25(OH)D metabolites and periodontitis

Table 2 shows the association between serum 25(OH)D metabolites and the prevalence of periodontitis. In the age-, gender-, and ethnicity-adjusted model, an inverse relationship was observed between total 25(OH)D and periodontitis. Each 1-point increase in SD of the log-transformed levels of 25(OH)D resulted in a 19% decrease in the risk of periodontitis (95% CI: 0.75–0.88, $p < .001$, model 1). After potential confounders, including lifestyle factors, BMI, and self-reported health conditions, were adjusted for, the results were not considerably affected. In comparison to the controls who did not have periodontitis, in participants with periodontitis, the multivariable-adjusted OR was 0.85 (95% CI: 0.78–0.93, $p = .006$, model 3). Additionally, the ORs for periodontitis risk significantly decreased across the quantile of the 25(OH)D levels (p for trend = .006, model 3). Compared to the participants in the lowest quantile of 25(OH)D levels, those in the highest quantile had an OR of 0.63 (95% CI: 0.51–0.78, $p = .004$, model 3) after adjusting for multiple confounders. The cubic spline model indicated a potential non-linear relationship between 25(OH)D and periodontitis (p for non-linearity = .008, Figure 3a).

The relationship between 25(OH)D_3 and periodontitis was similar to that between 25(OH)D and periodontitis across all three models (Table 2). Every 1 point increase in the SD of the log-transformed levels of 25(OH)D_3 resulted in a decrease of 12% in the risk of periodontitis (95% CI: 0.80–0.97, $p = .031$, model 3) in the multivariable-adjusted model. We also found a statistically significant decline in ORs across the quantiles of 25(OH)D_3 levels (p for trend = .020, model 3). Moreover, a similar non-linear relationship was observed between 25(OH)D_3 and periodontitis (p for nonlinearity = .019, Figure 3b). However, no association was found between the C3-epi-25(OH)D_3 levels and periodontitis risk after controlling for potential confounders (Table 2).

3.3 | MR of 25(OH)D metabolites and periodontitis

We first used the fixed-effects IVW method to pool effect estimations from individual genetic instruments. There was no association between a 1 point SD increase in the log-transformed levels of 25(OH)D and periodontitis (OR = 1.02, 95% CI: 0.90–1.16, $p = .732$). This indicated that there was no proof of a causal relationship between the levels of 25(OH)D and periodontitis risk. Similar findings were obtained for the association of 25(OH)D_3 (OR = 1.04, 95% CI: 0.93–1.17, $p = .472$) and C3-epi-25(OH)D_3 (OR = 1.11, 95% CI: 0.87–1.41, $p = .400$) with the risk of periodontitis (Figure 4, Table S3). We also performed a variance heterogeneity test to examine the robustness of the IVW results, which showed that there was low proof of heterogeneity (Table S4).

3.4 | Horizontal pleiotropy assessment and sensitivity analysis

The intercept estimated from MR-Egger regression did not deviate significantly from zero (Table S4); thus, there was no apparent horizontal pleiotropy. To test for potential bias from horizontal pleiotropy, several sensitivity analyses were used to investigate the relationship between the levels of 25(OH)D metabolites and periodontitis. The estimates were similar to those obtained with the IVW method when all SNPs were used (Figure 4 and Table S3). The consistent effect across the multiple methods demonstrated that our results were robust against bias from horizontal pleiotropy.

TABLE 2 Odds ratio (95% CI) for prevalent periodontitis according to serum 25(OH)D metabolite concentrations among participants in NHANES 2009–2012

	Serum 25(OH)D metabolite concentrations (nmol/L)[a]				
	Quantile 1	Quantile 2	Quantile 3	Quantile 4	Per 1 SD increment in log-transformed level
25(OH)D					
Median (range)	34.8 (8.3–45.8)	54.9 (45.8–62.8)	71.4 (62.8–81.4)	94.1 (81.4–375.0)	
No. of cases/total	1055/1817	921/1815	861/1805	788/1809	3625/7426
Model 1[b]	1.00	0.68 (0.58–0.80)***	0.63 (0.52–0.77)***	0.57 (0.47–0.69)***	0.81 (0.75–0.88) ***
Model 2[c]	1.00	0.70 (0.57–0.86)**	0.66 (0.52–0.84)**	0.63 (0.52–0.77)***	0.85 (0.78–0.92) **
Model 3[d]	1.00	0.68 (0.54–0.85)*	0.64 (0.50–0.83)*	0.63 (0.51–0.78)**	0.85 (0.78–0.93)**
$25(OH)D_3$					
Median (range)	30.8 (5.2–41.6)	50.6 (41.6–59.0)	67.2 (59.0–77.5)	90.0 (77.5–373.7)	
No. of cases/total	1032/1812	925/1811	875/1812	793/1811	3625/7426
Model 1[b]	1.00	0.71 (0.60–0.83)***	0.73 (0.60–0.89)**	0.63 (0.52–0.77)***	0.85 (0.79–0.92) ***
Model 2[c]	1.00	0.75 (0.60–0.94)*	0.75 (0.59–0.94)*	0.72 (0.59–0.87)**	0.89 (0.81–0.97) *
Model 3[d]	1.00	0.73 (0.56–0.94)*	0.71 (0.55–0.93)*	0.71 (0.56–0.88)*	0.88 (0.80–0.97)*
C3-epi-$25(OH)D_3$					
Median (range)	1.2 (1.1–2.0)	2.6 (2.0–3.1)	3.8 (3.1–4.7)	6.3 (4.7–37.8)	
No. of cases/total	967/1815	956/1810	866/1817	836/1804	3625/7426
Model 1[b]	1.00	0.96 (0.77–1.19)	0.79 (0.65–0.96)*	0.79 (0.63–1.00)	0.91 (0.83–0.99)*
Model 2[c]	1.00	1.00 (0.78–1.27)	0.83 (0.66–1.04)	0.82 (0.63–1.05)	0.92 (0.84–1.00)
Model 3[d]	1.00	0.96 (0.73–1.26)	0.81 (0.62–1.05)	0.84 (0.62–1.12)	0.93 (0.84–1.03)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; NHANES, National Health and Nutrition Examination Survey.

[a]ORs of periodontitis comparing quantile (Q2 to Q4) of 25(OH)D metabolites with Q1 or per 1 SD increase of 25(OH)D metabolites, estimated from weighted logistic regression models.

[b]Model 1: adjusted for age, gender, and ethnicity.

[c]Model 2: further adjusted for BMI, education level, family income-to-poverty ratio, smoking status, alcohol consumption, leisure-time physical activity, and healthy eating index.

[d]Model 3: further adjusted for self-reported health conditions, including hypertension, diabetes, hypercholesterolaemia, cardiovascular disease, and cancer.

*$p < .05$; **$p < .01$; ***$p < .001$.

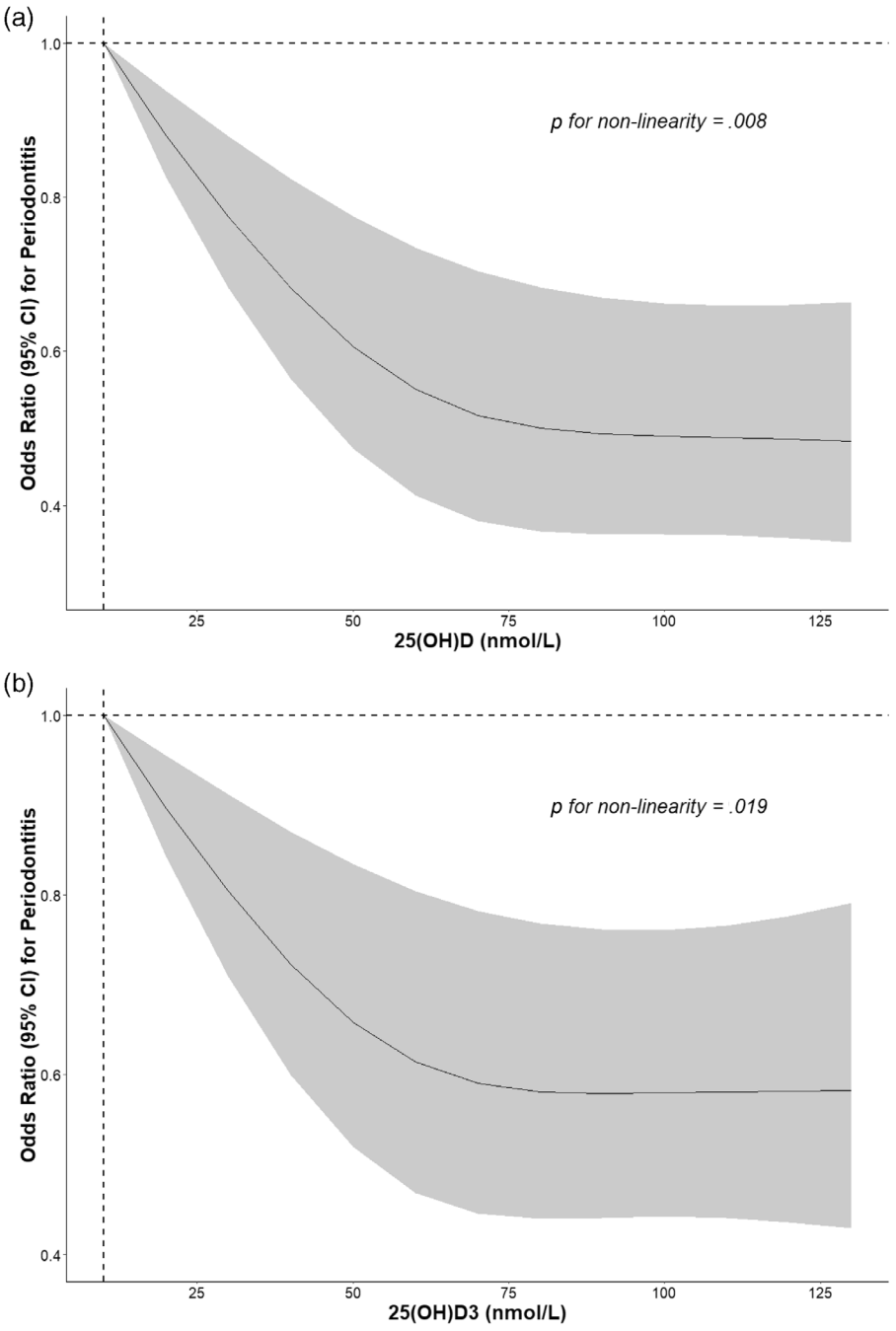

FIGURE 3 Association of serum 25(OH)D (a) and 25(OH)D_3 (b) with periodontitis among participants in NHANES 2009–2012. Odds ratios (ORs) were adjusted for age, gender, ethnicity, body mass index, education level, family income-to-poverty ratio, smoking status, alcohol consumption, leisure-time physical activity, healthy eating index, and self-reported healthy conditions (hypertension, diabetes, hypercholesterolemia, cardiovascular disease, and cancer). The solid line represents ORs, and the shaded areas represent 95% confidence intervals. Knots were set at 25th, 50th, and 75th. The reference values for 25(OH)D and 25(OH)D_3 were 10.0 nmol/L (OR = 1.0). 25(OH)D, 25-hydroxyvitamin D; NHANES, National Health and Nutrition Examination Survey; OR, odds ratio

With regard to 25(OH)D and 25(OH)D_3, we restricted our MR instruments to SNPs close to genes that affect vitamin D synthesis and metabolism directly. A total of eight SNPs for 25(OH)D levels and four SNPs for 25(OH)D_3 levels were detected within or beside the *GC*, *CYP24A1*, *CYP2R1*, and *DHCR7* genes, and explained 2.6% and 4.2% of the phenotypic variation, respectively. Again, neither the IVW method nor the five sensitivity analyses indicated a causal link between 25(OH)D and 25(OH)D_3 levels and periodontitis risk (Table S3).

To alleviate further concerns about horizontal pleiotropy, we examined whether the selected instruments for the MR analyses were associated with other phenotypes with the help of PhenoScanner. We excluded rs11723621/rs4588 (*GC* gene), rs10832289 (*CYP2R1* gene), and rs6127099/rs17216707 (*CYP24A1* gene), which are related to white blood cell count, hip circumference, and glomerular filtration rate, respectively, at a genome-wide level of significance. Five of the remaining genetic instruments accounted for 0.9% of the 25(OH)D variance, and the two remaining variants accounted for 1.2% of the 25(OH)D_3 variance. We found that the causal effect estimate remained largely unchanged, although the CIs had widened further (Table S3).

We also examined the reverse impact of periodontitis risk on 25(OH)D and 25(OH)D_3 levels using periodontitis-related genetic IVs (Table S5). The causal effect of periodontitis risk was not statistically

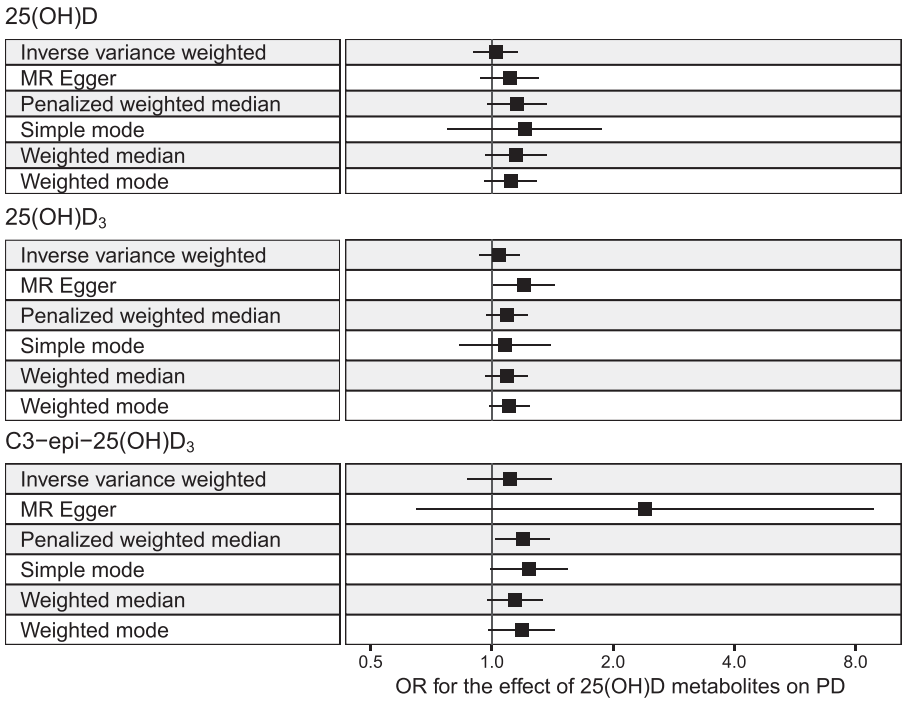

FIGURE 4 Forest plot of the Mendelian randomization study investigating the effect of 25(OH)D metabolites on periodontitis.

significantly associated with a 1-SD increase in the level of 25(OH)D ($\beta = -.003$, 95% CI: −0.023 to 0.017, $p = .787$) or 25(OH)D_3 ($\beta = -.025$, 95% CI: −0.065 to 0.015, $p = .223$), ruling out the probability of reverse causation.

4 | DISCUSSION

To the best of our knowledge, the present study is the first to provide a comprehensive investigation of the association of vitamin D status with periodontitis risk based on a combination of large-scale observational study data and MR analysis of large-scale genetic data. We investigated the association between periodontitis and metabolites of 25(OH)D, including 25(OH)D_3 and C3-epi-25(OH)D_3, and their potential causal relationship. The results showed that total 25(OH)D and 25(OH)D_3 had an inverse association with periodontitis, but C3-epi-25(OH)D_3 did not show any association with periodontitis. We also observed a potential non-linear relationship between periodontitis and total 25(OH)D and 25(OH)D_3. However, in contrast to the observational findings, the results of the wide-ranging MR study did not support an association between 25(OH)D or its subtypes with periodontitis risk. Specifically, increase in these variables was not association with protection against periodontitis. This lack of evidence for a causal relation was confirmed by our sensitivity analyses and genetic instrument selection.

The inverse association between total 25(OH)D and periodontitis observed in the present large-scale study is in line with the results of numerous other studies (Perayil et al., 2015; Ebersole et al., 2018; Machado et al., 2020). In the present study, we also observed a potential non-linear relationship (threshold effect) between periodontitis and total 25(OH)D. As far as we know, very few studies have reported this non-linear association before. The non-linear relationship might explain the inconsistent association of 25(OH)D with periodontitis reported by previous observational studies. However, in contrast to the observational findings, our present wide-ranging MR study did not support the association between 25(OH)D and periodontitis risk. Our results confirm the recent MR findings that no causal link is present between 25(OH)D and periodontitis (Baumeister et al., 2021). And the recent MR study of the causal relationship between vitamin D and periodontitis supports our conclusions (Z. Hu et al., 2022). However, our analysis provides more solid evidence than the previous study because our study includes various sensitivity methods to preclude the possibility of bias from horizontal pleiotropy and uses a bi-directional MR method to eliminate reverse causality between periodontitis and 25(OH)D concentration. With regard to the variation between the observational and MR results, adjusting the confounding effects of adiposity, diet, and physical activity (with only one inaccurate measure used to represent these variables) at the baseline may only have moderately decreased their effects on the observational results. Importantly, MR provides a higher level of evidence; therefore, there was no causal relationship between 25(OH)D and periodontitis.

Consistent with the results for total 25(OH)D, 25(OH)D_3 was also found to have a reverse correlation and non-linear relationship with periodontitis, and this is also in agreement with previous studies (Dietrich et al., 2004; Wang et al., 2019). With regard to the underlying mechanism, 25(OH)D_3 may modulate periodontal inflammation and bone absorption by inhibiting the production of interleukin (IL)-8 and monocyte hemoattractant protein (MCP)-1 (Andrukhov et al., 2014; Nakashyan et al., 2017). However, contrary to the observational studies, our MR study (again) showed that there was no causal relationship between 25(OH)D_3 and periodontitis. Thus, our results further support the previous evidence that there is no causal relationship between 25(OH)D and periodontitis.

To our knowledge, neither observational studies nor MR studies have examined the association between periodontitis and C3-epi-25(OH)D_3 (which is an isomer of 25(OH)D_3). This is probably because the epimers of 25(OH)D_3 have only recently been detected in human paediatric and adult populations (Messerlian et al., 2000; Djekic-Ivankovic et al., 2017). Additionally, the technical difficulties involved in the measurement of low concentrations of 25(OH)D_3 make it difficult to include this variable in large-scale investigations. Considering the potential involvement of C3-epi-25(OH)D_3 in bone metabolism (Holick et al., 1980; Haddad et al., 1993), it is important to study the relationship between this 25(OH)D_3 isomer and periodontitis.

However, we found that C3-epi-25(OH)D_3 was not associated with periodontitis in either the observational or the MR studies. Thus, the findings based on the biochemical measurements of 25(OH)D metabolites and their genetically predicted concentrations are conflicting. Based on the present findings, along with the results of randomized controlled trials, vitamin D supplementation cannot be recommended for the prevention of periodontitis.

The strengths of the present study are its large sample size, the inclusion of several 25(OH)D metabolites, and the use of genetic information as an instrumental variable. Importantly, the MR analysis allowed the evaluation of the causal link between 25(OH)D metabolites and periodontitis, and this approach is less vulnerable to bias from reverse causation and confounding compared with traditional observational studies. There are also several limitations of this study that should be mentioned. First, in our MR study, we cannot entirely exclude horizontal pleiotropy, that is, a link between the outcome of interest and the MR instrument through pathways other than the suggested exposure, which is a typical problem in MR studies and a cause of bias. However, it should be noted that the MR-Egger intercepts in the statistical analysis revealed no indication of pleiotropy. A second limitation is the possibility of a weak instrument bias, especially with the sensitivity analyses which were limited to smaller genetic instrument sets. Third, as seasons affect the levels of vitamin D (i.e., they are higher after exposure to sunlight), reducing this effect by calculating the average effect of 25(OH)D on periodontitis across all seasons may have influenced the results. Furthermore, MR analysis estimates the lifetime effect of exposure and not the effect at a specific time. Therefore, it is not clear whether vitamin D supplementation for preventing periodontitis at a particular time in life for a specific duration would be useful. Fifth, our observational data indicate a potential non-linear link between periodontitis and 25(OH)D metabolites. However, in the MR study, only a linear causal association was examined, so non-linear causality cannot be ruled out. Additionally, our observational and genetic data did not originate from the same samples, as we used a multi-ethnic U.S. population for the cross-sectional study and individuals of European descent for the MR study. A future study on a study population of the same ethnicity is needed to preclude potential confounding factors for population heterogeneity. Finally, because of differences in allele frequencies and disease or exposure rates across ancestries, IVs obtained from European populations might not be present in other populations. Therefore, the generalizability of the genetic analysis is limited to individuals of European descent. In the future, these findings need to be confirmed with observational, experimental, and MR studies on 25(OH)D metabolites in other populations.

5 | CONCLUSION

In conclusion, our MR study does not indicate a causal link between periodontitis and genetically defined increased levels of 25(OH)D and its metabolites, even though the observational studies indicated a strong association between 25(OH)D, as well as 25(OH)D_3, and periodontitis. The observational findings could be biased as a result of uncontrolled confounders. Therefore, based on the present findings, there is no indication that vitamin D supplementation may be useful for the prevention of periodontitis.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to the conception and design of the study. Wenjing Li and Qiwen Zheng have been involved in data analysis, data interpretation, drafting the manuscript, and revising it critically. Xuliang Deng, Changqing Zeng, and Mingming Xu have been involved in designing the study and revising the manuscript critically. Xuliang Deng gave the final approval of the version to be published.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest concerning this article's authorship and publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at https://wwwn.cdc.gov/nchs/nhanes/Default.aspx (NHANES), https://cnsgenomics.com/content/data (total 25(OH)D), https://doi.org/10.6084/m9.figshare.12611822.v1 (25(OH)D3 and C3-epi-25(OH)D3), and https://data.bris.ac.uk/data/dataset/2j2rqgzedxlq02oqbb4vmycnc2 (periodontitis).

ETHICS STATEMENT

Each cohort included in this study received its respective institutional research ethics board's approval to enroll patients and all participants provided written informed consent. All information used for this study is publicly available as deidentified GWAS summary statistics.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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