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The role of vitamin D in metabolic and cardiovascular health

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The role of vitamin D in metabolic and cardiovascular health

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Editorial: The role of vitamin D in metabolic and cardiovascular health

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Editorial on the Research Topic
The role of vitamin D in metabolic and cardiovascular health

In the last few decades, interest in vitamin D (VitD) has grown significantly since numerous studies have suggested that besides its well-established roles in bone metabolism (1), it could have other important roles in organism, including roles in immunity, endocrine, cardiovascular, and reproductive system (2–7). Many studies indicated that VitD status is inversely associated with the incidence of several metabolic diseases and conditions, including obesity (8, 9), insulin resistance (10–14), metabolic syndrome (15–17), dyslipidemia (13, 18, 19), diabetes (10, 11, 20–22), non-alcoholic fatty liver disease (NAFLD) (23–25), and cardiovascular diseases (7, 26–28). However, the findings were often inconsistent, and the cause/effect relationships particularly remained to be confirmed, as well as the molecular pathways of these associations. Moreover, the relationship of VitD with metabolic and cardiovascular disorders seems to be bidirectional: e.g., obesity could worsen VitD deficiency (8), and *vice versa*, VitD deficiency could aggravate obesity and related metabolic and cardiovascular complications (insulin resistance, defects in insulin secretion, disordered metabolism of lipids, hepatic steatosis, and gut dysbiosis) (7, 9) by multiple mechanisms, many of which are still undiscovered and unclarified. Additionally, since in the above-mentioned disorders and diseases there is also chronic inflammation and increased oxidative stress, the role of VitD as immunomodulator and anti-oxidative agent has been proposed as one of the mechanisms by which VitD can influence these conditions (29–34).

This Frontiers Research Topic "*The role of vitamin D in metabolic and cardiovascular health*" focused on epidemiological research on associations of VitD with metabolic and cardiovascular health, particularly in specific population groups, and pathophysiological pathways of these associations, as well as possible confounding factors which modulate these associations. The Research Topic welcomed also articles on the role of VitD as one of supporting therapeutics in cardiovascular and metabolic diseases, as well as immunomodulator.

In this Research Topic there are 13 papers covering the above-mentioned aspects.

Chen Y. C. et al. compared the risk for VitD deficiency across different categories of metabolically healthy normal weight (MHNW) to metabolically unhealthy overweight/obese insulin resistant (MUO) subjects, by studding 6,655 Chinese adults. The study confirmed the

highest risk for VitD deficiency among the MUO subjects, but also indicated some gender and age- related differences: among men, the increased risk was noted particularly in MUO men >50 years old, while in younger men, the risk was highest among metabolically healthy obese (MHO) men. In contrast, among women, in both age subgroups the highest risk was represented among MUO women, but the stronger association was noted among younger women. This study indicated possible gender-influenced associations of VitD status with obesity and adverse cardiometabolic and inflammatory profiles.

Similarly, Yin X. et al. analyzed the associations of VitD status with HOMA-IR (Homeostatic Model Assessment of Insulin Resistance), as a robust measure of insulin resistance, in 6,079 American adults without diabetes and other chronic diseases, by using the data from the National Health and Nutrition Examination Surveys (NHANES). The study also confirmed the negative associations between serum VitD concentrations and HOMA-IR, which remained significant after multiple adjustments for many possible confounders, including age, gender, race/ethnicity, and body mass index (BMI). Nevertheless, the further stratification analyses showed some racial/ethical differences: in people with Non-Hispanic Black origin this inverse association between VitD and HOMA-IR was not observed, which indicates the need for further studies focused on ethnic/racial disparities.

Song et al. examined the possible additive effects of obesity and VitD status on the all-cause, cardiovascular and cancer-related mortality, by using the data from the NHANES surveys. In the models adjusted for multiple confounders (including age, gender, race/ethnicity, smoking, and BMI), an independent effect of VitD both insufficiency and deficiency on all mortality rates was confirmed, with deficiency having stronger effect. Interestingly, the effect of VitD deficiency overcame the effect of obesity on all mortality rates. The highest risk for overall and cardiovascular mortality was observed among VitD deficient obese subjects, while for cancer mortality among VitD deficient normal weight subjects, indicating different mechanisms of associations of VitD with mortality in different conditions.

Similarly, Chen X. et al. examined the possible effects of VitD status on the all-cause, cardiovascular and cancer-related mortality among subjects with hyperlipidemia, by using the data from the NHANES surveys. In the models adjusted for multiple confounders (including age, gender, race/ethnicity, smoking, and BMI), serum VitD level was identified as an independent factor for all-cause and cardiovascular mortality, but no association was found with malignancy-specific mortality among these subjects. Particularly serum VitD levels <25 ng/ml were associated with a higher risk for all-cause and cardiovascular mortality, indicating the need for monitoring of VitD levels and correcting VitD insufficiency/deficiency among hyperlipidemic subjects.

Zheng et al. using the NHANES data found a significant negative correlation between serum VitD levels and the risk of frailty in older people.

Shree et al. performed a meta-analysis on the association of serum VitD levels and polymorphism in the VitD receptor (VDR) gene with celiac disease, showing that reduced serum level of 25(OH)D and rs2228570-T polymorphism of *Fok1* T-allele of VDR gene could be implicated in pathophysiology of this autoimmune disease. Zhou et al. examined the association between serum VitD levels and plasma myeloperoxidase (MPO) levels, as a marker of oxidative stress, in 6,414 Chinese women and men. After adjusting for multiple confounders, the study found that circulating 25(OH)D was negatively associated with MPO levels.

Yin W. J. et al. examined 3,713 pregnant Chinese women in the second trimester of pregnancy, their serum VitD levels, biochemical and clinical indicators of cardiovascular risk and inflammation, and the inflammatory potential of their diet, using the empirical dietary inflammatory pattern (EDIP) score. The study revealed that serum VitD levels mediated significant proportion of the association between the dietary inflammatory potential (i.e., EDIP score) and cardiovascular risk in pregnant women. At the same time, the circulating marker of inflammation, high-sensitivity C-reactive protein (hs-CRP), mediated significant proportion of the association between serum VitD levels and cardiovascular risk, indicating significance of anti-inflammatory effect of VitD in the prevention of cardiometabolic disturbances related to pro-inflammatory diets.

Yang et al. performed a meta-analysis on the effects of VitD supplementation on the circulating lipid levels in subjects with prediabetes, and found that VitD supplementation might beneficially affect triglyceride levels in these subjects, while no significant effects on total cholesterol, HDL-cholesterol and LDL-cholesterol were found. The study revealed that particularly longer duration of treatment (more than 1 year), with doses which correct VitD deficiency/insufficiency, are required to improve triglyceride levels. However, just a few studies were included, and more research on that topic is necessary.

An interesting article by Lee et al. focused on the effect of VitD supplementation on hypercalciuria/urolithiasis prevention in 140 children with epilepsy undergoing ketogenic dietary therapy (KDT). It is known that ketogenic diets relate to increased risk for hypercalciuria/urolithiasis, while the role of VitD on this risk is less clear. Interestingly, the study showed an inverse association of serum VitD levels with the urinary calcium/urinary creatinine ratio, a marker of hypercalciuria. The study also pointed-out that the serum VitD levels >40 ng/mL and the vitamin D3 supplementation doses >50 IU/kg are probably needed for preventing hypercalciuria related to KDT.

Bernardo et al. studied the combined effect of obesity (induced by a high-fat diet) and VitD dietary depletion on metabolic profile and progression of kidney damage in an experimental model of ischemia/reperfusion kidney injury in rats. The study pointed out both independent and additive effects of obesity and VitD depletion on exacerbation of multiple metabolic and inflammatory changes, and progression of functional, hemodynamic, and morphological kidney alterations.

Gázquez et al. studied the effect of VitD supplementation during pregnancy in rats by using different VitD metabolites. The study showed that the monohydroxylated form of VitD, 25(OH)D3, given orally provided better VitD availability compared to vitamin D3: it doubled 25(OH)D3 concentrations in maternal and fetal blood. No adverse effects on pregnancy and fetus were shown. Moreover, 25(OH)D3 had an additional effect on the expression of VDR, fatty acid translocase (FAT), and scavenger-receptor class B type-1 (SR-B1) in maternal liver; and

VDR and glutamate decarboxylase GAD67 in fetal brain, which requires further investigation.

Finally, Hanel et al. compared the gene-regulatory potential of three different VitD metabolites: 25(OH)D3, 25(OH)D2, and 1,25(OH)2D3 in human peripheral blood mononuclear cells (PBMCs), and found that although monohydroxylated metabolites can have similar effect on expression of 206 common target genes, their effective concentrations were in the range of supra-physiological concentrations and were 600-fold higher than effective concentrations for 1,25(OH)2D3, indicating 600-fold lower effectiveness.

In summary, the articles in this Research Topic confirm the independent and additive role of VitD in pathophysiology of cardiometabolic and autoimmune diseases, and emphasizes the need for further research in this field. Particularly studies in different population groups, studies on pathophysiological mechanisms, and well-controlled randomized trials with VitD as preventive and therapeutic agent, are needed.

Author contributions

IŠ wrote the manuscript. MD-I and JD-M revised, co-wrote, and edited the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Calcifediol During Pregnancy Improves Maternal and Fetal Availability of Vitamin D Compared to Vitamin D3 in Rats and Modifies Fetal Metabolism

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The fetus depends on the transplacental transfer of vitamin D. Calcifediol (25-OH-D3) is the vitamin D metabolite that crosses the placenta. Previously, oral 25-OH-D3 improved serum 25-OH-D3 compared to vitamin D3 in non-pregnant subjects, although no studies are available in pregnant women. We evaluated the availability of oral 25-OH-D3 compared to vitamin D3 during pregnancy, as well as, their levels in the fetus and effect on metabolism-related proteins. Twenty female rats per group were fed with 25 μg/kg of diet of vitamin D3 (1,000 UI vitamin D/kg diet) or with 25 μg/kg diet of 25-OH-D3. We analyzed 25-OH-D3 levels in maternal and fetal plasma; protein levels of vitamin D receptor (VDR), fatty acid translocase (FAT), and scavenger-receptor class B type-1 (SR-B1) in both maternal liver and placenta; and protein levels of VDR and Glutamate decarboxylase (GAD67) in fetal brain. 25-OH-D3 doubled the concentration of 25-OH-D3 in both maternal and fetal plasma compared to vitamin D3. In addition, maternal liver VDR, FAT, and SR-BI increased significantly in the 25-OH-D3 group, but no changes were found in the placenta. Interestingly, 25-OH-D3 decreased GAD67 expression in the fetal brain and it also tended to decrease VDR (P = 0.086). In conclusion, 25-OH-D3 provided better vitamin D availability for both mother and fetus when administered during pregnancy compared to vitamin D3. No adverse effects on pregnancy outcomes were observed. The effects of 25-OH-D3 on the expression of VDR and GAD67 in fetal brain require further investigation.

Keywords: availability, calcidiol, calcifediol, pregnancy, vitamin D

Abbreviations: 25-OH-D3, hydroferol; FAT/CD36, fatty acid translocase; GAD, glutamate decarboxylase; GAD67, glutamic acid decarboxylase 67; PBS-T, phosphate saline buffer with 0.05% Tween-20; SEM, standard error of the mean; SR-B1, scavenger receptor class B type-1; VDR, vitamin D receptor; Vitamin D3, cholecalciferol.

INTRODUCTION

Maternal vitamin D insufficiency, during both pregnant and non-pregnant states, is a common issue and a significant problem in public global health (1). Supplementation of food with vitamin D or the use of vitamin D supplements is the most universal strategy to improve vitamin status. Cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) are the most widely used compounds. While the use of vitamin D3 and vitamin D2 has been supported by historical data and practicality, calcifediol (25-OH-D3) should be evaluated as an alternate oral supplement during pregnancy. Evidence is mounting that it is a more bioavailable form of vitamin D in the non-pregnant state (2, 3).

Oral supplementation with 25-OH-D3 resulted in a more rapid increase in serum 25-OH-D3 compared to oral vitamin D3 in non-pregnant subjects (3). This is consistent with a higher intestinal absorption rate for 25-OH-D3 (4, 5), that may have important advantages when intestinal absorption capacity is decreased due to disease. In addition, as oral 25-OH-D3 is more potent than vitamin D3, lower dosages are needed to achieve desired therapeutic effects (6). There is still no consensus on the vitamin D activity (IU units) conversion factor for 25-OH-D3 and much less is known in the pregnant population (2, 3, 6, 7). Hemodilution may lead to differential responses to vitamin D supplementation between pregnant women. Since some women of reproductive age receive 25-OH-D3 supplementation, it is also important to evaluate the efficiency and risks of such supplementation during pregnancy.

Clinical research investigating the role of vitamin D in human health and disease has relied on the measurement of total 25-OH-D3 in serum or plasma to assess vitamin D status. However, 25-OH-D3 is an inactive form of vitamin D that requires further hydroxylation in the kidneys into 1,25-(OH)2-D3, the active form of vitamin D. However, the active metabolite 1,25-(OH)2-D3 cannot cross the placenta, but 25-OH-D3 readily crosses (8, 9). As the placenta expresses the enzyme 1-α-hydroxylase, it may synthesize 1,25-(OH)2-D3, which seems to play an immunomodulatory role within fetal tissue (9).

The activation of vitamin D receptor (VDR) is heavily dependent on the binding of 1,25-(OH)2-D3 to the receptor (10). The VDR-1,25-(OH)2-D3 complex then translocates into the nucleus to activate DNA transcription. Better understanding of the mechanisms involved in the placental transfer and fetal availability of key nutrients are essential to provide more solid dietary advice to pregnant women.

Fatty acid translocase (FAT/CD36) is a fatty acid carrier placed in the plasma membrane of several tissues, including placenta, and may transport vitamin D and other lipophilic compounds (11). In addition, FAT/CD36 is essential for the very low-density lipoprotein (VLDL) exportation from the liver and its deletion is related to liver steatosis, obesity and non-alcoholic fatty liver disease (12–14). By other hand, scavenger receptor-B1 (SR-B1) is the main receptor of high-density lipoprotein (HDL) in the liver and is pivotal for the uptake of cholesterol from peripheral tissues back into the liver and cholesterol reverse transportation to feces (14). SR-B1 is involved in the cellular uptake of vitamin D (15) but its role in vitamin D tissue storage and the status of vitamin D is currently not known.

In this study, we aimed to compare for the first time the bioavailability of 25-OH-D3 and vitamin D3 administrated during pregnancy. We explored their effects on serum status and on several proteins related to vitamin D transport and metabolism measured in maternal, placenta, and fetal tissues. 25-OH-D3 supplementation is of major interest because it could likely be supplemented at a lower dose than vitamin D3 in order to achieve desirable efficacy in both pregnant women and their babies.

MATERIALS AND METHODS

Animals and Study Design

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Murcia (N° A13180105) and conform to the ARRIVE guidelines for animal research (16). Animals received humane treatment in accordance with the European Union guidelines for the care and use of laboratory animals. Female adult Sprague Dawley rats (7 weeks of age) were supplied by the Animal Laboratory Service of the University of Murcia. Animals were housed individually with *ad libitum* access to food and water in a humidity and temperature-controlled ($22 \pm 1°C$) room on a 12 h light/dark cycle.

Forty female rats of 7 weeks of age were fed a modified version of AIN-93M diet (17) for 10 days. This modified diet provided by the Abbott Nutrition was deficient in vitamin D, vitamin E and folic acid to ensure similar vitamin basal status in all the animals (Modified AIN-93 Vitamin Mix at 10 g/kg of diet without Vitamin E, Vitamin D, or Folic Acid). Animal weight was recorded every week. After this nutritional deprivation period, the female rats were split into two groups (n = 20 each group) and each group of rats were fed a particular test diet for 4 weeks: (1) The control group received commercial AIN-93G diet with 25 μg/kg diet of vitamin D3 at 40 UI/g Vitamin D3 (1,000 UI vitamin D/kg of diet) and (2) the calcifediol experimental group received the AIN-93G diet but with 25 μg/kg of 25-OH-D3 (Merck, Germany) instead of vitamin D3. The commercial AIN-93 G diet used in both control and experimental groups contained vitamin E and folic acid according usual AIN-93G composition. Subsequently, the female rats were mated (1:1) with male rats. Once fecundation took place (by sperm presence in vaginal smear under the microscope), male rats were removed. Female rats were then allocated to appropriate cages and continued to be fed with their assigned test diets throughout the pregnancy. At day 20 of gestation, rats were anesthetized with a mixture of 5 mg ketamine hydrochloride, 0.25 mg chlorobutanol and 1 mg xylazine per 100 g body weight. Maternal blood was extracted by heart puncture and fetal blood by decapitation. Maternal liver, placenta, and fetal brain were also collected (4 placentas and 4 fetal brain were pooled per rat). Additionally, blood samples were also collected from the tail at different stages of the study: (1) at the start of the study (a subset of n = 4 animals), (2) after the nutritional deprivation period (another subset of n = 4 animals) and (3) after introduction of respective test diets for 4 weeks and

right before mating (n = 7 controls with Vitamin D3 and n = 7 with 25-OH-D3 diet) (**Figure 1**). Blood was collected in EDTA-coated tubes and centrifugated at 1,400 g for 10 min at 4°C to obtain plasma. Plasma and tissues were frozen in liquid nitrogen and stored at −80°C until analysis.

Plasma Vitamin D Analyses

25-OH-D3 was analyzed in the plasma of the animals by direct competitive immunoluminometric assay using coated magnetic microparticles in a LIAISON® XL automated analyzer (DiaSorin S.p.A., Saluggia, Italy). The plasma levels of 25-OH-D3 were analyzed using the tail blood samples collected mentioned above. The 25-OH-D3 levels of both maternal and fetal plasma collected at the end the study were also analyzed. Maternal blood was extracted by heart puncture and fetal blood by decapitation at day 20 of gestation.

Protein Extracts for Western Blotting

Protein extracts were obtained by homogenizing 30 mg of placental tissue, maternal liver or fetal brain in 0.3 mL ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptin) from Cell Signaling Technology (MA, United States). Phenylmethanesulfonyl fluoride solution 1 mM was added to the lysis buffer before homogenization (18). Samples were homogenized using a Tissue Lyser LT device (Qiagen Iberia SL, Madrid, Spain). Protein lysates were obtained from the supernatant after 15 min centrifugation at 10.000 g 4°C. Protein concentration was quantified by Bradford assay (19) and samples were stored at −80°C until Western blot analysis.

Western Blot Analysis

The primary antibodies used were: rabbit monoclonal against FAT/CD36 (Abcam, Cambridge, United Kingdom, Ref: ab17044) 1:250 in maternal liver and 1:200 in placenta; rabbit monoclonal anti-VDR (Abcam, Cambridge, United Kingdom, Ref: ab109234) 1:500 in maternal liver, 1:200 in placenta and 1:400 in fetal brain; rabbit monoclonal antibody against SR-B1 (Abcam, Cambridge, United Kingdom, Ref:ab217318) 1:700; rabbit monoclonal antibody against glutamic acid decarboxylase 67 (GAD67) (Abcam, Cambridge, United Kingdom, Ref: ab108626) 1:700, and mouse monoclonal anti-β-actin (Sigma-Aldrich, MO, United States, Ref:A5316). The secondary antibodies used were anti-mouse (Santa Cruz Biotechnology, TX, United States, sc 516102), anti- rabbit (Santa Cruz Biotechnology, TX, United States, sc-2357) and anti-goat (Sigma-Aldrich, MO, United States, Ref: SAB3700295-1MG) polyclonal antibodies conjugated with horseradish peroxidase.

The protein extracts (15 μg protein) diluted in sample buffer were resolved on 10% polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). Membranes were blocked in phosphate saline buffer with 0.05% Tween-20 (PBS-T) containing 2% bovine serum albumin for 1 h at room temperature. Thereafter, membranes were incubated with primary antibodies overnight at 4°C. Blots were then washed with PBS-T and probed for 1 h at room temperature with the correspondent secondary antibodies conjugated with horseradish peroxidase. Finally, membranes were stripped with Tris/HCl buffer pH 2.3 containing beta-mercaptoethanol 0.1 M and re-probed with anti-beta-actin to perform loading controls. Immunoblot signals were detected using a chemiluminescence kit according to the manufacture's instruction (Pierce ECL 2 Western Blotting Substrate; Thermo Fisher Scientific, MA, United States) (20). Density of all bands was determined by densitometry using Image Quant LAS 500 software (GE Healthcare, CA, United States). Relative protein expression data were normalized against β -actin level.

Statistical Analysis

Shapiro-Wilk normality test was used to check normal distribution of continuous variables. The results were expressed as mean ± standard error of the mean (SEM). The two experimental groups were compared using unpaired t-test analyses. ANOVA test was also applied for multiple testing followed by *post-hoc* Bonferroni in the comparisons of **Figure 2**. Pearson correlations were also performed. Chi^2 analysis of qualitative data were also analyzed. The statistical analyses were evaluated by the SPSS® 24.0 software package (IBM Corp., NY, United States). A p-value 0.05 was considered to be statistically significant.

RESULTS

Maternal, fetal, and placental weights were not significantly different between the groups fed 25-OH-D3 diet and the control group that was fed vitamin D3 (**Table 1**). The number of fetuses per dam was also similar between both diet groups. In addition, no differences were found in the abortion rate between both groups. There was also no difference in fertility rate. In summary, pregnancy-related outcomes are comparable between the study groups (**Table 1**).

The 10-day deprivation period resulted in significant decreases in plasma levels of 25-OH-D3 compared to the baseline level detected at the start of the study (Day 0) (**Figure 2**). The animals were split into two groups and were fed different diets for 4 weeks (Day 40). At Day 40, plasma levels of 25-OH-D3 were significantly higher in the 25-OH-D3 group than in the vitamin D3 control group. Furthermore, the level of 25-OH-D3 in the 25-OH-D3 group was higher than the baseline level detected at the study entry (**Figure 2**). In contrast, vitamin D3 group plasma 25-OH-D3 levels were not different from baseline levels. The data showed that 25-OH-D3 supplementation resulted in higher levels of 25-OH-D3 in plasma than vitamin D3 supplementation in non-pregnant animals.

At delivery, maternal plasma levels of 25-OH-D3 were significantly higher in the 25-OH-D3 group compared to those of the vitamin D3 group (**Figure 3A**). The maternal plasma concentration of 25-OH-D3 group was almost two times higher than that found in the vitamin D3 group. Fetal plasma 25-OH-D3 levels were also significantly higher in the 25-OH-D3 group compared to those of the vitamin D3 group (**Figure 3B**). The levels of fetal plasma 25-OH-D3 in the 25-OH-D3 group were

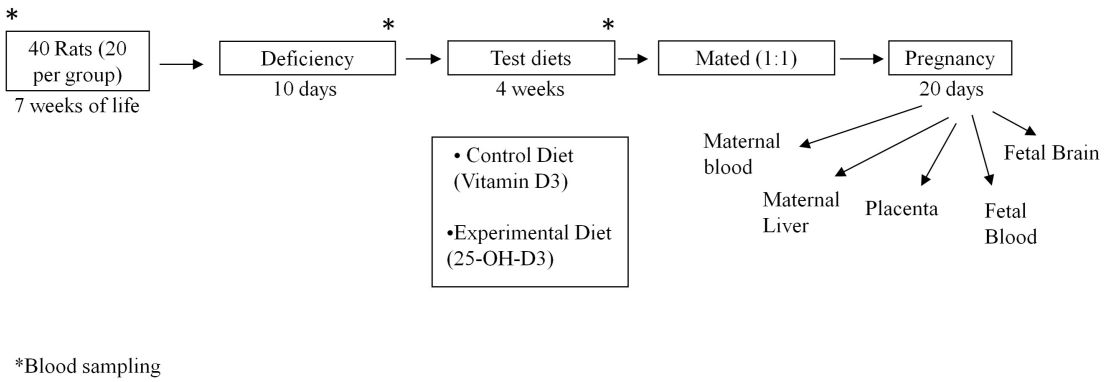

FIGURE 1 | Experimental design. *Blood sample collected at such time of the experiment.

about 1.6× higher than those detected in the vitamin D3 group (50 ng/mL in experimental group vs. 80 ng/mL in control). Thus, the results showed that 25-OH-D3 supplementation had higher potency in raising vitamin D status in both maternal and fetal plasma during pregnancy compared to vitamin D3 supplementation. There was a significant correlation between the levels of 25-OH-D3 in maternal and fetal plasma (r = 0.555, P = 0.005). 25-OH-D3 is the vitamin D metabolite that crosses the placenta. The ratio of fetal to maternal 25-OH-D3 concentrations at delivery was similar in all groups (25-OH-D3 fetal/maternal plasma: 3.51 ± 0.46 in the 25-OH-D3 group vs. 3.56 ± 0.13 in the vitamin D3 group, p = 0.908).

With regards to vitamin D metabolism, pregnant rat dams supplemented with 25-OH-D3 had significantly higher VDR hepatic protein levels compared to the vitamin D3 group (**Figure 4A**). The increase in protein levels were also observed for SR-B1 ($p < 0.05$) and FAT/CD36 (p = 0.059) (**Figure 4A**). The findings showed that the higher vitamin D levels in pregnant rat dams corresponded with higher expressions of vitamin D metabolism-related proteins, such as VDR which is a known receptor for 1,25-(OH)2-D3.

Interestingly, the protein levels for VDR, SR-B1, and FAT/CD36 in the placentas were similar in the two groups (**Figure 4B**). This result suggested that placental transfer of 25-OH-D3 from the mother to the fetus was not affected, highlighting the importance of achieving an optimal maternal 25-OH-D3 concentration for increasing fetal serum vitamin D status.

Due to the remarkable results observed in maternal liver, we decided to analyze VDR levels in fetal brain. Surprisingly, maternal 25-OH-D3 supplementation tended to decrease VDR expression in fetal brain (p = 0.086) although the differences were not statistically significant." This is probably due to a negative feedback mechanism to protect the fetal brain (**Figure 4C**). In addition, we also detected lower expression of GAD67, which is an established marker of GABAergic neurons, in the fetal brain by maternal 25-OH-D3 supplementation during pregnancy (**Figure 4C**). These results suggested that the impacts caused by the increase in vitamin D status in the fetuses were systemic.

DISCUSSION

We report for the first time during pregnancy that supplementation with 25-OH-D3 dramatically increased plasma 25-OH-D3 in both the dams and fetuses compared to D3 with important perturbations in the levels of vitamin D relevant proteins in both mother and fetus. For this study, we did not adjust the diets for vitamin D activity since the conversion rate for 25-OH-D3 remains uncertain. Vitamin D3 has a recognized biological activity of 40 IU per microgram. This conversion factor has been used to achieve 1,000 IU/kg in both the vitamin D and 25-OH-D3 diets. Nevertheless, it should be noted that commercial hydroferol (25-OH-D3) has been reported to be 60 IU per microgram. Therefore, there is a possibility that 25-OH-D3 conversion factor should be 60 IU per microgram or more instead of 40 IU per microgram. This could also explain the results obtained by this study.

Our study showed that 25-OH-D3 supplementation increased the level of 25-OH-D3 in both maternal and fetal serum two

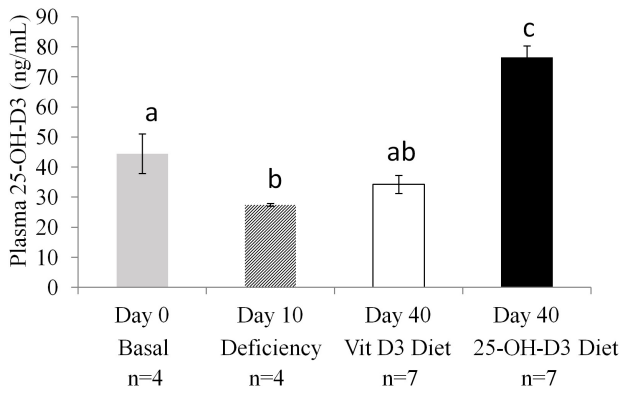

FIGURE 2 | Plasma 25-OH-Vitamin D3 levels in non-pregnant animals at different time points: at study entry (Basal n = 4), after a deficiency diet (n = 4) and after 4 weeks of feeding with test diets containing 25-OH-D3 (experimental diet n = 7) or vitamin D3 (n = 7) before mating. ANOVA, $P < 0.001$. Values not sharing a common superscript letter are significantly different ($P < 0.05$).

TABLE 1 | Animal characteristics and dietary intake.

	Experimental (n = 18)	Control (n = 16)	P
Maternal weight (g)			
At the start of the study	163.99 ± 3.08	169.02 ± 5.14	0.408
After deficiency (day 10)	192.68 ± 3.79	195.74 ± 4.50	0.604
At delivery	346.14 ± 8.13	355.69 ± 6.77	0.384
Dietary intake before pregnancy (g/d)	11.73 ± 0.31	12.12 ± 0.35	0.416
N° fetus	11.17 ± 1.01	12.81 ± 0.56	0.177
Fetal weight (g)	3.76 ± 0.19	3.34 ± 0.16	0.097
Placental weight (g)	0.56 ± 0.05	0.50 ± 0.01	0.202
Abortions per rat	0.28 ± 0.14	0.13 ± 0.09	0.361
No pregnant rats (n/%)*	2 (10%)	1 (6%)	0.647

Media ± SEM or n (%). Significance level set at $P < 0.05$ by t-test.
**Differences evaluated by Chi^2.*

times more effectively than supplementation with a matching level of vitamin D. The higher availability in fetal 25-OH-D3 occurred without differences in VDR, FAT/CD36, or SR-B1 protein expressions in placental tissue. The results on the relationship between placental VDR and 25-OH-De are scarce and contradictory. In mice, placental VDR expression was significantly up-regulated in vitamin D3-pretreated animals supporting anti-inflammatory effects against lipopolysaccharide in the placenta (21). However, in adolescents, placental VDR expression was inversely associated with neonatal 25(OH)D (P = 0.012) and maternal 25(OH)D (P = 0.080) while positively with neonatal 1,25(OH)2D (P = 0.006) (22). In gestational diabetes, low vitamin D was reported in serum while higher levels of placental VDR; in fact, low serum levels could even up-regulate the placenta VDR gene expression *via* negative feedback regulation, such that the increase in the bioavailability of vitamin D might compensates for the deficiency (23). Placenta is a key organ of transfer for 25-OH-D3 that even expresses the enzyme 1-α-hydroxylase, to synthesize 1,25-(OH)2-D3 and hence to regulate inflammatory processes. For this reason, the protein levels of VDR in placenta may differ to those in maternal liver. In addition, no changes in pregnancy outcomes or fetal weight were found between the groups in the present study.

Although 25-OH-D3 or calcifediol has been widely used for dietary supplementation of vitamin D, it is not approved for use in pregnant women as there is lack of safety data from randomized controlled trials. An additional problem with the use of 25-OH-D3 is that vitamin D3 and calcifediol are not equipotent (6) and there is no consensus on the conversion factor that should be used for 25-OH-D3 to calculate vitamin D activity (IU units). Conversion-factor estimates of 1.4 and 5-fold-increase arose in two intervention studies with patients who required vitamin D treatment (2, 24). A study conducted in winter in older adults reported that each microgram of oral 25-OH-D3 was about five times more effective in raising serum 25-OH-D3 in older adults than an equivalent amount of vitamin D3 (2); oral supplementation with 25 μg calcifediol reached 134.6 ± 26 nmol/L 25-OH-D3 in serum compared to 69.0 ± 8.7 nmol/L using the same dose of vitamin D3 (2). In contrast, Bischoff-Ferrari et al. (7) showed that in healthy postmenopausal women (n = 10 women per group), oral supplementation of 25-OH-D3 increased plasma 25-OH-D3 levels three times more effectively than a matched dose of vitamin D3. 25-OH-D3 supplementation rapidly and safely elevated serum 25-OH-D3 concentrations in a dose-dependent manner to improve vitamin D status in different populations (3); a daily dose of 10 mg of 25-OH-D3 maintained serum 25-OH-D3 concentrations between 75 and 100 nmol/L (3). However, data that help to define the conversion factor for 25-OH-D3 during pregnancy are lacking. The data generated by this study will provide another piece of information to help define the intake of vitamin D and circulating level of 25-OH-D3 in pregnant women that is adequate to improve fetal development and prevent maternal complications.

In the present study, dietary 25-OH-D3 significantly increased VDR protein in maternal liver. We also observed 25-OH-D3-mediated increases in maternal liver levels of the fatty acid transporter FAT/CD36 and the cholesterol carrier SR-B1. These findings suggest changes in the transport of lipophilic nutrients such as vitamin D in this organ. Higher FAT and SR-B1 protein levels in the maternal liver might result in higher uptake of 25-OH-D3, which may in turn increase its activity. Since the actions of vitamin D are mediated by VDR that binds 1,25(OH)2D3, this could also support higher active form of vitamin D in the maternal liver. Recently, Kiourtzidis et al. (25) reported in mice deficient in SR-B1 (Srb1−/−) or in CD36 (Cd36−/−) that received triple-deuterated vitamin D3 (vitamin D3-d3), they had significantly lower levels of 25-OH-D3-d3 in serum and tissues than in wild type animals; this study also confirmed that SR-B1 is not only crucial for the hepatic uptake of HDL cholesterol but also for the uptake of vitamin D into the liver to synthesize 25-(OH)-D, the primary biomarker of vitamin D status (25).

Interestingly, low serum levels of 25-(OH)-D have been observed in patients suffering obesity or non-alcoholic liver diseases compared to those of healthy subjects (26–28). CD36 levels are increased in several studies of NAFLD where they correlate with hepatic liver content (29, 30) but not in all (12). Despite higher SR-B1 or FAT/CD36 could favor higher vitamin D tissue uptake in these patients, the large amount of fat stored in tissue might reduce their final levels of 25-OH-D3 in serum. Whether vitamin D supplementation improves NAFLD has remained controversial in clinical trials (31–33). In mice, vitamin D supplementation alleviated NAFLD by activating VDR, whereas hepatic-specific knockout of VDR abolished the ameliorative effects of vitamin D on NAFLD (34). The higher levels on liver VDR by supplementation with 25-OH-D3 vs. vitamin D3 in the present study could be of major interest.

Placenta is a key organ that mediates nutrient transfer. It is important to note that 1,25-(OH)-2D does not practically cross the placental tissue, while its inactive precursor 25-(OH)-D readily crosses the tissue to the fetal compartment (8, 9). In the present study, the administration of 25-OH-D3 did not change the expression of FAT, SR-B1 in the placenta by the type of supplement. The lipid transport in the placenta and the cholesterol uptake/efflux is different than in the liver which could explain the differences between tissues. Nevertheless, this

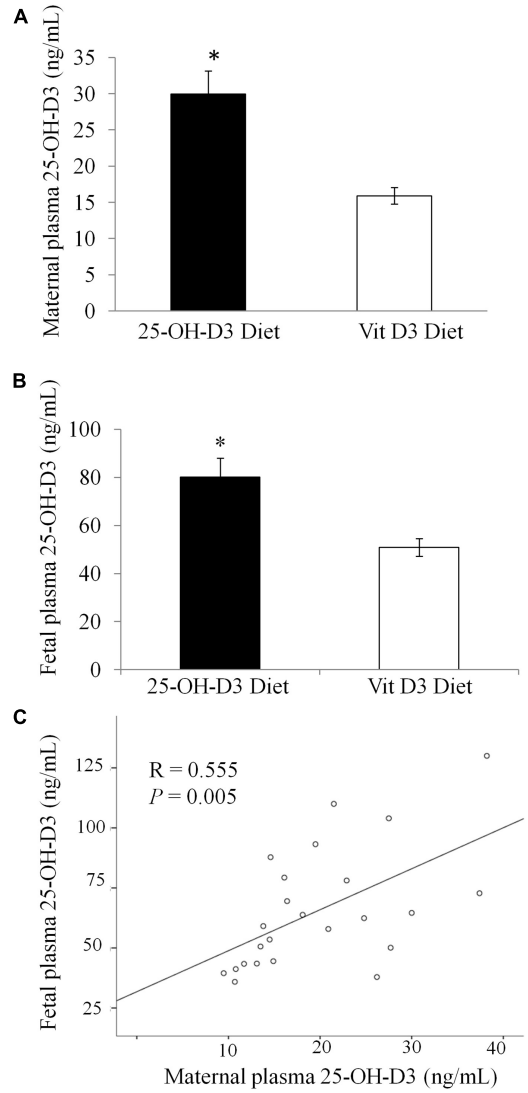

FIGURE 3 | Concentration of 25-OH-Vitamin D3 in plasma at delivery in rats treated during pregnancy with 25-OH-D3 (experimental diet) or vitamin D3 control diet. **(A)** Concentration of 25-OH-Vitamin D3 in maternal plasma at delivery ($P < 0.001$). **(B)** 25-OH-Vitamin D3 in fetal plasma at delivery ($P = 0.003$). **(C)** Correlation between the levels of 25-OH-D3 in maternal and fetal plasma. *T*-test significant differences $^*P < 0.05$.

is the first study on the effect of the different types of vitamin D supplements in these placental carriers.

Vitamin D receptor is the single known regulatory mediator of hormonal 1,25-(OH)2-D3 in higher vertebrates (10). It acts in the nucleus of vitamin D target cells to regulate the expression of genes whose products control diverse cell type-specific biological functions that includes mineral homeostasis. However, as VDR expression emerged in other tissues, it became clear that vitamin D action in many cellular targets was unrelated to mineral regulation, suggesting additional vitamin D hormone functions (35). One surprising finding here is the trend of down-regulation of VDR in the fetal brain ($p = 0.086$) in the 25-OH-D3 group when compared to the vitamin D3 control group. Despite this result, was not statistically significant this down regulation could be a self-protection mechanism triggered by the high vitamin D level in the circulation (**Figure 3C**). In addition, 25-OH-D3 supplementation reduced the expression of glutamate decarboxylase GAD67, one of the GABAergic neuronal markers. Glutamate decarboxylase (GAD) is localized only in presynaptic terminals of GABAergic inhibitory neurons. There are two common forms of GAD-GAD65 and GAD67. These isoforms are encoded by independent genes with different subcellular localizations. GAD67 is localized in the cell soma of inhibitory neurons. GAD67 knocked-out mice have reduced GABA levels throughout the brain, a reduction in GAD activity, and severe cleft palate which leads to death within 24 h after birth (36). In this study, we found that the expression of VDR in fetal brain was positively associated with GAD67 ($R = 0.391$, $P = 0.033$). The relationship between Vitamin D and GABAergic neurons had been reported previously. GABA-Aα4 (37) and GABA B receptor 1 (38) expression was decreased in vitamin D deficient animals. However, some other studies have reported no difference in GABA transmission (39, 40), although the discrepancies between studies could be due to differences in the brain regions analyzed. We found changes in fetal vitamin D metabolism, beyond the serum levels of 25-OH-D3, that should be investigated in the fetus since it is in active neurodevelopment.

There is consensus that adequate vitamin D is necessary during pregnancy for maintaining both maternal calcium homeostasis and fetal bone development. There are also ongoing discussions about the potential effects of vitamin D levels on pregnancy outcomes, such as preterm birth, gestational diabetes, preeclampsia risk, and also on children's long-term health outcomes such as asthma and neurodevelopment (41–43). Hajhashime et al. (44) showed that direct sunlight exposure for 30 min daily (30% of body surface area) for 10 weeks can provide 25-OH-D3 levels of almost 20 ng/mL (up from 15.09 ng/mL) in the plasma of pregnant women with vitamin D deficiency. However, the same study also showed that dietary supplementation of vitamin D3 at 4,000 IU per day for 10 weeks increased 25-OH-D3 plasma level to 31.27 ng/mL (up from 15.95 ng/mL), which is significantly higher than the level achieved by sun exposure. On a side note, increase in sun exposure is associated with increase in cancer risk. Therefore, dietary supplementation of Vitamin D is a much more feasible intervention strategy than sun exposure for addressing vitamin D deficiency issues in pregnant women.

25-OH-D3 supplementation in pregnant women is of major interest because it could be likely achieve desirable maternal and fetal blood levels at lower doses than Vitamin D3. In fact, some endocrinologists are using it in pregnant women even though it is not approved for use in pregnancy due to the lack of safety data. Therefore, it is important to study the bioavailability of the different forms of vitamin D in during pregnancy. It is not feasible to extrapolate from other study populations due to the physiological changes and hemodilution that occur during pregnancy.

In conclusion, 25-OH-D3 is more potent than Vitamin D3 in raising the vitamin D status in pregnant rats Supplementation with 25-OH-D3 increased maternal and fetal 25-OH-D3 plasma

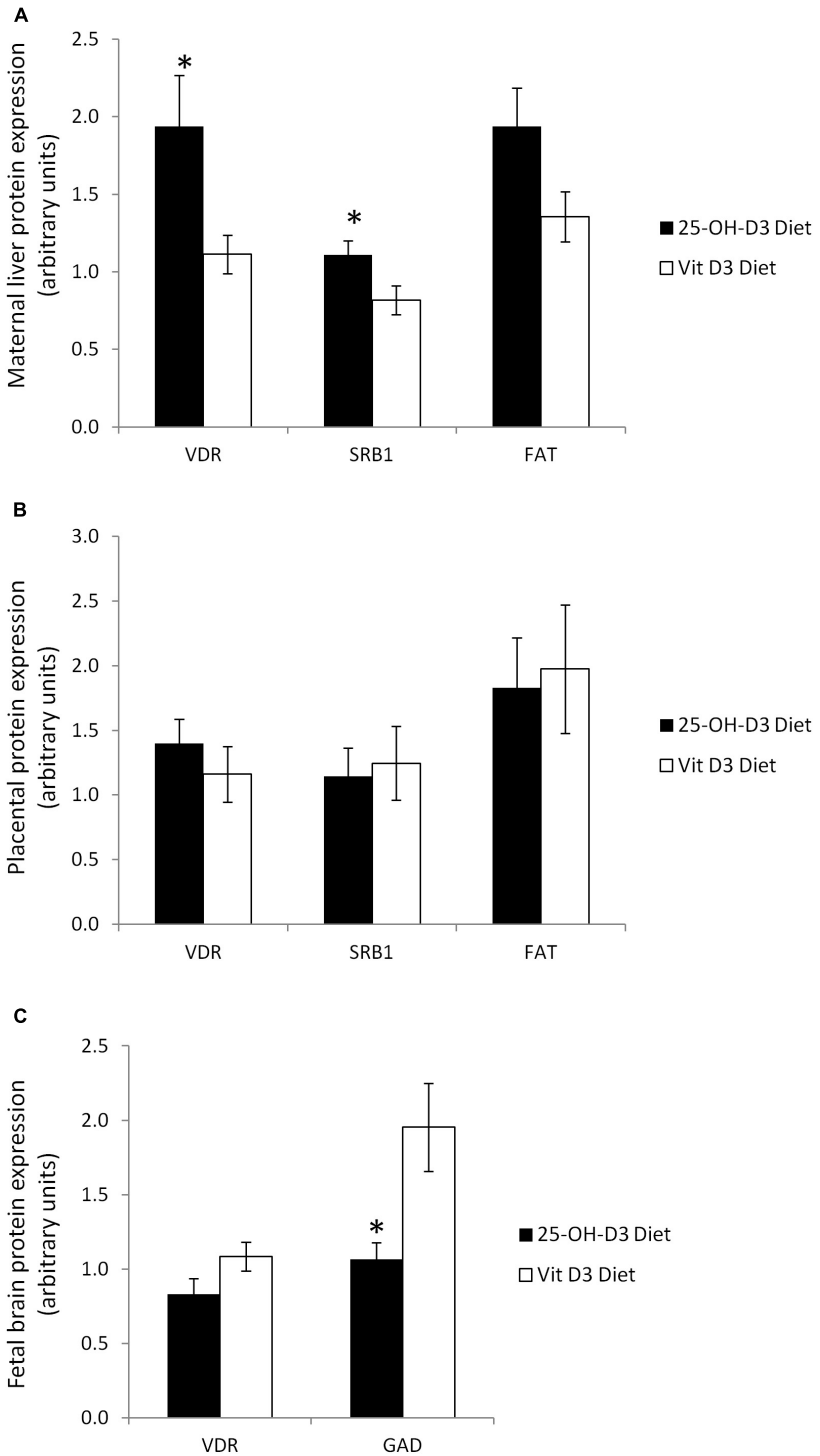

FIGURE 4 | Vitamin D related proteins in several tissues at delivery in rats treated during pregnancy with 25-OH-D3 (experimental diet) or vitamin D3 (control diet). **(A)** Maternal liver: vitamin D receptor (VDR), fatty acid translocase (FAT/CD36) ($P = 0.059$), and Scavenger Receptor 1 (SR-B1); **(B)** placenta: VDR, FAT/CD36, and SR-B1; **(C)** fetal brain at delivery: VDR ($P = 0.086$) and glutamate decarboxylase (GAD). *T*-test significant differences $^*P < 0.05$.

concentrations by nearly two times compared to vitamin D3. 25-OH-D3 supplementation also increased VDR levels and some lipid carriers in maternal liver as SR-B1 and FAT/CD36, but this increase was not found in the placenta. In contrast, maternal 25-OH-D3 decreased fetal brain VDR and GAD. Thus, supplemental 25-OH-D3 improved maternal and fetal vitamin D status better

than vitamin D3. Its effects on fetal tissues should be further explored in future studies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Murcia (N° A13180105).

AUTHOR CONTRIBUTIONS

AG supervised the animal experiment, performed vitamin D analysis, and contributed to wrote the manuscript. MS-C performed Western blot analysis. EL conceived the study, designed and conducted the research, and had primary responsibility for the final content. AB, RR, and JC revised the manuscript. All authors have read and approved the final version of the manuscript.

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Gene-Regulatory Potential of 25-Hydroxyvitamin D_3 and D_2

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Human peripheral blood mononuclear cells (PBMCs) represent a highly responsive primary tissue that is composed of innate and adaptive immune cells. In this study, we compared modulation of the transcriptome of PBMCs by the vitamin D metabolites 25-hydroxyvitamin D_3 (25(OH)D_3), 25(OH)D_2 and 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$). Saturating concentrations of 1,25(OH)$_2D_3$, 25(OH)D_3 and 25(OH)D_2 resulted after 24 h stimulation in a comparable number and identity of target genes, but below 250 nM 25(OH)D_3 and 25(OH)D_2 were largely insufficient to affect the transcriptome. The average EC_{50} values of 206 common target genes were 322 nM for 25(OH)D_3 and 295 nM for 25(OH)D_2 being some 600-fold higher than 0.48 nM for 1,25(OH)$_2D_3$. The type of target gene, such as primary/secondary, direct/indirect or up-/down-regulated, had no significant effect on vitamin D metabolite sensitivity, but individual genes could be classified into high, mid and lower responders. Since the 1α-hydroxylase CYP27B1 is very low expressed in PBMCs and early (4 and 8 h) transcriptome responses to 25(OH)D_3 and 25(OH)D_2 were as prominent as to 1,25(OH)$_2D_3$, both vitamin D metabolites may directly control gene expression. In conclusion, at supra-physiological concentrations 25(OH)D_3 and 25(OH)D_2 are equally potent in modulating the transcriptome of PBMCs possibly by directly activating the vitamin D receptor.

Keywords: vitamin D, transcriptome, PBMCs, target genes, 1α,25-dihydroxyvitamin D3, 25-hydroxyvitamin D3, 25-hydroxyvitamin D2

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INTRODUCTION

Although humans can produce vitamin D_3 endogenously when they expose their skin to UV-B (1), predominant indoor lifestyle as well as insufficient UV indices in Northern latitudes (above 38°) at winter times (2), suggest the supplementation of the vitamin for at least a part of the year (3). Humans and animals use the cholesterol precursor 7-dehydrocholesterol as the substrate for vitamin D_3 synthesis, while fungi and yeast produce vitamin D_2 on the basis of the sterol ergosterol (4). Vitamin D_2 and vitamin D_3 differ only in the structure of their side chain, but in human intestine the uptake vitamin D_3 seems to be more effective (5). Nevertheless, both forms of vitamin D are used for food fortification and direct supplementation (6).

Vitamin D_2 and vitamin D_3 are metabolized in the liver by the CYP (cytochrome P450) enzymes CYP2R1 and CYP27A1 to 25(OH)D_2 and 25(OH)D_3, respectively (7). The sum of the serum concentrations of both metabolites [or sometimes only 25(OH)D_3] is traditionally used as biomarker for the vitamin D status of an individual (8, 9). A vitamin D status below 50 nM increases the risk for musculoskeletal disorders, such as rickets in children and osteomalacia as well as fractures in adults (10). Moreover, insufficient 25(OH)D serum levels are associated with

a number of immune-related diseases, such as rheumatoid arthritis (11), multiple sclerosis (12), type I diabetes (13) and inflammatory bowel disease (14). In addition, vitamin D deficiency raises the risk for severe consequence of microbe infections in tuberculosis, influenza or COVID-19 (coronavirus disease) (15–17). Therefore, one should aim for serum 25(OH)D levels in the range of 75–100 nM, i.e., 30–40 ng/ml (18). In contrast, a vitamin D status of above 250 nM should be avoided, in order to prevent deleterious effects of hypercalcemia (19).

The biologically most active form of vitamin D_3 and D_2 are $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$, respectively, which function in sub-nanomolar concentrations as nuclear hormones (20). For endocrine purposes $1,25(OH)_2D$ is synthesized in the kidneys by the enzyme CYP27B1 using 25(OH)D as a substrate (21), while for paracrine use $1,25(OH)_2D$ is produced also in CYP27B1 expressing keratinocytes and immune cells (22). Since $1,25(OH)_2D$ is the natural, high affinity ($K_D = 0.1$ nM) ligand of the nuclear receptor VDR (vitamin D receptor) (23, 24), vitamin D affects the activity of hundreds of genes in VDR expressing tissues (25). Thus, the physiological functions of vitamin D are associated with changes of the transcriptome of multiple tissues and cell types by ligand-activated VDR (26). The vitamin D-triggered transcriptome has been studied *in vitro* in a number of cell culture models, such as THP-1 monocytic leukemia cells (27–29), as well as in PBMCs (30, 31). Primary cells like PBMCs are a natural mixture of innate and adaptive immune cells like monocytes, natural killer cells, T and B cells. They are far closer to the human *in vivo* situation than cell lines and can be obtained with minimal harm to the donor (32).

The affinity of VDR for $25(OH)D_3$ is 100- to 1,000-fold lower than for $1,25(OH)_2D_3$ (33, 34), which parallels with the fact that the serum concentration of $25(OH)D_3$ is some 1,000-fold higher than that of $1,25(OH)_2D_3$ (0.05–0.15 nM) (35). This relation raised already earlier the question, whether $25(OH)D_3$ has the potential to act as a nuclear hormone that directly activates the VDR (36). The molecule $1,25(OH)_2D_3$ has three hydroxyl groups, each of which is specifically contacted by a pair of polar amino acids within VDR's ligand-binding pocket (37, 38). In contrast, 25(OH)D lacks the hydroxyl group at carbon 1 and therefore binds with lower affinity to the receptor, but takes the same agonistic conformation within the ligand-binding pocket (39).

In this study, we analyzed the transcriptome-wide effects of $25(OH)D_3$ and $25(OH)D_2$ in comparison to that of $1,25(OH)_2D_3$ in freshly isolated human PBMCs. We will demonstrate that $25(OH)D_3$ and $25(OH)D_2$ are equally potent in modulating the transcriptome of PBMCs and cannot exclude the possibility that both vitamin D metabolites directly activate the VDR.

MATERIALS AND METHODS

Sample Collection

Peripheral blood was collected from a single healthy individual (male, age 57 years) the vitamin D responsiveness of whom had already been characterized in the VitDbol trial (NCT02063334) (32, 40). The ethics committee of the Northern Savo Hospital District had approved the study protocol (#9/2014). The individual gave written informed consent to participate in the study and the experiments were performed in accordance with relevant guidelines and regulations.

PBMC Isolation and Stimulation

PBMCs were isolated immediately after collecting 100 ml peripheral blood using Vacutainer CPT Cell Preparation Tubes with sodium citrate (Becton Dickinson) according to manufacturer's instructions. After washing with phosphate-buffered saline, the cells were grown at a density of 0.5 million/ml in 5 ml RPMI1640 medium supplemented with 10% charcoal-depleted fetal calf serum, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified 95% air/5% CO_2 incubator and treated for 4, 8 or 24 h either with $1,25(OH)_2D_3$ (0.1, 1 and 10 nM) $25(OH)D_3$ (100, 250, 500, 750, and 1,000 nM), $25(OH)D_2$ (100, 250, 500, 750, and 1,000 nM) or solvent (0.1% EtOH). All experiments were performed in three repeats. Deconvolution of RNA-seq data using the algorithm CIBERSORTx (41) and its LM6 signature matrix estimated the relative amounts of B cells (7%), $CD8^+$ T cells (32%), $CD4^+$ T cells (20%), natural killer cells (21%) and monocytes/macrophages (20%) within the PBMC pool.

RNA-Seq Data Generation and Processing

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche) according to manufacturer's instructions. RNA quality was assessed on an Agilent 2100 Bioanalyzer system (RNA integrity number $\geq$ 8). rRNA depletion and cDNA library preparation were performed using the New England Biolabs kits NEBNext rRNA Depletion, NEBNext Ultra II Directional RNA Library Prep for Illumina and NEBNext Multiplex Oligos for Illumina (Index Primers Sets 1 and 2) according to manufacturer's protocols. RNA-seq libraries went through quality control on an Agilent 2100 Bioanalyzer and were sequenced on a NextSeq 500 system (Illumina) at 75 bp read length using standard protocols at the Gene Core facility of the EMBL (Heidelberg, Germany). The libraries were sequenced as four batches. Fastq files of the 66 libraries can be found at Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo) with accession number GSE199273.

Single-end, reverse-stranded cDNA sequence reads were aligned to the reference genome (version GRCh38) with Ensembl annotation (version 103) by using default settings of the nf-core/rnaseq STAR-Salmon pipeline (version 3.0) (http://doi.org/10.5281/zenodo.4323183) (42). The number of nucleotide sequence reads are shown in **Supplementary Table 1**. Ensembl gene identifiers were annotated with gene symbol, description, genomic location and biotype by accessing the Ensembl database (version 104) *via* the R package *BiomaRt* (version 2.46.0) (43). Ensembl gene identifiers missing HGNC gene symbol annotation, Entrez ID, genomic location information or being mitochondrially encoded were removed from the datasets. When a gene name appeared more than once, the entry with the highest average gene counts was kept.

Transcriptome Data Analysis

Differential gene expression analysis was computed in R (version 4.1.2) in the Rocky Linux 8.5 operating system using the tool *EdgeR* (version 3.36.0) (44). The analysis focused

on the 19,142 protein-coding genes, in order to reduce transcriptional noise expected by non-coding genes. Read counts were normalized for differences in library size to counts per million (CPM). Genes with very low expression were filtered out by applying the function *FilterByExpr()*, in order to mitigate the multiple testing problem and to not interfere with the statistical approximations of the *EdgeR* pipeline. This requirement was fulfilled by 12,939 genes. After filtering, library sizes were recomputed and trimmed mean of M-value normalization was applied. The transcriptome data structure was explored *via* the dimensionality reduction method multidimensional scaling (MDS) (**Supplementary Figure 1**). MDS was computed *via* *EdgeR*'s function *plotMDS()*, in which distances approximate the typical $\log_2$ fold change (FC) between the samples. This distance was calculated as the root mean square deviation (Euclidean distance) of the largest 500 $\log_2$FCs between a given pair of samples, i.e., for each pair a different set of top genes was selected. The inspection of the MDS plot showed that (i) the time-dependent divergence from the native transcriptome state and (ii) the concentration-dependent modulation by treatment with 1,25(OH)$_2$D$_3$, 25(OH)D$_3$ or 25(OH)D$_2$ are the two principal factors driving differences in the gene expression profiles of PBMCs. The gene-wise statistical test for differential expression was computed using the generalized linear model quasi-likelihood pipeline (45). The empirical Bayes shrinkage was robustified against outlier dispersions as recommended (45). The *glmTreat* approach was used to test for differential expression relative to FC $>$ 1.5 at the early time points (4 and 8 h) and FC $>$ 2 at 24 h. Taking all treatments together, 553 genes with a Benjamini-Hochberg corrected *p*-value [i.e., false discovery rate (FDR)] $<$ 0.05 and a total trimmed mean of M-value normalized CPM count $>$ 10 (i.e., the sum of the average gene expression level of the reference 10 nM 1,25(OH)$_2$D$_3$ and EtOH-treated samples at each time point) were considered as vitamin D targets (**Supplementary Table 2**). Mean-Difference (MA) plots (**Figures 1A**, **3A**; **Supplementary Figure 2**) for the 19 different treatment conditions were generated with *vizzy* (version 1.0.0, https://github.com/ATpoint/vizzy).

Dose Response Analysis

The effect of vitamin D metabolites on the change in mRNA levels (absolute $\log_2$FC) was modeled with the three-parameter log-logistic function LL.3 from the R package *drc* (46) having lower limit fixed at 0. The estimated relative EC_{50} values and their standard errors were retrieved from the curve fits *via* the *summary()* function. The quality of fitting was checked by manual inspection. EC_{50} values were reported only for those 206 genes, for which the value could be estimated for all three vitamin D metabolites. Pairwise comparisons of EC_{50} values between different metabolites as well as groups of target genes were performed using Tukey's test implemented in the R package *multcomp* (version 1.4.18) and family-wise error rate (FWER)-adjusted *p*-values retrieved. Comparisons with a FWER $<$ 0.05 were considered as significant. The code of the analysis can be found at https://github.com/andreahanel/2022_Doseresponse.

RESULTS

Transcriptome-Wide Responses to 1,25(OH)$_2$D$_3$, 25(OH)D$_3$, and 25(OH)D$_2$

In order to obtain maximal responses of the transcriptome, PBMCs from an healthy individual were treated immediately after isolation for 24 h with either 10 nM 1,25(OH)$_2$D$_3$, 1,000 nM 25(OH)D$_3$, 1,000 nM 25(OH)D$_2$ or solvent (0.1% EtOH). In three repeats RNA-seq was performed on the basis of total RNA. As in comparable studies (31, 47, 48), strict statistical thresholds of FDR $<$ 0.05 and FC $>$ 2 were applied. This resulted in 313 genes (75 up-regulated, 238 down-regulated) responding to 1,25(OH)$_2$D$_3$, 365 target genes of 25(OH)D$_3$ (98 up-regulated, 267 down-regulated) and 385 genes modulated by 25(OH)D$_2$ (67 up-regulated, 318 down-regulated) (**Figure 1A**). For all three vitamin D metabolites the genes *CAMP* (cathelicidin antimicrobial peptide), *FN1* (fibronectin 1), *LOXHD1* (lipoxygenase homology PLAT domains 1) and *SLC24A4* (solute carrier family 24 member 4) were the top up-regulated and the genes *CXCL10* (C-X-C motif chemokine ligand 10), *STEAP4* (STEAP4 metalloreductase), *CXCL9* and *OLFM1* (olfactomedin 1) the most down-regulated.

In addition to saturating ligand concentrations, PBMCs were treated for 24 h with 0.1 and 1 nM 1,25(OH)$_2$D$_3$, with 100, 250, 500, and 750 nM 25(OH)D$_3$ as well as with 100, 250, 500, and 750 nM 25(OH)D$_2$. This resulted in 7 and 179 target genes for 0.1 and 1 nM 1,25(OH)$_2$D$_3$, no targets for 100 and 250 nM 25(OH)D$_3$, 322 and 392 responding genes for 500 and 750 nM 25(OH)D$_3$, no targets for 100 nM 25(OH)D$_2$ as well as 30, 342, and 397 genes that reacted on a stimulation with 250, 500, and 750 nM 25(OH)D$_2$ (**Supplementary Figure 3A**). These in total 526 different genes were filtered with a list of 662 genes, which were detected by time course analysis of treatment of PBMCs with 1,25(OH)$_2$D$_3$ (31). This led to 389 confirmed vitamin D target genes, of which 6, 150, and 254 responded to 0.1, 1 and 10 nM 1,25(OH)$_2$D$_3$, 263, 294, and 293 to 500, 750, and 1,000 nM 25(OH)D$_3$ as well as 25, 278, 307, and 299 to 250, 500, 750, and 1,000 nM 25(OH)D$_2$ (**Supplementary Figure 3B**). Venn diagrams indicated that there are 237 common targets of 500, 750, and 1,000 nM 25(OH)D$_3$ as well as 231 common targets of 500, 750, and 1,000 nM 25(OH)D$_2$ (**Figure 1B**). From both lists 206 genes are identical with the 254 targets responding to 10 nM 1,25(OH)$_2$D$_3$.

The 206 common targets represent a stable set of genes responding to variant concentrations of all three tested vitamin D metabolites (**Figure 1C**). Combined box plots and violin plots monitored the overall change in the expression of these 206 genes with raising metabolite concentrations. Interestingly, 250 nM of both 25(OH)D$_3$ and 25(OH)D$_2$ were insufficient for general gene regulation, while 500 nM of both vitamin D metabolites was clearly above this threshold.

In summary, both by number as well as by most responsive target genes the transcriptome-wide responses to saturating concentrations of 1,25(OH)$_2$D$_3$, 25(OH)D$_3$ and 25(OH)D$_2$ are very comparable. At concentrations of 250 nM and below, 25(OH)D$_3$ and 25(OH)D$_2$ are largely insufficient to significantly modulate the expression of vitamin D target genes.

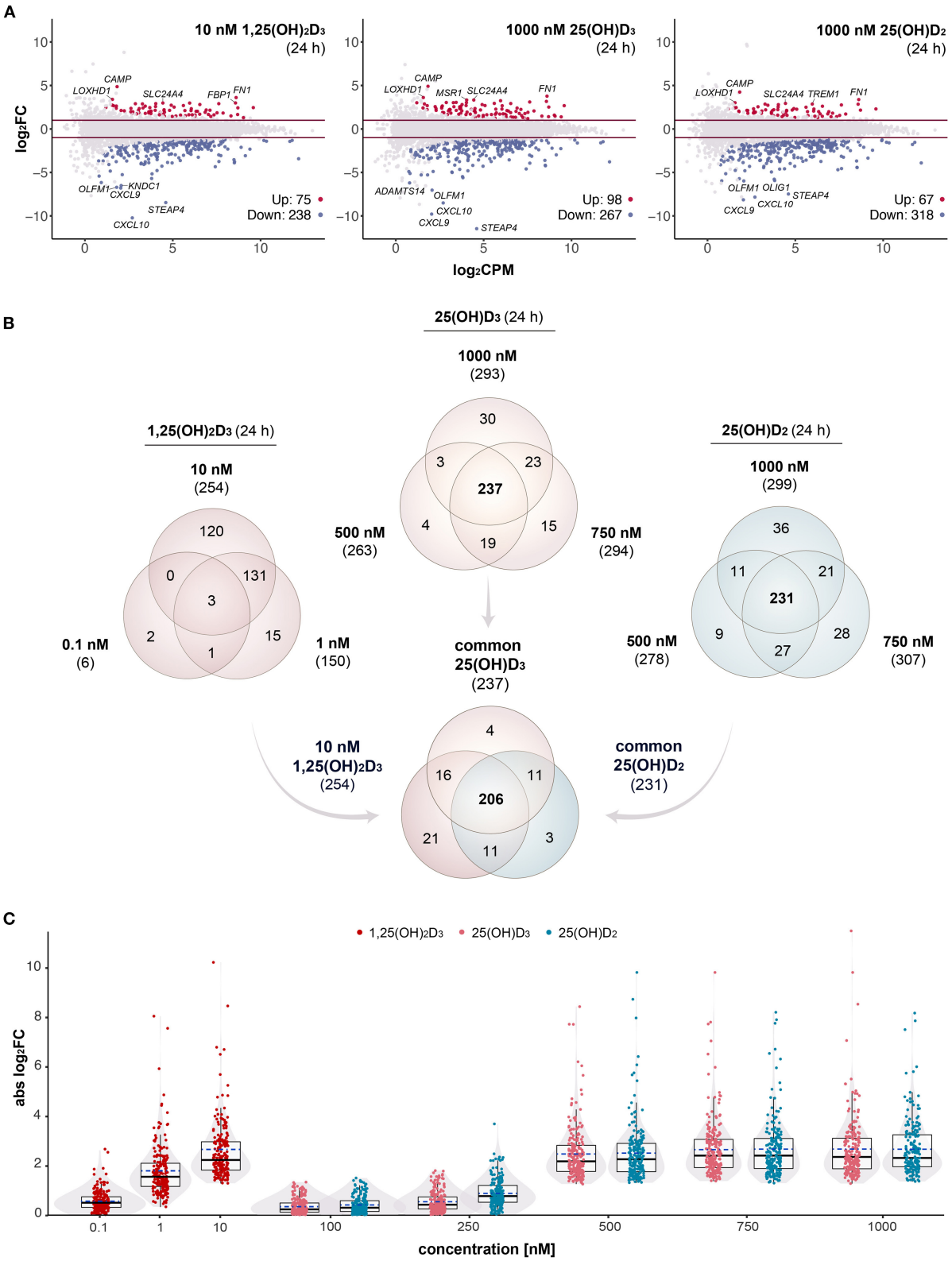

FIGURE 1 | Gene-regulatory potential of vitamin D metabolites. MA plots display the genome-wide transcriptional response of PBMCs treated for 24 h with 10 nM $1,25(OH)_2D_3$, 1000 nM $25(OH)D_3$ or 1000 nM $25(OH)D_2$ in comparison to solvent (0.1% EtOH) **(A)**. For each gene, the change of expression (log_2FC) between

(Continued)

FIGURE 1 | treated and control samples is shown in relation to its mean expression level (log_2CPM). Differential expression analysis was performed as a pairwise comparison per each concentration by using *glmTreat* test. Significantly (FDR < 0.05) up- and down-regulated genes are highlighted in red and blue, respectively. Horizonal lines (red) indicate the applied statistical testing threshold (absolute FC > 2). The top 5 responsive genes (up- and down-regulated) are labeled. Venn diagrams show the overlap of vitamin D target genes per metabolite **(B)**. The relations between all treatments and concentrations (at 24 h) are provided in **Supplementary Figure 3**. Box and violin plots summarize the distribution of the magnitude of expression change (absolute log_2FC) of the 206 common genes for each vitamin D metabolite and concentration **(C)**. Solid lines within the boxes indicate medians, while dashed lines mark the mean.

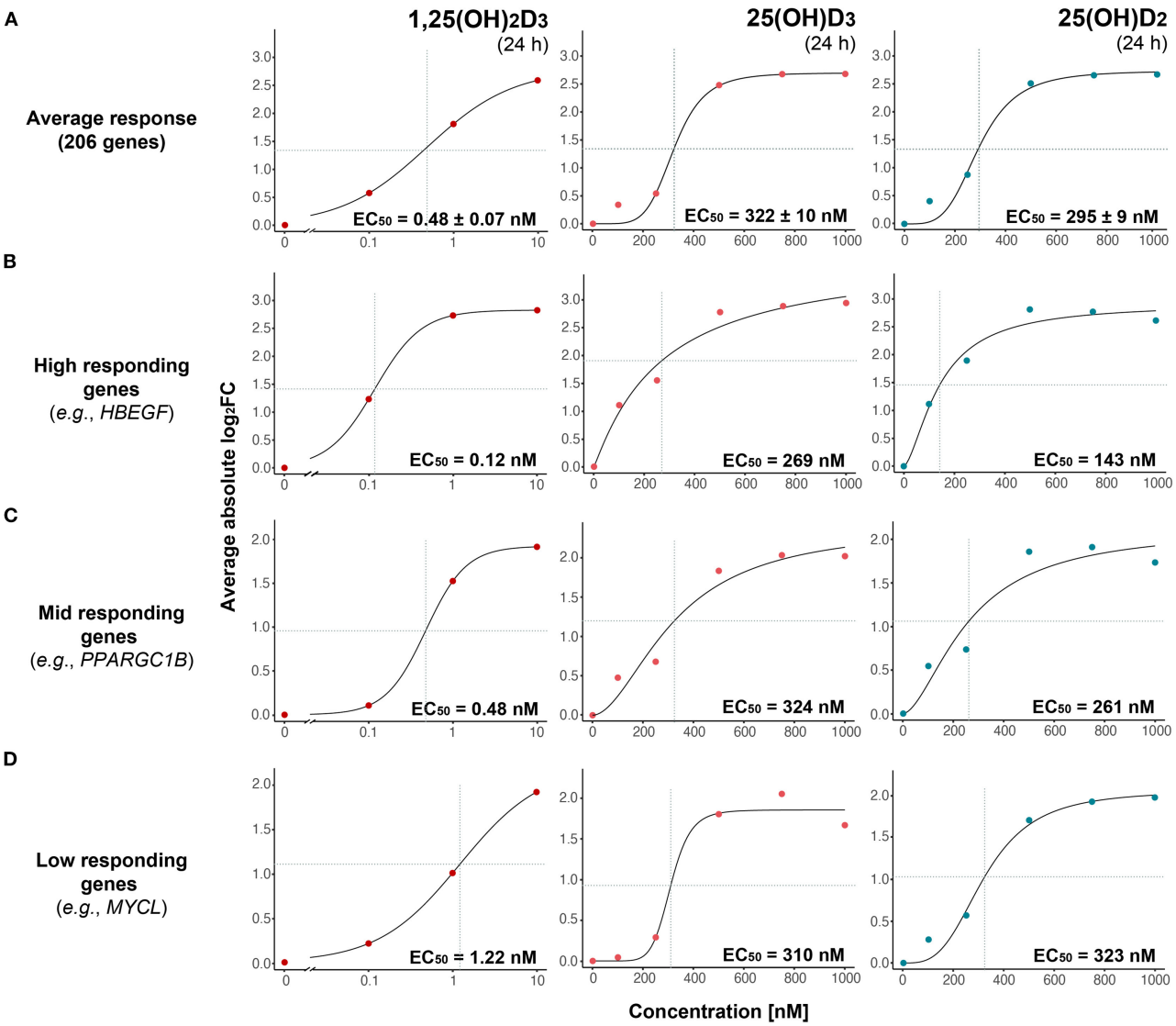

FIGURE 2 | Gene-specific response to vitamin D metabolites. The magnitude of expression change (absolute log_2FC) as a function of vitamin D metabolite concentration is modeled with a three-parameter log-logistic model based on the average of 206 common targets **(A)** or indicated individual genes **(B–D)**. Genes are segregated into high-, mid- and low-responding based on their estimated EC_{50} values in response to $1,25(OH)_2D_3$. Standard errors of the EC_{50} estimates are indicated.

Gene-Specific Sensitivity to Vitamin D Metabolites

Plotting the FC of the 206 common vitamin D target genes over vitamin D metabolite concentration and using the three-parameter log-logistic model, determined the EC_{50} values 0.48 nM for $1,25(OH)_2D_3$, 322 nM for $25(OH)D_3$ and 295 nM for $25(OH)D_2$ (**Figure 2A**). There is statistically no significant difference (*FWER* > 0.05, Tukey's test) between the potencies of $25(OH)D_3$ and $25(OH)D_2$, but their average EC_{50} is some 640-fold higher than that of $1,25(OH)_2D_3$.

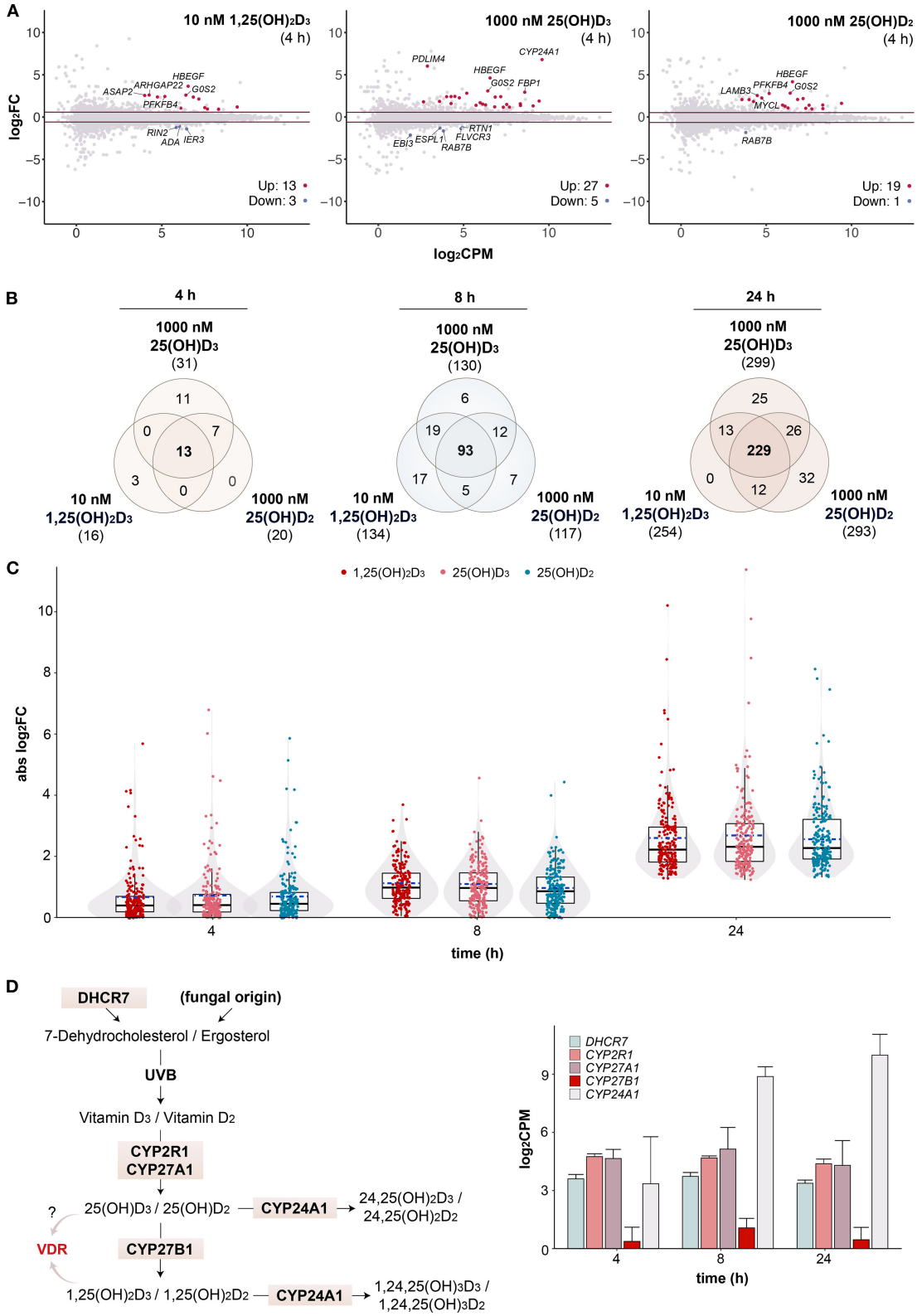

FIGURE 3 | Changes in transcriptional profiles over time. MA plots show early gene expression changes in PBMCs treated for 4 h with 10 nM $1,25(OH)_2D_3$, 1000 nM $25(OH)D_3$ or 1000 nM $25(OH)D_2$ in comparison to solvent (0.1% EtOH, i.e., time-matched control) **(A)**. For each gene, difference in expression (log_2FC) between

(Continued)

FIGURE 3 | treated and control samples is shown in relation to its mean expression level (log_2CPM). Genes were tested for differential expression relative to an absolute FC > 1.5 using *glmTreat* method. Horizonal lines (red) indicate the applied statistical testing threshold. The top 5 most responsive up- and down regulated genes (if any; FDR < 0.05) are highlighted. Venn diagrams show the overlap of vitamin D target genes per time point **(B)**. The relations between all treatments are provided in **Supplementary Figure 5**. Box and violin plots summarize the distribution of the magnitude of expression change (absolute log_2FC) of the 206 common genes for each vitamin D metabolite and time point **(C)**. Solid lines within the boxes indicate medians, while dashed lines mark the mean. Please note that the data of the 24 h time point serve as a reference and are identical to those presented in **Figure 1C**. A map of the human vitamin D metabolism pathway marks key enzymes [**(D), left**]. The mean of $1,25(OH)_2D_3$-treated and untreated mRNA expression of the five indicated genes is displayed in log_2-scale as columns for all three time points [**(D), right**]. Error bars indicate standard deviation.

Based on the reference dataset (31), the 206 common targets were subdivided either into 85 primary targets (25 up- and 60 down-regulated) and 121 secondary targets (17 up- and 104 down-regulated) or into 94 direct targets (31 up- and 63 down-regulated) and 112 indirect targets (11 up- and 101 down-regulated). For $1,25(OH)_2D_3$ the EC_{50}-values of the eight different categories varied from 0.29 to 0.73 nM but did not differ significantly (*FWER* > 0.05, Tukey's test) between each other (**Supplementary Figure 4**). Similarly, for $25(OH)D_3$ the range was 318 to 389 nM and for $25(OH)D_2$ 192 to 313 nM, but the difference was not statistically significant (*FWER* > 0.05, Tukey's test).

Since neither gene categories nor up- or down-regulation allowed a distinction of the sensitivity of vitamin D target genes to vitamin D metabolites, the dose responses of the 206 genes were investigated on an individual gene level. Manual inspection of the dose response curves provided for 130 genes convincing fits for all three vitamin D metabolites. Interestingly, for $1,25(OH)_2D_3$ the EC_{50}-values ranged from 0.10 nM [*ENPP2* (ectonucleotide pyrophosphatase/phosphodiesterase 2)] to 2.39 nM [*LMNA* (lamin A/C)], for $25(OH)D_3$ from 121 nM [*NXPH4* (neurexophilin 4)] to 461 nM [*ENTPD7* (ectonucleoside triphosphate diphosphohydrolase 7)] and for $25(OH)D_2$ from 132 nM (*SLC11A1*) to 421 nM [*STAB1* (stabilin 1)] (**Supplementary Table 2**). The wide range of gene-specific sensitivity to $1,25(OH)_2D_3$ allowed the categorization of the representative 130 vitamin target genes into 59 high responders (EC_{50} value range from 0.10 to 0.39 nM), 59 mid responders (0.41 to 1.06 nM) and 12 low responders (1.20 to 2.39 nM). Representative genes for the three categories are *HBEGF* (heparin binding EGF like growth factor) as high responding gene (**Figure 2B**), *PPARGC1B* (PPARG coactivator 1 beta) as mid responding gene (**Figure 2C**) and *MYCL* (MYCL proto-oncogene, BHLH transcription factor) as low responding gene (**Figure 2D**). Importantly, the far smaller range of the gene-specific EC_{50} values for $25(OH)D_3$ and $25(OH)D_2$ did not allow a categorization of the vitamin D target genes by their response to these metabolites.

Taken together, the average EC_{50} values of the response of vitamin D target genes to $25(OH)D_3$ and $25(OH)D_2$ are not significantly different and are in the order of 300 nM, i.e., some 600-fold higher as those for $1,25(OH)_2D_3$. Categorization of the target genes into primary/secondary, direct/indirect or up-regulated/down-regulated does not allow any significant distinction in their response to the three vitamin D metabolites. However, individual target genes can be classified by their response to $1,25(OH)_2D_3$ (but not to $25(OH)D_3$ and $25(OH)D_2$) as high, mid and low responding genes.

Time Course Response of Vitamin D Target Genes

In order to investigate whether $25(OH)D_3$ and $25(OH)D_2$ may activate vitamin D signaling without enzymatic conversion by CYP27B1 to $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$, respectively, the transcriptome-wide response to saturating concentrations of all three vitamin D metabolites was assessed by RNA-seq at earlier time points. After 4 h stimulation with 10 nM $1,25(OH)_2D_3$, 16 genes (13 up- and 3 down-regulated) passed the statistical threshold (FDR < 0.05, FC > 1.5), with 1,000 nM $25(OH)D_3$ 32 genes (27 up- and 5 down-regulated) and with 1,000 nM $25(OH)D_2$ 20 genes (19 up- and 1 down-regulated) (**Figure 3A**). Common top up-regulated genes were *HBEGF* and *G0S2* (G0/G1 switch 2). After filtering with the reference dataset of vitamin D target genes in PBMCs (**Supplementary Figure 5**) (31), Venn diagrams indicated that there are 13 common genes (out of 31 in total) of the three vitamin D metabolites responding already after 4 h, 93 (out of 159 in total) after 8 h and 229 (out of 337 in total) after 24 h (**Figure 3B**). As observed in previous time course studies (29, 31), at earlier time points there are more up- than down-regulated genes, since the process of up-regulation is more straightforward and quicker than that of down-regulation. When comparing the response of the 206 common target genes to the three vitamin D metabolites, there was no significant difference in the response to 10 nM $1,25(OH)_2D_3$, 1,000 nM $25(OH)D_3$ or 1,000 nM $25(OH)D_2$ at 4, 8 and 24 h (**Figure 3C**).

The enzymes DHCR7 (7-dehydrocholesterol reductase), CYP2R1, CYP27A1, CYP27B1 and CYP24A1 are critical nodes in the vitamin D metabolism pathway (**Figure 3D**, left). Therefore, the relative mRNA expression of the genes encoding for these enzymes were extracted from the transcriptome datasets and compared at the time points 4, 8 and 24 h (**Figure 3D**, right). *DHCR7*, *CYP2R1* and *CYP27A1* show comparable mid-range expression, while the average expression of *CYP27B1* is 10- to 32-fold lower. In this way, *CYP27B1* belongs to the 5% lowest expressed genes in PBMCs. Since the *CYP24A1* gene is a well-known vitamin D target gene (49), its expression is even 671-fold higher than that of *CYP27B1*. For comparison, the relative expression values of all five genes in PBMCs of 12 participants of our VitDHiD study (50) are displayed (**Supplementary Figure 6**). The individuals were ranked by increasing *CYP27B1* expression, which varied by a factor of 12.5, but being in average some 200-fold lower as the

CYP24A1 expression. Thus, in vitamin D-triggered PBMCs the synthesis of $1,25(OH)_2D$ on the basis of 25(OH)D is far less prominently covered by enzyme expression as the degradation of 25(OH)D and $1,25(OH)_2D$ to 24,25(OH)D and $1,24,25(OH)_3D$, respectively.

In summary, already at early time points (4 and 8 h) the PBMC transcriptome responds to a stimulation with saturating concentrations of $25(OH)D_3$ and $25(OH)D_2$ as prominently as to $1,25(OH)_2D_3$. The expression of CYP27B1 protein in PBMCs is very likely too low for an efficient conversion of $25(OH)D_3$ and $25(OH)D_2$ into $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$, respectively, in particular in the presence of highly expressed CYP24A1 protein. However, it cannot be excluded that metabolic formation of $1,25(OH)_2D$ is partly contributing to the results obtained.

DISCUSSION

The focus of this study was to compare the gene regulatory potential of the vitamin D metabolites $25(OH)D_3$ and $25(OH)D_2$ to each other and in reference to $1,25(OH)_2D_3$. At supra-physiological concentrations of 10 nM $1,25(OH)_2D_3$ (100-times the natural serum concentration) and 1,000 nM 25(OH)D (10-times the recommended serum level) the response of the PBMC transcriptome is very comparable, i.e., the expression of some 300 genes is significantly modulated showing after 24 h stimulation some 3-times more down-regulation than up-regulation. Moreover, the target gene lists of $25(OH)D_3$, $25(OH)D_2$ and $1,25(OH)_2D_3$ are to 85% identical, i.e., our experimental series had relatively low transcriptional noise. These observations suggest that all three vitamin D metabolites use identical mechanisms in the modulation of vitamin D target gene expression. Form a structural point of view this is obvious, since 25(OH)D will bind in the same agonistic VDR conformation as $1,25(OH)_2D$ (51).

The 206 common vitamin D target genes, on which we focus in this study, represent the majority of the vitamin D sensitive part of the PBMC transcriptome, although (in order to further reduce transcriptional noise) they had been filtered by a reference dataset from a recent $1,25(OH)_2D_3$ time course study in PBMCs (31). The estimation of average EC_{50} values of 322 nM for $25(OH)D_3$ and 295 nM for $25(OH)D_2$ compared to 0.48 nM for $1,25(OH)_2D_3$ is the first report on the sensitivity of the transcriptome to vitamin D metabolites. These results provide an additional argument that there is no difference in the response of the transcriptome to $25(OH)D_3$ and $25(OH)D_2$. Moreover, our findings indicate with the factor of ∼600 a good estimation of the relative gene regulatory potential of 25(OH)D compared to $1,25(OH)_2D$. Since serum levels of 25(OH)D are even 1,000-times higher than that of $1,25(OH)_2D$, this supports the option that 25(OH)D can directly modulate the expression of vitamin D target genes. However, 25(OH)D concentrations in the order of 300 nM are far higher than the recommended 100 nM. Therefore, irrespective of the mechanism of action, for persons with a normal vitamin D status the transcriptome as a whole may not be regulated by 25(OH)D. However, there are a few very sensitive genes, such as *NXPH4*, *SLC11A1*, *ADGR3* (adhesion G protein-coupled receptor G3), *G0S2*, *HBEGF*, and *PMEPA1* (prostate transmembrane protein, androgen induced 1), which showed EC_{50} values for 25(OH)D below 150 nM. Thus, in healthy persons with a very high vitamin D status, a few genes may be directly affected by elevated 25(OH)D serum levels.

In addition to the control of the vitamin D status of healthy individuals through careful sun exposure and vitamin D_3 or D_2 supplementation, there are clinical settings, where supplementation with higher doses of $25(OH)D_3$ or $25(OH)D_2$ are recommended (52). These patients may reach, at least for a limited time, far higher 25(OH)D serum levels than healthy individuals. Moreover, $25(OH)D_3$ is used as a food supplement in animal farming, *e.g.*, for accelerating the growth of chicken (53). Also in these settings elevated $25(OH)D_3$ serum levels may be reached. Thus, there are a few scenarios, in which larger parts of the vitamin D-dependent transcriptome may be affected by 25(OH)D supplementation.

Studying the transcriptome's sensitivity to treatments with vitamin D metabolites led to the interesting observation that vitamin D target genes can be distinguished in high, mid and low responders. This suggests that not all vitamin D target genes respond equally to a stimulation with a given concentration of a VDR ligand. High responding genes, the best known of which are *HBEGF* (54) and *G0S2* (55), get activated at already at 5-times lower levels than the average of all genes, while low responding genes, such as *LMNA* (56) and *STAB1* (48), need for their response up to 5-times higher $1,25(OH)_2D_3$ concentrations than the mean. Interestingly, high responding genes tend to be primary targets that are directly regulated by activated VDR binding to enhancers in the vicinity of the gene's transcription start sites (57), while low responding genes are preferentially secondary targets that are regulated by transcription factors encoded by primary target genes (31). This adds a new characteristic to the description of vitamin D target genes, the mechanistic basis and physiological meaning of which needs to be further explored in the future.

For the main aim of this study, the comparison of the gene regulatory potential $25(OH)D_3$ and $25(OH)D_2$, it does not matter, if the observed effects on the PBMC transcriptome are explained either (i) by the enzymatic conversion of 25(OH)D into $1,25(OH)_2D$ during the 4–24 h duration of stimulation phase, which then activates the VDR, (ii) by a direct binding of 25(OH)D to the VDR or (iii) a combination of both. The very low expression of the *CYP27B1* gene, in particular in relation to *CYP24A1* expression, in PBMCs of one person used in this study is representative for other individuals. This calls into question, whether there was enough 1α-hydroxylase activity to convert within 4 h a sufficient amount of 25(OH)D into $1,25(OH)_2D$, which then stimulated primary vitamin D target genes. For example, in order to reach a $1,25(OH)_2D$ level of 10 nM, 1% of the 1,000 nM 25(OH)D pool need to be handled within 4 h. However, as it is typical for *in*

vitro cell culture stimulation experiments, supra-physiological concentrations of $1,25(OH)_2D_3$ and 25(OH)D are compared. In fact, the tight regulation of the $1,25(OH)_2D_3$ level *in vivo via* the molecule's rapid degradation by the enzyme CYP24A1 (58), indicates that concentrations used *in vitro* most likely never occur *in vivo*.

In conclusion, $25(OH)D_3$ and $25(OH)D_2$ are equally potent in modulating the transcriptome of PBMCs and regulate the same set of vitamin D target genes as the most potent VDR ligand, $1,25(OH)_2D_3$. However, in order to observe consequences of the gene regulatory potential of 25(OH)D, concentrations of 300 nM or higher need to be available. This is three times the recommended serum level, i.e., it does not apply to healthy individuals with a regular vitamin D status.

DATA AVAILABILITY STATEMENT

Fastq files of the raw data can be found at GEO with accession number GSE199273.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Northern Savo Hospital District had approved the study protocol (#9/2014). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AH performed RNA-seq data analysis. CC did RNA isolation and RNA-seq library preparation. CV synthesized the tested compounds. AH and CC wrote the manuscript, which was reviewed by CV. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.910601/full#supplementary-material

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Association between metabolic body composition status and vitamin D deficiency: A cross-sectional study

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This study aimed to investigate the risk of vitamin D deficiency in a relatively healthy Asian population, with (i) metabolically healthy normal weight (MHNW) (homeostasis model assessment-insulin resistance [HOMA-IR] < 2. 5 without metabolic syndrome [MS], body mass index [BMI] < 25), (ii) metabolically healthy obesity (MHO) (HOMA-IR < 2.5, without MS, BMI ≥ 25), (iii) metabolically unhealthy normal weight (MUNW) (HOMA-IR ≥ 2.5, or with MS, BMI < 25), and (iv) metabolically unhealthy obesity (MUO) (HOMA-IR ≥ 2.5, or with MS, BMI ≥ 25) stratified by age and sex. This cross-sectional study involved 6,655 participants aged ≥ 18 years who underwent health checkups between 2013 and 2016 at the Chang Gung Memorial Hospital. Cardiometabolic and inflammatory markers including anthropometric variables, glycemic indices, lipid profiles, high-sensitivity C-reactive protein (hs-CRP), and serum 25-hydroxy vitamin D levels, were retrospectively investigated. Compared to the MHNW group, the MHO group showed a higher odds ratio (OR) [1.35, 95% confidence interval (CI) 1.05–1.73] for vitamin D deficiency in men aged < 50 years. By contrast, in men aged > 50 years, the risk of vitamin D deficiency was higher in the MUO group (OR 1.44, 95% CI 1.05–1.97). Among women aged < and ≥ 50 years, the MUO group demonstrated the highest risk for vitamin D deficiency, OR 2.33 vs. 1.54, respectively. Our study revealed that in women of all ages and men aged > 50 years, MUO is associated with vitamin D deficiency and elevated levels of metabolic biomarkers. Among men aged < 50 years, MHO had the highest OR for vitamin D deficiency.

KEYWORDS

metabolic body composition, obesity, vitamin D deficiency, inflammatory marker, cardiometabolic marker

Introduction

The prevalence of obesity has been increasing worldwide. Obesity-related disorders have been widely studied. Research has focused on visceral fat accumulation, which is recognized as a cardiometabolic risk factor (1). Adipocyte hypertrophy results in unbalanced blood flow, local hypoxia, inflammatory macrophage infiltration, increased synthesis, and release of pro-inflammatory mediators [such as tumor necrosis factor-alpha [TNF-α], interleukin [IL]-6, and IL-8], and tissue inflammation (2, 3). Adipokines such as TNF and IL-6, which are secreted from visceral fat, may contribute to the development of atherosclerosis (4, 5). Body mass index (BMI) is a convenient tool for assessing the extent of overweight and obesity. However, BMI has limitations in the evaluation of body composition and metabolic status (6–8).

Some obese individuals appear to be protected from the development of metabolic disturbances or complications; thus, they are referred to as metabolically healthy obesity (MHO) (9). Individuals with MHO have lower visceral fat values than individuals with a similar body fat percentage (10). In addition, despite having excessive body weight, individuals with MHO demonstrate normal blood pressure, lipid profile, insulin sensitivity, inflammatory markers such as C-reactive protein (CRP) (11–13), and favorable levels of liver enzymes, which may reflect lower liver fat content (14) without significantly increased risks of diabetes and cardiovascular diseases (15, 16). Several mechanisms have been hypothesized to explain MHO. For example, high mitochondrial transcription and low inflammation levels in subcutaneous adipose tissue are associated with lower liver fat and MHO levels (17). In addition, differences in visceral fat accumulation, birth weight, adipose cell size, gene expression encoding markers of adipose cell differentiation (11) and lipolysis (18) were suggestive of MHO phenotype development.

By contrast, some individuals with normal weight but metabolic disturbances or complications were defined as metabolically unhealthy normal weight (MUNW) groups. Such individuals might be characterized by higher body fat percentage, visceral fat and insulin levels, increased adipocyte size, and predisposition to type 2 diabetes mellitus (T2DM), hyperlipidemia, and cardiovascular diseases compared with patients with a similar BMI (19–24). Central fat distribution, lower physical activity energy expenditure, and lower peak oxygen uptake appeared to be predisposing factors for MUNW. In addition, a cognitive attitude toward food and lifestyle plays a role in insulin sensitivity in MUNW (25).

Vitamin D involves in a variety of processes in human body. In addition to calcium (Ca) homeostasis and bone metabolism, vitamin D plays an important role in multiple organs and has many physiological functions (26). Recent studies suggest that low vitamin D levels are a risk factor not only for osteoporosis, sarcopenia, and frailty, but also for infection, autoimmune diseases, and other cardiometabolic diseases, such as hypertension (27, 28), diabetes (29) and metabolic syndrome (30–37). Vitamin D is mediated by the vitamin D receptor (VDR), which regulates the transcription of several target genes (38). VDR has been identified in a large variety of cell types, including monocytes, cardiomyocytes, pancreatic beta cells, vascular endothelial cells, neurons, immune cells, and osteoblasts (39). Recent studies have demonstrated that VDR and vitamin D-metabolizing enzymes are expressed in adipocytes (40).

Evidence has shown that vitamin D inhibits the expression of adipogenic transcription factor genes, leading to a significant reduction in lipid accumulation and adipocyte apoptosis (2, 41). Obese individuals tend to have lower vitamin D levels (42–46), predisposing them to the development of comorbidities. Increased sequestration by the white adipose tissue reduces vitamin D bioavailability (47, 48). Vitamin D deficiency is also associated with the dysregulation of white adipose tissue and blood levels of inflammatory factors, including CRP and IL-10 (2). Several cross-sectional and cohort studies have found a positive correlation between vitamin D levels, beta cell function (49), and insulin sensitivity. Patients with low vitamin D levels appear to have a high risk of insulin resistance. The potential role of vitamin D deficiency in insulin resistance has been associated with inherited gene polymorphisms, including vitamin D-binding protein, vitamin D receptor, and the vitamin D 1 alpha-hydroxylase gene (30, 50–54).

After a thorough literature search, we found that the association between vitamin D deficiency and metabolic body composition status remains controversial. Furthermore, little has been reported about cardiometabolic markers among the different metabolic phenotypes with respect to sex and age (55). Since male and female have different features of adiposity distribution, which may affect vitamin D bioavailability, coupled with changes in metabolism due to the loss of protection from hormones after menopause, we aimed to compare the impact of metabolic phenotypes on the risk of vitamin D deficiency stratified by sex and age. We hypothesized that (i) metabolically unhealthy obesity (MUO) is an independent risk factor for vitamin D deficiency; (ii) in male participants of all ages and female participants aged $>$ 50 years, cardiometabolic markers have incremental trends among the healthy, MHO, MUNW, and MUO groups.

Materials and methods

Study design and participants

We retrospectively obtained data from adult participants (age $\geq$ 18 years) who underwent health checkups between 2013 and 2016 at Chang Gung Memorial Hospital. The exclusion criteria were as follows: (i) fasting $<$ 12 h; (ii) pregnancy;

(iii) conditions that may affect the metabolic status, such as hyperthyroidism or hypothyroidism, malignancy, chronic hepatitis, liver cirrhosis, hypothalamic disease, pituitary gland, or adrenal gland diseases; (iv) parathyroid gland disease or intake of medications that may affect vitamin D level; (v) high sensitivity (hs)-CRP > 10 mg/L, which may indicate acute infection status; and (vi) participants with incomplete data and history. In total, 6,655 participants were included in the analysis. Informed consent was not obtained because all data were accessed anonymously in the setting of retrospective records.

Data collection

Trained nurses used a standardized questionnaire to collect information on patients' medical and personal histories. Completion of the questionnaire was followed by anthropometric measurement, including body weight (kg), height (centimeter, cm), waist circumference (cm), and blood pressure (mmHg). Body height and weight were measured using calibrated meters and scales, according to a standardized protocol. BMI was calculated as body weight divided by the square of body height (kg/m^2). Waist circumference was measured midway between the lowest rib and iliac crest. Blood pressure was measured using an automated sphygmomanometer three times after the participants were seated for at least 15 min.

Laboratory data included total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C, mmol/L), high-density lipoprotein-cholesterol (HDL-C, mmol/L), triglyceride (TG, mmol/L), fasting blood glucose (FBG, mmol/L), hs-CRP (μg/mL), and insulin, which were determined using enzymatic, spectrophotometric, or colorimetric methods. Between 2013 and 2014, serum 25(OH)D levels were quantitatively determined using an electrochemiluminescence assay (ECLIA) performed on a Roche Cobas E601 immunoassay system (Roche Diagnostics, Mannheim, Germany); the unit of measurement was ng/mL. After 2015, it was determined using a chemiluminescent microparticle immunoassay (CMIA) on an Abbott I2000SR immunoassay system (Abbott Diagnostics, Illinois, USA), and the measurement unit was nmol/L. All the data were entered into an electronic database under strict quality control.

Participants who fulfilled at least three of the five criteria described by the Third Adult Treatment Panel (ATP III) of the National Cholesterol Education Program (NCEP) were defined as having metabolic syndromes. The five factors are high blood pressure (systolic blood pressure ≥ 130 mmHg and diastolic pressure ≥ 85 mmHg), under treatment, or already diagnosed with hypertension); high serum TG (≥ 1.7 mmol/L or under treatment); decreased HDL-C (< 1.03 mmol/L for males and < 1.29 mmol/L for females or under treatment); hyperglycemia (FBG ≥ 5.6 mmol/L, under treatment, or previously diagnosed with diabetes mellitus); and abdominal obesity (waist circumference ≥ 90 cm for males and ≥ 80 cm for females).

Definition of homeostasis model assessment-insulin resistance

HOMA-IR index was used to quantify the extent of insulin resistance. The cutoff value of HOMA-IR as an indicator of metabolic syndrome was based on two recent studies in Asian populations (26, 27). The HOMA-IR index formula was as follows:

$$\text{fasting insulin (mIU/L)} \times \frac{FBG(mmol/L)}{22.5}$$

Phenotypes of metabolic body composition status

All participants were categorized into four metabolic phenotypes:

(i) metabolically healthy normal weight (MHNW) (HOMA-IR < 2.5, without MS, BMI < 25), (ii) MHO (HOMA-IR < 2.5, without MS, BMI ≥ 25), (iii) MUNW (HOMA-IR ≥ 2.5 or with MS, BMI < 25), and (iv) MUO (HOMA-IR ≥ 2.5 or with MS, BMI ≥ 25).

Definition of vitamin D deficiency

A serum vitamin D level < 20 ng/mL is defined as vitamin D deficiency according to the Endocrine Society Clinical Practice Guidelines (56).

Statistical analysis

The mean ± standard deviation (SD) was used for continuous variables and the number (%) was used for categorical variables. The independent *t*-test and chi-square tests were used to compare differences between sexes for continuous and categorical variables, respectively. Analysis of variance and chi-square tests were used to compare the differences among different metabolic states (MHNW, MHO, MUNW, and MUO) for continuous and categorical variables, respectively. Additionally, linear contrast in the analysis of variance and the Cochran-Armitage test were used to determine the linear trend across metabolic states for continuous and categorical variables, respectively. Bonferroni *post hoc* comparisons were performed for pairwise analyses of the study groups. Multiple logistic regression models were used to explore the relationship between the metabolic phenotypes and vitamin D deficiency.

We chose the mean arterial pressure, TG/HDL-C ratio, and hs-CRP level as covariates. Sex, age, and HOMA-IR were grouped variables; thus, they were not adjusted for. Neither were FBG and insulin levels adjusted for because they were highly correlated with HOMA-IR. Metabolic body composition was a variable of interest; therefore, BMI and waist-to-height ratio were not adjusted for. The LDL-C level was not adjusted for because of its collinearity with TC. All statistical analyses were conducted using International Business Machine (IBM) Statistical Product and Service Solutions Statistics (SPSS, IBM Corp., Armonk, NY, USA). Statistical significance was set at a P-value < 0.05.

Results

Demographics of the study participants

A total of 6,655 participants were enrolled in this study. The main characteristics of the study participants, stratified by age ($<$ 50 and $\geq$ 50 years), are shown in Table 1. In the $<$ 50 years group (n = 2,589), the mean age and BMI of the male participants were slightly higher than that of the female participants. Mean arterial pressure (MAP), TC, TG, LDL-C, hs-CRP, insulin, and HOMA-IR were significantly higher in men than that in women ($P < 0.001$). The proportion of MHO and MUO were higher in men than that in women (30.2% vs. 13.0% and 17.3% vs. 4.0%, respectively). The prevalence of vitamin D deficiency was significantly higher in women than that in men (35.5% vs. 31.2%, $P = 0.025$; Table 1).

In the $\geq$ 50 years group (n = 4,066), the mean $\pm$ SD age of the participants was 58.6 $\pm$ 6.9 for men and 58.2 $\pm$ 6.5 for women. The mean $\pm$ SD BMI was 24.3 $\pm$ 3.1 for men and 24.2 $\pm$ 3.2 for women. No significant difference was observed in the mean $\pm$ SD age or BMI between men and women (Table 1).The MAP, FBG, TG, and hs-CRP levels were significantly higher in men than that in women, while TC, LDL-C, and insulin levels were significantly higher in women. HOMA-IR levels and vitamin D deficiency were not significantly different between the sexes. The proportion of MHO and MUO were higher in male than that in female. Vitamin D deficiency was observed in 22.7% of men and 24.5% of women, without a significant difference ($P = 0.18$).

Characteristics of men according to metabolic phenotypes

The baseline characteristics of men according to metabolic phenotypes stratified by age are presented in Table 2. The male participants were classified into four groups: MHNW, MHO, MUNW, and MUO.

Among the four metabolic groups of men aged $<$ 50 years, there were significant incremental trends in TC, TG, insulin, and HOMA-IR levels. The MHO group had the lowest vitamin D level (28.2 ng/mL) and the highest prevalence of vitamin D deficiency (35.8%, Table 2).

Among men aged $>$ 50 years, there was no significant difference in TC, LDL-C, and hs-CRP levels among the four groups. Individuals with MUNW had the highest FBG, TG, and TG/HDL-C levels. Insulin and HOMA-IR levels showed an incremental trend among the four groups. The percentage of vitamin D deficiency also showed an increasing trend among the four groups ($P = 0.009$, Table 2).

Characteristics of women according to metabolic phenotypes

The women were classified into four groups: MHNW, MHO, MUNW, and MUO. Table 3 shows the characteristics of the women according to the four metabolic phenotypes stratified by age.

Among the four metabolic groups of women aged $<$ 50 years, the levels of metabolic biomarkers, including FBG, TC, TG, LDL-C, insulin, and HOMA-IR, showed significant incremental trends. The MUO group had the highest MAP level, lowest vitamin D level (22.8 ng/mL), and the highest prevalence of vitamin D deficiency (47.4%). However, there was no significant difference in vitamin D levels and prevalence of vitamin D deficiency among the four groups ($P = 0.085$ and $P = 0.13$, respectively, Table 3).

When considering women aged $>$ 50 years, the MAP, TG, hs-CRP, insulin, and HOMA-IR levels showed significant incremental trends. However, TC and LDL-C levels were not significantly different among the four groups. The MUO group had the lowest vitamin D level (29.3 ng/mL) and the highest prevalence of vitamin D deficiency (29.2%). There was no significant difference in vitamin D levels and prevalence of vitamin D deficiency among the four groups ($P = 0.091$ and $P = 0.066$, respectively, Table 3).

Association between metabolic phenotypes and vitamin D deficiency

The associations between the metabolic phenotypes and vitamin D deficiency are shown in Table 4. Compared with MHNW, the MHO group showed a higher odds ratio (OR) for vitamin D deficiency in men aged $<$ 50 years, which remained statistically significant after adjusting for cardiometabolic factors, including MAP, TG/HDL-C, and hs-CRP [1.35, 95% confidence interval (CI) 1.05–1.73]. By contrast, in men aged $>$ 50 years, the risk of vitamin D deficiency was greater in the MUO group (OR 1.44, 95% CI 1.05–1.97) followed by MUNW (OR 1.53, 95% CI 0.96–2.43, $P = 0.076$) with borderline significance

TABLE 1 Main characteristics of the participants by age and sex.

Characteristics	<50 years old				≥ 50 years old			
	Total ($N = 2,589$)	Men ($n = 1,629$)	Women ($n = 960$)	*P*-value	Total ($N = 4,066$)	Men ($n = 2,210$)	Women ($n = 1,856$)	*P*-value
Age, years	40.9 ± 6.0	41.0 ± 5.8	40.7 ± 6.2	0.232	58.4 ± 6.7	58.6 ± 6.9	58.2 ± 6.5	0.097
BMI (kg/m^2)	23.8 ± 3.5	24.8 ± 3.4	22.2 ± 3.0	<0.001	24.2 ± 3.2	24.3 ± 3.1	24.2 ± 3.2	0.496
Waist-to-height ratio	0.50 ± 0.05	0.51 ± 0.05	0.48 ± 0.05	<0.001	0.52 ± 0.05	0.52 ± 0.05	0.53 ± 0.05	<0.001
Mean arterial pressure (mmHg)	86.1 ± 12.8	89.8 ± 12.1	79.8 ± 11.3	<0.001	91.4 ± 13.3	92.2 ± 13.0	90.4 ± 13.5	<0.001
Fasting glucose (mmol/L)	5.4 ± 1.3	5.5 ± 1.6	5.11 ± 0.67	<0.001	5.7 ± 1.6	5.8 ± 1.8	5.6 ± 1.3	<0.001
Total cholesterol (mmol/L)	5.2 ± 1.0	5.3 ± 1.0	4.9 ± 0.9	<0.001	5.4 ± 1.0	5.3 ± 1.0	5.5 ± 1.0	<0.001
TG (mmol/L)	1.5 ± 1.3	1.9 ± 1.5	0.95 ± 0.78	<0.001	1.5 ± 1.1	1.6 ± 1.3	1.35 ± 0.89	<0.001
LDL-C (mmol/L)	3.23 ± 0.89	3.40 ± 0.91	2.94 ± 0.78	<0.001	3.42 ± 0.88	3.39 ± 0.88	3.46 ± 0.87	0.008
HDL-C (mmol/L)	1.27 ± 0.31	1.17 ± 0.27	1.44 ± 0.31	<0.001	1.29 ± 0.32	1.20 ± 0.30	1.39 ± 0.31	<0.001
TG / HDL-C	1.4 ± 1.6	1.8 ± 1.8	0.74 ± 0.93	<0.001	1.3 ± 1.3	1.5 ± 1.5	1.09 ± 0.93	<0.001
hs-CRP (μg/mL)	1.7 ± 3.9	2.1 ± 4.5	1.1 ± 2.6	<0.001	2.4 ± 6.2	2.6 ± 6.8	2.1 ± 5.3	0.019
Insulin (mIU/L)	7.1 ± 4.0	7.6 ± 4.4	6.3 ± 3.0	<0.001	6.6 ± 4.2	6.4 ± 4.2	6.9 ± 4.1	<0.001
HOMA-IR	1.7 ± 1.2	1.9 ± 1.4	1.46 ± 0.79	<0.001	1.7 ± 1.4	1.7 ± 1.5	1.7 ± 1.3	0.578
Metabolic phenotypes				<0.001				0.049
MHNW, *n* (%)	1,543 (59.6)	790 (48.5)	753 (78.4)		2,255 (55.5)	1,219 (55.2)	1,036 (55.8)	
MHO, *n* (%)	617 (23.8)	492 (30.2)	125 (13.0)		1,127 (27.7)	625 (28.3)	502 (27.0)	
MUNW, *n* (%)	109 (4.2)	65 (4.0)	44 (4.6)		215 (5.3)	99 (4.5)	116 (6.3)	
MUO, *n* (%)	320 (12.4)	282 (17.3)	38 (4.0)		469 (11.5)	267 (12.1)	202 (10.9)	
25(OH)D (ng/mL)	28.8 ± 14.7	30.0 ± 15.5	26.8 ± 13.1	<0.001	33.9 ± 16.8	35.9 ± 18.3	31.5 ± 14.4	<0.001
Vitamin D deficiency, *n* (%)	850 (32.8)	509 (31.2)	341 (35.5)	0.025	955 (23.5)	501 (22.7)	454 (24.5)	0.180

BMI, body mass index; TG, Triglycerides; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, Homeostasis Model Assessment-Insulin Resistance; 25(OH)D, 25-OH-Vitamin D; MHNW, Metabolically healthy normal weight; MHO, metabolically healthy obesity; MUNW, metabolically unhealthy normal weight; MUO, metabolically unhealthy obesity.

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TABLE 2 Baseline characteristics of men according to metabolic phenotypes stratified by age.

Characteristics	MHNW	MHO	MUNW	MUO	*P*-value	*P*-trend
Men < 50 years old (*n* = 1,629)						
Number	790	492	65	282		
Age, years	40.9 ± 5.9	41.5 ± 5.6	42.3 ± 5.4	40.4 ± 6.2	0.015	0.653
BMI (kg/m^2)	22.2 ± 2.0	27.1 ± 1.8^{a}	23.3 ± 1.4^{a,b}	28.4 ± 2.6^{a,b,c}	<0.001	<0.001
Waist-to-height ratio	0.48 ± 0.04	0.54 ± 0.03^{a}	0.51 ± 0.03^{a,b}	0.56 ± 0.04^{a,b,c}	<0.001	<0.001
Mean arterial pressure (mmHg)	85.8 ± 10.9	92.3 ± 11.9^{a}	91.4 ± 12.1^{a}	96.5 ± 11.7^{a,b,c}	<0.001	<0.001
Fasting glucose (mmol/L)	5.3 ± 1.1	5.2 ± 0.53	6.9 ± 3.3^{a,b}	6.5 ± 2.5^{a,b}	<0.001	<0.001
Total cholesterol (mmol/L)	5.22 ± 0.95	5.31 ± 1.03	5.47 ± 1.02	5.54 ± 1.02^{a,b}	<0.001	<0.001
TG (mmol/L)	1.5 ± 1.0	2.1 ± 1.7^{a}	2.2 ± 1.5^{a}	2.6 ± 1.7^{a,b}	<0.001	<0.001
LDL-C (mmol/L)	3.33 ± 0.85	3.41 ± 0.92	3.59 ± 0.99	3.53 ± 1.02^{a}	0.006	0.001
HDL-C (mmol/L)	1.25 ± 0.30	1.11 ± 0.21^{a}	1.13 ± 0.31^{a}	1.06 ± 0.20^{a}	<0.001	<0.001
TG / HDL-C	1.3 ± 1.1	2.0 ± 2.3^{a}	2.2 ± 1.8^{a}	2.6 ± 1.9^{a,b}	<0.001	<0.001
hs-CRP (μg/mL)	1.6 ± 3.8	2.3 ± 4.4^{a}	2.2 ± 5.6	3.4 ± 5.8^{a,b}	<0.001	<0.001
Insulin (mIU/L)	5.3 ± 2.03	7.1 ± 1.96^{a}	12.3 ± 6.4^{a,b}	13.7 ± 5.0^{a,b,c}	<0.001	<0.001
HOMA-IR	1.23 ± 0.51	1.64 ± 0.47^{a}	3.5 ± 2.1^{a,b}	3.8 ± 1.8^{a,b}	<0.001	<0.001
25(OH)D (ng/mL)	31.4 ± 16.7	28.2 ± 14.4^{a}	31.5 ± 15.2	29.1 ± 13.6	0.002	0.352
Vitamin D deficiency, *n* (%)	234 (29.6)	176 (35.8)	20 (30.8)	79 (28.0)	0.071	0.782
Men ≥ 50 years old (*n* = 2,210)						
Number	1,219	625	99	267		
Age, years	59.1 ± 7.1	58.0 ± 6.5^{a}	57.7 ± 6.3	58.0 ± 7.1	0.002	0.034
BMI (kg/m^2)	22.2 ± 2.0	26.9 ± 1.6^{a}	23.6 ± 1.2^{a,b}	28.0 ± 2.4^{a,b,c}	<0.001	<0.001
Waist-to-height ratio	0.49 ± 0.04	0.56 ± 0.03^{a}	0.52 ± 0.03^{a,b}	0.57 ± 0.05^{a,b,c}	<0.001	<0.001
Mean arterial pressure (mmHg)	89.4 ± 12.7	95.2 ± 12.3^{a}	92.0 ± 11.8	97.8 ± 13.4^{a,b,c}	<0.001	<0.001
Fasting glucose (mmol/L)	5.5 ± 1.3	5.6 ± 1.1	8.4 ± 3.6^{a,b}	7.1 ± 2.4^{a,b,c}	<0.001	<0.001
Total cholesterol (mmol/L)	5.28 ± 0.98	5.25 ± 0.96	5.43 ± 1.01	5.29 ± 1.06	0.423	0.366
TG (mmol/L)	1.4 ± 1.27	1.7 ± 0.9^{a}	2.3 ± 2.0^{a,b}	2.0 ± 1.28^{a,b}	<0.001	<0.001
LDL-C (mmol/L)	3.36 ± 0.87	3.42 ± 0.86	3.43 ± 0.94	3.42 ± 0.96	0.390	0.356
HDL-C (mmol/L)	1.27 ± 0.32	1.13 ± 0.24^{a}	1.10 ± 0.25^{a}	1.07 ± 0.22^{a,b}	<0.001	<0.001
TG / HDL-C	1.3 ± 1.6	1.6 ± 1.0^{a}	2.4 ± 2.5^{a,b}	2.1 ± 1.7^{a,b}	<0.001	<0.001
hs-CRP (μg/mL)	2.6 ± 8.0	2.2 ± 3.9	3.2 ± 4.9	3.1 ± 6.4	0.207	0.134
Insulin (mIU/L)	4.6 ± 2.0	6.3 ± 2.1^{a}	10.8 ± 4.0^{a,b}	13.4 ± 6.5^{a,b,c}	<0.001	<0.001
HOMA-IR	1.11 ± 0.51	1.55 ± 0.52^{a}	3.7 ± 1.6^{a,b}	4.1 ± 2.5^{a,b,c}	<0.001	<0.001
25(OH)D (ng/mL)	37.0 ± 19.0	35.3 ± 17.2	32.9 ± 16.8	33.4 ± 18.0^{a}	0.005	0.002
Vitamin D deficiency, *n* (%)	261 (21.4)	136 (21.8)	28 (28.3)	76 (28.5)	0.040	0.009

BMI, body mass index; TG, Triglycerides; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, Homeostasis Model Assessment-Insulin Resistance; 25(OH)D, 25-OH-Vitamin D; MHNW, Metabolically healthy normal weight; MHO, metabolically healthy obesity; MUNW, metabolically unhealthy normal weight; MUO, metabolically unhealthy obesity.
^{a,b,c} significant post-hoc comparisons vs. MHNW, MHO, and MUNW, respectively.

in multivariable analysis compared with that of the MHNW group (Table 4).

In women aged < 50 years, the MUO group demonstrated the highest risk for vitamin D deficiency (OR 2.33, 95% CI 1.13–4.81) compared to the MHNW group after adjusting for MAP, TG/HDL-C, and hs-CRP levels. In women aged > 50 years, the MUO group demonstrated the highest risk for vitamin D deficiency (OR 1.54, 95% CI 1.08–2.21) followed by MHO (OR 1.28, 95% CI 0.996–1.648, $P = 0.053$) with borderline significance compared to that of the MHNW group in multivariable analysis.

Discussion

Our logistic findings implied that in women of all ages and men aged > 50 years, MUO was associated with vitamin D deficiency. Among men aged < 50 years, MHO had the highest OR for vitamin D deficiency. Subcutaneous adipose

TABLE 3 Baseline characteristics of women according to metabolic phenotypes stratified by age.

Characteristics	MHNW	MHO	MUNW	MUO	*P*-value	*P*-trend
Women < 50 years old (*n* = 960)						
Number	753	125	44	38		
Age, years	40.1 ± 6.3	43.0 ± 5.2[a]	42.0 ± 5.6	44.1 ± 5.4[a]	<0.001	0.001
BMI (kg/m^2)	21.2 ± 2.1	26.8 ± 1.8[a]	22.6 ± 1.4[a,b]	27.7 ± 1.9[a,c]	<0.001	<0.001
Waist-to-height ratio	0.46 ± 0.041	0.54 ± 0.044[a]	0.49 ± 0.038[a,b]	0.56 ± 0.035[a,b,c]	<0.001	<0.001
Mean arterial pressure (mmHg)	78.0 ± 10.2	85.6 ± 12.7[a]	84.3 ± 9.9[a]	91.4 ± 13.4[a,b,c]	<0.001	<0.001
Fasting glucose (mmol/L)	5.0 ± 0.51	5.2 ± 0.50[a]	5.7 ± 1.0[a,b]	6.0 ± 1.6[a,b]	<0.001	<0.001
Total cholesterol (mmol/L)	4.82 ± 0.85	5.02 ± 1.10	5.04 ± 0.99	5.25 ± 0.88[a]	0.002	0.005
TG (mmol/L)	0.85 ± 0.68	1.15 ± 0.63[a]	1.29 ± 0.59[a]	1.85 ± 1.90[a,b,c]	<0.001	<0.001
LDL-C (mmol/L)	2.87 ± 0.74	3.13 ± 0.86[a]	3.22 ± 0.89[a]	3.26 ± 0.78[a]	<0.001	0.002
HDL-C (mmol/L)	1.48 ± 0.30	1.31 ± 0.27[a]	1.29 ± 0.25[a]	1.22 ± 0.23[a]	<0.001	<0.001
TG / HDL-C	0.64 ± 0.83	0.94 ± 0.67[a]	1.10 ± 0.72[a]	1.7 ± 2.2[a,b,c]	<0.001	<0.001
hs-CRP (μg/mL)	0.89 ± 2.4	1.6 ± 2.4[a]	1.1 ± 1.3	3.1 ± 5.0[a,b,c]	<0.001	<0.001
Insulin (mIU/L)	5.5 ± 2.1	6.9 ± 2.1[a]	11.7 ± 2.2[a,b]	14.3 ± 4.1[a,b,c]	<0.001	<0.001
HOMA-IR	1.24 ± 0.49	1.60 ± 0.51[a]	2.91 ± 0.52[a,b]	3.7 ± 1.2[a,b,c]	<0.001	<0.001
25(OH)D (ng/mL)	26.6 ± 13.3	28.5 ± 12.7	28.4 ± 11.6	22.8 ± 10.5	0.085	0.099
Vitamin D deficiency, *n* (%)	273 (36.3)	39 (31.2)	11 (25.0)	18 (47.4)	0.130	0.917
Women ≥ 50 years old (*n* = 1,856)						
Number	1,036	502	116	202		
Age, years	57.7 ± 6.3	58.5 ± 6.3	59.7 ± 7.7[a]	59.6 ± 7.0[a]	<0.001	<0.001
BMI (kg/m^2)	22.1 ± 1.8	27.0 ± 1.9[a]	23.3 ± 1.3[a,b]	28.5 ± 2.6[a,b,c]	<0.001	<0.001
Waist-to-height ratio	0.50 ± 0.041	0.57 ± 0.040[a]	0.53 ± 0.036[a,b]	0.59 ± 0.045[a,b,c]	<0.001	<0.001
Mean arterial pressure (mmHg)	87.2 ± 13.2	93.2 ± 12.8[a]	94.7 ± 12.7[a]	97.7 ± 12.4[a,b]	<0.001	<0.001
Fasting glucose (mmol/L)	5.3 ± 1.0	5.4 ± 0.71	7.0 ± 2.6[a,b]	6.4 ± 1.7[a,b,c]	<0.001	<0.001
Total cholesterol (mmol/L)	5.53 ± 0.99	5.50 ± 0.99	5.59 ± 1.11	5.57 ± 0.98	0.749	0.367
TG (mmol/L)	1.2 ± 0.73	1.4 ± 0.79[a]	1.8 ± 1.3[a,b]	1.9 ± 1.2[a,b]	<0.001	<0.001
LDL-C (mmol/L)	3.42 ± 0.86	3.50 ± 0.85	3.54 ± 1.02	3.53 ± 0.92	0.137	0.089
HDL-C (mmol/L)	1.46 ± 0.32	1.35 ± 0.28[a]	1.27 ± 0.25[a]	1.23 ± 0.27[a,b]	<0.001	<0.001
TG / HDL-C	0.90 ± 0.79	1.13 ± 0.79[a]	1.58 ± 1.46[a,b]	1.65 ± 1.18[a,b,c]	<0.001	<0.001
hs-CRP (μg/mL)	1.8 ± 5.7	2.4 ± 4.2	2.6 ± 7.8	3.0 ± 3.9[a]	0.006	0.005
Insulin (mIU/L)	5.1 ± 1.97	6.6 ± 2.0[a]	12.0 ± 5.6[a,b]	13.4 ± 6.0[a,b,c]	<0.001	<0.001
HOMA-IR	1.2 ± 0.50	1.6 ± 0.49[a]	3.6 ± 1.91[a,b]	3.7 ± 1.87[a,b]	<0.001	<0.001
25(OH)D (ng/mL)	31.9 ± 14.7	31.1 ± 14.6	32.5 ± 13.9	29.3 ± 12.8	0.091	0.075
Vitamin D deficiency, *n* (%)	238 (23.0)	135 (26.9)	22 (19.0)	59 (29.2)	0.066	0.124

BMI, body mass index; TG, Triglycerides; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, Homeostasis Model Assessment-Insulin Resistance; 25(OH)D, 25-OH-Vitamin D; MHNW, Metabolically healthy normal weight; MHO, metabolically healthy obesity; MUNW, metabolically unhealthy normal weight; MUO, metabolically unhealthy obesity.
[a,b,c] significant posthoc comparisons vs. MHNW, MHO, and MUNW, respectively.

tissue can store large amounts of fat-soluble vitamin D (57); thus, leading to less vitamin D entering the blood circulation. Greater subcutaneous fat in women, which is related to estrogen, has a greater influence on serum vitamin D concentration than visceral fat tissue (58). Among men aged < 50 years, metabolically unhealthy participants tended to have more visceral fat; the metabolically healthy obese group tended to have more subcutaneous fat, thus leading to the current findings. Lifestyle differences may also contribute to sex and age differences. Younger females generally work indoors and often intentionally avoid sunshine, whereas older male and female might take vitamin D supplements and have more time to engage in outdoor activities (59). To the best of our knowledge, this is a novel study demonstrating cardiovascular risk factors and vitamin D deficiency according to sex, age, and metabolic body composition status in a large Chinese population. The study results provide physicians with useful information regarding vitamin D deficiency and

TABLE 4 Association between metabolic phenotypes and vitamin D deficiency stratified by age and sex.

	Number	Vitamin D deficiency, *n* (%)	Univariable analysis		Multivariable analysis[#]	
			OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Men **< 50 years** old						
MHNW	790	234 (29.6)	1		1	
MHO	492	176 (35.8)	1.32 (1.04–1.68)	0.022	1.35 (1.05–1.73)	0.019
MUNW	65	20 (30.8)	1.06 (0.61–1.83)	0.846	1.08 (0.62–1.89)	0.778
MUO	282	79 (28.0)	0.92 (0.68–1.25)	0.611	0.95 (0.68–1.31)	0.743
Men **≥ 50 years** old						
MHNW	1,219	261 (21.4)	1		1	
MHO	625	136 (21.8)	1.02 (0.81–1.29)	0.863	0.997 (0.784–1.269)	0.982
MUNW	99	28 (28.3)	1.45 (0.92–2.29)	0.114	1.53 (0.96–2.43)	0.076
MUO	267	76 (28.5)	1.46 (1.08–1.97)	0.013	1.44 (1.05–1.97)	0.022
Women **< 50 years** old						
MHNW	753	273 (36.3)	1		1	
MHO	125	39 (31.2)	0.80 (0.53–1.20)	0.275	0.94 (0.61–1.43)	0.757
MUNW	44	11 (25.0)	0.59 (0.29–1.18)	0.134	0.66 (0.33–1.34)	0.253
MUO	38	18 (47.4)	1.58 (0.82–3.04)	0.169	2.33 (1.13–4.81)	0.022
Women **≥ 50 years** old						
MHNW	1,036	238 (23.0)	1		1	
MHO	502	135 (26.9)	1.23 (0.97–1.58)	0.093	1.281 (0.996–1.648)	0.053
MUNW	116	22 (19.0)	0.78 (0.48–1.28)	0.329	0.86 (0.52–1.41)	0.546
MUO	202	59 (29.2)	1.38 (0.99–1.94)	0.058	1.54 (1.08–2.21)	0.018

OR, odds ratio; CI, confidence interval;BMI, body mass index; TG, Triglycerides; HDL-C, high-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein; MHNW, Metabolically healthy normal weight; MHO, metabolically healthy obesity; MUNW, metabolically unhealthy normal weight; MUO, metabolically unhealthy obesity.
[#]adjusted for mean arterial pressure + TG/HDL-C ratio and hs-CRP.

both age- and sex-specific intervention methods to decrease cardiometabolic risk.

A small population study found that obese individuals had significantly lower serum 25-hydroxy vitamin D (25[OH]D) levels than normal-weight participants, regardless of metabolic phenotypes (60). Patchaya et al. performed a retrospective chart review of outpatient medical records. Patients aged > 18 years with BMI > 30 kg/m^2 were enrolled and divided into two groups: MHO and MUO. They found no significant differences in the 25(OH)D levels between individuals with MHO and MUO. In addition, there was a negative correlation between 25(OH)D levels and adiposity markers (BMI, body weight, and waist circumference), but not between 25(OH)D levels and lipid parameters or HOMA-IR (61). An Iranian population-based study found that 25(OH)D levels were lower in patients with MUO than in those with MHO. Reduced vitamin D concentrations were associated with cardiometabolic and inflammatory markers in MUO compared with MHO. This study did not find a correlation between serum 25(OH)D levels and BMI in obese participants, but it was negatively correlated with waist circumference. Adipose tissue distribution has been hypothesized to be associated with the bioavailability of 25(OH)D (62). Another cross-sectional study recruited 111 healthy adults without diabetes. After adjusting for age, sex, and body fat percentage, 25(OH)D was no longer associated with insulin sensitivity, 2 h glucose, or hs-CRP but remained associated with fasting glucose. The authors interpreted that the association between vitamin D and cardiometabolic risk among healthy adults without diabetes is largely mediated by adiposity (63).

The most commonly mentioned mechanisms explaining the low vitamin D level in individuals with obesity include (i) less sun exposure, (ii) negative feedback from an increased 1,25(OH)D concentration, (iii) sequestration of vitamin D within adipose tissue, and (iv) volumetric dilution resulting in lower 25(OH)D concentration (64).

We also found that for hs-CRP levels, in both sexes aged < 50 years, the highest value was observed in the MUO group, followed by the MHO group, with statistical significance. However, among men aged > 50 years, the MUNW group had the highest hs-CRP level, followed by the MUO group, without statistical significance. Among women aged > 50 years, the MUO group had the highest hs-CRP level, followed by the MUNW group (*P* for trend = 0.005). Hs-CRP, which represents inflammation status, is more influenced by obesity in the younger population, and it is more

frequently correlated with metabolically unhealthy status in the older population.

Vitamin D affects adipogenesis, apoptosis, oxidative stress, inflammation, and lipid metabolism (65). Hypponen et al. reviewed the evidence of calcitriol-induced inhibition of many of the adverse effects of obesity. For example, calcitriol suppresses the secretion of pro-inflammatory cytokines, stimulates the secretion of anti-inflammatory cytokines from macrophage-infiltrated adipose tissue, and upregulates insulin growth factor 1 (IGF-1) secretion, which has protective effects against metabolic syndrome. Calcitriol also promotes insulin secretion from islet beta cells, suppresses the overactivity of the renin-angiotensin system in islets, and protects against apoptosis. In obesity-related dyslipidemia, calcitriol can reduce hepatic TG synthesis (66).

Some studies have demonstrated the effects of vitamin D supplementation. A 12-week randomized controlled trial revealed that improvement of vitamin D status in T2DM patients resulted in the amelioration of systemic inflammatory markers, such as hs-CRP and IL-6 (67). A recent meta-analysis demonstrated that vitamin D improves serum levels of TC, TG, and LDL in patients with T2DM (68). A case-control study that focused on men with spinal cord injury demonstrated that even a small increase in vitamin D intake may improve TC independent of lean mass. Vitamin D adjusted for total dietary intake, was positively correlated with carbohydrate profile parameters (69). Another Mendelian randomization study found that a 25 nmol/L higher concentration was associated with a 14% lower risk of T2DM (70). Wenclewska et al. found that among patients suffering from metabolic disturbances and T2DM, supplementation with 2,000 IU vitamin D for 3 months reduced the level of oxidative deoxyribonucleic acid (DNA) damage, HOMA-IR, and TG/HDL ratio (71).

Our study focused on a large Asian population, stratified by age and sex. We evaluated cardiometabolic biomarkers and the risk of vitamin D deficiency among the four metabolic phenotypes. We combined both HOMA-IR and metabolic syndrome criteria to define metabolic health or unhealthy status, which makes it more indicative of morbidity and mortality and provides relatively convincing results.

Nevertheless, this study has several limitations. First, the cross-sectional study design makes it impractical to establish causal relationships. Second, we did not record participants' lifestyles, including physical activity, sun exposure, dietary habits, and use of vitamin D supplements. Third, our study participants were relatively healthy or had better health awareness; therefore, they may not represent the general population. Further research is warranted to elucidate the potential protective or anti-inflammatory effects of vitamin D in different obesity phenotypes.

In conclusion, in a relatively healthy population, our data revealed that in women of all ages and men aged $>$ 50 years, the MUO group had the highest OR for vitamin D deficiency. Among men aged $<$ 50 years, the highest OR for vitamin D deficiency was observed in the MHO group. The inflammatory biomarker hs-CRP is more strongly correlated with obesity in younger adults, and it is more correlated with metabolically unhealthy status in older individuals.

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 human participants were reviewed and approved by Institutional review board of Chang Gung Memorial Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Y-CC and W-CL conceived the study concept, design and contributed equally to this study. WY, H-YH, and X-JX were responsible for data collection. Y-CC drafted the manuscript. W-CL and J-YC interpreted the data. J-YC revised the final manuscript. All the authors approved the final version of the manuscript.

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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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Adequate 25(OH)D moderates the relationship between dietary inflammatory potential and cardiovascular health risk during the second trimester of pregnancy

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Background: Pro-inflammatory diets play an important role in developing cardiovascular disease (CVD). Vitamin D has been demonstrated to have an anti-inflammatory effect and promote cardiovascular health (CVH). However, it is unclear whether adequate vitamin D during pregnancy protects against poor CVH caused by pro-inflammatory diets.

Objective: To investigate the association of pro-inflammatory diets with the cardiovascular risk (CVR) among pregnant women and whether such association was modified by vitamin D status.

Methods: The study was based on a prospective birth cohort that included 3,713 pregnant women between 16 and 23 gestational weeks. In total, 25(OH)D concentrations and high-sensitivity C-reactive protein (hs-CRP) were measured from the collected blood. The dietary inflammatory potential was evaluated using the empirical dietary inflammatory pattern (EDIP) score based on a validated food frequency questionnaire. Gestational CVR was evaluated using the CVR score based on five "clinical" CVR metrics, including body mass index, blood pressure, total cholesterol, glucose levels, and smoking status.

Results: The proportion of women with a CVR score >0 was 54.3%. We observed a positive association between the EDIP score and CVR score. Compared with the lowest quartile, the CVR score (β = −0.114, 95% CI, −0.217, −0.011) and hs-CRP levels (β = −0.280, 95% CI, −0.495, −0.065) were lower in the highest quartile (*P* for trend <0.05). Increased CVR connected with high EDIP score was observed only in women with 25(OH)D concentrations

<50 nmol/L (RR = 1.85; 95% CI: 1.35, 2.54). Mediation analysis revealed that the proportion of association between the EDIP score and CVR score mediated by 25(OH)D was 28.7%, and the proportion of the association between 25(OH)D and the CVR score mediated by hs-CRP was 21.9%.

Conclusion: The higher dietary inflammatory potential was associated with an increased CVR during pregnancy by promoting inflammation. Adequate vitamin D could exert anti-inflammatory effects and modify such association.

KEYWORDS

vitamin D, cardiovascular health, pregnant women, dietary inflammatory potential, nutrients

Introduction

Cardiovascular disease (CVD) accounted for 40% of the deaths and is the leading cause of death and premature death in China (1). Pregnancy poses an immense challenge to women's metabolic function and cardiometabolic stressors and is more susceptible to cardiovascular damage (2). Recent evidence suggests that the mother's cardiovascular health (CVH) during pregnancy was significantly associated with the later cardiometabolic health among women and offspring (3).

Inflammation has been implicated in CVD etiology (4), and increasing inflammation may lead to a poor gestational CVH. The higher dietary inflammatory potential that leads to increased inflammation levels was associated with a higher risk of CVD (5). A diet intervention study found that high-inflammation levels moderated the effects of a diet intervention to control CVD (6, 7). Thus, interventions to reduce inflammation and thus protect CVH applicable to pregnant women are required, and vitamin D supplementation is an attractive target.

Vitamin D can regulate inflammation and is generally deficient during pregnancy (8). Previous intervention experiments have demonstrated that daily vitamin D supplementation will decrease systemic inflammatory markers such as high-sensitivity C-reactive protein (hs-CRP) (9). A similar relationship was found in our earlier study of high serum 25(OH)D concentrations during pregnancy which were inversely related to hs-CRP levels (10). Moreover, a recent large-scale population meta-analysis has confirmed that a low vitamin D level increases CVD risk (11). These studies indicate that vitamin D may inhibit inflammation and promote gestational CVH. So far, however, there has been a little discussion about whether adequate 25(OH)D moderates the relationship between dietary inflammatory potential and cardiovascular risk (CVR) during pregnancy.

Therefore, in this study, we tested the relationship between dietary inflammatory potential and CVR during pregnancy and whether such a relationship was modified by 25(OH)D concentrations. It could conceivably be hypothesized that 1) high-dietary inflammatory potential was associated with increased CVR during pregnancy by promoting inflammation, and 2) adequate 25(OH)D concentrations may modify such association by inhibiting inflammation.

Methods

Study participants and design

The data of this study was from a prospective birth cohort study. In the cohort study, a total of 4,216 pregnant women aged 18 to 45 years, with gestational ages from 16 to 23 weeks, were recruited in three hospitals (The First Affiliated Hospital of Anhui Medical University, Anhui Women and Child Health Care Hospital, and The First People's Hospital of Hefei City) from March 2018 and June 2021. The exclusion criteria included the following: missing blood samples, severe anemia, abnormal liver, renal, or thyroid function, ongoing infections (e.g., cervicovaginal infection and periodontal infection), and incomplete CVR data during pregnancy. In addition, pregnant women with hs-CRP concentrations >10 mg/L were excluded (12), as these likely indicate acute inflammatory response. Given that blood pressure (BP) is a component of gestational CVR, we did not exclude participants with eclampsia or pre-eclampsia.

At recruitment, weight and height were measured by well-trained staff using standardized procedures. The participants completed a structured questionnaire including sociodemographic characteristics, lifestyle, and perinatal health status through face-to-face interviews or medical records. Each participant completed a validated food frequency questionnaire (FFQ) at recruitment. Then, study nurses collected samples of the venous blood. At last, we obtained 3,713 pregnant women's complete data, including blood samples

(**Supplementary Figure 1**). The ethical approval was granted by the Ethics Committee of Anhui Medical University (20180092), and informed consent was obtained from each participant.

Dietary assessment

The nutrition information of the participants was assessed using an FFQ at 16–23 gestational weeks, pregnant women's self-reported food intake frequency, and serving size in the past month. Specified serving sizes are described by using natural portions (e.g., 1 tomato) or standard weight and volume measures of the servings commonly consumed (13). With responses ranging from "never" to "1 time a day or more", answers followed: never = 0 times/day; one to two times a week = 0.2/day; three to six times a week = 0.6/day; more than once per day = 1/day.

Assessment of the dietary inflammatory potential

The dietary inflammatory potential was assessed by the empirical dietary inflammatory pattern (EDIP) score. The development of the EDIP score was based on the previous studies (5, 14). It is based on circulating concentrations of 3 systemic inflammatory biomarkers, including interleukin-6, C-reactive protein (CRP), and tumor necrosis factor-α receptor 2 (TNFα-R2), to assess the overall subversive potential of diets. In brief, plasma levels of interleukin 6, TNFα-R2, and CRP were regressed on 39 pre-defined food groups by using reduced-rank regressions and stepwise linear regressions, selecting 18 food groups most predictive of these biomarkers. The EDIP was calculated as the weighted sum of these 18 food groups with weights (i.e., the contributions of each food to the overall score) equal to the coefficients from the stepwise regression. So, the food group with negative values suggests that these are anti-inflammatory foods. In this study, pizza was omitted because of the traditional Chinese-feeding habits. Therefore, dietary intakes of 17 food groups were used to calculate the EDIP score, including refined grains, processed meat, red meat, organ meat, other fish, other vegetables, high-energy beverage, low-energy beverages, tomatoes, organ meat, green leafy vegetables, fruit juice, beer wine, tea, coffee, snacks, and dark yellow vegetable. The EDIP calculation, including the average daily intake of each food group, was first divided by a specific group of servings (13) to determine its information; these values were then multiplied by its particular inflammatory coefficient (15) and compared to add, the final value is adjusted by dividing by 1,000 (**Supplementary Table 1**). The EDIP score was represented as pro-inflammatory diets with a higher score and anti-inflammatory diets with a lower score.

Assessment of gestational cardiovascular risk

Gestational CVR was evaluated using the CVR score at 24 to 28 gestational weeks. The CVR score model can be an effective and straightforward tool for the cardiovascular disease forecasting and warning. The CVR score model was based on the five "clinical" CVR metrics (body mass index [BMI], BP, total cholesterol [TC] level, smoking status, and blood glucose level. Each CVR metric was classified as ideal (0 points), intermediate (1 point), or poor (2 points). Increased CVR was defined as more than 0 points. The detailed classification criteria are as follows: BMI (kg/m^2): ideal: ≤28.4, intermediate: 28.5–32.9, poor: ≥33. BP (mmHg): ideal: systolic blood pressure (SBP) <120 and diastolic blood pressure (DBP) <80, intermediate: SBP 120-139, or DBP 80–89, poor: SBP ≥140 or DBP ≥90. TC (mg/dL): ideal: <260, intermediate: 260–299, poor: ≥300. Blood glucose (mg/dl): ideal: non-gestational diabetes mellitus (GDM), poor: GDM: fasting ≥92, 1-h oral glucose tolerance test (OGTT) ≥180, 2-h OGTT ≥ 153 (3). The results of four "clinical" CVR metrics (BMI, BP, TC, and blood glucose) were obtained from the hospitals at 24 to 28 gestational weeks. Thresholds of gestational BMI at 24 to 28 gestational weeks were defined by The HAPO cohort (16) accounting for gestational weight gain and pre-pregnancy BMI. Therefore, thresholds are appropriately higher than those for the non-pregnant adults. The smoking status was obtained from the questionnaires.

In addition, we also conducted two new CVR score models for sensitivity analysis. One was based on the five "clinical" CVR metrics (BMI, BP, triglyceride [TG] level, smoking status, and blood glucose level) and the other was based on the other metrics (pre-pregnancy BMI, BP, TC level, smoking status, and blood glucose level). The detailed classification criteria of TG and pre-pregnancy BMI are as follows: TG (mg/dl): ideal: <220, intermediate: 220–299, poor: ≥300. Pre-pregnancy BMI (kg/m^2): ideal: ≤24.9, intermediate: 25–29.9, poor: ≥30 (17). The correlation coefficient among CVR score models were shown in the **Supplementary Table 2**.

Laboratory analyses

The venous blood was collected from pregnant women at 16–23 gestational weeks. The blood samples were used to measure hypersensitive C-reactive protein, and 25(OH)D concentrations. The blood samples were centrifuged at 4°C and 2,056 × g for 5 min, quickly refrigerated at 4°C within 1 h, and then transferred to −80°C refrigerators within 8 h for long-term storage. The 25(OH)D and hs-CRP concentrations were determined using commercial chemiluminescence immunoassay kits (DiaSorin Stillwater, MN, United States) and turbidimetric inhibition immunoassay kits (Leadman biochemistry, Beijing, China) by well-trained researchers. The

coefficient of variation (CV) between and within classes is less than 10%. Serum 25(OH)D concentrations were divided into two groups (<50 nmol/L and ≥50 nmol/L) (18).

Statistical analysis

Demographic characteristics and clinic data were compared between different EDIP scores groups using the ANOVA for continuous variables and Chi-square analysis for the categorical variables. Variables were represented by the percentage or means (standard deviations, SDs).

Based on the restricted cubic spline hazard model, the association between EDIP score and increased CVR was shown. Based on the cubic curve-fitting models, the association of EDIP score with CVR score and hs-CRP or between hs-CRP and CVR score was shown.

Stratified analyses were used to estimate the association of EDIP scores with increased CVR according to serum 25(OH)D concentrations. We also conducted *post hoc* sensitivity analyses for the association between EDIP and gestational CVR based on the other CVR score models (included TG instead of TC or included pre-pregnancy instead of BMI at 24 to 28 gestational weeks). The analyses were performed using SPSS version 26.0 software (IBM Corp, Armonk, NY, United States). With a two-tailed *P*-value of <0.05 is considered significant.

Results

Attrition analyses showed that the distributions of the sociodemographic characteristics, perinatal health status, and pregnancy lifestyle factors in nonparticipants did not differ from the participants. At the baseline, the average participant age was 29.1 (SD = 4.2) years, and the mean pre-pregnancy BMI was 21.5 (SD = 2.9) kg/m^2. The proportion of women with increased CVR was 54.3%. **Table 1** shows the baseline characteristics of the study participants according to the EDIP score. The education and sedentary time differed across 3 groups divided by the EDIP score ($P < 0.05$).

In a cubic curve-fitting model fully adjusted for potential confounders, CVR score increased significantly with the increasing EDIP score in low and intermediate EDIP groups (**Figure 1A**). There was a significant positive association between the EDIP score and increased CVR (**Figure 1B**) or hs-CRP levels (**Figure 1C**). In addition, there was a significant positive association between hs-CRP levels and the CVR score (**Figure 1D**).

In multiple linear regression models, the β (95% CI) of CVR score and hs-CRP levels were −0.114 (−0.217, −0.011) and −0.280 (−0.495, −0.065) in the highest quartile compared with the lowest quartile of 25(OH)D (*P* for trend of <0.05), respectively (**Figure 2**).

Table 2 compares the difference in 25(OH)D and hs-CRP levels and found that 25(OH)D concentrations were the lowest and hs-CRP levels were the highest in the high EDIP group (*P* for trend <0.05). The further stratified analysis found that 25(OH)D concentrations were the lowest and hs-CRP levels were the highest in the high EDIP group when 25(OH)D concentrations were <50 nmol/L. Increased CVR connected with high EDIP scores was observed only in women with 25(OH)D concentrations <50 nmol/L (RR = 1.85; 95% CI: 1.35∼2.54) (**Table 2**). Sensitivity analyses produced similar results (**Supplementary Table 3**).

The role of hs-CRP and 25(OH)D in the association between EDIP score and CVR score were evaluated by the structural equation models. As shown in **Figure 3**, mediation analysis revealed that the proportion of association between the EDIP score and CVR score mediated by 25(OH)D was 28.7%. The proportion of the association between 25(OH)D concentrations and the CVR score mediated by hs-CRP was 21.9%. In addition, the proportion of the association between the EDIP score and the CVR score mediated by hs-CRP was 13.6%.

Discussion

To our knowledge, this is the first study to evaluate the role of vitamin D status in the association between dietary inflammatory potential and gestational CVH. We observed that the EDIP score was positively associated with the CVR score in a dose-response fashion, independent of traditional risk factors. We also found that the association was significantly modified by serum 25(OH)D concentrations, while the association between high EDIP scores and increased CVR appeared to be attenuated among the participants with sufficient serum 25(OH)D concentrations.

The relationship between dietary inflammation and CVH has been gradually recognized in recent years. An anti-inflammatory diet, rich in fiber, antioxidants, and long-chain-3 polyunsaturated fatty acids, may positively impact CVH (19, 20). A randomized controlled trial also showed that adherence to a Mediterranean diet (MeDiet) could reduce the incidence of cardiovascular disease by 30%, compared with the control diet (21). Conversely, a prospective study did not support the protective effect of high-dietary antioxidant levels on CVH (22). Another multicenter randomized study trial also found that the MeDiet in pregnancy did not reduce CVD risk (23). Conclusions based on these investigations were inconsistent, the leading cause may be the dietary indices such as Alternate Mediterranean Diet, Dietary Approaches to Stop Hypertension (DASH), and Alternative Healthy Eating Index, which generally assessed the whole dietary quality rather

TABLE 1 Characteristics of the study population.

Characteristics	All (n = 3713)	EDIP score[1]			P value[2]
		Low (n = 929)	Intermediate (n = 1858)	High (n = 926)	
CVR					
Ideal BMI, n (%)	3412(91.9)	858(92.4)	1720(92.6)	834(90.2)	0.062
Ideal blood pressure, n (%)	3042(81.9)	781(84.1)	1521 (81.9)	740(79.9)	0.067
Ideal total cholesterol level, n (%)	2886(77.7)	730(78.6)	1449(77.9)	707(76.3)	0.478
Ideal glucose level, n (%)	3080(83.0)	787(84.7)	1544(83.1)	749(81.0)	0.088
Non-smokers, n (%)	3686(99.3)	922(99.2)	1847(99.4)	917(99.0)	0.536
CVR score, M ± SD	1.0 ± 1.1	0.9 ± 1.1	1.0 ± 1.1	1.0 ± 1.2	0.033
25(OH)D concentration, M ± SD, nmol/L	38.54 ± 16.31	39.45 ± 17.00	38.42 ± 16.41	37.90 ± 15.36	0.110
Hs-CRP concentration, M ± SD, mg/L	3.23 ± 2.34	3.02 ± 2.15	3.24 ± 2.36	3.44 ± 2.49	0.001
Sociodemographic characteristics					
Age, M ± SD, years	29.1 ± 4.2	29.1 ± 4.4	29.2 ± 4.1	29.1 ± 4.3	0.748
Urban residence, n (%)	3442(92.7)	861(92.7)	1727(92.9)	854(92.2)	0.786
Bachelor's degree and above, n (%)	928(25.0)	192(20.7)	501(27.0)	235(25.4)	0.001
Household income >8000 yuan/m, n (%)	873(23.5)	231(24.9)	431(23.2)	211(22.8)	0.517
Perinatal health status					
Pre-pregnancy BMI, M ± SD, kg/m^2	21.5 ± 2.9	21.5 ± 3.0	21.4 ± 2.8	21.6 ± 3.0	0.092
Primipara, n (%)	1380(37.2)	337(36.3)	685(36.9)	358(38.7)	0.529
Excessive GWG[3], n (%)	1865(50.2)	481(51.8)	913(49.1)	471(50.9)	0.383
Family history of diabetes[4], n (%)	336(9.1)	75(8.1)	180(9.7)	81(8.8)	0.350
Family history of hypertension[4], n (%)	1241(33.4)	305(32.8)	628(33.8)	308(33.3)	0.871
Pregnancy lifestyle factors, n (%)					
Physical activity (≥ 3 days/week)	1683(45.3)	395(42.5)	861(46.3)	427(46.1)	0.138
Outdoor time (≥ 60 min/day)[5]	1155(31.1)	286(30.8)	588(31.6)	281(30.3)	0.760
Sedentary time (≥ 4 h/day)	2507(67.5)	597(64.3)	1254(67.5)	656(70.8)	0.010
Vitamin D supplementation≥ 3 days/week	2011(54.2)	501(53.9)	1018(54.8)	492(53.1)	0.701

BMI, Body Mass Index; EDIP, Empirical dietary inflammation pattern. GDM, gestational diabetes mellitus; GWG, gestational weight gain, CVR, cardiovascular risk.
[1]EDIP: Low, (< P_{25}); Intermediate, (P_{25}-P_{75}); High, (≥ P_{75}).
[2]P-value was from the analysis of variance (for means) or chi-square (for proportions).
[3]Excessive GWG: GWG > P_{50}
[4]A family history of hypertension or diabetes was defined as either parent having hypertension or diabetes.
[5]Outdoor time means time spent outdoors in the daytime.

than dietary inflammatory potential. Notably, we used a diet index EDIP, which strengthened the evaluation of dietary inflammatory potential. EDIP shares only a few foods with other dietary indexes (thus explaining its moderate correlation) and emphasizes unique inflammation-related foods. In addition, our findings are consistent with the Nurses' Health Study (NHS) cohort that a higher dietary inflammatory potential, as revealed by the higher EDIP scores, was associated with an increased risk of CVD (5). Our findings also found that a systemic inflammatory marker (hs-CRP) played a mediating role in such association. In a study of diet interventions to prevent CVD, high-inflammation marker levels moderated the effects of the DASH (7). Accordingly, decreased inflammation may lead to consequently improved gestational CVH. Thus, interventions to reduce inflammation and thus protect CVH applicable to the pregnant women are needed, and vitamin D supplements are an attractive target.

Vitamin D is an everyday nutritional supplement during pregnancy and may exhibit several anti-inflammatory effects (24, 25). In this study, our findings showed adequate 25(OH)D concentrations were associated with lower hs-CRP levels. In addition, we found that adequate 25(OH)D concentrations may modify gestational CVH by influencing hs-CRP levels. We also found that the inverse association between 25(OH)D concentrations and the CVR score could be mediated by hs-CRP levels. Recent evidence shows that serum 25(OH)D concentrations are negatively correlated with systemic inflammatory markers such as CRP (25). The previous study also found that high-serum 25(OH)D concentrations may reduce CVD risk through modulation of inflammatory processes, which was similar to our study (26). In the present study, we also found that there was no significant association between high EDIP and increased CVR among participants with sufficient

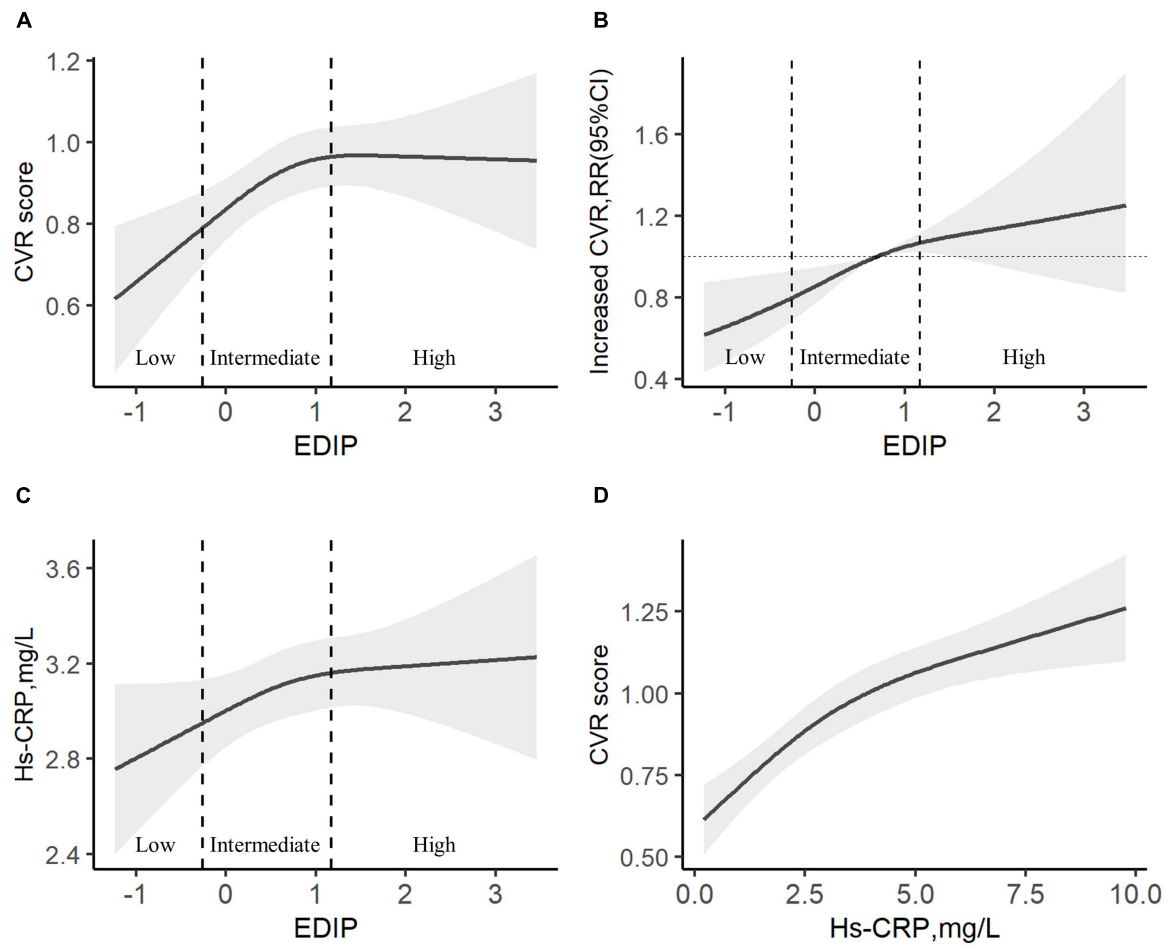

FIGURE 1
The association among EDIP scores, CVH, and hs-CRP. **(A)** A cubic curve-fitting model of the curvilinear association between EDIP and CVR score. **(B)** A restricted cubic spline hazard of the curvilinear association between EDIP and Increased CVR. **(C)** A cubic curve-fitting model of the curvilinear association between EDIP and hs-CRP. **(D)** A cubic curve-fitting model of the curvilinear association between hs-CRP and CVR score. All models were adjusted for age, residence, education, income, pre-pregnancy BMI, parity, gestational weight gain, family history of diabetes and hypertension, physical activity, outdoor time, sedentary time, and vitamin D supplementation frequency. Increased CVR, CVR score >0 points. CVR, cardiovascular risk, EDIP, Empirical dietary inflammation pattern.

serum 25(OH)D concentrations. On the one hand, this modification may be through the anti-inflammatory effects of vitamin D. On the other hand, 25(OH)D concentrations may also directly mediate the association between high-EDIP scores and increased gestational CVR, which is also confirmed by our results.

In addition, this study found that vitamin D deficiency is common during pregnancy, and 78.4% of women had 25(OH)D concentrations <50 nmol/L. However, the majority of developing nations, including China, do not offer vitamin D deficiency screening during pregnancy, and most pregnant women also do not follow the recommendation regarding vitamin D supplementation. Our study suggests that vitamin D supplementation during pregnancy may have potential benefits on the gestational CVH.

The mechanisms underlying the vitamin D effect on the association between inflammatory dietary patterns and CVD risk remain unclear. Several potential mechanisms may explain the relations. For example, $25(OH)D_3$ as an anti-inflammatory compound can inhibit nuclear factor kappa beta (NF–κB) activation through increased vitamin D receptor (VDR) expression. So, vitamin D deficiency can induce inflammation of the blood vessel walls and promote atherosclerosis by enhancing NF–κB activation (27). In addition, vitamin D deficiency can increase inflammation, enhance inflammatory cytokines expression, and inhibit VDR expression and activity. This may lead to enhanced signaling of downstream inflammatory signaling cascades resulting in various CVD (28).A previous study suggests that high 25(OH)D concentrations may reduce CVD risk by modulating immune function and inflammatory

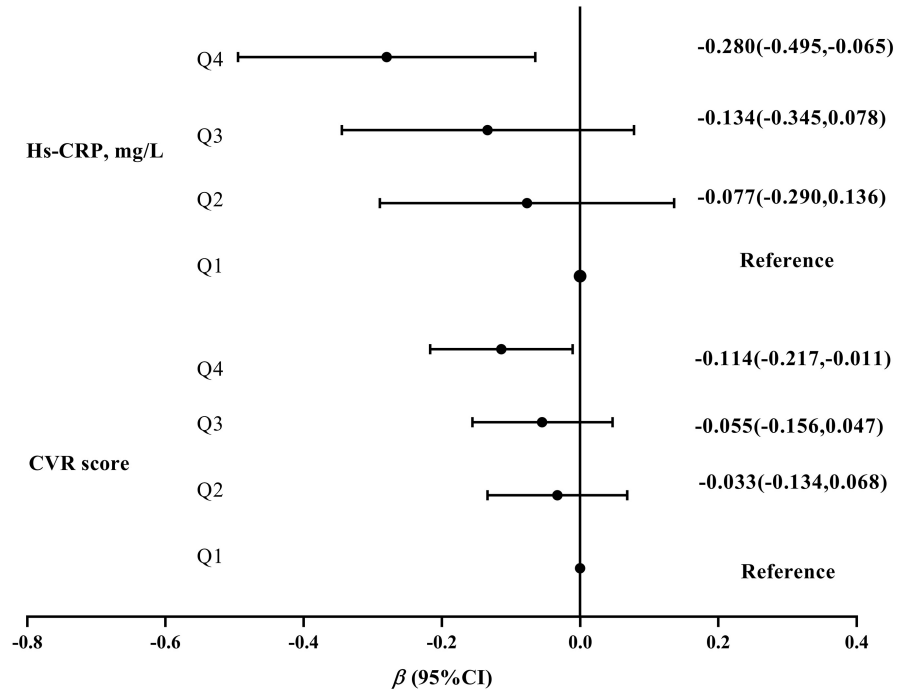

FIGURE 2
The association of 25(OH)D concentrations with CVR scores and hs-CRP levels. 25(OH)D concentrations were divided into four groups by the quartile (Q1\Q2\Q3\Q4). All models were based on the multiple linear regression and adjusted for age, residence, education, income, pre-pregnancy BMI, parity, gestational weight gain, family history of diabetes and hypertension, physical activity, outdoor time, sedentary time, and vitamin D supplementation frequency. CVR, cardiovascular risk.

TABLE 2 The association between EDIP and increased CVR stratified by vitamin D status.[1]

Groups	n (%)	25(OH)D[2], nmol/L M ± SD	Hs-CRP[3], mg/L M ± SD	Increased CVR[4]	
				n (%)	*RR*[1] (95% *CI*)
Overall					
Low EDIP	929 (25.0)	39.45 ± 17.00	3.02 ± 2.15	465 (50.1)	1.00
Intermediate EDIP	1858(50.0)	38.42 ± 16.41	3.24 ± 2.36	1025(55.2)	**1.23 (1.05, 1.44)**
High EDIP	926 (25.0)	37.90 ± 15.36	3.44 ± 2.49	525 (56.7)	**1.31 (1.09, 1.58)**
25(OH)D ≥ 50 nmol/L					
Low EDIP	200 (25.0)	64.78 ± 15.16	2.64 ± 1.96	87 (43.5)	1.00
Intermediate EDIP	400(49.9)	62.44 ± 13.59	3.16 ± 2.28	203 (50.7)	1.33 (0.94, 1.88)
High EDIP	201 (25.1)	60.64 ± 8.44	3.31 ± 2.21	101 (50.2)	1.33 (0.90, 1.98)
25(OH)D < 50 nmol/L					
Low EDIP	733 (25.2)	32.50 ± 9.19	3.12 ± 2.20	380 (51.8)	**1.41 (1.03, 1.94)**
Intermediate EDIP	1458(50.1)	31.83 ± 9.53	3.27 ± 2.38	822 (56.4)	**1.70 (1.26, 2.29)**
High EDIP	721 (24.7)	31.57 ± 9.72	3.49 ± 2.51	422 (58.5)	**1.85 (1.35, 2.54)**

CVR, cardiovascular risk, EDIP, Empirical dietary inflammation pattern.
[1]These models were adjusted for age, residence, education, income, pre-pregnancy BMI, parity, gestational weight gain, family history of diabetes and hypertension, physical activity, outdoor time, sedentary time, and vitamin D supplementation frequency.
[2]P for trend across 3 groups (EDIP) was 0.041, P for trend across 6 groups [EDIP * 25(OH)D concentrations] was <0.001.
[3]P for trend across 3 groups (EDIP) was < 0.001, P for trend across 6 groups [EDIP * 25(OH)D concentrations] was <0.001.
Increased CVR, (cardiovascular risk score >0 point).

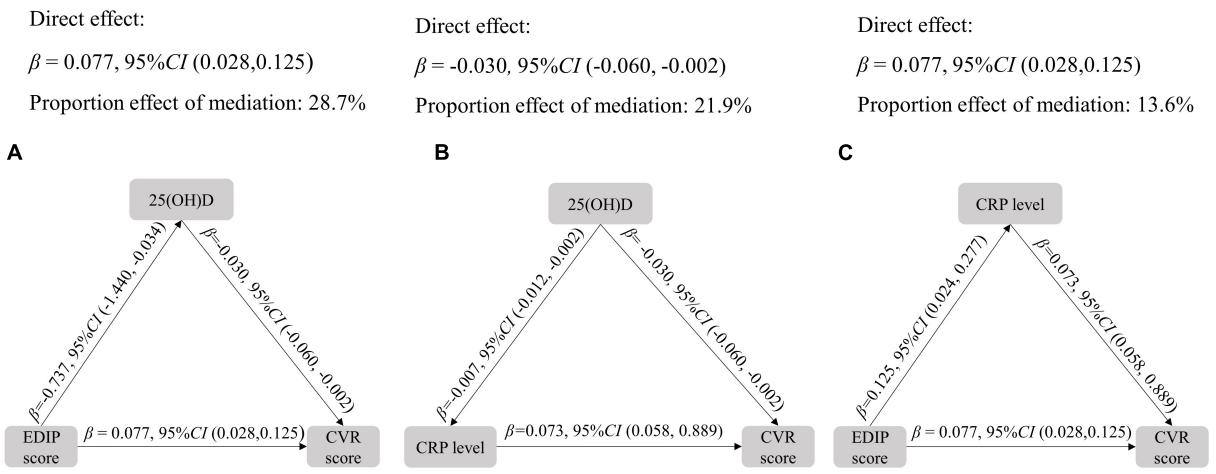

FIGURE 3
Mediation analysis among EDIP score, CVR score, and hs-CRP. All models adjusted for age, residence, education, income, pre-pregnancy BMI, parity, gestational weight gain, family history of diabetes and hypertension, physical activity, outdoor time, sedentary time, and vitamin D supplementation frequency. CVR, cardiovascular risk, EDIP, Empirical dietary inflammation pattern.

processes (26). In addition, laboratory and animal study data indicated that 25(OH)D inhibits vascular smooth muscle cell proliferation and vascular calcification, controls volume homeostasis and blood pressure *via* regulation of the renin-angiotensin-aldosterone system and exerts anti-inflammatory effects (29–31). These findings indicate that vitamin D regulates blood pressure by acting on the endothelial and smooth muscle cells and thus plays an essential anti-inflammatory role in CVH. These anti-inflammatory effects of vitamin D may modify the association between a high EDIP score and increased gestational CVR.

This is the first study examining the moderating effect of vitamin D on the relationship between a pro-inflammatory diet and gestational CVH. In addition, the EDIP, a validated, empirically developed, food-based tool, was used to strongly assess the dietary inflammation potential. Although a single inflammation biomarker was measured in this study, the significant correlation between hs-CRP levels and the EDIP score supports the validity of EDIP evaluation. To sum up, we adjusted for broad sociodemographic characteristics; the sample size was relatively large and reduced residual confounding.

Study limitations

First, our research cannot draw causality, and it takes longer to verify cardiovascular events. In addition, our findings need to be confirmed in the randomized clinical trials. Second, self-reported FFQ diet data of the pregnant women may have measurement errors, which usually weakens the actual connection. Third, we did not consider the effect of the participants' salt intake on CVH. Furthermore, the data on CVR was not collected at the baseline, and we are not able to assess the CVR status at the baseline of the included individuals. Hence, the temporality and the causality between diet and CVR are compromised in this study. In addition, only hs-CRP was measured for inflammation biomarkers. Hence, inflammatory status of individuals cannot be evaluated comprehensively. Moreover, a caution should be taken when interpreting this study results, since previous studies (32–37) have shown that components of the CVR score in this study (BMI, blood pressure, total cholesterol, glucose levels, and smoking status, and also triglyceride levels) are in inverse association with vitamin D levels, and therefore, a higher CVR score should be automatically associated with lower vitamin D levels in our study. To sum, our research was conducted only on the pregnant Chinese women. Therefore, our research results may need to be extended to other populations for verification.

Conclusion

In sum, our research indicates that the regulation of chronic inflammation may be a potential mechanism linking dietary patterns and gestational CVR, and vitamin D may have anti-inflammatory effects to reduce cardiovascular risk caused by the pro-inflammatory foods. Reducing the inflammation potential of the diet among pregnant women may provide an effective strategy for promoting CVH. Future studies need to verify the potential protective effects of vitamin D supplementation during pregnancy on cardiovascular health induced by a pro-inflammatory diet.

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 human participants were reviewed and approved by the Ethics Committee of Anhui Medical University (No. 20180092). The patients/participants provided their written informed consent to participate in this study.

Author contributions

W-JY performed the experiments and was responsible for the collection and compilation of data, analysis of data, and writing the manuscript. L-JY contributed to the compilation of the data and helped wrote the manuscript. LW, F-CD, QL, and LZ were responsible for collecting clinical data and contributing to clinical assessments. R-XT and X-MJ designed the study and assisted with the data collection. PZ was the guarantor of this work designed and supervised the study and revised the manuscript. All authors read and approved the final manuscript.

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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/fnut.2022.952652/full#supplementary-material

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Association between serum 25-hydroxyvitamin d and myeloperoxidase: A cross-sectional study of a general population in China

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Background: Several studies have found a strong association between cardiovascular diseases and myeloperoxidase (MPO) as a marker of oxidative stress. Although the anti-inflammatory effects of vitamin D in adults have been validated, evidence about the relationship between MPO and 25(OH)D is lacking. This study aimed to investigate the relationship between MPO and 25(OH)D in the general Chinese population.

Methods: From November 2018 to August 2019, a total of 6414 subjects were enrolled in a tertiary referral hospital in China, which included 3,122 women and 3,292 men. The dependent and independent variables were MPO and 25(OH)D, respectively. The confounders included age, sex, body mass index, waist-hip ratio, smoking status, alcohol drinking status, calcium, and parathyroid hormone concentration.

Results: In the fully adjusted model, we found that MPO decreased by 0.12 (95% CI −0.16, −0.08), ng/mL for each unit (1 nmol/L) increase in 25(OH)D. When 25(OH) D was divided into quartiles, compared with Q1 (< 41.4 nmol/L), the adjusted beta coefficients (β) of MPO in Q2–Q4 were −2.29 (95% CI, −4.31 to −0.27), −4.76 (95% CI, −6.83 to −2.69), and −6.07 (95% CI, −8.23 to −3.92), respectively (*P* for the trend < 0.0001). When 25(OH) D was divided according to clinical severity, compared with the severely deficient (< 30 nmol/L) group, the adjusted beta coefficients (β) of MPO in the insufficient (≥ 30, < 50 nmol/L) and sufficient groups (≥ 50 nmol/L) were −2.59 (95% CI, −5.87 to 0.69) and −5.87 (95% CI, −9.17 to −2.57), respectively (*P* for the trend < 0.0001).

Conclusion: After adjusting for age, sex, BMI, waist-hip ratio, smoking status, alcohol status, calcium, and PTH, circulating 25(OH)D was negatively associated with MPO.

KEYWORDS

myeloperoxidase, association, cross-sectional study, cardiovascular diseases, 25-dihydroxyvitamin D

Introduction

The mortality rate of cardiovascular disease remains high in the world. In 2020, Cardiovascular Diseases (CVDs) were responsible for approximately 19.1 million deaths (1). In China, CVDs became the leading cause of all-age disability-adjusted life-years in 2017 (2). Given the heavy burden of CVDs, exploring risk factors and understanding the underlying mechanisms involved in CVDs are crucial to their prevention and potential therapeutic targets.

Previous studies have found that inflammation and oxidative stress contribute to major components of cardiovascular risk (3, 4). Myeloperoxidase (MPO), a member of the heme peroxidases superfamily that stored in leukocytes and macrophages, is a 146 kDa glycosylated homodimer protein that consists of two monomers (5). Upon leukocyte activation, the main function of MPO released from the cells is to produce reactive oxidants, such as hypochlorous acid and hypothiocyanous acid, to exert innate immune, and antibacterial effects (5, 6). Although MPO has an important physiological function, its maladjustment involved in oxidative stress and inflammation can cause severe tissue damage in several diseases (7). Several studies have found a strong association between MPO and CVDs; that is, elevated MPO is a biomarker for the occurrence and progression of atherosclerosis, coronary heart disease, hypertension, heart failure, and stroke (8–11). Additionally, given the inspiring results against CVDs through inhibition of MPO in animal models (12), we may anticipate new therapeutic targets for the prevention and treatment of CVDs. Although knockout/knockdown of MPO gene expression and the use of some pharmacological treatment targeting MPO can exert cardiovascular protection *in vitro* and *in vivo*, more strategies for regulating MPO are needed, especially in the general population (12).

Vitamin D is a fat-soluble steroid hormone that can be synthesized by sunlight or supplemented through diet. In clinical practice, 25(OH)D (circulating 25-dihydroxyvitamin D) is commonly used to assess vitamin D status in an individual (13). There is substantial evidence that a low 25(OH)D status significantly increases the risk of cardiovascular disease (14, 15). Moreover, 25(OH)D significantly correlates negatively with some systemic inflammatory parameters (for example, neutrophil-lymphocyte ratio, monocyte-lymphocyte ratio and C-reactive protein) in patients undergoing coronary angiography (16). More importantly, vitamin D acts as an antioxidant against oxidative stress and inflammation (17). Although some studies on the cardiovascular benefits of vitamin D are controversial, evidence suggests that vitamin D supplementation improves left ventricular function and inflammation in patients with heart failure (18, 19).

As far as we know, although a previous study found a link between vitamin D status and MPO in 66 obese children (20), there has been no exploration of the relationship between MPO and 25(OH)D despite vitamin D's anti-inflammatory benefits in adults. There is a need to further elucidate the relationship between vitamin D status and MPO in the general population. Therefore, we conducted a cross-sectional study in general populations (6,414 subjects) without cardiovascular events in China, assuming a negative relationship between 25(OH)D and MPO levels.

Materials and methods

Study design and participants

This is a population-based cross-sectional study among subjects undergoing routine health examinations at our hospital's health management center from November 2018 to August 2019. The West China Hospital, a tertiary hospital with three subcenters in Sichuan, provides over 60,000 routine physical examinations annually (21, 22). Participants were enrolled into the study if they fulfilled the following inclusion criteria: voluntarily go to the Health Management Center of West China Hospital of Sichuan University for health examination between November 2018 and August 2019; aged over 18 years; willing to sign an informed consent form. The following criteria demanded their exclusion: (1) incapability to provide informed consent; (2) missing circulating 25(OH)D and MPO measurements; (3) history of hypertension, diabetes, hyperuricemia, chronic heart disease, and hyperlipidemia; and (4) history of use of blood sugar, blood pressure and serum lipid lowering agents. Finally, of the 19,920 consecutive individuals, we excluded those without 25(OH)D and MPO measurements ($n = 12,676$) and those with high-risk factor for cardiovascular diseases ($n = 830$). The study enrolled 6,414 participants in total (**Figure 1**). The study protocol was approved by the local Ethics Committee of West China Hospital, Sichuan University (No. 2018-303) and informed consent was obtained from all participants. The study was conducted according to the guidelines of the Declaration of Helsinki.

Demographic data

Demographic and lifestyle information on participants was collected by a trained interviewer through standard procedures as previously reported (21). Specifically, never smoking was defined as self-reported smoking fewer than 100 cigarettes, current smoking was defined as smoking in the past 30 days, and former smoking was defined as not smoking in the past 30 days. Current drinking was defined as one alcohol-unit at least once a week for more than 6 months, former drinking was defined as abstinence from drinking for at least half a year, and never drinking was defined as drinking monthly or less. Self-reported family history of cardiovascular disease was defined as

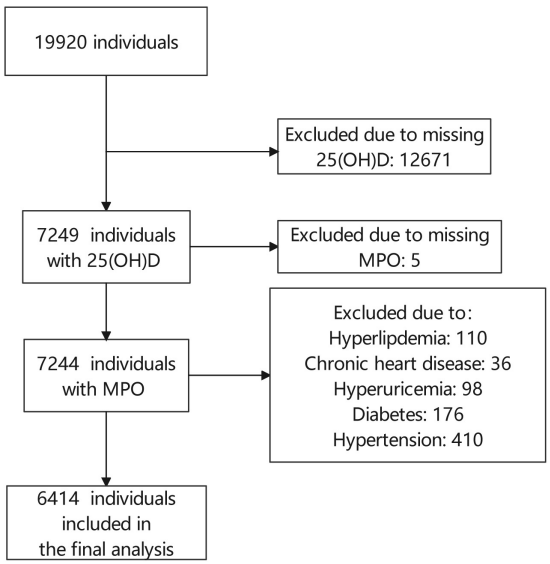

FIGURE 1
Flow chart of study participants.

a coronary heart disease, stroke, or peripheral vascular disease in a first-degree relative. Sex, age, smoking, drinking status, self-reported family history of cardiovascular disease, and medical history can be obtained from medical records.

Anthropometric measurements

Height, weight, waist circumference, and hip circumference were obtained by trained nurses. We measured waist and hip circumference with a flexible and inextensible tape to the nearest 0.1 cm by trained nurses. The waist circumference was measured midway between the anterior superior iliac crest and the 12th costal margin and the hip circumference was measured horizontal around the maximum gluteal circumference in a standing position (23). The body mass index (BMI) was obtained by formula BMI = (weight in kilograms)/(height (in meters)^2). In accordance with the World Health Organization (WHO), central obesity for the Asian population is defined as a waist-to-hip ratio of more than 0.9 for men and more than 0.8 for women (24).

Determination of laboratory measurements

After overnight fasting, blood samples were collected into 10 mL EDTA tubes from cubital vein by trained nurses (21). All blood samples were analyzed in strict accordance with standard laboratory test methods in the clinical laboratory of the West China Hospital certified by the China National Accreditation Board.

Serum parathyroid hormone (PTH) concentrations were measured using electrochemiluminescence immunoassays (Cobas®8000-e602 modular analyzer, Roche Diagnostics Ltd., Rotkreuz, Switzerland). Serum calcium concentrations were measured on the Cobas 8000-c701 clinical chemistry analyzer. Serum CRP concentrations were measured on a IMMAGE800 analyzer (Beckman Coulter, Inc., United States).

To measure serum 25(OH)D, an enzyme-linked immunosorbent assay (ELISA) was used (Immunodiagnostic Systems, IDS Ltd., London, United Kingdom) as per the manufacturer's instructions (25). Using a commercial enzyme-linked immunosorbent assay kit (EACHY, Suzhou, China), myeloperoxidase concentrations were determined in plasma samples using standard methods (**Supplementary Methods**).

Statistical analysis

Normality of continuous variables was checked by Kolmogorov–Smirnov (KS) test and normal Q-Q plots. For normally distributed continuous variables, the mean ± *SD* is shown; the median and interquartile range (IQR) for non-normally distributed continuous variables are shown. When analyzing normally distributed continuous variables, one-way analysis of variance (ANOVA) with appropriate parametric representation was used. Categorical variables expressed as percentages were compared using the chi-square test. When analyzing non-normally distributed data, Wilcoxon signed ranking was used. As long as variables were categories, McNemar and Yates's correction tests were performed.

Using an unadjusted and a multivariate-adjusted linear and logistic model, regression coefficient and corresponding 95% confidence intervals (CI) were reported by using unadjusted (crude model), minimally adjusted (adjusted model I), and fully adjusted analysis (adjusted model II) according to STROBE guidelines (26). Specifically, the unadjusted model did not correct for any variables. Model I correct for age (years) and sex. In Model II, age, sex, BMI (kg/m^2), waist-hip ratio, smoking status, alcohol status, calcium (mmol/L) and PTH (pg/dL) were controlled. To better understand the relationship between MPO and vitamin D, 25(OH)D concentrations were categorized into a categorical variable by quartile and the predefined categories as follows: sufficient (≥ 50 nmol/L), insufficient (30–50 nmol/L), and severely deficient (<30 nmol/L) (27). Furthermore, the non-linear association between 25(OH)D and MPO was explored using a generalized additive model (GAM) model and smooth curve fitting. A sensitivity analysis was conducted by subgroup and interaction analysis to explore the effects of possible modifiers on the 25(OH)D-MPO relationship. When exploring elevated MPO and vitamin D deficiency and insufficiency, we assessed unmeasured confounding by

calculating E value (28). The E-value quantifies the required magnitude of an unmeasured confounder that could negate the observed association between MPO and vitamin D deficiency and insufficiency.

Multiple imputation was implemented by chained equations (MICE) to generate five datasets with complete data for missing covariates. Using the standard multiple imputation Rubin's rules, multivariable and GAM analyses were performed on the combined imputed datasets.

Two-tailed *P*-value < 0.05 was considered statistically significant unless otherwise stated. Statistical analysis was performed using R version 4.0.[1]

Results

A total of 6,414 subjects were included in the cross-sectional study, which included 3,122 women and 3,292 men. The characteristics of the study participants were grouped into four quantiles, Q1–Q4, depending on the levels of 25(OH)D, as described in Table 1. Between all quintiles of 25(OH)D groups, significant differences were observed in age, sex, BMI, waist-hip ratio, smoking status, alcohol status, calcium, PTH, and MPO. Higher serum 25(OH) D levels were more common in subjects who were older, male, current smokers and drinkers, and had higher serum calcium, lower PTH, and MPO levels. The characteristics of those individuals excluded due to exclusion criteria in the final analysis did not differ substantially from those included (Supplementary Table 1).

As vitamin D deficiency improved (from severely deficient to insufficient and sufficient 25(OH)D groups), MPO showed a decreasing trend in both men and women (*P* for trend < 0.0001) (Figure 2). The non-linear dose–response curve conducted by GAM demonstrated that the association between 25(OH)D and MPO was linear after adjusting for the confounding variables (Figure 3). Then, the association between 25(OH)D and MPO was observed by univariate and multivariate models, as reported in Table 2. In the crude model, we found that MPO decreased by 0.11 ng/mL for each unit (1 nmol/L) increase in 25(OH)D; the same trend was seen in Model I and Model II after adjusting for other confounding variables. Based on statistical and clinical practice, we then transformed the 25(OH)D level into categorical variables for multivariable analysis as stated in the Methods. There was a strong negative correlation between serum 25(OH) D levels and MPO after controlling for age, sex,

1 http://www.r-project.org/

TABLE 1 Characteristics of the study participants according to serum 25(OH)D concentrations.

		25(OH)D, nmol/L				
		Q1	Q2	Q3	Q4	
	Total	(< 41.4)	(41.41 < 52.0)	(52.0 < 64.6)	(≥ 64.6)	*P*-value
No. of participants	6,414	1,652	1,610	1,583	1,569	
Age (years)	46.45 ± 10.55	44.12 ± 10.46	45.25 ± 10.18	47.05 ± 10.45	49.54 ± 10.31	<0.001
Sex						<0.001
Women	3,122 (48.67%)	1,081 (65.44%)	816 (50.68%)	675 (42.64%)	550 (35.05%)	
Men	3,292 (51.33%)	571 (34.56%)	794 (49.32%)	908 (57.36%)	1,019 (64.95%)	
BMI, kg/m^2	23.56 ± 3.23	23.29 ± 3.46	23.70 ± 3.30	23.77 ± 3.11	23.47 ± 2.99	<0.001
Waist-hip ratio	0.85 ± 0.07	0.83 ± 0.08	0.85 ± 0.08	0.86 ± 0.07	0.86 ± 0.07	<0.001
Smoking status, *N* (%)						<0.001
Never	4,613 (71.92%)	1,296 (78.45%)	1,177 (73.11%)	1,096 (69.24%)	1,044 (66.54%)	
Former	279 (4.35%)	40 (2.42%)	48 (2.98%)	76 (4.80%)	115 (7.33%)	
Current	1,522 (23.73%)	316 (19.13%)	385 (23.91%)	411 (25.96%)	410 (26.13%)	
Alcohol status, *N* (%)						<0.001
Never	3,567 (55.61%)	1,092 (66.10%)	908 (56.40%)	803 (50.73%)	764 (48.69%)	
Former	52 (0.81%)	7 (0.42%)	14 (0.87%)	12 (0.76%)	19 (1.21%)	
Current	2,795 (43.58%)	553 (33.47%)	688 (42.73%)	768 (48.52%)	786 (50.10%)	
Family history of cardiovascular disease, *N* (%)	285 (4.44%)	77 (4.66%)	63 (3.91%)	71 (4.49%)	74 (4.72%)	0.675
Calcium (mmol/L)	2.33 ± 0.09	2.31 ± 0.09	2.32 ± 0.08	2.33 ± 0.09	2.34 ± 0.08	<0.001
Parathyroid hormone (pg/dL)	6.28 ± 2.07	6.88 ± 2.41	6.40 ± 2.01	6.05 ± 1.84	5.75 ± 1.77	<0.001
CRP (mg/L)	1.91 (1.36–2.89)	1.86 (1.29–2.95)	1.94 (1.38–2.93)	1.91 (1.40–2.82)	1.92 (1.38–2.85)	0.197
25(OH)D, nmol/L	54.42 ± 18.34	34.16 ± 5.66	47.01 ± 3.06	58.35 ± 3.59	79.38 ± 13.82	<0.001
MPO, ng/ml	25.68 (19.06–35.31)	27.37 (21.44–37.21)	26.35 (19.43–36.78)	24.82 (17.48–34.34)	23.77 (17.30–32.76)	<0.001

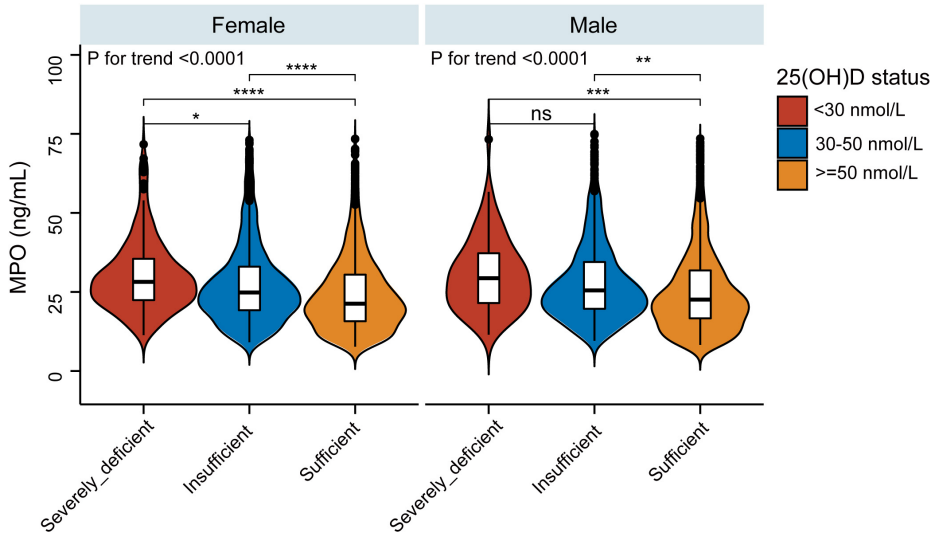

FIGURE 2
Violin and boxplot representing relative MPO levels between the sufficient, insufficient, and severely deficient 25(OH)D groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

BMI, waist-hip ratio, smoking status, alcohol status, calcium and PTH. When 25(OH) D was divided into quartiles, compared with Q1 (< 41.4 nmol/L), the adjusted beta coefficients (β) of MPO in Q2–Q4 were −2.29 (95% CI, −4.31 to −0.27), −4.76 (95% CI, −6.83 to −2.69), and −6.07 (95% CI, −8.23 to −3.92), respectively, with P for the trend < 0.0001. When 25(OH) D was divided according to clinical severity, compared with the severely deficient (<30 nmol/L) group, the adjusted beta coefficients (β) of MPO in the insufficient (≥ 30, < 50 nmol/L) and sufficient groups (≥ 50 nmol/L) were −2.59 (95% CI, −5.87 to 0.69) and −5.87 (95% CI, −9.17 to −2.57), respectively, with P for the trend < 0.0001.

Further assessment of possible moderating factors on the association between 25(OH)D and MPO was achieved through subgroup and interaction analyses. None of the variables, including age (< 60 vs. ≥ 60 years; P for interaction = 0.8706), sex (P for interaction = 0.2848), smoking status (Past/Current vs. Never; P for interaction = 0.331), drinking status (Past/Current vs. Never; P for interaction = 0.4406), BMI (< 24 vs. ≥ 24 kg/m^2; P for interaction = 0.2997), or central obesity (yes vs. no; P for interaction = 0.6745), significantly modified the 25(OH)D-MPO relationship (Table 3 and Figure 4).

To exclude the potential biased effect of missing data, we further performed sensitivity analysis using multiple imputation. As shown in Supplementary Table 2, no significant difference was observed between the created complete data and preimputation data. The relationship between MPO and 25(OH)D was still linear in the pro-imputation data (Supplementary Figure 1). After combining the pro-imputation, we still found a significant negative trend between 25(OH)D and MPO (Supplementary Table 3). After excluding those with thyroid-related diseases (n = 31), family history of cardiovascular disease (n = 285) and the those with lowest 1% or 2.5% of 25(OH)D levels respectively, the association between 25(OH)D and MPO did not change (Supplementary Tables 4, 5, 7). When MPO was divided into quartiles, Supplementary Table 6 showed that compared with Q1 (<18.35 ng/ml), the adjusted odds ratio (OR) for vitamin D deficiency and insufficiency (25(OH)D < 50 nmol/L) in Q2-Q4 were 1.48 (95% CI, 1.26–1.73), 1.78 (95% CI, 1.52–2.09), and 1.77 (95% CI, 1.51–2.08), respectively, with P for the trend < 0.0001. To assess unmeasured confounding by calculating E value, we found that confounders having a relative risk association = 2.94 with both elevated MPO and vitamin D deficiency and insufficiency to deviate our conclusions (Supplementary Figure 2).

Discussion

Our aim was to investigate whether circulating 25(OH)D was independently associated with MPO. Since the increase in MPO can promote the occurrence and development of cardiovascular diseases and vitamin D is a well-documented protective factor of cardiovascular risk, it is very important to explore the relationship between the two indicators. To our knowledge, the present study is the first population-based report of an independent relationship between 25(OH)D and MPO after adjustment for confounders in Chinese adults.

Numerous studies have linked vitamin D deficiency to metabolic disorders, such as increased levels of inflammation, oxidative stress, reactive oxygen species (ROS) production,

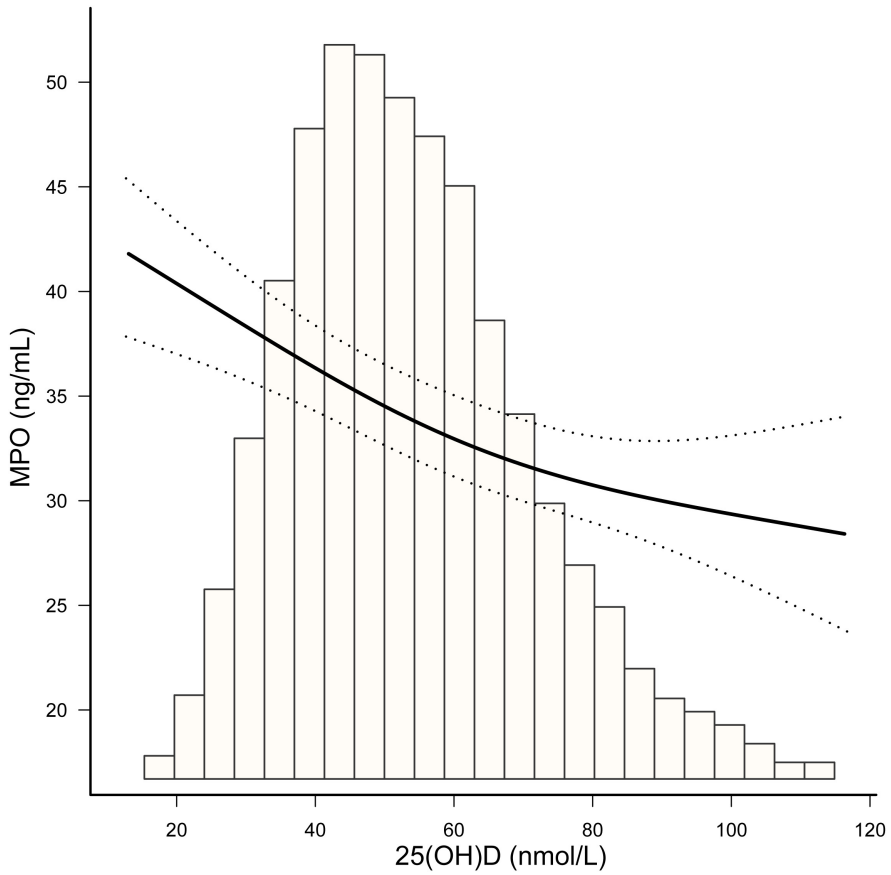

FIGURE 3
The smooth curve fitting presented linear associations between serum 25(OH)D concentrations and MPO among participants. Adjustment for: age (years), sex, BMI, waist-hip ratio, smoking status, alcohol status, calcium (mmol/L) and parathyroid hormone (pg/dL).

TABLE 2 Effect of 25(OH)D concentrations on MPO.

Variables	*N*	Crude model β (95% CI) *P*-value	Adjusted model I* Adjusted β (95% CI) *P*-value	Adjusted model II** Adjusted β (95% CI) *P*-value
25(OH)D, nmol/L				
Continuous	6,414	−0.11 (−0.14, −0.07) < 0.0001	−0.10 (−0.14, −0.06) < 0.0001	−0.12 (−0.16, −0.08) < 0.0001
Categories				
< 30	370	Ref	Ref	Ref
≥ 30, < 50	2,533	−1.66 (−4.60, 1.27) 0.2669	−1.63 (−4.57, 1.32) 0.2791	−2.59 (−5.87, 0.69) 0.1215
≥ 50	3,511	−4.80 (−7.68, −1.91) 0.0011	−4.56 (−7.49, −1.62) 0.0023	−5.87 (−9.17, −2.57) 0.0005
P for trend		<0.0001	<0.0001	<0.0001
Quartiles				
Q1 (< 41.4)	1,652	Ref	Ref	Ref
Q2 (≥ 41.41, < 52.0)	1,610	−1.67 (−3.51, 0.18) 0.0771	−1.75 (−3.61, 0.10) 0.0641	−2.29 (−4.31, −0.27) 0.0262
Q3 (≥ 52.0, < 64.6)	1,583	−4.01 (−5.86, −2.15) < 0.0001	−4.01 (−5.90, −2.13) < 0.0001	−4.76 (−6.83, −2.69) < 0.0001
Q4 (≥ 64.6)	1,569	−5.28 (−7.14, −3.42) < 0.0001	−5.11 (−7.04, −3.17) < 0.0001	−6.07 (−8.23, −3.92) < 0.0001
P for trend		<0.0001	<0.0001	<0.0001

Adjust I model adjust for: Age (years), sex.
Adjust II model adjust for: Age (years), sex, BMI, Waist-hip ratio, Smoking status, Alcohol status, Calcium (mmol/L) and Parathyroid hormone (pg/dL). The β-values indicate unstandardized regression coefficients. 95% CI indicates 95% confidence interval.

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TABLE 3 Effect size of 25(OH)D on MPO in prespecified and exploratory subgroups.

	No of participants	Median (Q1–Q3)	Adjusted β (95% CI)	P for interaction
Sex				0.2848
Male	3,042	25.2 (18.4–35.3)	−0.10 (−0.15, −0.04)	
Female	2,848	25.5 (18.6–35.4)	−0.14 (−0.20, −0.08)	
Age				0.8706
< 60	5,248	25.4 (18.5–35.7)	−0.14 (−0.18, −0.09)	
≥ 60	642	25.0 (18.4–33.9)	−0.12 (−0.23, −0.01)	
Smoke				0.331
Never	4,198	25.4 (18.6–35.2)	−0.11 (−0.16, −0.06)	
Past/Current	1,692	25.1 (18.4–35.7)	−0.15 (−0.22, −0.08)	
Alcohol				0.4406
Never	3,248	25.6 (18.9–35.7)	−0.10 (−0.16, −0.05)	
Past/Current	2,642	25.0 (18.1–35.0)	−0.14 (−0.20, −0.08)	
BMI				0.2997
< 24	3,367	25.2 (18.4–35.3)	−0.10 (−0.16, −0.05)	
≥ 24	2,523	25.5 (18.7–35.3)	−0.15 (−0.21, −0.08)	
Central obesity				0.6745
No	3,169	25.5 (18.5–36.6)	−0.11 (−0.17, −0.06)	
Yes	2,721	25.1 (18.5–34.3)	−0.13 (−0.19, −0.07)	

The β-values indicate unstandardized regression coefficients. 95% CI indicates 95% confidence interval.

insulin resistance, endothelial dysfunction, and disruption of blood sugar and lipids, which contribute to an increased risk of cardiovascular disease (29–31). Recently, Cătoi et al. (32) conducted a cross-sectional study to investigate the association between 25(OH)D and markers of oxidative stress in 47 patients with type 2 diabetes. They found that compared to those with serum 25(OH)D greater than 20 ng/mL, interleukin 6, total oxidant status and oxidative stress index were significantly higher in the 25(OH)D less than 10 ng/mL and 25(OH)D between 10 and 20 ng/ml group. Codoñer-Franch et al. (20) designed a pioneering observational study to explore the relationship between vitamin D status and MPO in 66 obese Caucasian children from 7 to 14 years old. Consistent with our results, they also found that the MPO in the 25(OH)D insufficient group (<20 ng/m) was higher than that in the 25(OH)D (≥ 20 ng/mL) sufficient group among children. However, the above studies also discussed the relationship between 25(OH)D and other oxidative stress and inflammation indexes. In addition, the small sample size prevented them from formulating a confound-correcting model to satisfy these oxidative stress indicators and 25(OH)D. Specifically, they only adjusted for age, sex and sexual maturity status, but other factors that affect vitamin D metabolism, such as BMI, PTH, lipid levels and calcium levels, should also be considered. However, due to different research focuses, Codoñer-Franch and associates did not discuss the above issues in depth.

Several factors influence the relationship between oxidative stress and vitamin D. According to STROBE guidelines, subgroup and interaction analyses are helpful to reveal the underlying truths (26). Smoking and alcohol consumption have previously been reported to cause oxidative stress through the production of ROS and reactive nitrogen species (RNS) (33, 34). In addition, obesity is associated not only with systemic inflammation and oxidative stress but also with vitamin D deficiency (35–37). A previous study reported significant interactions between acute symptoms and oxidative stress status in patients undergoing coronary angiography (16). In our study, however, we did not find that smoking, alcohol consumption, or obesity affected the MPO-25(OH)D correlation. The reason may be that the population we included was the general population without hypertension, diabetes and hyperlipidemia, in which the risk factors for cardiovascular disease could not be synergistic with oxidative stress.

MPO, a pro-oxidant enzyme, may be a promising target for cardiovascular diseases. Previous studies have shown that MPO-catalyzed nitrification and chlorination can target apolipoprotein A-I (APOA-I), a major component of high-density lipoprotein (HDL) (38). Oxidation of HDL and APOA-I inhibits cholesterol efflux in macrophages, as well as proliferation and migration of vascular smooth muscle cells, resulting in atherosclerotic plaque instability (39, 40). In addition, the activation of MPO can lead to the production of metalloproteinases and the transformation of fibroblasts into myofibroblasts, ultimately contributing to the synthesis, and degradation of collagen (41). It is well established that vitamin D acts as an antioxidant by removing excess ROS. In our study, we found significant inverse correlation between 25(OH)D and MPO, which partly reflects the importance of

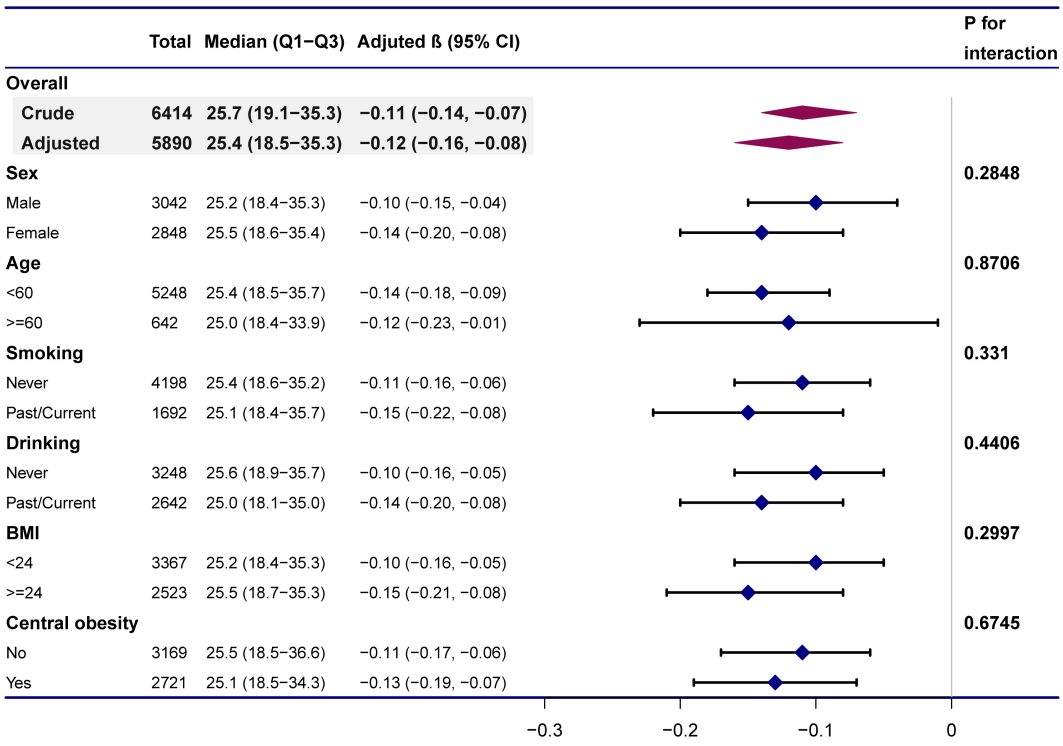

FIGURE 4
Subgroup analyses of the effect of 25(OH)D concentrations on MPO. Adjustment for: age (years), sex, BMI, waist-hip ratio, smoking status, alcohol status, calcium (mmol/L), and parathyroid hormone (pg/dL) except the stratification variable in each case.

improvement for vitamin D status. The possible mechanism is that 1,25-dihydroxy vitamin D3 binds to vitamin D receptors to form nuclear receptor-ligand complexes, functioning as transcription factors to regulate the expression of more than 200 genes, including many oxidative stress-related enzymes (42, 43). Consistently, previous studies have also found that vitamin D supplementation significantly increases catalase activity, a hemoprotein evaluation of antioxidant status (44).

Continuing controversy surrounds the range and recommendations for blood concentrations of 25(OH)D. The guidelines from The Institute of Medicine of The National Academies recommend that serum 25(OH)D concentrations greater than 50 nmol/L meet the needs of most people; when the concentration exceeds 125 nmol/L, attention should be given (45). In contrast, according to the Endocrine Society Practice Guidelines, the adequate reference range of sufficient 25(OH)D is 50–250 nmol/L (46). Indeed, in most studies, the upper limit of the safe range for vitamin D is usually defined as 250 nmol/L (47). In our study, 25(OH)D above 125 nmol/L was detected in only 20 individuals. However, 45.2% of the included individuals had 25(OH)D below 50 nmol/L, which shows the importance of vitamin D supplementation in the general population.

Previous meta-analyses have demonstrated that long-term vitamin D supplementation can reduce levels of inflammation and oxidative stress, thereby contributing to cardiovascular protection (48, 49). In a mouse model of periprosthetic joint infection, Hegde et al. (50) found that higher MPO was exhibited in mice fed a vitamin D-deficient diet than in those fed an adequate vitamin D diet. Interestingly, when mice fed a vitamin D-deficient diet were given an adequate vitamin D diet again after surgery, their MPO levels recovered to levels comparable to those of mice on a normal vitamin D diet. Another *in vitro* study indicated that adding vitamin D to the human neutrophil culture medium reduced MPO release by 22% (51). Another interesting observation on the efficacy of vitamin D supplementation on MPO in patients with type 2 diabetes was conducted by Cojic et al. (44). They found that compared with metformin group, vitamin D supplementation plus metformin group resulted in a significant decrease in MPO and a significant increase in antioxidative enzyme activity after 6 months. Therefore, there is reason to believe vitamin D supplementation can lower MPO levels and thus play a cardiovascular protective role in general population. Nevertheless, the mechanisms by which vitamin D affects MPO require further research.

To enhance the level of vitamin D in serum, several exogenous supplementation regimens and lifestyles are recommended for general population. First, since over 90% production of the vitamin D derived from sunshine, it requires short, regular exposures to sunlight without sunscreen (52,

53). However, individuals with darker skin, older age and who live in areas with less sun exposure should consider taking exogenous vitamin D supplementation. Due to the limited supply of vitamin D-fortified foods in most parts of the world, cholecalciferol of 1,000–2,000 IU per day or Ergocalciferol of 50,000 IU per month is recommended for general adults (54, 55).

The strengths of our research are mainly in the following aspects. First, large sample sizes and standardized survey and measurement procedures improve the accuracy and validity of the results. Second, since we focused on exploring the relationship between MPO and vitamin D status, we adopted a more rational strategy for dealing with confounding factors. Third, the GAM model was applied to explore the non-linear relationship in our study. Fourth, subgroup and interaction analyses were performed to further analyze potential factors influencing the relationship between MPO and vitamin D status. Finally, we use multiple imputation to address the impact of missing variables on the results. The above mentioned provides a basis for understanding the mechanism by which vitamin D exerts its protection against oxidative stress from another perspective and for the design of future intervention trials to prevent cardiovascular diseases.

However, our study has some limitations. First, a causal relationship between MPO and vitamin D status cannot be established due to the nature of the cross-sectional study; further long-term follow-up and intervention studies will help provide evidence regarding the effect of vitamin D on MPO. Second, although we corrected for some major confounding factors, bias due to unmeasured confounders was not excluded. We used sensitivity analysis excluding those with thyroid-related diseases, family history of cardiovascular disease and the those with lowest 1% or 2.5% of 25(OH)D levels, respectively, and found that the results of sensitivity analysis did not change primary result (**Supplementary Tables 4, 5, 7**). Besides, in a sensitivity analysis exploring elevated MPO and vitamin D deficiency and insufficiency, we assessed unmeasured confounding by calculating E value. The results showed that it is unlikely that any unmeasured confounders could explain the association between elevated MPO and vitamin D deficiency/insufficiency (**Supplementary Table 6** and **Supplementary Figure 2**). Third, because we did not include individuals with hypertension, diabetes, hyperlipidemia and cardiovascular diseases, our conclusions cannot be extrapolated to the above-mentioned population. Fourth, in addition to MPO, future studies need to investigate the relationship between vitamin D status and other inflammatory and oxidative stress markers in a large sample of the general population to provide a complete and an overall association and correlation picture. Fifth, the information concerning regular administration of vitamin D supplementation by the participants was not obtained. However, a previous cross-sectional epidemiological survey showed low consumption of vitamin D-related foods (only 18.44% of women consumed more than 250 g of milk) and about 5% women reported taking a vitamin supplement in Sichuan, which reflects low intake of vitamin D supplements (55). In addition, if someone took vitamin D supplementation, there would be fewer cases of vitamin D deficiency/insufficiency, making it more difficult to identify the association between increased MPO and vitamin D deficiency/insufficiency, thus biasing the results toward the null. Of note, the potential resulting from vitamin D supplementation would bias toward to the null and thus result in an underestimation of the association between MPO and vitamin D deficiency/insufficiency.

Conclusion

Our data have demonstrated that after adjusting for age, sex, BMI, waist-hip ratio, smoking status, alcohol status, calcium and PTH, circulating 25(OH)D is negatively associated with MPO. Further prospective studies and clinical trials are needed to confirm the potential causal relationships.

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 human participants were reviewed and approved by the West China Sichuan University Hospital Research Committee (No. 2018-303). The patients/participants provided their written informed consent to participate in this study.

Author contributions

JZ collected the data and conducted statistical analyses as well as wrote the manuscript. RL, TB, and WJ involved in fruitful discussions. JZ and YH edited the manuscript. YH manages the entire project. A consensus was reached among all authors regarding the final draft of the manuscript.

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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/fnut.2022.948691/full#supplementary-material

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Association of hypercalciuria with vitamin D supplementation in patients undergoing ketogenic dietary therapy

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Background: Ketogenic dietary therapy (KDT) is used as an effective treatment for epilepsy. However, KDT carries the risk of bone health deterioration; therefore, vitamin D supplementation is required. Vitamin D replacement therapy in KDT has not been established because it may be related to hypercalciuria/urolithiasis, which are common adverse effects of KDT. Hence, this study aimed to evaluate the dose-dependent association between vitamin D_3 and hypercalciuria/urolithiasis in patients undergoing KDT and dose optimization for renal complications.

Materials and methods: Overall, 140 patients with intractable childhood epilepsy started 3:1 KDT (lipid to non-lipid ratio) at the Severance Children's Hospital from January 2016 to December 2019. Regular visits were recommended after KDT initiation. Participants were assessed for height, weight, serum 25-hydroxyvitamin D (25-OH-D_3) level, parathyroid hormone level, and ratio of urinary excretion of calcium and creatinine (Uca/Ucr). Kidney sonography was conducted annually. Patients who already had urolithiasis and were taking hydrochlorothiazide before KDT, failed to maintain KDT for 3 months, did not visit the pediatric endocrine department regularly, did not take prescribed calcium and vitamin D3 properly, or needed hospitalization for $>$ 1°month because of serious medical illness were excluded. Data from patients who started diuretic agents, e.g., hydrochlorothiazide, were excluded from that point because the excretion of calcium in the urine may be altered in these patients.

Result: In total, 49 patients were included in this study. Uca/Ucr ratio significantly decreased with increasing levels of 25-OH-D_3 (p = 0.027). The odds ratio for hypercalciuria was 0.945 (95% confidence interval, 0.912–0.979; p = 0.002) per 1.0 ng/mL increment in 25-OH-D_3 level. Based on findings

of receiver operating characteristic curve analysis and Youden's J statistic, the cut-off 25-OH-D_3 level for preventing hypercalciuria was > 39.1 ng/mL at 6 months. Furthermore, the vitamin D_3 supplementation dose cut-off was > 49.5 IU/kg for hypercalciuria prevention.

Conclusion: An inverse relationship between Uca/Ucr ratio and 25-OH-D_3 level was noted, which means that vitamin D supplementation is helpful for preventing hypercalciuria related to KDT. We suggest that the recommended 25-OH-D_3 level is > 40 ng/mL for hypercalciuria prevention and that KDT for children with epilepsy can be optimized by vitamin D_3 supplementation at 50 IU/kg.

KEYWORDS

vitamin D, vitamin D deficiency, ketogenic diet, hypercalciuria, urolithiasis

Introduction

Since 1921, ketogenic dietary therapy (KDT) has been considered a well-known non-pharmacologic anti-convulsant treatment for both children and adults with multi-drug resistant epilepsy (1). KDT is based on the fact that lipophilic compounds known as ketone bodies, such as acetoacetate, acetone, and beta-hydroxybutyrate, can cross the blood–brain barrier and act as direct anticonvulsants (2). It is also known that intermediate chain triglycerides, such as decanoic acid, can directly inhibit α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and that KDT increases adenosine level and inhibits DNA methylation, which are the known key mechanisms of KDT for treating epilepsy (3). However, KDT without careful management may accompany various side effects such as gastrointestinal symptoms, hepatic dysfunction, dyslipidemia, growth retardation, urolithiasis, pancreatitis, and cardiac abnormalities, especially when it is used together with high-dose anti-epileptic drugs (AEDs) (1).

Bone health deterioration is one of the most common clinical issues in patients undergoing KDT. Patients with intractable epilepsy usually have prolonged exposure to high-dose AEDs and are at risk of vitamin D deficiency (4). Furthermore, KDT is a diet prone to being deficient in essential nutrients, including calcium, phosphorus, magnesium, vitamin K, and vitamin D. Additionally, ketone bodies produced by KDT also induce acidification, which converts active vitamin D into an inactive form (1). Therefore, KDT can lead to calcium and vitamin D deficiencies and worsen bone vulnerability, causing osteoporosis; hence, calcium and vitamin D supplementation are needed. Given the possible risks and complications of KDT, patients on KDT are advised to visit an endocrinologist regularly to ensure adequate calcium and vitamin D supplementation.

Meanwhile, hypercalciuria and urolithiasis are also common complications of KDT, which are caused by increased bone demineralization due to acidosis. Acidosis induces hypocitraturia, which in turn increases free calcium, and increases the less soluble uric acid levels (5). As calcium is the most frequent component of urinary calculi and the major constituent of approximately 75% of the stones, an increase in the urinary excretion of calcium is the most common risk factor for urolithiasis (6, 7). Low urine volume and hypercalciuria increase Randall's plaque formation, which is specific to calcium oxalate stone formation (8, 9). Consequently, urolithiasis may develop in children undergoing KDT (10, 11). Although hypercalciuria and urolithiasis are not absolute contraindications for KDT or indications for cessation of KDT (1), they may cause poor compliance and treatment failure, as the presence of kidney stones may lead to severe abdominal pain or dysuria, which may reduce the patient's quality of life.

Calcium intake and vitamin D supplementation have been thought to be risk factors for hypercalciuria because they can increase intestinal absorption of calcium and cause hypercalcemia, even without KDT (12). However, as mentioned above, calcium and vitamin D supplementation are required for patients undergoing KDT. In addition, as vitamin D levels in patients with urolithiasis are lower than those in the normal population, vitamin D deficiency is thought to increase the occurrence of kidney stone formation (13). Therefore, whether calcium intake and vitamin D supplementation worsen hypercalciuria and promote kidney stone formation as well as the optimal level of 25-hydroxyvitamin D (25-OH-D_3) and appropriate use of vitamin D supplementation in patients with KDT remain controversial.

Considering the above-mentioned arguments, in this study, we aimed to establish the correlation between vitamin D_3 dose and the occurrence of hypercalciuria/urolithiasis in patients undergoing KDT. In addition, we evaluated the optimal dose to minimize renal complications.

Materials and methods

Participants

Overall, 140 patients with intractable childhood epilepsy were started on KDT at a 3:1 lipid to non-lipid ratio in the pediatric neurology department of Severance Children's Hospital, Seoul, South Korea from January 2016 to December 2019. All patients were referred to the pediatric endocrine department for monitoring the endocrinologic adverse effects of KDT, such as growth retardation, dyslipidemia, multivitamin deficiency, and hypothyroidism. Among these patients, those who maintained KDT for > 3 months and regularly visited the pediatric endocrine department for monitoring were included in this study. Regular outpatient visits were recommended at 1, 3, 6, and 12 months, unless there were medical issues. Patients who already had urolithiasis and received hydrochlorothiazide before KDT, failed to maintain KDT for 3 months, did not take the prescribed calcium and vitamin D_3 supplementation properly, or did not undergo endocrinological follow-up studies including biochemical laboratory tests, were excluded. Additionally, we excluded patients requiring prolonged hospitalization due to serious illness after KDT initiation, since the changes in their systemic condition and changes in treatment such as AEDs and KDT could significantly alter their clinical aspects. Further, data from patients who started diuretic agents, such as hydrochlorothiazide, were eliminated from that point in time because the excretion of calcium in the urine may be altered in these patients. Finally, 49 patients were included in this study. Among those who continued KDT, 6-month data were obtained from 38 patients and 1-year data were obtained from 22 patients.

The type and dosage of AEDs were not changed significantly during KDT, and the formulations were modified to contain as little carbohydrates as possible. Patients on KDT were supplemented with multivitamins, L-carnitine, calcium, and vitamin D_3 (cholecalciferol). We used a combination tablet containing 100 mg of calcium and 1,000 IU of vitamin D_3 per pill for calcium and vitamin D supplementation. Daily doses of calcium and vitamin D_3 were approximated based on weight: 0.5 tablets (elemental 50 mg of calcium and 500 IU of vitamin D_3) for a bodyweight up to 10 kg, 1.0 tablet (100 mg of elemental calcium and 1,000 IU of vitamin D_3) for a bodyweight of 10–20 kg, 1.5 tablets (150 mg of elemental calcium and 1,500 IU of vitamin D_3) for a bodyweight of 20–40 kg, and 2 tablets (200 mg of elemental calcium and 2,000 IU of vitamin D_3) for a bodyweight of > 40 kg. If the serum 25-OH-D_3 level was < 20 ng/mL or > 50 ng/mL, the doses of the calcium and vitamin D_3 complex were increased or decreased by 0.25 tablets (25 mg of elemental calcium and 250 IU of vitamin D_3).

The Institutional Review Board of Severance Hospital Clinical Trial Center (subject no. 4-2020-0549) approved this study. Because this was a retrospective study that analyzed only the results obtained during the general course of medical treatment, the need for informed consent was waived. We complied with the Declaration of Helsinki to protect participant rights and personal information.

Data collection

Patients' heights and weights were measured at each visit. Further, levels of serum calcium (mg/dL), phosphorus (mg/dL), alkaline phosphatase (mg/dL), 25-OH-D_3 (ng/mL), parathyroid hormone (PTH, pg/mL), urinary excretion of calcium (Uca, mg/dL), and creatinine (Ucr, mg/dL) in spot urine samples, and urine osmolality were assessed at each visit to identify whether hypercalciuria or any side effects of vitamin D_3 supplementation had occurred. Serum calcium, phosphate, and alkaline phosphatase levels were measured using Hitachi chemistry autoanalyzer 7600-110 (Hitachi Ltd., Tokyo, Japan) at the central laboratory of Severance Hospital. Serum 25-OH-D_3 level was determined using a radioimmunoassay (DiaSorin, Inc., Stillwater, MN, United States; intraassay CV < 4.1%, inter-assay CV < 7.0%). The serum PTH concentration was measured at our hospital using a second-generation PTH assay (Elecsys PTH; Roche Diagnostics, Mannheim, Germany) on the Cobas e801 immunoassay analyzer (Roche Diagnostics). Serum osteocalcin level was measured using an electrochemiluminescence immunoassay (Elecsys N-MID Osteocalcin; Roche Diagnostics; intraassay CV < 1.8%, inter-assay CV < 3.3%), and the urinary N-terminal telopeptide was calculated by competitive immunoassay (Vitros^{TM} NTx reagent pack; Ortho-clinical Diagnostics, Inc., Rochester, NY, United States). Urinary calcium excretion (calculated as the ratio of urine calcium level to creatinine level) of the patients was measured by random urine tests using an automated urine chemistry analyzer AU5800 (Beckman Coulter, Fullerton, CA, United States) and LIAISON system (DiaSorin, Saluggia, Italy) at every admission before the initiation of each cycle. The criteria for hypercalciuria were applied differently by age according to the Uca/Ucr ratio (≥ 0.86 for up to 7 months old, ≥ 0.60 for 7–18 months old, ≥ 0.42 for 19 months to 6 years old, and ≥ 0.20 for > 6 years old) (14). The status of the serum 25-OH-D_3 level was classified as deficient (< 20 ng/mL), insufficient (20–30 ng/mL), and sufficient (> 30 ng/mL) (15).

Statistical analysis

All statistical analyses were performed using SAS version 9.4 (SAS Inc., Cary, NC, United States) and R package version 3.6.3.[1] Continuous variables are presented as means and standard deviation (SD). Linear regression analysis was performed to investigate the factors that affect the Uca/Ucr ratio at each

1 http://www.R-project.org

visit. To consider the longitudinal structure of data (i.e., four assessment time points), linear mixed-effect models were used to investigate the factors affecting the Uca/Ucr ratio. An autoregressive model (1) correlation structure was assumed among repeated measures of all longitudinal analyses. A logistic regression model was used to investigate factors affecting the occurrence of hypercalciuria at each visit. Moreover, since the occurrence of hypercalciuria was measured at each visit, data were analyzed using the generalized estimating equation model to identify factors affecting the occurrence of hypercalciuria throughout the study period. The results are indicated by odds ratios and confidence intervals. The analysis results of the entire period were adjusted by time effect. To obtain the cut-off value, receiver operating characteristic curve analysis and Youden's J statistic were used. Statistical significance was set at $p < 0.05$.

Results

Of the 49 participants enrolled in this study, 31 (63.3%) were boys. All participants were diagnosed with intractable epilepsy, and their seizures began at a mean age of 2.1 ± 2.4 years (range, 0.0–11.7 years). At KDT initiation, the mean age was 4.3 ± 3.2 years (range, 0.3–14.1 years), which was an average of 2.2 ± 2.1 years (0.1–9.5 years) after the onset of seizures (**Table 1**). The participants received an average of 2.4 ± 1.1 AEDs (range, 0–5 AEDs). The average level of 25-OH-D_3 was 22.4 ng/mL, with 21 (42.9%) patients being deficient in 25-OH-D_3, whereas 19 (38.8%) had insufficient levels, and nine (18.4%) had sufficient levels before KDT initiation. One patient had hyperparathyroidism and 25-OH-D_3 deficiency, and among five patients with hypoparathyroidism, four had insufficient 25-OH-D_3 levels and one had deficient levels. Although 11 (22.4%) patients already met the definition of hypercalciuria before KDT initiation, they were enrolled in this study because none of them had taken hydrochlorothiazide in accordance with the judgment of the pediatric nephrologists. The patients took an average of 49.9 IU/kg (range, 19.6–102.6 IU/kg) vitamin D_3 supplementation (**Table 1**).

Three months after KDT initiation with an average vitamin D_3 supplementation of 50.8 IU/kg, only one patient had hypercalcemia (serum calcium, 11.4 mg/dL; normal range, 8.5–10.5 mg/dL). The patient was administered 55.6 IU/kg of vitamin D_3 and 100 mg of elemental calcium, and their 25-OH-D_3 level increased from 30.84 to 46.7 ng/mL and PTH level was low at 7.8°pg/mL. In addition, he had hypercalciuria, with a Uca/Ucr ratio of 2.56. Therefore, we reduced the supplemental doses of vitamin D_3 and calcium by half (vitamin D_3, 27.8 IU/kg; elemental calcium, 50 mg/kg). Although his 25-OH-D_3 level remained similar (43.4 ng/mL) during the follow-up observation, hypercalcemia, and hypercalciuria resolved (Uca/Ucr ratio = 0.19) without any medication. Hyperparathyroidism was not observed in any patient, whereas hypoparathyroidism was identified in 17 (34.7%) patients; however, there was no association between PTH level and Uca/Ucr ratio (**Table 2**). Hypercalciuria was observed in 27 (55.5%) patients; however, no factors affected Uca/Ucr ratio (**Table 2**). Moreover, the risk of hypercalciuria decreased as the dose of vitamin D_3 supplementation increased (odds ratio = 0.950; $p = 0.014$) (**Table 3**).

Six months after KDT initiation with an average vitamin D_3 supplementation dose of 44.5 IU/kg, calcium and phosphorus levels of all participants were within the normal range. There were 13 (34.0%) patients with hypoparathyroidism and none with hyperparathyroidism. Hypercalciuria was observed in 19 (50.0%) patients. Age at KDT initiation, height, weight, and 25-OH-D_3 levels were negatively associated with Uca/Ucr ratio (**Figure 1** and **Table 2**). Additionally, an increased dose of vitamin D_3 supplementation (odds ratio = 0.956; $p = 0.028$) and a sufficient 25-OH-D_3 level (odds ratio = 0.888; $p = 0.010$) decreased the risk of hypercalciuria (**Figure 2** and **Table 3**). The optimal level of 25-OH-D_3, which minimizes the occurrence of hypercalciuria with maximum sensitivity and specificity, was 39.14 ng/mL (**Figure 3A**), and the optimal dose of vitamin D_3 supplementation was 49.47 IU/kg (**Figure 3B**).

One year after KDT initiation with an average vitamin D_3 supplementation dose of 35.3 IU/kg, calcium and phosphorus levels of all participants were within the normal range, and six (27.3%) patients had hypoparathyroidism. Further, nine patients (40.9%) had hypercalciuria. The dose of vitamin D_3 supplementation and 25-OH-D_3 levels did not affect the Uca/Ucr ratio, whereas height and 25-OH-D_3 levels were negatively associated with Uca/Ucr ratio (**Table 2**). However, none of these factors increased the risk of hypercalciuria occurrence (**Table 3**).

In the analysis of the overall follow-up period in which the time variable was corrected through the linear mixed model, the increased dose of vitamin D_3 supplementation (odds ratio = 0.976; $p = 0.043$) and increased 25-OH-D_3 level (odds ratio = 0.945; $p = 0.002$) decreased the risk of hypercalciuria, consistent with the trend shown at 3 and 6 months.

Urolithiasis developed in three patients (6.1%): two boys and one girl. Patient 1 was a 3-month-old boy for whom vitamin D_3 (60.98 IU/kg) was prescribed and discontinued 6 months after KDT initiation because of kidney stone formation. Patient 2 was a 7-year-old girl for whom 45.66 IU/kg of vitamin D_3 was prescribed. She was diagnosed as having urolithiasis 6 months after KDT initiation; however, vitamin D_3 supplementation was continued. This was because (1) her 25-OH-D_3 level was only 21.39 ng/mL, which was only slightly higher than the lower recommended limit of vitamin D_3, and (2) up to 40–60% of kidney stones in children are reported to be non-calcium-based; considering the lower vitamin D levels, it was unlikely that her urolithiasis was calcium-based (14). Patient 3 was a 19-month-old boy who was diagnosed with urolithiasis 9 months after KDT initiation and vitamin

TABLE 1 Characteristics of children with intractable epilepsy at ketogenic dietary therapy initiation.

	Baseline (N = 49)	At 3 months (N = 47)	At 6 months (N = 38)	At 12 months (N = 21)
Sex M: F (n)	31:18	30:17	25:13	14:7
Age (years)	4.3 ± 3.2 (0.3–14.1)	4.6 ± 3.2 (0.6–14.4)	4.7 ± 3.3 (0.8–14.6)	4.4 ± 2.4 (1.3–10.4)
Current AED (n)	2.4 ± 1.1 (0.0–5.0)			
Age at first seizure (year)	2.1 ± 2.4 (0.0–11.7)	2.1 ± 2.4 (0.0–11.7)	2.1 ± 2.5 (0.0–11.7)	1.7 ± 2.0 (0.0–7.5)
Duration of seizure (year)	2.2 ± 2.1 (0.1–9.5)	2.2 ± 2.1 (0.1–9.5)	2.6 ± 2.1 (0.6–10.0)	2.7 ± 1.5 (1.1–7.5)
Height (cm)	100.9 ± 22.2 (64.0–170.1)	103.1 ± 21.9 (65.7–170.0)	105.8 ± 21.7 (71.0–171.0)	103.3 ± 16.8 (78.5–142.5)
Height SDS	−0.03 ± 1.15 (−3.15–1.98)	−0.32 ± 0.91 (−2.44–1.46)	−0.24 ± 0.96 (−1.98–1.66)	−0.42 ± 0.97 (−2.43–1.51)
Weight (kg)	17.5 ± 9.2 (7.1–51.0)	18.2 ± 9.4 (6.9–57.8)	18.9 ± 10.3 (8.7–61.2)	16.8 ± 5.8 (9.0–33.5)
Weight SDS	−0.09 ± 1.31 (−3.20–2.95)	−0.27 ± 1.31 (−3.15–3.47)	−0.36 ± 1.22 (−2.63–2.09)	−0.14 ± 1.02 (−1.90–2.32)
BMI	16.4 ± 2.3 (12.3–24.2)	16.4 ± 2.4 (12.3–22.9)	16.0 ± 2.0 (13.3–20.9)	16.3 ± 1.7 (12.6–20.0)
Serum variables				
Calcium (mg/dL)	9.7 ± 0.5 (8.9–10.5)	9.6 ± 0.6 (8.3–11.4)	9.6 ± 0.5 (8.8–10.6)	9.5 ± 0.5 (8.8–10.4)
Phosphorus (mg/dL)	5.3 ± 0.6 (4.1–6.8)	4.7 ± 0.5 (3.1–6.1)	4.8 ± 0.5 (3.8–6.0)	4.8 ± 0.6 (3.7–5.8)
ALP (mg/dL)	231.7 ± 89.2 (68.0–499.0)	186.6 ± 57.6 (66.0–321.0)	196.7 ± 80.6 (52.0–441.0)	205.2 ± 85.4 (97.0–433.0)
PTH (pg/mL)	26.4 ± 12.0 (8.0–75.7)	17.8 ± 7.0 (6.8–33.6)	20.1 ± 8.8 (6.1–42.2)	19.6 ± 5.3 (9.0–28.1)
25-OH-D_3 (ng/mL)	22.4 ± 9.0 (9.8–49.1)	35.5 ± 9.9 (10.1–58.8)	33.9 ± 9.9 (11.8–55.0)	29.9 ± 8.5 (12.3–48.8)
Deficiency, n (%)	21 (42.9%)	3 (6.1%)	2 (5.4%)	1 (4.5%)
Insufficiency, n (%)	19 (38.8%)	8 (16.3%)	12 (32.4%)	10 (45.5%)
Sufficiency, n (%)	9 (18.4%)	38 (77.6%)	23 (62.2%)	9 (50.0%)
Not checked			1	1
Urinary excretion				
Calcium	8.9 ± 9.2 (0.0–39.8)	31.1 ± 22.8 (2.2–95.6)	25.3 ± 21.1 (3.3–83.4)	26.3 ± 17.4 (0.5–59.5)
Creatinine	57.8 ± 38.3 (3.6–178.9)	72.0 ± 52.8 (3.6–264.5)	79.5 ± 69.6 (3.8–399.0)	83.5 ± 57.7 (12.2–244.0)
Uca/Ucr	0.26 ± 0.38 (0.00–1.63)	0.6 ± 0.6 (0.0–2.8)	0.5 ± 0.4 (0.0–2.1)	0.4 ± 0.3 (0.0–1.4)
Hypercalciuria (n, %)	11, 22.4%	27, 57.4%	19, 50.0%	9, 42.9%
Vitamin D_3 supplementation (IU/kg)		50.8 ± 18.3 (15.4–102.6)	44.5 ± 20.4 (0.0–81.3)	35.1 ± 17.4 (0.0–66.1)

25-OH-D_3, 25-hydroxyvitamin D; AED, anti-epileptic drugs; ALP, alkaline phosphatase; BMI, body mass index; BSA, body surface area; PTH, parathyroid hormone; SDS, standard deviation score; Uca/Ucr, urinary excretion of calcium/urinary excretion of creatinine ratio.

TABLE 2 Factors affecting the ratio of urinary excretion of calcium (Uca) to urinary excretion of creatinine ratio (Ucr) using longitudinal mixed-effect models.

	Month 3			Month 6			Month 12			Overall		
	β	SE	*P*-value	β	SE	*P*-value	β	SE	*P*-value	β	SE	*P*-value
Sex (ref = M)	0.088	0.188	0.640	0.159	0.100	0.118	0.291	0.140	0.045	−0.119	0.167	0.487
Age at KDT initiation	−0.038	0.028	0.180	−0.034	0.014	**0.024**	−0.036	0.021	0.086	−0.029	0.030	0.349
Seizure onset age	−0.036	0.037	0.333	−0.039	0.020	0.053	−0.035	0.028	0.219	−0.021	0.038	0.589
Height (cm)	−0.006	0.004	0.142	−0.006	0.002	**0.009**	−0.006	0.003	**0.049**	−0.006	0.004	0.159
Weight (kg)	−0.015	0.009	0.128	−0.012	0.005	**0.017**	−0.013	0.007	0.096	−0.014	0.012	0.254
Vitamin D (IU/kg)	−0.002	0.005	0.655	−0.002	0.002	0.352	−0.006	0.003	0.076	−0.002	0.004	0.622
25-OH-D_3 (ng/mL)	−0.007	0.010	0.486	−0.011	0.005	**0.027**	−0.015	0.007	**0.028**	−0.016	0.009	0.093
PTH	−0.020	0.011	0.0778	−0.004	0.007	0.5772	−0.010	0.009	0.2499	−0.008	0.005	0.1153
Osteocalcin	−0.0006	0.0015	0.7057	−0.0003	0.0005	0.6081	−0.0004	0.0003	0.2393	−0.0003	0.0004	0.4157
NTx	0.0003	0.0002	0.1123	0.00004	0.0002	0.8660	−0.0004	0.0003	0.2856	0.0003	0.0001	0.0080

25-OH-D_3, 25-hydroxyvitamin D; β, beta coefficient; KDT, ketogenic dietary therapy; SE, standard error; PTH, parathyroid hormone; NTx, N-telopeptide; Uca, urinary excretion of calcium; Ucr, urinary excretion of creatinine; ref, reference; M, male.
Statistically meaningful data are shown in bold.

TABLE 3 Odds ratio of factors related to the occurrence of hypercalciuria.

	Month 3		Month 6		Month 12		Overall	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Sex (ref = M)	1.008 (0.299, 3.403)	0.989	1.745 (0.437, 6.972)	0.431	0.429 (0.057, 3.223)	0.410	1.213 (0.515, 2.857)	0.658
Age at beginning KDT	1.047 (0.869, 1.261)	0.629	0.896 (0.727, 1.105)	0.303	1.187 (0.814, 1.733)	0.374	1.030 (0.869, 1.221)	0.733
Seizure onset age	1.072 (0.831, 1.382)	0.594	0.861 (0.645, 1.148)	0.307	1.318 (0.800, 2.172)	0.279	1.028 (0.814, 1.300)	0.814
Height (cm)	1.012 (0.985, 1.041)	0.379	0.980 (0.950, 1.011)	0.208	1.010 (0.962, 1.060)	0.691	1.004 (0.981, 1.027)	0.756
Weight (kg)	1.009 (0.947, 1.076)	0.779	0.953 (0.878, 1.034)	0.249	1.042 (0.898, 1.209)	0.587	0.998 (0.942, 1.058)	0.952
Vitamin D (IU/kg)	0.950 (0.911, 0.990)	**0.014**	0.956 (0.918, 0.995)	**0.028**	1.005 (0.956, 1.056)	0.857	0.976 (0.954, 0.999)	**0.043**
25-OH-D_3 (ng/mL)	0.963 (0.901, 1.029)	0.267	0.888 (0.812, 0.971)	**0.010**	0.955 (0.851, 1.072)	0.436	0.945 (0.912, 0.979)	**0.002**

25-OH-D_3, 25-hydroxyvitamin D; KDT, ketogenic dietary therapy; OR, odds ratio; CI, confidence interval; ref, reference; M, male.
Statistically meaningful data are shown in bold.

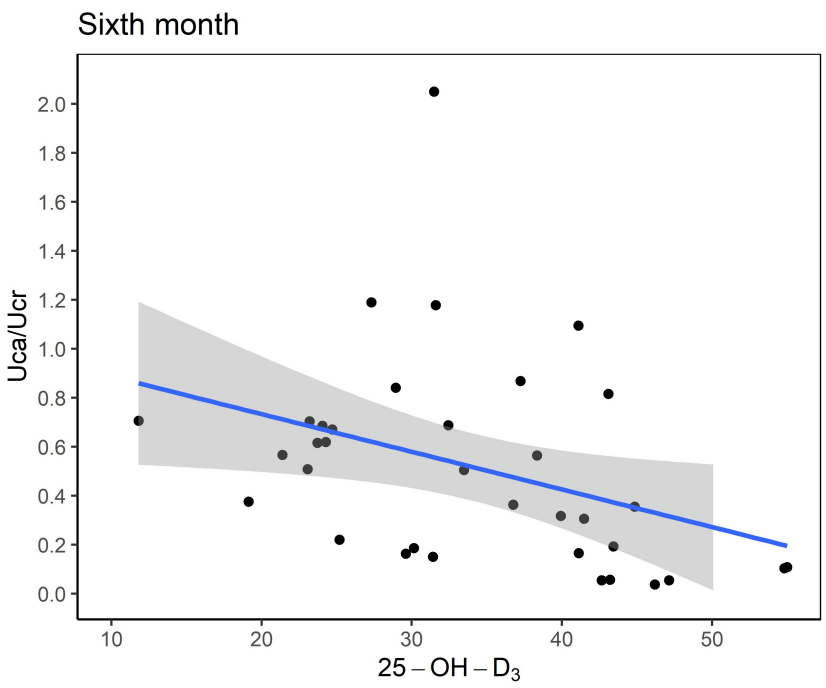

FIGURE 1
Scatter plot between the urinary excretion of calcium (Uca) to urinary excretion of creatinine (Ucr) ratio and serum vitamin D level (ng/mL). Uca, urinary excretion of calcium; Ucr, urinary excretion of creatinine.

D_3 supplementation (51.02 IU/kg), even though he did not develop hypercalciuria during the follow-up period. Before KDT initiation, these three patients had neither hypercalciuria nor urolithiasis, and all children with documented stones were first managed medically with increased fluids and urine alkalization using oral potassium citrate to yield a urine pH of 6.5. All three patients reached remission within 2 years with the aid of medical treatment and did not require lithotripsy for their kidney stones. For patients 2 and 3, vitamin D_3 supplementation was continued for the remission of urolithiasis.

Discussion

To our best knowledge, this is the first study to assess the relationship between several clinical variables, including vitamin D_3 dose, serum 25-OH-D_3 level, and occurrence of hypercalciuria/urolithiasis in pediatric KDT patients. We found that serum 25-OH-D_3 level and hypercalciuria have an inverse correlation, and as 25-OH-D_3 level rises by 1.0 ng/mL, Uca/Ucr ratio decreases by 0.011. The optimal serum 25-OH-D_3 level for preventing hypercalciuria was > 39.1 ng/mL, and the cut-off vitamin D_3 supplementation dose was > 49.5 IU/kg.

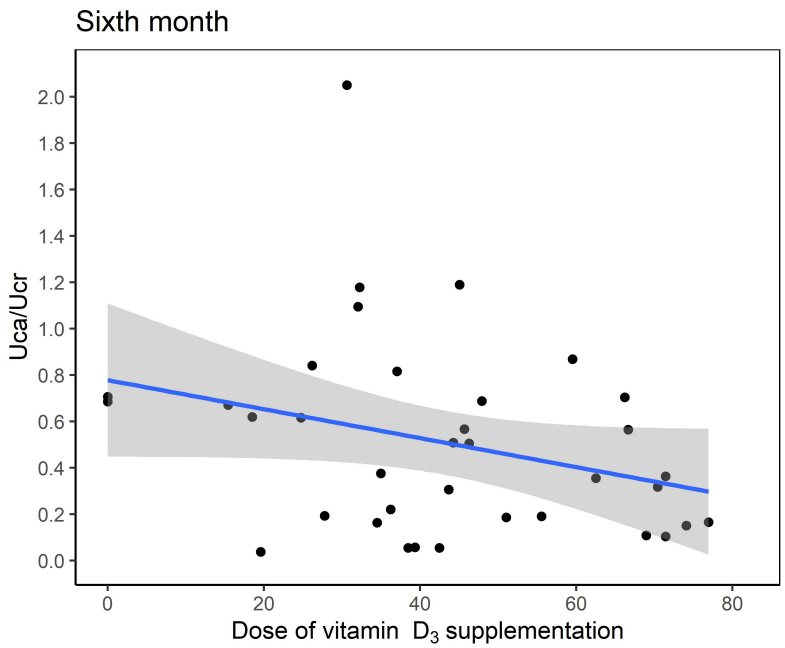

FIGURE 2
Scatter plot between the urinary excretion of calcium (Uca) to urinary excretion of creatinine (Ucr) ratio and dose of vitamin D_3 supplementation at 6 months after ketogenic dietary therapy initiation.

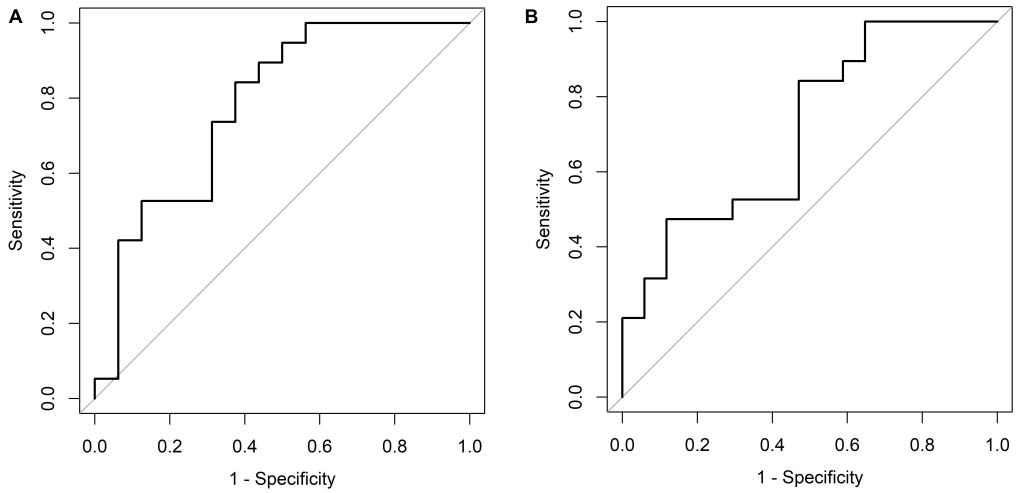

FIGURE 3
(A) Receiver operating characteristic plot [6 months, 25-OH-D_3 level (ng/mL)]. Cut point: ≤ 39.14, > 39.14. Area under the curve (AUC): 0.7796 (0.6179, 0.9413). **(B)** Receiver operating characteristic plot [6 months, vitamin D_3 supplementation dose (IU/kg)]. Cut point: ≤ 49.47, > 49.47. Area under the curve (AUC): 0.7121 (0.5399, 0.8843).

Kidney stone formation is a complex process, which includes urine supersaturation and nucleation, growth, aggregation, and retention of crystals in the kidney (13). KDT can cause kidney stone formation, and the incidence of urolithiasis in children undergoing KDT is 1.4–7% (5, 10, 16). This might be due to hyperuricemia, which increases calcium excretion related to metabolic acidosis, or urine acidification, which results in uric acid supersaturation and decreased urinary citrate concentration (5). According to the "free-particle theory" and "fixed-particle theory," supersaturated urine is the key process involved in kidney stone formation because the formation and growth of crystals occurs within highly saturated urine (11, 17).

It is unclear whether vitamin D supplementation or high serum 25-OH-D_3 level increases the risk of hypercalciuria or kidney stone formation. Many physicians are hesitant to treat vitamin D deficiency in patients with kidney stones because of concerns that vitamin D_3 supplementation increases

urinary calcium excretion. This hesitation might be because the most prevalent type of kidney stone is calcium based, and vitamin D increases intestinal calcium absorption and then urinary calcium excretion (18). Calcitriol binds to vitamin D receptors in enterocytes and increases calcium absorption (19). In addition, intestinal calcium absorption is increased in absorptive hypercalciuria (20), and calcitriol serum levels are also correlated with urinary calcium excretion (21). According to a systematic review and meta-analysis, increased circulating calcitriol was associated with kidney stones, and among patients with urolithiasis, circulating 25-OH-D_3 levels were markedly higher in hypercalciuria than in normocalciuria (22). Therefore, vitamin D is often cited as a risk factor for hypercalciuria and kidney stones (19).

However, several studies have shown that vitamin D supplementation is not associated with urolithiasis. In a prospective study, despite supplementation with high-dose vitamin D_3 (mean daily dose, 3,440 IU) in healthy controls to maintain 25-OH-D_3 levels within 30–88 ng/mL for 6 months, no hypercalcemia or hypercalciuria was noted (23). Another study on patients with urolithiasis showed that hypercalciuria or significant changes in urinary calcium excretion did not occur when 50,000 IU of vitamin D_3 was administered each week, and there was no relation between 25-OH-D_3 level change and urinary calcium excretion (24). A large systematic review and meta-analysis study found that vitamin D supplementation may increase the risk of hypercalcemia and hypercalciuria, but did not increase the risk of kidney stone formation, regardless of the duration of supplementation, dosage, co-supplementation with calcium, and baseline 25-OH-D_3 level (12). In a large cohort study, there was also no association found between vitamin D_3 intake and incidence of kidney stones (25).

Furthermore, despite being controversial, vitamin D deficiency may be a predisposing factor for kidney stone formation. Several studies have shown that vitamin D deficiency is more prevalent in patients with kidney stone formation than in those without (26, 27). There are several hypotheses, as follows: first, secondary hyperparathyroidism caused by vitamin D deficiency can lead to urolithiasis. Second, there are several risk factors shared between vitamin D deficiency and urolithiasis, including obesity and decreased dietary calcium intake. Third, vitamin D deficiency might be responsible for inducing oxidative stress and inflammation in the kidney, which can cause urolithiasis (13).

Vitamin D deficiency causes a decrease in the absorption of dietary calcium, resulting in secondary hyperparathyroidism, which attempts to maintain serum calcium by mobilizing calcium from the bones by increasing osteoclastic activity. These processes decrease bone mineral density. Moreover, hyperparathyroidism increases phosphorus wasting in the kidneys, which results in a low normal or low serum phosphorus level. This results in an inadequate calcium-phosphorus product, causing a mineralization defect in the bones. Consequently, vitamin D deficiency results in osteopenia and osteoporosis (15).

In addition to bone health, vitamin D has various health benefits, as vitamin D receptors exist in most tissues and cells and active vitamin D influences the expression levels of more than 200 genes (28). Vitamin D deficiency causes muscle weakness, whereas increased 25-OH-D_3 level markedly improves performance speed and proximal muscle strength (29). Further, vitamin D has recently been found to be a key factor in the immune system, as (1) it induces the production of antimicrobial peptides and cytokines, (2) it simulates autophagy for controlling intracellular infections, and (3) vitamin D signaling promotes innate immune response. Thus, vitamin D deficiency is associated with susceptibility toward infections (30). In addition, active vitamin D has biological actions, including angiogenesis, renin production, insulin stimulation, macrophage cathelicidin production, and cellular proliferation inhibition (31). In chronic inflammatory diseases, such as type 2 diabetes and autoimmune diseases, vitamin D is supposed to play an important role in gene regulation (32), and supraphysiological doses of the active form of vitamin D may reduce excessive cell proliferation, even in cancer (31). Furthermore, vitamin D supplementation has been suggested to be potentially preventative against cardiovascular diseases through several mechanisms including upregulation of the renin-angiotensin-aldosterone system, blood pressure increase, and ventricular musculo-hypertrophy (33). In a meta-analysis of eight prospective cohort study, the group with the lower 20% of serum vitamin D levels was associated with increased cardiovascular mortality and all-cause mortality (34). Vitamin D deficiency is also correlated with dyslipidemia (35) and is thought to be more influential in high-fat diets such as KDT.

Given the known benefits of vitamin D in maintaining bone health and its potential benefits for cardiovascular, autoimmune, and neoplastic diseases, and given findings suggesting its safety, active vitamin D supplementation is required in patients undergoing KDT. In addition, we found out that maintaining adequate levels of vitamin D is helpful for hypercalciuria and urolithiasis. Serum 25-OH-D_3 level and Uca/Ucr ratio showed an inverse correlation during KDT, and although not statistically significant, Uca/Ucr ratio decreased with an increase in the dose of vitamin D_3 supplementation per weight. In addition, results at 6 months of KDT showed that 25-OH-D_3 level of < 39.1 ng/mL and inadequate vitamin D supplementation of < 49.5 IU/kg could also increase the risk of hypercalciuria. Therefore, it might be helpful to maintain sufficient serum levels of vitamin D (almost 40 ng/mL) and implement vitamin D supplementation (50 IU/kg) to prevent hypercalciuria.

Although there is no consensus on the optimal serum levels of 25-OH-D_3, vitamin D deficiency is defined with a 25-OH-D_3 level of < 20 ng/mL, relative insufficiency with levels between 20 and 29 ng/mL, and sufficient level is ≥ 30 ng/mL. Further, vitamin D poisoning is defined by 25-OH-D_3 level > 150 ng/mL

(15, 28). In a study involving adults, the maximum bone mineral density was achieved when the 25-OH-D_3 level reached $\geq$ 40 ng/mL (29). The recommended dose of vitamin D supplementation in children is approximately 400–1,000 IU per day to avoid deficiency and maintain the proper range and 1,000–2,000 IU per day with calcium supplementation for the treatment of vitamin D deficiency (15). According to our findings, the optimal 25-OH-D_3 level and supplemental doses are similar to those previously recommended by experts. Therefore, although it is necessary to adjust the vitamin D supplementation dose according to the patient's condition, it is better to actively supplement vitamin D than to hesitate due to concerns about hypercalciuria and urolithiasis.

This study has several limitations. First, it was a retrospective study conducted only with patients who received KDT of 3:1 ratio. Moreover, regular visits were difficult for candidates of this study due to severe neurological disorders and a higher risk of complication compared with normal children. For these reasons, a follow-up duration of $>$ 6 months was difficult for many patients, which was thought to be the reason for the absence of clear statistical association over the entire follow-up period. Statistically significant results were obtained only from data within 6 months of the optimal 25-OH-D_3 level and appropriate dose of vitamin D supplementation. Second, this study was conducted only on consecutive patients who were referred to the pediatric endocrine department of a single institute, suggesting a distortion in our conclusion owing to the inevitable selection bias. Third, we failed to consider the effects of the anticonvulsants and the supplemental nutrients used by the subjects. AEDs like carbonic anhydrase inhibitors may affect urinary calcium excretion. Herein, nine and seven patients were taking zonisamide and topiramate, respectively, during KDT. However, as these medications are often prescribed for intractable epilepsy, several other patients also used those medications before KDT. As we could not determine the effects of those medications, we did not exclude patients taking them. Though there were no prescription of important supplemental nutrients except multivitamins, personal checks for all the purchased supplements were not possible; hence, we were unable to consider the effects of other supplements.

Nevertheless, this study was meaningful because it gave suggestions for vitamin D supplementation to children on KDT, who are at risk of poor bone health and secondary osteoporosis. Some of the study results showed that contrary to the traditional belief, vitamin D supplementation can help reduce the risk of hypercalciuria during KDT. In addition, we believe that this study can provide a new perspective on the kidney-related side effects that are generally of concern when vitamin D supplementation is implemented. Hypercalciuria and urolithiasis are associated with dietary factors such as intake of fewer fruits and vegetables and more red meat and salt (13, 36), making it difficult to control the variables in a normal population. In this regard, our study has the advantage that it was conducted under the same, controlled dietary conditions. In addition, the results will be applicable to children undergoing KDT.

In conclusion, we recommend that all children on KDT receive 50 IU/kg of daily vitamin D supplementation and maintain a serum 25-OH-D_3 level of 40 ng/mL to minimize the incidence of hypercalciuria. Further studies with larger numbers of multicenter patients over a longer period of follow-up are required for more evidence and better recommendations.

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 human participants were reviewed and approved by the Institutional Review Board of Severance Hospital Clinical Trial Center. Written informed consent from the 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

ML and AK: conceptualization, methodology, and writing—original draft preparation. HL, KS, HSC, JS, and SK: formal analysis, investigation, resources, and data curation. HWC, H-CK, JL, HK, H-SK, and AK: writing—review and editing and supervision. All authors contributed to the article and approved the submitted version.

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 vitamin D3 levels and frailty in the elderly: A large sample cross-sectional study

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Background: Frailty is recognized as a cornerstone of geriatric medicine. Accurately screening and identifying frailty can promote better quality and personalized medical services for the elderly. Previous studies have shown that the association between vitamin D and frailty in the elderly population is still controversial. More research is needed to explore the association between them.

Materials and methods: We used three waves of data from the National Health and Nutrition Examination Survey (NHANES). Based on the widely accepted AAH FRAIL Scale, we measured and evaluated the participants' frailty from five aspects: fatigue, resistance, ambulation, illness, and loss of weight. All possible relevant variables are included. Machine learning XGboost algorithm, the Least Absolute Shrinkage Selection Operator (LASSO) regression and univariate logistic regression were used to screen variables, and multivariate logistic regression and generalized additive model (GAM) were used to build the model. Finally, subgroup analysis and interaction test were performed to further confirm the association.

Results: In our study, XGboost machine learning algorithm explored the relative importance of all included variables, which confirmed the close association between vitamin D and frailty. After adjusting for all significant covariates, the result indicated that for each additional unit of 25-hydroxyvitamin D3, the risk of frailty was reduced by 1.3% with a statisticaldifference. A smooth curve was constructed based on the GAM.

It was found that there was a significant negative correlation between 25-hydroxyvitamin D3 and the risk of frailty.

Conclusion: There may be a negative correlation between 25-hydroxyvitamin D3 and the risk of frailty. However, more well-designed studies are needed to verify this relationship.

KEYWORDS

disability, endocrinology, vitamin D, 25-hydroxyvitamin D3, frailty

Introduction

In the past few decades, with the rapid progress of medicine, life expectancy has increased significantly worldwide (1). As a result, the number of the elderly has increased, and it is expected that the population over the age of 60 will double in the next 30 years (2). However, because of the heterogeneity of human beings and the complexity of social economy and environment (3, 4), human aging is often heterogeneous (5). Therefore, scientists introduced the concept of frailty to better understand the heterogeneity of human aging (6).

Frailty is an age-related clinical condition, which is characterized by the reduction of individual homeostasis reserve and excessive vulnerability to endogenous and exogenous stressors (7, 8). This negative health condition between health and disability will lead to a disproportionate increase in adverse medical risk events relative to risk exposure factors (9). Frailty is favored by researchers because its contribution to the improvement of the traditional health care system largely conforms to the medical needs of the rapidly aging society (10). It is worth noting that frailty can be reversed (11). Accurately screening and identifying frail people or people who may develop into frailty is one of the key factors to provide them with high-quality medical treatment and care (12).

Frailty has a higher incidence rate in the elderly, and is more directly related to long-term nutritional status (13). The frailty of young people is often caused by major diseases and trauma, which is less related to nutrition (8, 14). Previous studies have shown that the prevalence of frailty among the elderly over 65 years old in the community is 10–20%, while the prevalence of frailty among the elderly over 85 years old in the community has risen to 30–45% (15).

In the past 30 years, the criteria for screening and identifying frailty have not been agreed. In several recent studies, it has been confirmed that there is no significant difference in prediction accuracy between frailty measurement tools such as Fried Phenotype, Edmonton FRAIL Scale and AAH Frailty Index (16). AAH FRAIL Scale, which has been widely proved to be efficient, was selected as the measure of frailty in our study (17).

Vitamin D has many functions, such as promoting the absorption of calcium and phosphorus, cell growth and differentiation, and regulating immune function (18, 19). 25-hydroxyvitamin D3 is the main circulating form of vitamin D, and because of its stable nature and long half-life, it is regarded as the is the best indicator of vitamin D level in the body (20). The activated form of vitamin D, 1,25-dihydroxyvitamin D3, has been shown to induce monocytes to differentiate into macrophages and reduce the release of inflammatory cells and chemokines (19). The differentiation and aging of cells, the level of immunity and inflammation in the body will affect the health status of the human body and the individual internal balance ability reserve to varying degrees.

Driven by the rapid development of computer processing power, memory and storage, machine learning algorithms are trained to efficiently obtain the required information from massive data (21). XGboost is an efficient implementation of the Gradient Boosting Decision Tree (GBDT) algorithm (22), which further improves the accuracy of intelligent prediction, avoids overfitting, improves the generalization of the algorithm, and further enhances the interpretability (23), thus becoming a widely accepted algorithm in machine learning and data mining (24). For the establishment of XGboost model and LASSO regression algorithms (25), we use the 10-fold cross validation method to obtain the model performance of the entire dataset (26). For cross validation, the dataset was divided into 10 folds, of which onefold was used as the test set and the rest as the training set; All the results of the 10 repetitions were taken as the average of the overall performance (27). According to the results, the optimal machine learning coefficients are selected.

Materials and methods

Data source

The present study analyzed respondent data from the National Health and Nutrition Examination Survey (NHANES), which was collected in three cycles (2007–2008, 2009–2010, and 2013–2014) by the Centers for Disease Control and Prevention

(CDC), National Center for Health Statistics (NCHS) in the USA.

Ethical considerations

All patient information in the database is anonymous, and all participants are aware of and consent to the data collection activities. The NHANES program ethical approval and informed consent signed by participants were obtained before NHANES collected data. No further ethical approval and informed consent are required for this study.

Study population

The data of NHANES database from the three cycles was selected. A total of 30,861 participants took the survey. Protocols used in the NHANES were approved by US National Center for Health Statistics Research Ethics Review Board, and written informed consent was provided by all participants.

According to the definition of the elderly by the United Nations and World Health Organization,[1,2] we included participants older than 65 years old.

The inclusion and exclusion criteria were as follows: (1) No lack of relevant data of all the indicators of the previously validated FRAIL Scale (**Appendix 1**). FRAIL Scale items in AAH) (2) No lack of data of relevant biochemical indexes such as serum vitamin D3 levels and all other biochemical covariates (see below). (3) No lack of data of general demographic characteristics of the participants, including race, age, income, use of alcohol and tobacco. (4) According to the standards of the World Health Organization and United Nations, people over 65 years old are defined as the elderly. Finally, a total of 527 participants were included in the study.

Study variables

The variables of each case were included in the study: (1) Patient demographics: gender, age, race, income, education level (2) Variables related to the FRAIL Scale index, including relevant questionnaire data related to fatigue, resistance, ambulation, illness, loss of weight (3) Relevant laboratory examination indicators: including urine albumin, urine creatinine, serum albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), total calcium, cholesterol, serum creatinine, gamma glutamyl transferase (GGT), serum glucose, refrigerated serum iron, lactate dehydrogenase (LDH), phosphorus, total bilirubin, total protein, uric acid, sodium, potassium, chloride, globulin, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride and 25-hydroxyvitamin D3. (4)The participants' body mass index (BMI), defined as the weight divided by the square of height (kg/m^2)(28). Finally, we included a total of 57 variables.

Evaluation criterion

Frailty

All included subjects were categorized into robust (scored 0), pre-frail (scored 1–2) and frail (scored 3–5) clusters according to the previously validated FRAIL Scale. A complete description of the FRAIL Scale items scoring criteria, and baseline prevalence are provided in **Appendix 1** (29).

Hypertension

The average value of three blood pressure measurements was calculated, and the mean blood pressure was used to assess whether the participants are hypertensive. The diagnostic criteria of hypertension are systolic pressure $\geq$ 140 mmHg and/or diastolic pressure $\geq$ 90 mmHg.

Drinking

We examined the classification of alcohol consumption in previous studies, which was finally divided into two levels. An alcoholic is defined as a person who drinks more than 12 drinks per year.

Vitamin D

Serum 25-hydroxyvitamin D3 concentrations were measured at the National Center for Environmental Health, CDC, Atlanta, GA using the DiaSorin RIA kit (Stillwater MN). Serum 25-hydroxyvitamin D3 status was classified into three levels: Participants with serum 25-hydroxyvitamin D3 $<$ 30 nmol/L were defined as deficiency; Participants with concentrations $>$ 30 but $<$ 50 nmol/L were defined as insufficiency; Participants with concentrations $>$ 50 nmol/L were considered as normal vitamin D status (30).

Statistical analysis

T-test, Mann–Whitney U test and weighted linear regression were used for continuous variables and Chi-square tests were used for categorical variables. LASSO regression was employed to screen variables, and multivariate logistic regression model was developed to analyze the significance of

1 https://previous.iiasa.ac.at/web/home/about/news/190227-aging.html

2 https://previous.iiasa.ac.at/web/home/research/researchPrograms/WorldPopulation/Meetings/181105-UNDESA.html

variables. The generalized additive model (GAM) can better fit the association between vitamin D3 and frailty and avoid the possibility of overfitting and underfitting (31, 32). Finally, we performed subgroup analysis in the presence of interaction effects for further exploration.

In addition, the XGboost algorithm is used to calculate, rank and output the most relevant and significant variables of the state of frailty of the participants, so as to verify the stability and reliability of our conclusions. We tested the collinearity in the regression model by calculating variance inflation factors (VIF) and excluded independent variables with VIF > 10.

All analyses were completed by using STATA version 15 (StataCorp LP, College Station, TX, USA) and R statistical software (R4.0.2).

Result

Characteristics of study population

In this study, 30,861 participants were obtained from the NHANES database. After excluding 23,344 participants with deficiency data, there were 7,517 participants including all the data items we needed. The 6,163 participants with missing data of any relevant items in the questionnaires ("Medical Conditions," "Physical Functioning," "Osteoporosis," "Mental Health-Depression Screener," "Alcohol Use," "Smoking and Weight History" questionnaires) and the 296 participants with missing laboratory test results were excluded. Because this study focused on the health management of the elderly, 531 participants under the age of 65 were excluded according to the definition of the elderly by WHO, and 527 participants were finally included in this trial (**Figure 1**).

The age, education level and income of the participants in the robust, pre-frail and frail groups were found to be different (**Table 1**). The average age of the frail patients was significantly higher than the average age of the patients in the pre-frail group ($p < 0.001$), and was significantly higher than the average age of the participants in the robust group ($p < 0.001$). There was no significant difference in gender and race among the three groups according to the Chi-square test (**Table 1**).

In addition, it was observed that there were significant differences in BMI, urinary albumin, ALP, BUN, serum creatinine, serum glucose, phosphorus, total protein, serum albumin, serum globulin and 25-hydroxyvitamin D3 levels among the three groups according to the weighted linear regression model. The average BMI of the pre-frail participants was significantly higher than that of the robust participants ($p = 0.001$), but participants with frailty were no different from other two groups in terms of BMI. However, the urinary albumin, BUN and serum creatinine in the frail group were significantly higher than those in the pre-frail group and the robust group. This demonstrated that the frailty of the elderly is closely related to renal function, and the inclusion and adjustment of serum biomarkers related to chronic kidney disease will greatly optimize our analysis. In addition, there were also significant differences in biomarkers related to systemic nutritional status and liver disease among the three groups. Serum albumin in the frail group was lower and globulin was higher than that in the pre-frail group and the robust group. There was no significant difference in the concentration triglycerides, LDL-cholesterol, HDL-Cholesterol and aminotransferases among the three groups (**Table 1**).

Single factor analysis: Univariate logistic regression

In order to further explore the relationship between 25 hydroxyvitamin D3 level and the occurrence of frailty, we included the robust group and the pre-frailty group into the non-frailty group, which corresponds to the frailty group.

Univariate logistic regression was performed to analyze the significance of associations between the occurrence of frailty and the serum levels of 25-hydroxyvitamin D3, other laboratory indicators, gender, age, race, education level, income level, and BMI. The serum levels of 25-hydroxyvitamin D3, iron, HDL cholesterol, LDL cholesterol and total cholesterol were negatively correlated with the occurrence of frailty, while the serum levels of GGT, serum creatinine, triglycerides and BMI were positively correlated with the occurrence of frailty. In addition, probability of frailty was higher in the low-income population and smoking population (**Table 2**).

Multivariate analysis: Least Absolute Shrinkage Selection Operator regression

As is shown in **Supplementary Table 1**, we tested the collinearity in the regression model and excluded three variables: cholesterol, total bilirubin, and total protein.

Figure 2 depicted the results of selection of variables by using LASSO regression. In **Figure 2B**, the red dots indicate the tuning parameter (Lambda) and the two dotted lines represent the two special Lambda (log) values selected in the LASSO model using 10-fold cross-validation, namely, value of lambda (log) that gives minimum mean cross validated error [Lambda. min (log)] and large value of lambda (log) such that error is within 1 standard error of the minimum [Lambda.1se (log)]. The variables selected by these two lambda values are the variables included in the corresponding optimization model. The Lambda min (log) was 0.0129 (-4.3513). The AUC value of Receiver Operating Characteristic (ROC) curves of the prediction model based on the variables screened by the Lambda values are 0.729 (**Figure 2C**), indicating that LASSO regression

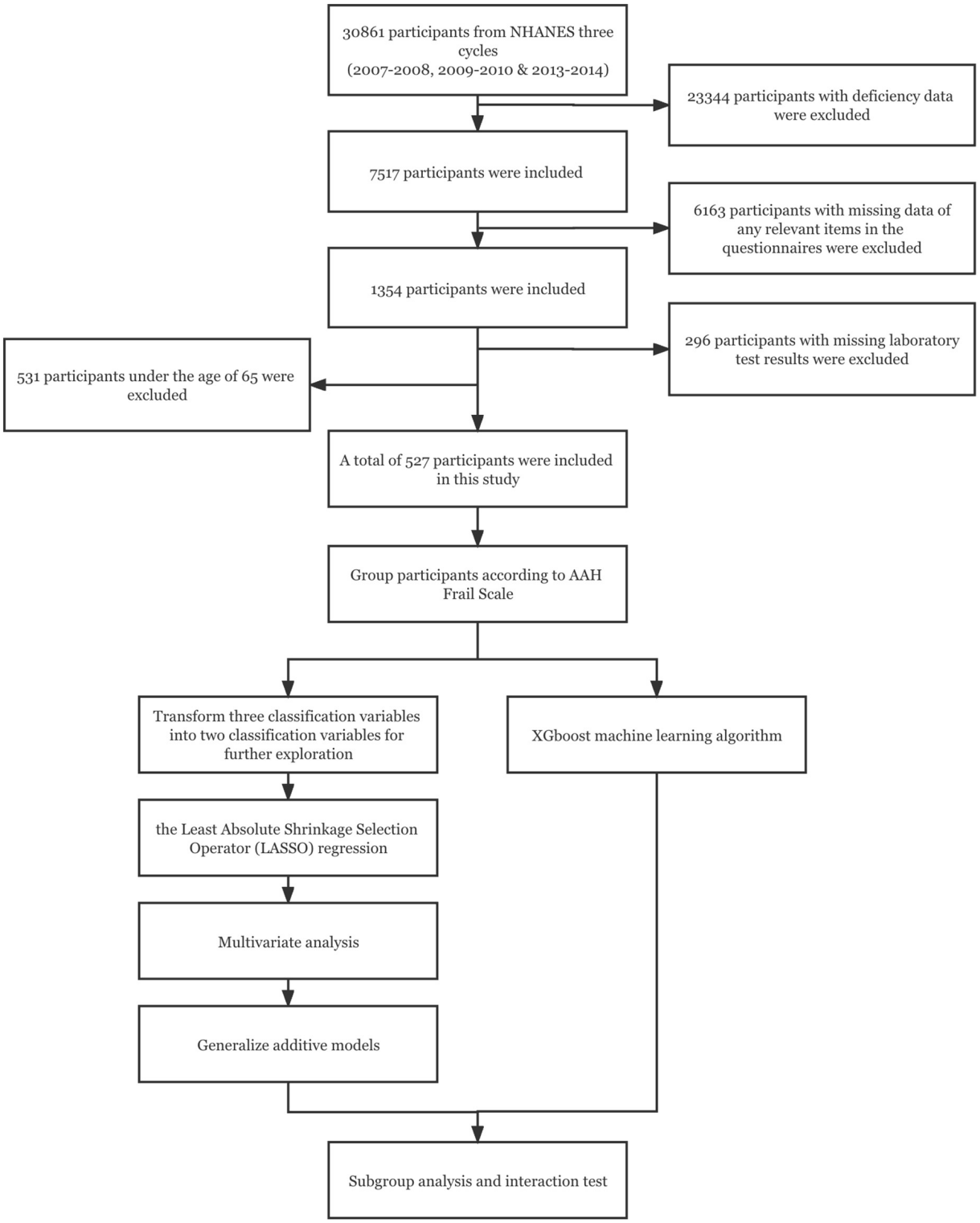

FIGURE 1
The work flow diagram.

can effectively screened out the variables with strong correlation with frailty.

According to the Lambda min (log) of 10-fold cross-validation, the selected variables are urine albumin, AST, BUN, total calcium, serum creatinine, GGT, refrigerated serum iron, chloride, triglyceride, LDL-cholesterol, serum globulin, sodium, age, race, income, smoking, alcohol use, BMI and 25-hydroxyvitamin D3 (**Supplementary Table 2**).

TABLE 1 Candidate variables and baseline characteristics of the participants.

Variables	Total	Robust	Pre-frail	Frail	*P*-value	*P*-value*	*P*-value**	*P*-value***
Sex					0.152	0.935	0.063	0.060
Male	338 (64.1%)	170 (65.1%)	148 (65.5%)	20 (50.0%)				
Female	189 (35.9%)	91 (34.9%)	78 (34.5%)	20 (50.0%)				
Age	72.2 ± 5.0	71.8 ± 4.7	72.1 ± 5.1	75.2 ± 5.3	< 0.001	0.452	< 0.001	< 0.001
Race					0.508	0.228	0.536	0.211
Mexican American	46 (8.7%)	23 (8.8%)	22 (9.7%)	1 (2.5%)				
Non-Hispanic Black	36 (6.8%)	14 (5.4%)	20 (8.8%)	2 (5.0%)				
Non-Hispanic White	356 (67.6%)	177 (67.8%)	149 (65.9%)	30 (75.0%)				
Other Hispanic	73 (13.9%)	37 (14.2%)	29 (12.8%)	7 (17.5%)				
Other race	16 (3.0%)	10 (3.8%)	6 (2.7%)	0 (0.0%)				
Education					0.152	0.217	0.042	0.175
Non-received higher education	312 (59.2%)	170 (65.1%)	148 (65.5%)	20 (50.0%)				
Received higher education	215 (40.8%)	91 (34.9%)	78 (34.5%)	20 (50.0%)				
Income					0.049	0.131	0.024	0.153
Earning less than $1000,000	485 (92.0%)	234 (89.7%)	211 (93.4%)	40 (100.0%)				
Earning more than or equal to $1000,000	42 (8.0%)	27 (10.3%)	15 (6.6%)	0 (0.0%)				
Smoking					< 0.001	0.001	< 0.001	0.082
Every day	74 (14.0%)	20 (7.7%)	43 (19.0%)	11 (27.5%)				
Some days	11 (2.1%)	6 (2.3%)	3 (1.3%)	2 (5.0%)				
Not at all	442 (83.9%)	235 (90.0%)	180 (79.6%)	27 (67.5%)				
Alcohol use					0.532	0.394	0.359	0.648
Yes	431 (81.8%)	218 (83.5%)	182 (80.5%)	31 (77.5%)				
No	96 (18.2%)	43 (16.5%)	44 (19.5%)	9 (22.5%)				
BMI (kg/M^2)	28.0 ± 5.3	27.2 ± 4.5	28.8 ± 5.6	28.3 ± 6.7	0.002	0.001	0.209	0.550
Albumin, urine (μg/mL)	75.4 ± 445.8	42.5 ± 172.2	65.9 ± 240.1	343.5 ± 1438.2	< 0.001	0.559	< 0.001	< 0.001
Creatinine, urine (mg/dL)	113.1 ± 65.2	110.9 ± 64.2	114.5 ± 66.0	120.2 ± 67.2	0.647	0.543	0.403	0.614
ALT (U/L)	22.2 ± 18.9	21.3 ± 11.1	23.6 ± 26.1	20.4 ± 8.9	0.332	0.179	0.785	0.326
AST (U/L)	25.4 ± 15.3	24.6 ± 8.1	26.8 ± 21.5	23.1 ± 7.3	0.172	0.111	0.569	0.160
GGT (U/L)	27.3 ± 26.5	24.5 ± 22.8	29.7 ± 28.7	32.0 ± 33.7	0.047	0.029	0.092	0.608
LDH (U/L)	136.6 ± 27.4	136.0 ± 25.2	136.4 ± 30.1	141.0 ± 26.3	0.556	0.864	0.281	0.328
ALP (U/L)	70.3 ± 24.0	69.0 ± 24.8	70.2 ± 21.5	79.5 ± 29.8	0.034	0.557	0.009	0.024
BUN (mmol/L)	5.8 ± 2.5	5.6 ± 2.0	5.7 ± 2.4	7.4 ± 4.4	< 0.001	0.545	< 0.001	< 0.001
Total calcium (mmol/L)	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	0.693	0.590	0.437	0.628
Creatinine, serum (μmol/L)	90.0 ± 42.5	86.3 ± 23.3	88.7 ± 27.6	121.9 ± 123.2	< 0.001	0.525	< 0.001	< 0.001
Glucose, serum (mmol/L)	6.1 ± 1.9	6.0 ± 1.8	6.1 ± 1.9	6.7 ± 2.1	0.149	0.595	0.051	0.099
Chloride (mmol/L)	103.5 ± 3.3	103.7 ± 3.2	103.4 ± 3.1	103.4 ± 4.2	0.682	0.395	0.684	0.962
Iron, refrigerated (μmol/L)	15.9 ± 5.5	16.5 ± 5.5	15.7 ± 4.8	13.2 ± 8.0	0.001	0.092	< 0.001	0.007
Phosphorus (mmol/L)	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	0.046	0.951	0.015	0.018
Uric acid (μmol/L)	352.0 ± 86.3	347.0 ± 83.8	355.7 ± 83.6	363.7 ± 113.7	0.364	0.269	0.254	0.587
Sodium (mmol/L)	139.6 ± 2.5	139.6 ± 2.6	139.6 ± 2.5	139.9 ± 1.9	0.681	0.767	0.383	0.480
Potassium (mmol/L)	4.1 ± 0.4	4.1 ± 0.4	4.1 ± 0.4	4.2 ± 0.5	0.596	0.554	0.512	0.336
Albumin, serum (g/L)	139.6 ± 2.5	41.9 ± 2.7	41.8 ± 2.8	40.8 ± 3.6	0.057	0.549	0.017	0.040
Globulin, serum (g/L)	29.2 ± 4.9	29.1 ± 4.7	29.2 ± 4.8	30.1 ± 5.8	0.426	0.828	0.193	0.241
HDL-Cholesterol (mmol/L)	1.4 ± 0.4	1.5 ± 0.4	1.4 ± 0.4	1.4 ± 0.3	0.142	0.057	0.321	0.980
Triglyceride (mmol/L)	1.4 ± 0.7	1.3 ± 0.6	1.5 ± 0.8	1.4 ± 0.7	0.071	0.022	0.588	0.495
LDL-cholesterol (mmol/L)	2.8 ± 1.0	2.9 ± 1.0	2.7 ± 1.0	2.4 ± 0.9	0.007	0.045	0.004	0.078
25-hydroxyvitamin D3 (nmol/L)	65.7 ± 28.0	70.0 ± 27.9	62.2 ± 27.5	57.1 ± 27.1	0.001	0.002	0.006	0.284

% for: Sex Race Education Income Smoking Alcohol-use. *P*-value was calculated by Chi-square test. Mean ± SD for: Age BMI (kg/m^2) Albumin, urine (μg/mL); Creatinine, urine (mg/dL); ALT (U/L); AST (U/L); ALP (U/L); BUN (mmol/L); Total calcium (mmol/L); Creatinine, serum (μmol/L) GGT (U/L); Glucose, serum (mmol/L); Iron, refrigerated (μmol/L) LDH (U/L); Phosphorus (mmol/L); Uric acid (μmol/L); Potassium (mmol/L) Chloride (mmol/L); Globulin, serum (g/L); HDL-Cholesterol (mmol/L); Triglyceride (mmol/L); LDL-cholesterol (mmol/L) Albumin, serum (g/L) Sodium (mmol/L); 25-hydroxyvitamin D3 (nmol/L). *P*-value was calculated by weighted linear regression model. *P*-value*: *P*-value for Robust-Pre-frail based on Fisher's Least Significant Difference (LSD) *post hoc* test. *P*-value**: *P*-value for Robust-Frail based on Fisher's Least Significant Difference (LSD) *post hoc* test. *P*-value***: *P*-value for Pre-frail-Frail based on Fisher's Least Significant Difference (LSD) *post hoc* test. BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GGT, gamma glutamyl transferase; LDH, lactate dehydrogenase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

The variables screened by LASSO regression were included into the multivariate logistic regression. In the fully adjusted model, it was found that the risk of frailty will be reduced by 1.3% for each additional unit of 25-hydroxyvitamin D3.

TABLE 2 Univariate analysis for frailty.

	Univariate logistic regression	
Variables	**OR (95% CI)**	***P*-value**
Sex		
Male	1	
Female	1.090 (0.763, 1.556)	0.636
Age	1.033 (0.998, 1.069)	0.066
Race		
Mexican American	1	
Non-Hispanic White	1.571 (0.649, 3.807)	0.316
Non-Hispanic Black	1.011 (0.547, 1.869)	0.971
Other Hispanic	0.973 (0.465, 2.035)	0.941
Other race	0.600 (0.187, 1.925)	0.390
Education		
Non-received higher education	1	
Received higher education	0.741 (0.523, 1.050)	0.091
Income		
Earning less than $1000,000	1	
Earning more than or equal to $1000,000	0.518 (0.269, 0.998)	0.049
Smoking		
Every day	1	
Some days	0.309 (0.085, 1.125)	0.074
Not at all	0.326 (0.189, 0.563)	< 0.001
Alcohol use		
Yes	1	
No	1.261 (0.809, 1.967)	0.306
BMI (kg/m^2)	1.060 (1.025, 1.097)	< 0.001
Albumin, urine (μg/mL)	1.001 (1.000, 1.002)	0.107
Creatinine, urine (mg/dL)	1.001 (0.998, 1.004)	0.432
ALT (U/L)	1.007 (0.993, 1.020)	0.319
AST (U/L)	1.009 (0.993, 1.026)	0.258
GGT (U/L)	1.010 (1.001, 1.018)	0.024
LDH (U/L)	1.001 (0.995, 1.008)	0.639
ALP (U/L)	1.005 (0.997, 1.012)	0.203
BUN (mmol/L)	1.067 (0.994, 1.145)	0.074
Total calcium (mmol/L)	2.000 (0.293, 13.660)	0.479
Creatinine, serum (μmol/L)	1.006 (1.000, 1.013)	0.048
Glucose, serum (mmol/L)	1.051 (0.957, 1.153)	0.299
Chloride (mmol/L)	0.977 (0.927, 1.029)	0.381
Iron, refrigerated (μmol/L)	0.960 (0.930, 0.991)	0.012
Phosphorus (mmol/L)	1.522 (0.518, 4.473)	0.445
Uric acid (μmol/L)	1.001 (0.999, 1.003)	0.188
Sodium (mmol/L)	1.018 (0.951, 1.090)	0.603
Potassium (mmol/L)	0.928 (0.598, 1.440)	0.739
Total protein (g/L)	0.998 (0.965, 1.031)	0.887
Globulin, serum (g/L)	1.010 (0.975, 1.047)	0.565
Albumin, serum (g/L)	0.963 (0.907, 1.023)	0.218
HDL-Cholesterol (mmol/L)	0.658 (0.433, 0.999)	0.049
Triglyceride (mmol/L)	1.326 (1.029, 1.710)	0.029
LDL-cholesterol (mmol/L)	0.791 (0.661, 0.945)	0.009
Total Cholesterol (mmol/L)	0.836 (0.718, 0.973)	0.020
Bilirubin, total (μmol/L)	0.996 (0.960, 1.033)	0.816
25-hydroxyvitamin D3 (nmol/L)	0.989 (0.983, 0.995)	< 0.001

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GGT, gamma glutamyl transferase; LDH, lactate dehydrogenase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

For sensitivity analysis, we transformed 25-hydroxyvitamin D3 from continuous variable to classified variables—sufficiency, insufficiency, and deficiency. The *P*-value of 25-hydroxyvitamin D3 trend in the two model is consistent with the result when 25-hydroxyvitamin D3 is a continuous variable. The risk for frailty was 1.5 and 2.2 times higher in vitamin D insufficient and deficient group, respectively, compared with vitamin D sufficient group (**Table 3**).

Generalized additive model

According to our sensitivity test, the correlation between vitamin D3 as a categorical variable and frailty is not completely consistent with the correlation between vitamin D3 as a continuous variable. Linear regression better describes the association between two continuous variables while controlling for other confounders, compared with logistic regression, but in case there in non-linear association, GAM is more applicable (33). GAM is a statistical model that fits data with a higher degree of freedom (34). Before modeling, it is not necessary to analyze the relationship between response variables and explanatory variables. Instead, the response variables and each explanatory variable are modeled separately and added to obtain GAM (35). After adjusting the statistically significant variables screened by LASSO regression in GAM, it was found that the level of 25-hydroxyvitamin D3 was significantly negatively correlated with the risk of frailty (**Figure 2D**).

Subgroup analysis

In order to better explain this result, we conducted subgroup analysis and interaction test (36). This study, stratified by gender, race, education level, income, BMI and alcohol use, verified whether the conclusion of the relationship between 25-hydroxyvitamin D3 level and the risk of frailty based on the overall population was still applicable to each subgroup. The results showed that except for the interaction between 25-hydroxyvitamin D3 and the risk of frailty in the sex subgroup of model 1, there was no interaction in other subgroups. Our conclusion is stable and reliable (**Figure 3**).

Machine learning using the XGBoost algorithm model

In the development and validation phase of the research, we input all the variables into the XGboost machine learning algorithm based on 10-fold cross validation method for the three classifications of states of frailty. These variables include sociodemographic data of participants and all relevant laboratory data. According to the relative importance of

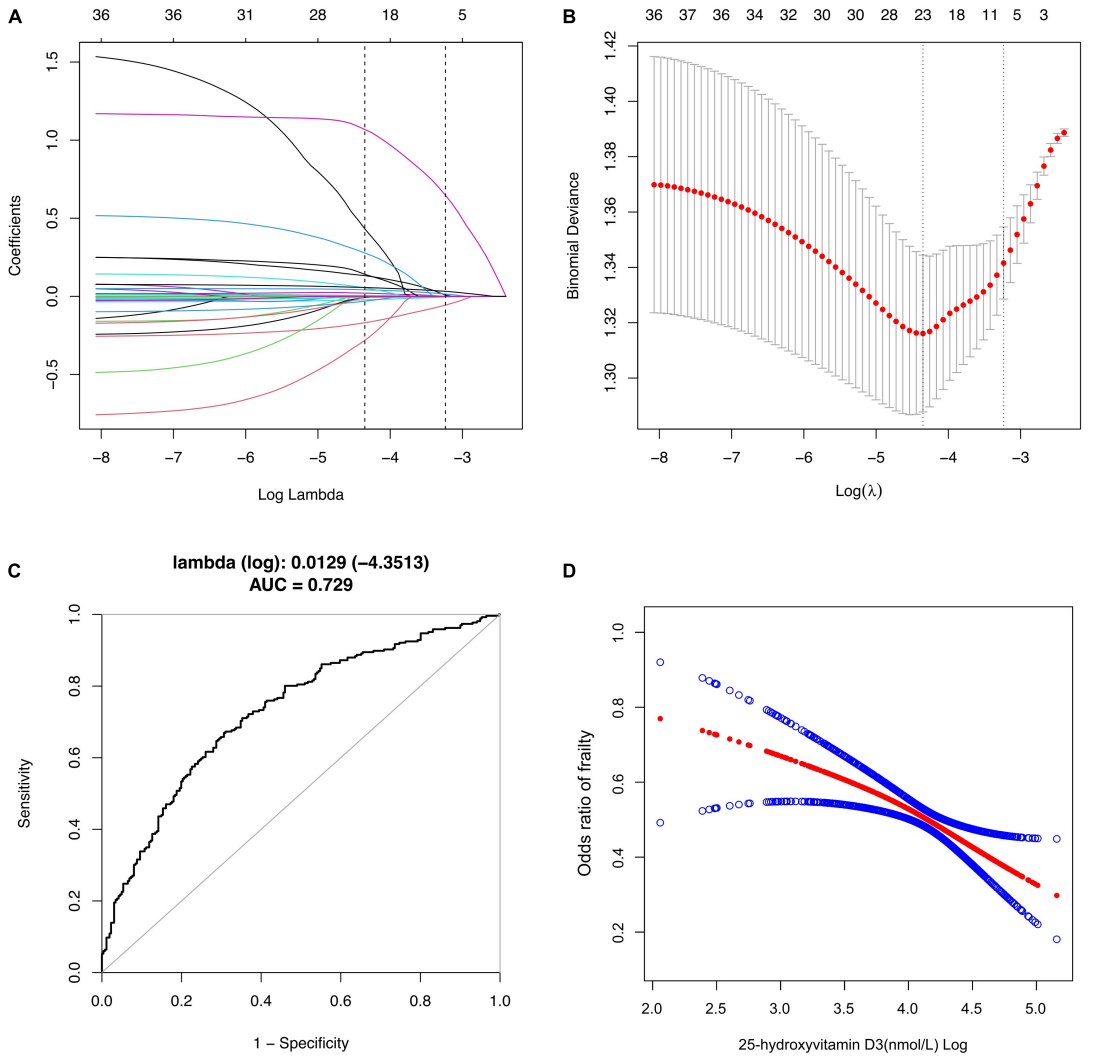

FIGURE 2
Selection of Variables using LASSO regression. **(A)** Lasso coefficient of 31 variables in model 1; **(B)** the optimal penalty coefficient [Lambda (log) = 0.0129] in the Lasso regression was identified with the minimum criterion; **(C)** Receiver operating characteristic (ROC) curves according to LASSO Regression; **(D)** The relationship between Vitamin D3 and Frailty. Solid rad line represents the smooth curve fit between variables according to GAM. Blue bands represent the 95% of confidence interval from the fit.

variables attached by XGboost algorithm, BMI, age, 25-hydroxyvitamin D3, cholesterol and urine albumin are the five most significant variables in the dataset (Figure 4).

Discussion

With the development of population aging, more and more elderly people are in a state defined as frailty due to the impairment of their physical and psychological functions, which has brought a heavy burden to individuals, families and society (5, 37). The frailty of the elderly is due to the cumulative decline of physiological function and the increase of physical and mental vulnerability related to aging, which reduces the ability of the elderly to effectively cope with diseases or trauma, and will lead to more adverse consequences: falls, delirium, incontinence, etc. (38).

The relationship between vitamin D and health has been a long-discussed topic (39, 40). In recent years, the relationship between vitamin D and frailty in the elderly has gradually attracted researchers' attention (41). Through in-depth analysis of the data of the U.S. National Health and Nutrition Examination Survey, this study shows that 25-hydroxyvitamin D3 is an important protective factor for the frailty of the elderly. Vitamin D can affect the body function through several mechanisms. Firstly, it can affect the body function through the potential mechanism of indirectly regulating calcium and phosphorus metabolism. Secondly,

TABLE 3 The association between VitD3 and Frailty in the multiple regression model.

Multivariate analysis		
Variables	**OR (95% CI)**	***P*-value**
Model 1	0.989 (0.983, 0.995)	< 0.001
Model 2	0.989 (0.982, 0.996)	0.002
Model 3	0.987 (0.979, 0.995)	< 0.001
25-hydroxyvitamin D3 status		
Sufficiency	Reference	
Insufficiency	1.506 (0.920, 2.466)	
Deficiency	2.244 (1.119, 4.497)	
P for trend	0.011	

Model 1: No adjustments have been made; Model 2: Adjustments were made for gender, age, race, smoking, alcohol use, income, and education level; Model 3: Adjustments were made for age, race, income, smoking, alcohol use, BMI, urine albumin, AST, BUN, serum creatinine, refrigerated iron, chloride, sodium, globulin, triglyceride, and LDL-cholesterol according to LASSO regression. BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GGT, gamma glutamyl transferase; LDH, lactate dehydrogenase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

it can directly regulate the transcription of genes related to calcium channels (42) and skeletal muscle proliferation and differentiation at the genome level by combining with vitamin D receptor (VDR) on skeletal muscle cells (43, 44).

Frailty also has a great impact on the perioperative period (45). In many clinical trials, it has been found that preoperative frailty and low serum 25 hydroxyvitamin D3 can directly increase the probability of postoperative mortality and serious complications, predicting a worse prognosis (46–48). In addition, Jim Bartley has shown that vitamin D can reduce the probability of postoperative pulmonary infection through anti-inflammatory effect (49). The above findings prove that it is beneficial to supplement appropriate amount of vitamin D during perioperative period. The identification and mitigation of patients' frailty is of great reference value to anesthesiology and surgery (50, 51). Our results confirmed that the level of 25-hydroxyvitamin D3 was negatively correlated with the occurrence of frailty, which can be used in clinical practice to improve the prognosis of patients with frailty.

The subgroup and interaction analysis confirmed that our conclusion is robust and reliable. Subgroup analysis showed that women were more likely to become frail than men, non-Hispanic Whites, people with higher education and low-income groups were more likely to be frail. Gender may affect the expression of aging related genes and the epigenetic changes of aging (52), including mitochondrial dysfunction in aging (53). According to the research of Therri Usher et al. the difference of frailty between races cannot be explained by obesity, chronic disease burden or low-income people (54).

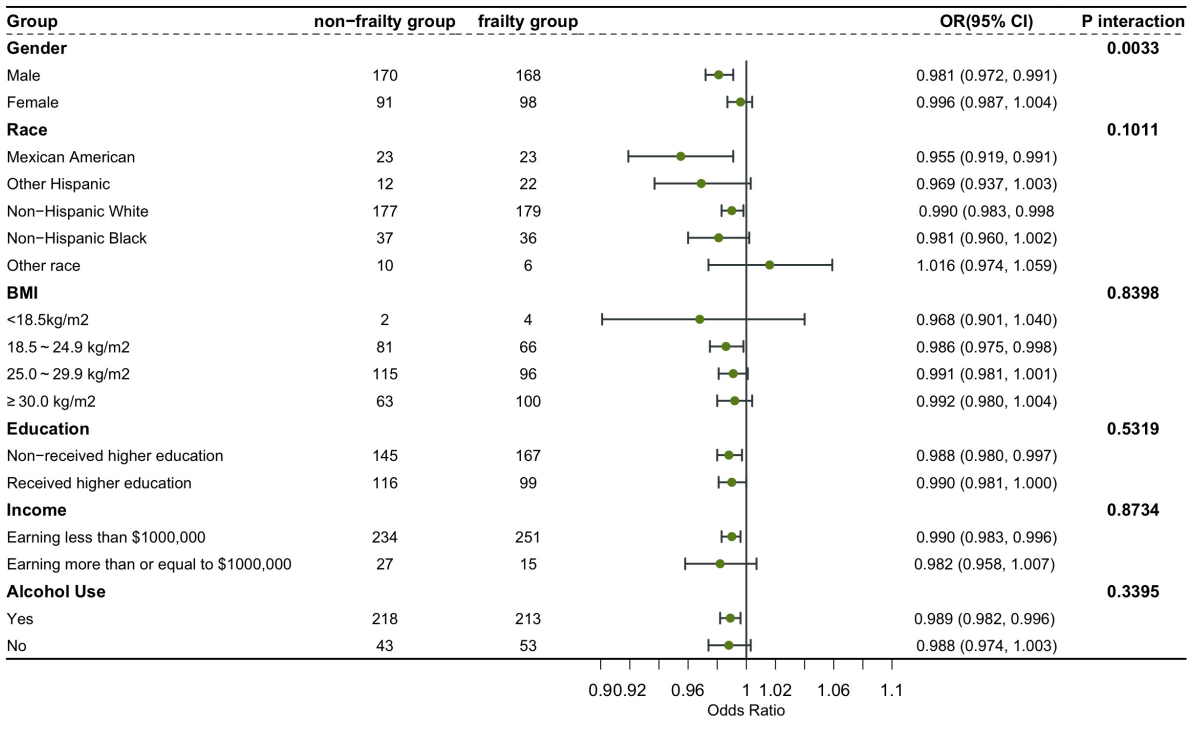

FIGURE 3
Subgroup analysis and interaction test.

More research on racial frailty needs to be carried out to implement targeted interventions. High-income groups may reduce the probability of frailty because of better lifestyle and better medical conditions (55, 56). In addition, it was found that the risk of frailty increased with the increase of BMI. However, according to previous meta-analysis (57), frailty is positively associated with both underweight and obesity. This may be because our analysis is based on the sample from the United States. As a developed economy, the United States has a high obesity rate (58), and there is also a certain interaction between BMI and household income level (59). It may be difficult for lower income people to maintain healthier living habits, which leads to weakness to a certain extent. In addition, our sample of people with lower BMI is also small, which may also lead to some bias.

Compared with previous studies, our study has certain advantages and innovation. Firstly, our study was based on the real-world population study in the United States, and 527 elderly people over 65 years old are included, forming a cross-sectional study with a large sample size. Secondly, we adopted XGboost algorithm and LASSO regression which have been proved to be extremely robust and efficient to screen and verify variables. Additionally, to avoid overfitting and underfitting, we built the GAM to fit the results, which demonstrated the non-linear negative correlation between the possibility of frailty and 25-hydroxyvitamin D3 levels. Finally, we performed subgroup analysis and interaction test to verify the reliability of the conclusions and extend the application scope of our conclusion.

In addition, our research has certain limitations; (1) It is difficult to distinguish causal relationship through cross-sectional study, and we will further apply prospective cohort study to deepen the conclusion in the future; (2) The sample of this study is based on the population survey data of the United States, so whether the conclusions of this paper can be extended to the population in other countries and regions needs further research and investigation; (3) The elderly often have chronic diseases, including chronic heart disease, chronic kidney disease, etc. Our study has included and adjusted biomarkers related to chronic kidney disease, chronic liver disease and other chronic diseases to a certain extent, but there are still some biomarkers of diseases that may lead to frailty have not been included and adjusted. (4) It is worth noting that the assessment of frailty in this paper is based on the AHH Frailty Scale, which may lead to some bias. Among a variety of frailty screening tools, frailty scale and frailty index have been proved to have the strongest predictive validity for disability and mortality (60), and their test efficiency has been confirmed in a variety of populations, including middle-aged women (61), African American people, and Mexican adults (62). However, a number of comparative studies and cohort studies have shown that although there is moderate consistency and strong positive correlation between a variety of frailty screening tools (60, 61, 63), there are still some differences in the frailty incidence rate, and these differences will affect our results to a certain extent. In the future, we will apply multiple frailty screening tools based on NHANES database, including Fried Frailty Phenotype, Frailty Index, FRAIL Scale, Clinical Frailty Scale, Time Up-and-Go Test, Tilburg Frailty Indicator, Groningen Frailty Indicator and Edmonton Frailty Scale, to conduct comparative research to comprehensively demonstrate the consistency, correlation, and bias among the multiple frailty screening tools for predicting the frailty of the elderly.

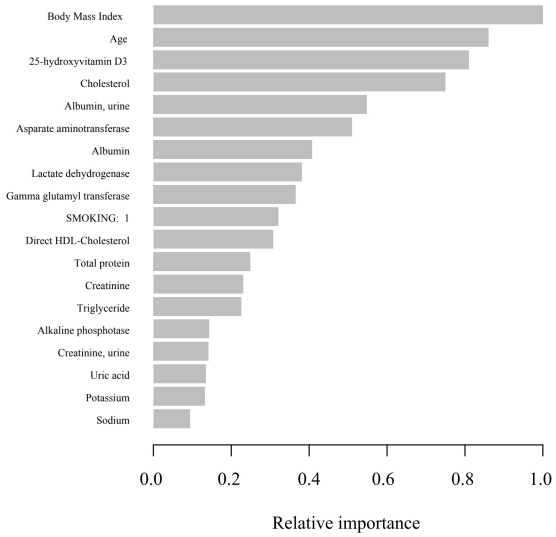

FIGURE 4
Relative importance of the selected variables using XGBoost and the corresponding variable importance score. *X*-axis indicates the importance score, which is the relative number of a variable that is used to distribute the data, *Y*-axis indicates the selected variable.

Conclusion

In conclusion, the results of this cross-sectional study based on the data of the U.S. National Health and Nutrition Examination Survey database show that there is a significant negative correlation between serum vitamin D3 levels and frailty in the elderly population. The confirmation and revelation of the negative correlation will be of great guiding significance to clinical work including perioperative management.

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 human participants were reviewed and approved by the Protocols used in the NHANES were approved by US National Center for Health Statistics Research Ethics Review Board, and written informed consent was provided by all participants information on this is available on the official NHANES site: https://wwwn.cdc.gov/Nchs/Nhanes/ (https://www.cdc.gov/nchs/nhanes/irba98.htm). And the data for the explored NHANES surveys are publicly available for exploration (https://www.cdc.gov/nchs/data_access/restrictions.htm). The patients/participants provided their written informed consent to participate in this study.

Author contributions

QX: project administration, validation data curation, and supervision. ZZ: conceptualization, methodology, data curation, formal analysis, and writing – original draft. WX, YQ, and FW: methodology and writing – review and editing. All authors contributed to the article and approved the submitted version.

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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/fnut.2022.980908/full#supplementary-material

SUPPLEMENTARY TABLE 1
Stepwise screening of collinearity of independent variables.

SUPPLEMENTARY TABLE 2
Coefficients assigned to variables according to LASSO regression.

SUPPLEMENTARY TABLE 3
Relative importance of the selected variables using XGBoost and the corresponding importance score of each variable.

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Appendix 1

FRAIL scale items in AAH

Fatigue: "How much of the time during the past 4 weeks did you feel tired?" 1 = All of the time, 2 = Most of the time, 3 = Some of the time, 4 = A little of the time, 5 = None of the time. Responses of "1" or "2" are scored as 1 and all others as 0. Baseline prevalence = 20.1%.

Resistance: "By yourself and not using aids, do you have any difficulty walking up 10 steps without resting?" 1 = Yes, 0 = No. Baseline prevalence = 25.5%.

Ambulation: "By yourself and not using aids, do you have any difficulty walking several hundred yards?" 1 = Yes, 0 = No. Baseline prevalence = 27.7%.

Illness: For 11 illnesses, participants are asked, "Did a doctor ever tell you that you have [illness]?" 1 = Yes, 0 = No. The total illnesses (0–11) are recoded as 0–4 = 0 and 5–11 = 1. The illnesses include hypertension, diabetes, cancer (other than a minor skin cancer), chronic lung disease, heart attack, congestive heart failure, angina, asthma, arthritis, stroke, and kidney disease. Baseline prevalence = 2.1%.

Loss of weight: "How much do you weigh with your clothes on but without shoes? [current weight]" "One year ago in (MO, YR), how much did you weigh without your shoes and with your clothes on? [weight 1 year ago]" Percent weight change is computed as: [(weight 1 year ago – current weight]/weight 1 year ago)] $\times$ 100. Percent change $>$ 5 (representing a 5% loss of weight) is scored as 1 and $<$ 5 as 0. Baseline prevalence = 21.0%.

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Association between vitamin D serum levels and insulin resistance assessed by HOMA-IR among non-diabetic adults in the United States: Results from NHANES 2007–2014

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Insulin resistance, a pathological response to insulin hormone in insulin-dependent cells, is characterized by the presence of high glucose and insulin concentrations. The homeostasis model of insulin resistance (HOMA-IR) is one of the most used indexes to estimate insulin resistance by assessing the fasting glucose and insulin levels. An association was observed between vitamin D levels and insulin resistance, which varied in different ethnic groups, and there is some evidence that vitamin D supplementation could contribute to the improvement of insulin resistance. This study assessed the association between 25-hydroxyvitamin D (25[OH]D) concentration and HOMA-IR in American adults aged 20 years and older, without diabetes and other chronic diseases that can influence insulin resistance. The data from the National Health and Nutrition Examination Survey (NHANES) 2007–2014 were used by exploiting the free and publicly-accessible web datasets. Linear regression models were performed to evaluate the association between serum 25(OH)D concentration and HOMA-IR, and a negative association was observed, which remained significant following the adjustment for age, gender, race/ethnicity, education, body mass index (BMI), physical activity, the season of examination, current smoking, hypertension, the use of drugs which can influence insulin resistance, serum bicarbonates, triglycerides, and calcium and

phosphorus levels. Only in non-Hispanic Blacks was this inverse association between vitamin D and HOMA-IR not observed in the fully adjusted model. Further studies are needed to explain the mechanisms of the observed ethnic/racial differences in the association of vitamin D levels with HOMA-IR.

KEYWORDS

vitamin D, 25-hydroxyvitamin D, insulin resistance, NHANES, cross-sectional

Introduction

Insulin resistance is identified as an underlying and partly modifiable pathogenic factor of type 2 diabetes mellitus (T2DM) and many related conditions (1). Even though hyperinsulinemic-euglycemic clamp is a gold standard for estimating insulin resistance, it is a quite expensive, invasive, and time-consuming method, which requires trained staff, and therefore, the homeostasis model of insulin resistance (HOMA-IR) presents one of the most simple and suitable substitutes to estimate IR, by assessing the fasting glucose and insulin levels (2).

Vitamin D is the collective name for vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) (3). Surveys from across the globe have shown that vitamin D deficiency was a global health problem that affects people of various ages and nationalities (4, 5). Numerous illnesses, including T2DM (6), obesity (7–9), metabolic syndrome (9, 10), chronic kidney disease (CKD) (11), infective diseases (including COVID-19) (12), autoimmune disorders (13), and infertility (14, 15), have been associated with insufficient vitamin D levels. Many cross-sectional surveys and meta-analyses indicated vitamin D deficiency to be inversely related to HOMA-IR (8, 16), and some meta-analyses (but not all) have shown that supplementation with vitamin D may help control glycemic response and can improve insulin resistance in patients with T2DM (17–19). Additionally, vitamin D receptor (VDR) polymorphisms are associated with insulin resistance and abnormal glucose metabolism, particularly in some ethnic groups (20, 21). Furthermore, a cross-sectional study in the USA, which was performed based on the National Health and Nutrition Examination Survey (NHANES) 2001–2006, found that Non-Hispanic Black people were at a greater risk for insulin resistance compared to White people (22), which may be due to lower serum vitamin D levels.

In this study, we aimed to examine the associations between 25-hydroxyvitamin D (25[OH]D) and HOMA-IR in American adults without diabetes and explore the factors that impact insulin resistance in particular ethnics, using the available data from NHANES 2007–2014, a large-scale and nationally representative cross-sectional surveys of the U.S. population. We hypothesized that the association between insulin resistance and vitamin D would differ across the ethnic groups.

Materials and methods

Data source

The National Health and Nutrition Examination Survey is an ongoing, health-related survey that assesses the nutritional and health status of the American population. Survey participants were recruited by a stratified multistage probability sampling method to ensure the sample was nationally representative (23).

The original study protocol was available on the website of the ethics review board of the national center for health statistics research (https://www.cdc.gov/nchs/nhanes/irba98.htm), which was further approved by the ethical review committee (protocol # 2005–06; protocol # 2011–17). The current study was based on the existing data retrieved from NHANES, and the details were extracted from the official website (24).

Study population

This study used public data retrieved from four cycles of NHANES (2007–2008, 2009–2010, 2011–2012, and 2013–2014). Adult patients aged 20 or older with available data for HOMA-IR and vitamin D were included. The exclusion criteria were the presence of Type 1 diabetes mellitus (T1DM) and T2DM (since in patients with diabetes, HOMA-IR may not be a representative indicator of insulin resistance due to diminished insulin secretion) (25), CKD, and the use of drugs that can influence insulin sensitivity, including antidiabetic drugs, glucose elevating agents, antineoplastics and anti-retroviral agents, adrenal cortical steroids, selective estrogen receptor

Abbreviations: AA, associate of arts; ANOVA, analysis of variance; BMI, body mass index; CKD, chronic kidney disease; GED, general educational development; HOMA-IR, homeostasis model of insulin resistance; IQR, interquartile range; NHANES, National Health and Nutrition Examination Survey; PAL, physical activity level; PTH, parathyroid hormone; ROS, reactive oxygen species; SD, standard deviation; T2DM, type 2 diabetes mellitus; VDR, vitamin D receptor; VIF, variance inflation factor; 25(OH)D, 25-hydroxyvitamin D.

modulators, parathyroid hormone and analogs, antiandrogens, aromatase inhibitors, calcimimetics, antipsychotics, other metabolic agents, bone resorption inhibitors (bisphosphonates, etc.), and niacin. Although some anti-hypertensive drugs, sex hormones (including contraceptives), and statins can influence insulin sensitivity, the subjects who used those medications were not excluded from the study, because a substantial number of the subjects were using these agents ($N = 1,081$, $N = 269$, and $N = 561$, respectively). Nevertheless, to account for their potential influence on insulin sensitivity, the usage of these drugs was included in covariates in our regression analyses. Participants with any covariates missing were excluded.

Type 2 diabetes mellitus is diagnosed based on plasma glucose levels, including either the fasting plasma glucose value or the 2-h plasma glucose value during a 75 g oral glucose tolerance test or the glycosylated hemoglobin A1c criteria (26). However, either doctor-diagnosed or self-reported diabetes is included for certain. The participants with impaired glucose tolerance or impaired fasting glucose, in case they were not using antidiabetic drugs, were included. CKD was diagnosed based on an increased albumin/creatinine ratio (≥30 mg/g) and a decreased estimated glomerular filtration rate (<60 ml/min/1.73m^2) (27). The data on the prescription medications were inquired and collected by trained interviewers.

Measurement

Plasma and serum samples for fasting plasma glucose, serum insulin, 25(OH)D, bicarbonates, total calcium, phosphorus, and triglycerides were obtained and stored in the Mobile Examination Center until shipped to the Centers for Disease Control and Prevention Environmental Health Laboratory (Atlanta, Georgia). The HOMA-IR model was used to evaluate insulin resistance, calculated using the following formula: fasting serum insulin (μU/L) × fasting plasma glucose (mmol/L)/22.5 (28). Concentrations of 25(OH)D3 and 25(OH)D2 in the serum samples were analyzed using super high-ultra performance liquid chromatography-tandem mass spectrometry. Total 25(OH)D (or vitamin D) was defined as the sum of 25(OH)D3 and 25(OH)D2. In terms of the serum total vitamin D levels, the participants were classified as deficient (<50 nmol/L), suboptimal (50–75 nmol/L), and sufficient (>75 nmol/L), as recommended by the American Endocrine Society (29).

Covariates

We tested all covariates if they were associated with HOMA-IR or vitamin D levels, and the significantly associated covariates were included in the adjusted linear regression models. The eligible covariates included age, gender, race/ethnicity, education, body mass index (BMI), physical activity level (PAL), the season of examination, current smoking, hypertension, the usage of antihypertensive drugs, sex hormones (30, 31), statins (32, 33), serum bicarbonates (34, 35), triglycerides (36, 37), and calcium and phosphorus levels (38–40). The race/ethnicity was divided into five groups: Mexican Americans, Other Hispanics, Non-Hispanic Whites, Non-Hispanic Blacks, and Other races/ethnicities (including Asians and mixes). Education levels were categorized as <9th grade, 9th−11th grade (including 12th grade with no diploma), high school graduate/general educational development (GED) or equivalent, college/associate of arts (AA) degree, college graduate or above, refused, and unknown. The season of examinations was classified into November to April and May to October. The current smokers were separated from the former and never smokers. Participants who reported smoking either some days or every day at the time of the interview were considered current smokers. Participants who smoked more than 100 cigarettes during their lifetime but did not smoke currently were former smokers. Body mass index (BMI, kg/m^2) was defined as body weight in kilograms divided by squared body height in meters. Physical activity level (PAL) scores were calculated to assess physical activity based on the different levels of activity, including vigorous (2 points) or moderate (1 point) work-related activity, vigorous (2 points) or moderate (1 point) leisure-time physical activity, and walking or bicycling for transportation (1 point). The minimum PAL score was 0, and the maximum PAL score was 5. Hypertension was defined as having systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 80 mmHg, which were measured on more than or equal to two occasions to acquire an average (41).

Statistical analysis

Median and interquartile range (IQR) were used to describe a non-normal distribution. The mean and standard deviation (SD) were used to describe a normal distribution. To compare the differences between various vitamin D status categories, the χ^2 test (for nominal data), the one-way analysis of variance (ANOVA) (for continuous variables with normal distribution), and the Kruskal-Wallis's test (for continuous variables with non-normal distribution) were used. In linear correlation analyses, any continuous variable that was not normally distributed underwent log 10 transformation to ensure its normal distribution (HOMA-IR, triglycerides). Pearson correlation coefficient (r) was used for normally distributed continuous variables, while Spearman correlation coefficient (r_s) was used for non-normally distributed continuous variables or ordered categorical variables. The Point-biserial correlation coefficient (r_{pb}) was used for dichotomous variables.

The association between total vitamin D and HOMA-IR was evaluated by employing the enter-type linear regression

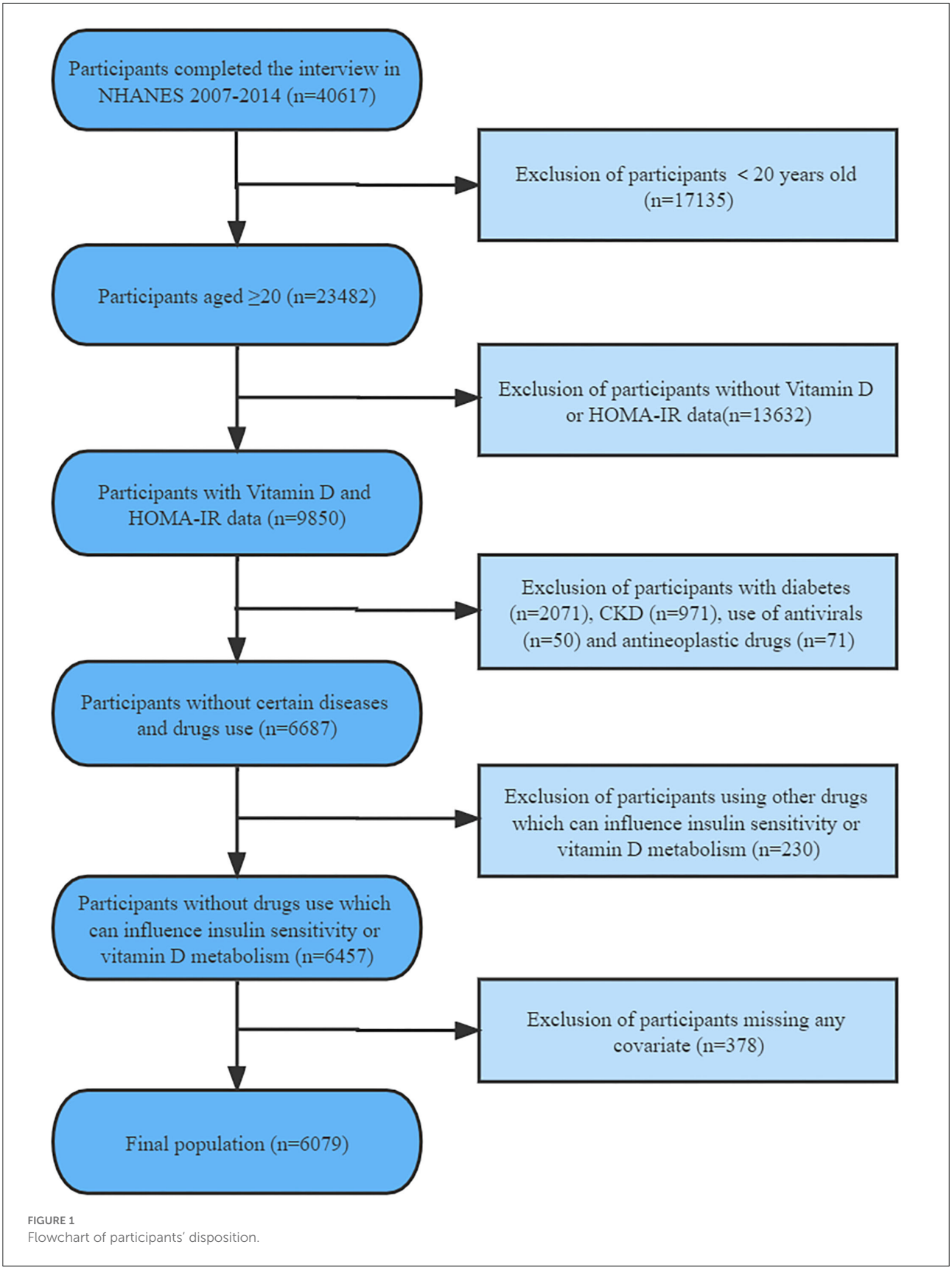

FIGURE 1
Flowchart of participants' disposition.

models. Standardized beta was utilized to compare the relative predictive strength of different covariates in the regression models. The variance inflation factor (VIF) was used to assess the multicollinearity of all covariates in the regression model. In linear regression analyses, HOMA-IR and triglycerides underwent log 10 transformation. Stratified regression analyses were used to account for differences between races. Two-tail $p < 0.05$ were considered statistically significant. All analyses were performed using Empower stats (http://www.empowerstats.net/cn/) and SPSS software Version 21.0.

Results

Baseline characteristics of participants

Following the exclusions, this study included a total of 6,079 participants aged 20 years or older (Figure 1). Baseline characteristics of the selected participants were classified according to varying serum vitamin D status categories as provided in Table 1.

The proportion of vitamin D deficiency did not differ between men and women; however, a higher proportion of vitamin D suboptimal and a lower proportion of vitamin D sufficient was found among men. The highest proportion of vitamin D deficiency was among the Non-Hispanic Blacks (63.25%), followed by Mexican Americans, other races-ethnicities (including Asians and mixes), other Hispanics, and finally, the Non-Hispanic Whites (15.25%). Meanwhile, the highest proportion of vitamin D sufficiency was observed among the non-Hispanic Whites (45.56%), while the lowest was among the non-Hispanic Blacks (11.62%).

As expected, a higher prevalence of vitamin D deficiency was observed in samples collected during the winter period (from November to April). With the exception of those who refused to answer or were left unknown about their education levels, the group with the most vitamin D deficiency belonged to the education groups of 9th−11th grade, whereas the group with the most vitamin D sufficiency was the group of college graduates or above. In the present study population, patients with hypertension and current smokers were more vitamin D deficient. Compared with the vitamin D sufficient subgroup, participants in the deficient subgroup were at the highest HOMA-IR.

Serum vitamin D levels were related to age, sex, race, educational level, BMI, PAL score, the season of examination, smoking status, usage of antihypertensive drugs, sex hormones, and statins, serum fasting insulin, triglycerides, bicarbonates, and calcium levels, as well as HOMA-IR. There was no association found between serum vitamin D levels and plasma fasting glucose, serum phosphorus, and hypertension (Table 1).

Relationship between serum 25(OH)D and HOMA-IR

Table 2 shows that all covariates, except age and serum calcium, were linearly related to HOMA-IR. Regarding the stratified racial analysis, insulin resistance was found to be different among various races: Mexican Americans and other Hispanics were more prone to higher HOMA-IR, while the Non-Hispanic Whites and other races/ethnicities (including Asiatic) were less susceptible, while the Non-Hispanic Blacks were in the middle (Table 2).

Linear regression analysis (Table 3) revealed that HOMA-IR was inversely associated with vitamin D levels prior to the adjustments for covariates (Model 1). The unadjusted model described only a small variance in HOMA-IR by using only vitamin D levels (2.8%). Following the adjustments of covariates that included age, gender, specific race/ethnicity, education, BMI, physical activity, the season of examination, current smoking, hypertension, the usage of antihypertensive drugs, sex hormones, and statins, as well as serum bicarbonates, calcium, and phosphorus levels (Model 2), this inverse association between vitamin D and HOMA-IR remained significant, although it decreased. This model explained a much higher variance in HOMA-IR (36.1%). The association of vitamin D with HOMA-IR in the fully adjusted model with added log-transformed triglycerides was even more significant since the standardized regression coefficient for vitamin D increased (Model 3). This model explained the highest percentage of variance in HOMA-IR (41.3%).

In stratified regression analyses (Table 4), only in the Non-Hispanic Blacks, there was no significant inverse association between vitamin D and insulin resistance in the fully adjusted model with serum triglycerides included (Model 3).

In the general population or ethnic subgroups, BMI contributed the most to HOMA-IR, as shown in Supplementary Tables S1, S2. The influence of vitamin D on HOMA-IR was the strongest among other races/ethnicities (including Asiatic) compared to Mexicans and other Hispanics and the Non-Hispanic Whites, while the association was not observed in the Non-Hispanic Blacks (Supplementary Table S2).

Discussion

The present study confirmed the inverse association between vitamin D and insulin resistance in accordance with many studies in different countries (42–45). However, the direct effect of vitamin D on insulin sensitivity is still controversial, since some meta-analyses indicated that vitamin D supplementation did not have the expected beneficial effects, which could be attributed to suboptimal dosing and short duration of follow-up (46, 47).

TABLE 1 Baseline characteristics of participants and distribution across different vitamin D categories.

	Total ($N = 6,079$)	Baseline serum vitamin D, nmol/L			ANOVA, Kruskal-Wallis's test or χ^2 test
		<50 ($N = 1,968$)	50-75 ($N = 2,335$)	>75 ($N = 1,776$)	*P* value
Age, Median (IQR)	43.00 (31.00–56.00)	38.00 (29.00–51.00)	42.00 (31.00–55.00)	48.00 (35.00–60.00)	<0.001
Serum vitamin D (nmol/L), Mean ± SD	63.08 ± 25.88	36.43 ± 9.18	62.27 ± 6.93	93.68 ± 20.29	<0.001
HOMA-IR, Median (IQR)	2.22 (1.40–3.66)	2.57 (1.56–4.23)	2.25 (1.44–3.66)	1.87 (1.20–3.01)	<0.001
Plasma fasting glucose (mmol/L), Mean ± SD	5.42 ± 0.54	5.42 ± 0.54	5.43 ± 0.53	5.39 ± 0.54	0.105
Serum fasting insulin (μU/L), Median (IQR)	9.28 (6.04–14.80)	10.78 (6.66–17.27)	9.45 (6.20–14.66)	7.88 (5.16–12.32)	<0.001
Serum triglycerides (mmol/L), Median (IQR)	1.08 (0.76–1.59)	1.02 (0.71–1.53)	1.14 (0.79–1.65)	1.07 (0.78–1.59)	<0.001
Serum bicarbonates (mmol/L), Mean ± SD	25.21 ± 2.10	25.02 ± 2.07	25.23 ± 2.06	25.39 ± 2.18	<0.001
Serum calcium (mmol/L), Mean ± SD	2.34 ± 0.08	2.33 ± 0.08	2.34 ± 0.08	2.36 ± 0.08	<0.001
Serum phosphorus (mmol/L), Mean ± SD	1.19 ± 0.17	1.19 ± 0.17	1.19 ± 0.17	1.19 ± 0.17	0.290
BMI (kg/m^2), Mean ± SD	28.10 ± 6.33	29.30 ± 7.32	28.18 ± 5.83	26.66 ± 5.41	<0.001
PAL score, Mean ± SD	1.74 ± 1.30	1.60 ± 1.31	1.76 ± 1.29	1.88 ± 1.29	<0.001
Gender (*N*, %)					<0.001
Men	3,037 (49.96)	962 (31.68)	1,285 (42.31)	790 (26.01)	
Women	3,042 (50.04)	1,006 (33.07)	1,050 (34.52)	986 (32.41)	
Race/Ethnicity (*N*, %)					<0.001
Mexican American	947 (15.58)	394 (41.61)	416 (43.93)	137 (14.47)	
Other Hispanics	667 (10.97)	193 (28.94)	349 (52.32)	125 (18.74)	
Non-Hispanic Whites	2,702 (44.45)	412 (15.25)	1,059 (39.19)	1,231 (45.56)	
Non-Hispanic Blacks	1,102 (18.13)	697 (63.25)	277 (25.14)	128 (11.62)	
Other races	661 (10.87)	272 (41.15)	234 (35.40)	155 (23.45)	
Season of examination (*N*, %)					<0.001
November to April	2,828 (46.52)	1,174 (41.51)	1,061 (37.52)	593 (20.97)	
May to October	3,251 (53.48)	794 (24.42)	1,274 (39.19)	1,183 (36.39)	
Education (*N*, %)					<0.001
<9th grade	514 (8.46)	180 (35.02)	236 (45.91)	98 (19.07)	
9–11th grade	878 (14.44)	328 (37.36)	338 (38.50)	212 (24.15)	
High school graduate/GED or equivalent	1,309 (21.53)	466 (35.60)	485 (37.05)	358 (27.35)	
College/AA degree	1,782 (29.31)	597 (33.50)	664 (37.26)	521 (29.24)	
College graduate or above	1,589 (26.14)	393 (24.73)	610 (38.39)	586 (36.88)	
Refused or unknown	7 (1.12)	4 (57.14)	2 (28.57)	1 (14.29)	
Hypertension (*N*, %)					0.262
Yes	1,592 (26.19)	541 (33.98)	602 (37.81)	449 (28.20)	
No	4,487 (73.81)	1,427 (31.80)	1,733 (38.62)	1,327 (29.57)	
Current smoking (*N*, %)					<0.001
Yes	1,328 (21.85)	490 (36.90)	487 (36.67)	351 (26.43)	
No	4,751 (78.15)	1,478 (31.11)	1,848 (38.90)	1,425 (29.99)	
Sex hormones (*N*, %)					<0.001
Yes	269 (4.43)	41 (15.24)	69 (25.65)	159 (59.11)	
No	5,810 (95.57)	1,927 (34.92)	2,266 (40.07)	1,617 (29.30)	
Statins (*N*, %)					<0.001
Yes	561 (9.23)	103 (18.36)	203 (36.19)	255 (45.45)	
No	5,518 (90.77)	1,856 (33.64)	2,128 (38.56)	1,534 (27.80)	
Antihypertensive drugs (*N*, %)					<0.001
Yes	1,081 (17.78)	275 (25.44)	398 (36.82)	408 (37.74)	
No	4,998 (82.22)	1,684 (33.69)	1,933 (38.68)	1,381 (27.63)	

AA, associate of arts; ANOVA, analysis of variance; BMI, Body Mass Index; GED, general educational development; HOMA-IR, the homeostasis model of insulin resistance; IQR, inter quartile range; N, number of participants; PAL, physical activity level; SD, standard deviation.

TABLE 2 Screening of covariates based on statistically significant association with log-transformed HOMA-IR.

Age[#b]	0.016	**BMI**[#a]	0.562**
Gender[#c]	−0.033*	**PAL score**[#b]	−0.132**
Race/Ethnicity		**Season of examination**[#c]	−0.052**
Mexican American[#c]	0.121**	**Current smoking**[#c]	0.047**
Other Hispanic[#c]	0.028*	**Hypertension**[#c]	−0.113**
Non-Hispanic White[#c]	−0.074**	**Antihypertensive drugs**[#c]	−0.138**
Non-Hispanic Black[#c]	0.014	**Sex hormones**[#c]	0.047**
Other races[#c]	−0.069**	**Statins**[#c]	−0.073**
Education[#b]	−0.104**	**Serum bicarbonate**[#a]	−0.116**
Less than 9th grade[#c]	0.046**	**Serum triglycerides (log–transformed)**[#a]	0.372**
9-11th grade[#c]	0.030*	**Serum calcium**[#a]	−0.016
High school graduate/GED or equivalent[#c]	0.029*	**Serum phosphorus**[#a]	−0.091**
College/AA degree[#c]	0.017		
College graduate or above[#c]	−0.100**		

**$P < 0.01$.
*$P < 0.05$.
[#a]Pearson's r.
[#b]Spearman's r_s.
[#c]Point-Biserial's r_{pb}.
AA, associate of arts; BMI, Body Mass Index; GED, general educational development; HOMA-IR, the homeostasis model of insulin resistance; PAL, physical activity level.

TABLE 3 Linear regression relationship for serum vitamin D and log-transformed HOMA-IR in models.

		Adjusted R^2	Standardized β	Non-standardized β (95% CI)
Vitamin D	Model 1[a]	0.028	−0.168	−0.002 (−0.002, −0.002)**
	Model 2[b]	0.361	−0.054	−0.001 (−0.001, 0.000)**
	Model 3[c]	0.413	−0.056	−0.001 (−0.001, 0.000)**

**$P < 0.01$.
[a]Model 1 is not adjusted.
[b]Model 2 adjusted for age, gender, specific race/ethnicity, education, BMI, physical activity, the season of examination, current smoking, hypertension, the usage of antihypertensive drugs, sex hormones, statins, serum bicarbonates, calcium, and phosphorus levels.
[c]Model 3 adjusted for age, gender, specific race/ethnicity, education, BMI, physical activity, the season of examination, current smoking, hypertension, the usage of antihypertensive drugs, sex hormones, statins, serum bicarbonates, log-transformed triglycerides, calcium, and phosphorus levels.
β, beta (regression coefficients); BMI, Body Mass Index; CI, confidence intervals; HOMA-IR, the homeostasis model of insulin resistance.

The mechanisms by which vitamin D can influence insulin sensitivity are various, and some of them are still unknown. Some studies showed that vitamin D by interacting with VDR in insulin-responsive tissues increased the transcription and number of insulin receptors (48, 49). Also, vitamin D can influence the extracellular calcium concentration and influx through the insulin-responsive cell, subsequently activating the glucose transporters, thus enhancing the response to insulin (50, 51). In addition, vitamin D could block the effect of inflammatory cytokines on insulin signaling by modulating the innate immune system and decreasing inflammatory cytokine secretion (52, 53). It is known that reactive oxygen species (ROS) can trigger insulin resistance (54), while vitamin D accelerates ROS catabolism by enhancing the synthesis of antioxidants and anti-inflammatory cytokines (55). Vitamin D can also modulate insulin sensitivity by activating peroxisome proliferator-activated receptors-δ, which reduces free fatty acid-induced insulin resistance (56, 57). Parathyroid hormone (PTH) can mediate insulin resistance by inhibiting insulin signaling and reducing glucose uptake, while vitamin D could exert an insulin-improving effect by reducing PTH levels (58). Moreover, higher PTH and vitamin D insufficiency can be jointly associated with higher HOMA-IR: the effect of PTH on insulin release from islets depends on vitamin D-related calcium and phosphorus (59, 60).

Regarding racial/ethnic differences in the association of vitamin D with HOMA-IR, one population-based investigation showed that the association between circulating 25(OH)D concentrations and insulin resistance did not differ within race (16). Conversely, other studies demonstrated that vitamin D was inversely associated with fasting insulin and insulin resistance in the Non-Hispanic Whites and Mexican Americans, but not in the Non-Hispanic Blacks (61, 62).

The reason for the lack of this association among the Non-Hispanic Blacks is still not clear. Black people have lower levels of vitamin D and higher levels of PTH compared to White people, so the negative association between vitamin D and insulin resistance should be stronger. However, in the Non-Hispanic Blacks, a decreased sensitivity to the effects of decreased vitamin D and elevated PTH was hypothesized (61, 63). Regarding 25(OH)D clearance, Black people had higher 25(OH)D clearance and lower 25(OH)D levels compared to White people, probably owing to lower levels of vitamin D binding protein (22, 62, 64, 65). The threshold for a sufficient 25(OH)D levels is the lowest among the Non-Hispanic Blacks (44), and the inverse association between 25(OH)D and PTH levels were only observed below a much lower cutoff point for vitamin D in Black people (66–68). As a result, the combined effect of PTH and vitamin D lacks in Black people.

In addition, in one study, it was observed that African Americans had significantly lower triglyceride levels for any given level of insulin sensitivity, compared with other

TABLE 4 Linear regression relationship for serum vitamin D and log-transformed HOMA-IR in stratification analysis of race/ethnicity.

	Non-standardized β (95% CI)		
	Model 1[a]	**Model 2[b]**	**Model 3[c]**
Mexican American	−0.003 (−0.004, −0.002)**	−0.001 (−0.002, 0.000)**	−0.001 (−0.002, 0.000)**
Other Hispanics	−0.002 (−0.004, −0.001)**	−0.001 (−0.002, 0.000)	−0.001 (−0.002, 0.000) ¥
Non-Hispanic Whites	−0.002 (−0.003, −0.002)**	−0.000 (−0.001, 0.000)*	−0.000 (−0.001, 0.000)*
Non-Hispanic Blacks	−0.001 (−0.002, 0.000)*	−0.000 (−0.001, 0.000)	−0.000 (−0.001, 0.000)
Other races	−0.002 (−0.003, −0.001)**	−0.001 (−0.002, 0.000)**	−0.001 (−0.002, −0.001)**

**$P < 0.01$.
*$P < 0.05$.
¥ $P = 0.051$.
[a]Model 1 is not adjusted.
[b]Model 2 adjusted for age, gender, education, BMI, physical activity, season of examination, current smoking, hypertension, the usage of antihypertensive drugs, sex hormones, statins, as well as serum bicarbonates, calcium, and phosphorus levels.
[c]Model 3 adjusted for age, gender, education, BMI, physical activity, season of examination, current smoking, hypertension, the usage of antihypertensive drugs, sex hormones, statins, as well as serum bicarbonates, log-transformed triglycerides, calcium, and phosphorus levels.
β, beta (regression coefficients); BMI, Body Mass Index; CI, confidence intervals; HOMA-IR, the homeostasis model of insulin resistance.

races/ethnicities (69, 70), and in another study, it was observed that low levels of triglyceride could slightly modify the association of 25(OH)D with insulin resistance (71), which probably could explain why there was no significant association between HOMA-IR and vitamin D in the Non-Hispanic Blacks. Nonetheless, even though adding the triglyceride levels in the regression model slightly increased the association between vitamin D and HOMA-IR (as assessed by standardized beta coefficients) in the whole studied sample, adding triglyceride levels in the model still did not make this association significant in the Non-Hispanic Blacks. Therefore, other factors can contribute more to the observed disparities in the Non-Hispanic Blacks. Various types of VDR genotypes and their related variants were related to the development of insulin resistance, which may potentially affect the individual response to vitamin D supplements (72, 73), and there probably could be racial disparities in the VDR polymorphism responsible for the lower association of vitamin D levels with HOMA-IR (21, 74–77). In addition, HOMA-IR mainly reflects hepatic insulin resistance, whereas vitamin D is more associated with insulin-mediated peripheral glucose uptake (78). Similarly, as for lower levels of serum triglycerides, intrahepatic fat, and intraperitoneal fat (70), it was shown that Black people have lower hepatic glucose production compared with other races/ethnicities, despite decreased whole-body insulin sensitivity and decreased peripheral (glucose disposal) and hepatic (suppression of glucose production) insulin sensitivity, compared with White people with the same body composition (79). Additionally, they have lower hepatic insulin clearance and increased insulin production, which probably could lead to increased insulin resistance, since chronic hyperinsulinemia can lead to insulin receptor desensitization (80, 81). Therefore, studies that include hyperinsulinemic-euglycemic clamp in the Non-Hispanic Blacks are needed to test if they will reveal different results compared with our study.

This study also found that Mexican Americans were more prone to be resistant to insulin, while non-Hispanic Whites were less susceptible. Mexican Americans have higher blood glucose levels and a greater family history of obesity, diabetes, and insulin resistance compared with the Non-Hispanic Whites (82). Mexican Americans with higher insulin levels were more likely to develop T2DM about 3–5 times more than the non-Hispanic Whites (82). A study about the genetics of variation in Mexican Americans demonstrated the importance of identifying HOMA-IR linkage on chromosome 12q24, as this region contained multiple candidate genes associated with obesity and diabetes (83).

This study has some potential limitations. The inherent properties of cross-sectional designs did not allow for verifying the causal relationships between vitamin D and insulin resistance. The study was limited to the non-diabetic population in the US, and the results cannot be extrapolated to the world; hence, larger multicenter analyses included would be more universally applicable. Additionally, HOMA-IR is only a substitute for a gold standard–a hyperinsulinemic-euglycemic clamp. The strengths are that we controlled for possible confounders and that we used a large scale and representative sample with precise super high-ultra performance liquid chromatography-tandem mass spectrometry for measurements of vitamin D serum levels.

In conclusion, race/ethnicity affected the negative association of vitamin D with insulin resistance assessed by HOMA-IR among the USA non-diabetic adults, as the negative association was not seen among the Non-Hispanic Blacks. While additional studies are required to verify the results of this study and explain the racial disparities, monitoring

serum 25(OH)D may be useful in detecting those with vitamin D deficiency, starting with timely and adequate supplementation to prevent possible negative metabolic consequences.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Review Board of the National Center for Health Statistics Research (https://www.cdc.gov/nchs/nhanes/irba98.htm). The patients/participants provided their written informed consent to participate in this study.

Author contributions

XY: conceptualization, methodology, formal analysis, investigation, and data curation, writing—original draft, writing—review and editing, visualization, supervision, and project administration. J-YC: formal analysis, software, validation, investigation, visualization, and writing—original draft. X-JH: programming, data curation, and writing—review and editing. JH-L, CH, WY, and N-XL: writing—original draft and writing—review and editing. W-CH: conceptualization. X-GG: project design and administration. All authors contributed to the article and approved the submitted version.

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/fnut.2022.883904/full#supplementary-material

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Association of serum total 25-hydroxy-vitamin D concentration and risk of all-cause, cardiovascular and malignancies-specific mortality in patients with hyperlipidemia in the United States

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Background: Vitamin D (VD) plays an important role in decreasing the risk of adverse events for various metabolic diseases. However, for patients with hyperlipidemia, the relationship between the main VD storage within the body known as serum 25-hydroxy-VD [25(OH)VD] and the risk of all-cause, cardiovascular and malignancies-specific mortality is still unclear.

Materials and methods: A total of 6740 participants above the age of 20 years with hyperlipidemia who completed the National Health and Nutrition Examination Survey (NHANES) between 2007 and 2016 and were followed up until 2019 were included in the study. The weighted Cox proportional hazards regression model and weighted competing risk regression model were used to evaluate the risk for all-cause, cardiovascular and malignancy-related mortality in relation to the serum 25(OH)VD. The model was adjusted according to age, gender, race, body mass index, lipids status, medication usage, the Charlson comorbidity index and healthy eating index. The last restricted cubic spline (RCS) method was used to present the relationship between hazard ratios (HR) associated with diverse cause-specified modalities and the serum 25(OH)VD levels.

Results: Serum 25(OH)VD was identified as an independent factor for mortality. Lower serum 25(OH)VD under the threshold of 25.6 and 25.2 ng/ml were significantly associated with a higher risk for all-cause and cardiovascular mortalities, respectively. However, no association was found between malignancy-specific mortality and serum 25(OH)VD.

Conclusion: Serum 25(OH)VD were identified as an independent factor associated with risks of all-cause and cardiovascular mortalities in patient with hyperlipidemia. Moreover, lower serum 25(OH)VD than 25.6 and 25.2 ng/mL

were, respectively, associated with a gradual increase in a risk for all-cause and cardiovascular mortality in patients with hyperlipidemia, and therefore regular monitoring of VD levels and correction of VD deficiency is recommended in those patients.

KEYWORDS

vitamin D, hyperlipidemia, NHANES, cardiovascular mortality, all cause mortality

Introduction

Hyperlipidemia (HL) is a common metabolic disease characterized by dysregulation of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triacylglycerol (TG), and cholesterol (CHL). Over 100 million people were diagnosed with hypercholesterolemia (total CHL levels > 240 mg/dL) in 2017, and another 31 million adults were diagnosed with elevated LDL-C levels in the United States (1). HL can reduce the patients' quality of life and increases their risk of developing cardiovascular disease by 2 to 3 times (2). Therefore, there is a need to develop treatment strategies to control blood lipid levels in patients with HL to improve their survival.

Studies have shown that several nutrients, such as vitamin D (VD), may have an important role in lowering lipid levels and in reducing the risk of mortality (3), especially in patients with hypertension (4) and type-2 diabetes (5). Therefore, VD supplements are often prescribed to patients with metabolic diseases suffering from VD deficiency. VD is stored in the body as 25-hydroxy-vitamin D [25(OH)VD], which encompasses both 25(OH)VD3 and 25(OH)VD2 forms (6). Because of its stable nature and long half-life, serum 25(OH)VD could be used as an optimal indicator for monitoring of VD levels. However, it is still debatable whether the administration of VD supplements could improve hypertension (7), insulin sensitivity (8), or lipid parameters (9). Therefore, the benefit of VD supplements on metabolic disorders is still unclear. In addition, relatively few studies evaluated the relationship between serum 25(OH)VD and the risk of mortality in patients with HL and VD deficiency. Furthermore, numerous factors can affect the normal VD levels, including age, diet, nutritional status, and underlying comorbidities. However, these factors have not been totally taken into account in current risk survival models.

Therefore, this study aimed to analyze the association between serum 25(OH)VD and the risk of all-cause and disease-specific (cardiovascular and malignancy) mortality in patients with HL in the United States (US) to guide the use of VD supplements in patients with HL.

Materials and methods

Data collection

The data was obtained from the National Health and Nutrition Examination database (NHANES). The NHANES was a national survey conducted by the National Center for Health Statistics to assess the health and nutritional status of adults and children in the US. The survey collected data on participants' demographics, socioeconomic status, diet, general health, medical examinations, and laboratory tests.

Participants above the age of 20 years with HL who completed the National Health and Nutrition Examination Survey (NHANES) between 2007 and 2016 and were followed up until 2019 were included in the study. HL was defined as a low-density lipoprotein-cholesterol (LDL-C) $\geq$ 130mg/dL (3.37 mmol/L), triglyceride (TG) $\geq$ 150mg/dL (1.7 mmol/L), total cholesterol (TC) $\geq$ 200 mg/dL (5.18mmol/L) or high-density lipoprotein-cholesterol (HDL-C) < 40mg/dL (1.04mmol/L) in males and 50 mg/dL (1.30mmol/L) in females (10). The participants' demographic information, including age at participating in the survey, ethnicity, gender, and body mass index (BMI), were extracted from the survey. Moreover, serum 25(OH)VD level, daily VD intake, comorbidities, and medication usage (in the past 30 days) were also extracted. The Charlson comorbidity index (CCI) were calculated according to questionnaire survey and examination, the healthy eating index (HEI) were calculated according to the HEI-2015 guidelines (11). Daily VD intake and dietary nutrients intake were calculated the average values using the data obtained from two 24h dietary recall interviews.

The follow-up survival data were obtained from the National Center for Health Statistics. The International Classification of Diseases version 10 (ICD-10) was used to classify the causes of death into

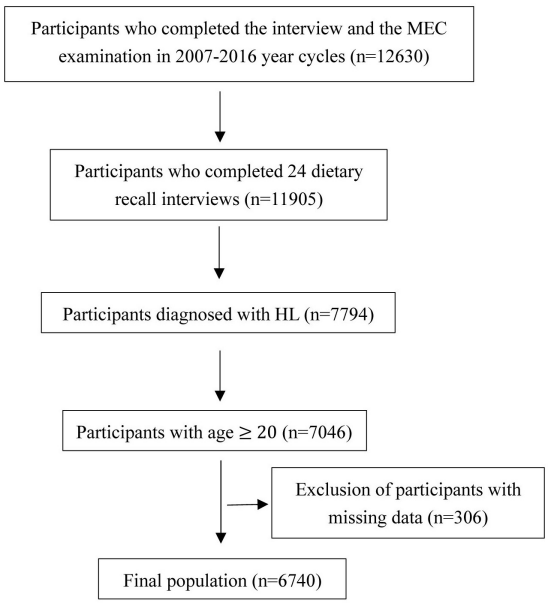

FIGURE 1
Flowchart of participants enrollment.

cardiovascular disease (ICD CODES I00-I09, I11, I13, I20-I51, and I60-I69) and malignancy-related (ICD coded C00-C97). Collection of data was shown in **Figure 1**.

Measurement methods of blood indicators

The serum 25(OH)VD was defined as the sum of serum 25(OH)D2, 25(OH)D3, and epi-25(OH)D3. All the measurements were acquired using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The serum levels of HDL-C, TC, and TG were tested using the Roche Modular P Chemistry Analyzer for enzymatic assays (12). LDL-C is calculated from measured values of TC, TG, and HDL-C according to the Friedewald calculation: [LDL-C] = [TC] – [HDL-C] – [TG/5] (13), where all values are expressed in mg/dL. The calculation is valid for TG levels less than or equal to 400 mg/dL, so only for subjects with TG levels lower of 400 mg/dl, were the data for LDL-C provided in the NHANES datasets.

Statistical analysis

Vitamin D status was defined as "deficient" [serum 25(OH)VD < 20 ng/ml], "insufficient" [serum 25(OH)VD = 20–30 ng/ml] and "sufficient" [serum 25(OH)VD > 30 ng/ml] (14). The serum 25(OH)VD levels were divided into five groups by the quintile values. The participants' age, race, gender, BMI, CCI, HEI, lipid status, the length of follow-up and medication usage were treated as covariates. Moreover, BMI was categorized into 3 groups: <25 (normal), 25–29.9 (overweight) and ≥30.0 (obesity) (15); HEI was categorized into 3 groups: <50.0 (poor dietary), 50.0–79.9 (need to improve) and ≥80.0 (healthy dietary) (16); VD intake was also categorized into 3 groups: <10 μg/d (less than the US Estimated Average Requirement, EAR), 10–15 μg/d and >15 μg/d (above the US Recommended Dietary Allowance, RDA) (17, 18). The continuous data were evaluated for normality. The association between continuous variables was calculated using the analysis of variance (ANOVA), and the Chi-square test was used to compare the categorical variables. The Pearson's correlation coefficient was used to calculate the correlation between the normally distributed continuous variables and the Spearman's correlation coefficient was used to calculate the correlation between the categorical variables and non-normally distributed continuous variables.

The risk for all cause-specific mortality in relation to the serum 25(OH)VD was estimated by the Cox proportional hazard (COXPH) model. For the malignancy/cardiovascular-specific mortalities, the competing risk regression (CRR) model was used to estimate the hazard ratios (HRs) and their 95% confidence intervals (95% CI). The sample weight was taken into account in the above models. The time dependent receiver operation curves (ROC) were calculated, then area under curve (AUC) of ROC by each time point were depicted for examining discriminative abilities of models, a bigger AUC curve indicated higher discriminative ability (19). Moreover, calibration curves were also depicted for tested models, the curves more closed to diagonal suggested the better calibration of models. Finally, the restricted cubic splines (RCS) with the unweighted Cox proportional hazard models were depicted to directly present the dose-response associations between serum 25(OH)VD and HRs of all cause and malignancy/cardiovascular-specific mortalities. Apart from including the length of follow-up, the models were adjusted for mortality covariates, including age, gender, race, the HEI and CCI scores, lipid status and medication usage. Since mortality in our study was a rare event, the Poisson's regression model was also used to estimate the incidence-rate mortality ratio. A two-sided *p*-value below 0.05 was considered statistically significant. The data were analyzed using the R software (version:4.2.1). The COX PHM was performed using the "rms" R package, and the Poisson's regression model was performed by the "lms" R package. The time-AUC of ROC and calibration curves were calculated using the "riskRegression" R package, The RCS model was built using the "survminer" R package, and the Pearson's correlation coefficient map was plotted *via* the "corrplot" R package.

Results

Characteristics of the participants

A total of 6740 participants were included in the study. The characteristics of the participants are summarized in Table 1. The included participants were 20-80 years old. Both genders were quite equally represented, and majority of the participants were non-Hispanic whites (46.8%). Almost 4/5 of the included participants were overweight or obese (77.1%). Almost half of the participants had a CCI score of 0 (45.2%), while based on the HEI scores, only small percentage of the participants (3.4%) were classified as healthy with an HEI score above 80. Only less than a third of the participants (29.8%) have received hypolipidemic medication treatment, such as statins (91.7%), fenofibrate (2.0%) and gemfibrozil (1.8%). The mean serum 25(OH)VD in the whole sample was 26.7 ng/mL, while the serum 25(OH)VD quintile levels were at 17.3, 23.2, 28.3, and 35.0 ng/mL. Only about 1/3 of the participants (34.7%) had VD sufficient status, while VD insufficiency and deficiency were detected in 36.6% and 28.6% of the participants, respectively. The non-Hispanic whites had the highest VD levels, while non-Hispanic Blacks and Mexican Americans had the lowest levels, with mean values of 30.6 $\pm$ 0.6, 21.1 $\pm$ 0.5 and 22.8 $\pm$ 0.4 ng/mL, respectively ($p < 0.01$). Obese people had the lowest vitamin D levels, compared with normal weight and overweight with mean values of 24.6 $\pm$ 0.4, 29.6 $\pm$ 0.4, and 27.4 $\pm$ 0.4 ng/mL, respectively ($p < 0.01$). The mean and median values of participants' daily VD intake were 4.7 $\pm$ 1.0 and 3.6 μg/d, respectively. The daily VD intake was less than the EAR (10 μg/d) in most of the participants (91.2%), while only 3.1% of the participants had intake above the RDA (15 μg/d). Participants with highest VD levels (higher quintiles) had higher HDL-C and lower TG levels. In general, with increase of serum 25(OH)VD, there was a trend for increased HDL-C and TC, and decreased TG and LDL-C levels; there was also a trend for increased HEI score, VD intake, and decreased BMI ($p < 0.01$). During the 51372 person-years of follow-up, 752 deaths were recorded (overall, in 11.2% of participants), of which 41.9% were due to cardiovascular disease, 31.3% were caused by malignancies, and 26.9% were attributed to other factors.

Correlations between variables in the present study

The results of the Pearson's and Spearman's inter-correlations between the included variables are summarized in Table 2. In summary, serum 25(OH)VD positively correlated with age, Non-Hispanic White race, CCI score, HEI score, HDL-C levels, TC levels, hypolipidemic drugs usage, while negatively correlated with male sex, other races except Non-Hispanic White, BMI, LDL-C and TG levels with statistical significance (Table 2). In regression models, the variance inflation factors between these variables were also calculated, and were less than 3 for all variables included: serum 25(OH)VD (1.28), gender (1.53), age (1.43), Non-Hispanic White (2.74), Non-Hispanic Black (2.55), other races (2.03) BMI (1.13), CCI (1.33), HEI (1.10), medication usage (1.15), HDL-C (1.81), LDL-C (1.87), TG (1.15), TC (2.04) and the length of follow-up (1.02), respectively. These findings suggest that the variables in the present study were independent, so multicollinearity was not an issue.

Association between serum 25-hydroxy-vitamin D with mortality risk for patients with hyperlipidemia

As shown in Table 3, serum 25(OH)VD levels were significantly associated with all-cause and cardiovascular-specific mortality in participants with HL. After fully adjusting the COXPH and CRR models for the potential confounders, the risk for all-cause mortality significantly increased with 25(OH)D levels below 23.1 ng/mL (model 2), while the risk for cardiovascular mortality significantly increased with 25(OH)D levels below 17.3 ng/mL (model 3). The risk for overall mortality increased in the first 2 quintiles, with gradual change, and then stabilized, while the risk for cardiovascular mortality significantly increased only in the first quintile. However, no significant association was noted between serum 25(OH)VD and the risk of malignancies-related mortality. The results were still robust after multivariable- adjusted estimation by different regression models. All models were tested their discriminative and calibration abilities, and the fully adjusted COXPH and CRR models presented better predictive performance (Figure 2).

Non-linear association between serum 25-hydroxy-vitamin D mortality hazard ratios

Restricted cubic spline (RCS) showed an "L-shape" association between the serum 25(OH)VD levels and all causes-specific and cardiovascular-specific mortality rates. The HRs for all causes and the cardiovascular-specific mortality significantly increased as serum 25(OH)VD decreased, at thresholds of 25.6 and 25.2 ng/mL, respectively. Above those thresholds, the HRs remained relatively stable for all mortalities. The similar association was noted between the malignancies-specific mortality HRs and serum 25(OH)VD, with the threshold below 25.6 ng/mL, but that

TABLE 1 The baseline of participants with hyperlipidemia according to serum 25(OH)VD.

	Serum 25(OH)VD (ng/mL)						
	Total	**<17.3**	**17.3–23.1**	**23.2–28.2**	**28.3–35.0**	**>35.0**	***p*-value**
Number of cases	6740	1343 (19.9)	1353 (20.0)	1352 (20.1)	1344 (19.9)	1348 (20.0)	
Age	53.1 (0.2)	48.3 (0.4)	49.5 (0.4)	52.4 (0.4)	55.0 (0.4)	60.6 (0.4)	
20–40	1787 (26.5)	481 (35.8)	463 (34.2)	362 (26.8)	299 (22.3)	182 (13.5)	<0.01
41–60	2482 (36.8)	513 (38.2)	515 (38.1)	517 (38.2)	507 (37.7)	430 (31.9)	
> 61	2471 (36.7)	349 (26.0)	375 (27.7)	473 (35.0)	538 (40.0)	736 (54.6)	
Gender							
Female	3994 (53.0)	808 (53.8)	772 (51.3)	729 (48.1)	756 (50.2)	929 (61.8)	<0.01
Male	3539 (47.0)	695 (46.2)	732 (48.7)	788 (51.9)	749 (49.8)	575 (38.2)	
Race/ethnicity							
Non-Hispanic White	3119 (46.3)	296 (22.0)	453 (33.5)	623 (46.1)	817 (60.8)	930 (69.0)	<0.01
Non-Hispanic Black	1192 (17.7)	504 (37.5)	257 (19.0)	189 (14.0)	121 (9.0)	121 (9.0)	
Mexican American	1017 (15.1)	279 (20.8)	280 (28.2)	233 (17.2)	146 (10.9)	79 (5.9)	
Others[1]	1412 (21.0)	264 (19.7)	363 (26.8)	307 (22.7)	260 (19.4)	218 (16.2)	
BMI	30.0 (0.2)	32.1 (0.3)	30.9 (0.2)	29.7 (0.2)	29.3 (0.2)	28.1 (0.2)	
<25.0	1544 (22.9)	234 (17.4)	220 (16.3)	307 (22.7)	344 (25.6)	439 (32.6)	<0.01
25.0–29.9	2297 (34.1)	373 (27.8)	478 (35.3)	461 (34.1)	488 (36.3)	497 (36.9)	
=30.0	2899 (43.0)	736 (54.8)	655 (48.4)	584 (43.2)	512 (38.1)	412 (30.6)	
CCI	1.2 (1.4)	1.1 (1.4)	1.2 (1.3)	1.3 (1.3)	1.6 (1.1)	1.3 (1.3)	
0	3046 (45.2)	685 (51.0)	684 (50.5)	630 (46.6)	591 (44.0)	456 (33.8)	<0.01
1	1465 (21.7)	258 (19.2)	309 (22.8)	284 (21.0)	288 (21.4)	326 (24.2)	
2	909 (13.5)	172 (12.8)	159 (11.8)	189 (14.0)	182 (13.5)	207 (15.4)	
=3	1320 (19.6)	228 (17.0)	201 (14.9)	249 (18.4)	283 (21.1)	359 (26.6)	
HDL (mg/dL)	52.4 (0.2)	50.1 (0.4)	49.3 (0.4)	50.6 (0.4)	53.6 (0.5)	58.4 (0.5)	<0.01
LDL (mg/dL)	121.8 (0.5)	123.6 (1.0)	123.0 (1.0)	121.9 (1.0)	121.3 (1.0)	119.2 (1.0)	<0.01
TG (mg/dL)	133.1 (0.8)	133.1 (1.9)	134.3 (1.9)	135.7 (1.8)	133.5 (1.8)	129.1 (1.7)	<0.01
TC (mg/dL)	200.8 (0.5)	200.2 (1.2)	199.3 (1.2)	199.6 (1.2)	201.6 (1.2)	203.4 (1.2)	<0.01
Healthy eatig index	54.5 (0.2)	50.9 (0.3)	53.4 (0.2)	54.8 (0.2)	55.7 (0.2)	57.6 (0.2)	
<50.0	2613 (38.8)	659 (49.1)	557 (41.2)	510 (37.7)	484 (36.0)	403 (29.9)	<0.01
50.0–79.9	3899 (57.9)	661 (49.2)	759 (56.1)	794 (58.7)	811 (60.3)	874 (64.8)	
=80.0	228 (3.4)	23 (1.7)	37 (2.7)	48 (3.6)	49 (3.7)	71 (5.3)	
Vitamin D intake (μg/d)	4.7 (1.0)	3.6 (0.9)	4.5 (1.0)	5.1 (0.9)	5.2 (1.0)	5.0 (0.9)	
Median	3.6	2.7	3.4	4.0	4.0	3.8	
<10	6146 (91.2)	1289 (96.0)	1246 (92.1)	1203 (89.0)	1193 (88.8)	1215 (90.1)	<0.01
10–15	384 (5.7)	34 (2.5)	71 (5.3)	98 (7.3)	102 (7.6)	79 (5.9)	
>15	210 (3.1)	20 (1.5)	36 (2.7)	51 (3.8)	49 (3.7)	54 (4.0)	
Medication usage							<0.01
No	2378 (35.3)	633 (47.1)	589 (43.5)	512 (37.9)	398 (29.6)	246 (18.3)	
hypolipidemic medications	2010 (29.8)	291 (21.7)	310 (22.9)	383 (28.3)	449 (33.4)	577 (42.8)	
Others[2]	2352 (34.9)	419 (31.2)	454 (33.6)	457 (33.8)	497 (37.0)	525 (39.0)	
Outcomes							<0.01
Alive	5988 (88.8)	1189 (88.5)	1226 (90.6)	1201 (88.8)	1200 (89.3)	1172 (86.9)	
Cardiovascular mortality	315 (4.7)	68 (5.1)	62 (4.6)	71 (5.3)	52 (3.9)	62 (4.6)	
Malignancies-specified mortality	235 (3.5)	53 (4.0)	32 (2.4)	48 (3.6)	48 (3.6)	54 (4.0)	
Other causes	202 (3.0)	33 (2.5)	33 (2.4)	32 (2.4)	44 (3.3)	60 (4.5)	
Length of follow-up							<0.01
7–12 years	4206 (62.4)	872 (64.9)	867 (64.1)	882 (65.2)	840 (62.5)	745 (17.7)	
3–6 years	2534 (37.6)	471 (35.1)	486 (35.9)	470 (34.8)	504 (37.5)	603 (23.8)	

Measurement data were as recorded means (SE) and count data were numbers (percentage); 1, Other races indicated Multi-Racial population and Hispanics; 2, indicated drugs except hypolipidemic medications. CCI, Charlson Comorbidity Index; HEI, healthy eating index; BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

TABLE 2 The correlation coefficients between variables in the present study.

	Serum 25 (OH)VD	Male	Age	Non-Hispanic White	Non-Hispanic Black	Mexican American	Other races[1]	CCI	BMI	hypolipidemic medication usage	HEI	HDL-C	LDL-C	TG	TC	Length of follow-up
Serum 25(OH)VD	1.000	**−0.046**	**0.259**	**0.353**	**−0.263**	**−0.156**	**−0.049**	**0.114**	**−0.203**	**0.153**	**0.137**	**0.212**	**−0.033**	**−0.027**	**0.045**	**0.065**
Male	**−0.046**	1.000	−0.001	0.023	**−0.035**	−0.003	0.007	0.009	**−0.077**	**−0.127**	**−0.099**	**−0.290**	**−0.030**	**0.109**	**−0.103**	−0.007
Age	**0.259**	−0.001	1.000	**0.187**	**−0.038**	**−0.128**	**−0.081**	**0.431**	**−0.072**	**0.265**	**0.179**	**0.189**	**−0.129**	**−0.031**	**−0.049**	**0.031**
Non-Hispanic White	**0.353**	0.023	**0.187**	1.000	**−0.430**	**−0.391**	**−0.478**	**0.137**	**−0.041**	**0.159**	**−0.060**	0.020	**−0.052**	**0.041**	**−0.024**	**−0.042**
Non-Hispanic Black	**−0.263**	**−0.035**	**−0.038**	**−0.430**	1.000	**−0.195**	**−0.239**	−0.027	0.141	**0.023**	**−0.030**	**0.079**	**0.031**	**−0.187**	−0.002	0.014
Mexican American	**−0.156**	−0.003	**−0.128**	**−0.391**	**−0.195**	1.000	**−0.217**	**−0.076**	**0.049**	**−0.139**	−0.005	**−0.080**	0.009	**0.091**	**0.005**	**−0.029**
Other races[1]	**−0.049**	0.007	**−0.081**	**−0.478**	**−0.239**	**−0.217**	1.000	**−0.076**	**−0.124**	**−0.093**	**0.106**	−0.028	0.028	**0.045**	**0.027**	**0.064**
CCI	**0.114**	0.009	**0.431**	**0.137**	**−0.027**	**−0.076**	**−0.076**	1.000	**0.118**	**0.276**	**0.008**	−0.030	−0.175	**0.062**	−0.143	**0.036**
BMI	**−0.203**	**−0.077**	**−0.072**	**−0.041**	**0.141**	**0.049**	**−0.124**	**0.118**	1.000	**0.064**	**−0.112**	−0.257	−0.042	**0.127**	**−0.095**	**0.028**
hypolipidemic medication usage	**0.153**	**−0.127**	**0.265**	**0.159**	0.023	**−0.139**	**−0.093**	**0.276**	**0.064**	1.000	**0.035**	0.079	−0.009	**0.033**	**0.033**	0.004
HEI	**0.137**	**−0.099**	**0.179**	**−0.060**	**−0.030**	−0.005	**0.106**	**0.008**	**−0.112**	**0.035**	1.000	0.141	−0.023	**−0.061**	0.015	**−0.027**
HDL-C	**0.212**	**−0.290**	**0.189**	0.020	**0.079**	**−0.080**	−0.028	−0.030	−0.257	0.079	0.141	1.000	0.113	**−0.394**	**0.358**	0.012
LDL-C	**−0.033**	**−0.030**	**−0.129**	**−0.052**	**0.031**	0.009	0.028	−0.175	−0.042	−0.009	−0.023	0.113	1.000	**0.046**	**0.923**	**−0.043**
TG	**−0.027**	**0.109**	**−0.031**	**0.041**	**−0.187**	**0.091**	**0.045**	**0.062**	**0.127**	**0.033**	**−0.061**	**−0.394**	**0.046**	1.000	**0.205**	**−0.106**
TC	**0.045**	**−0.103**	**−0.049**	**−0.024**	−0.002	**0.005**	**0.027**	**−0.143**	**−0.095**	**0.033**	0.015	**0.358**	**0.923**	**0.205**	1.000	−0.054
Length of follow-up	**0.065**	−0.007	**0.031**	**−0.042**	0.014	**−0.029**	**0.064**	**0.036**	**0.028**	0.004	**−0.027**	0.012	**−0.043**	**−0.106**	−0.054	1.000

The correlations among continuous variables were calculated the Pearson correlation coefficients and categorical variables were calculated the Spearman correlation coefficients; 1, other races indicated Multi-Racial population and Hispanics; Bold font means $p < 0.05$. CCI, Charlson Comorbidity Index; HEI, healthy eating index; BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

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TABLE 3 Association between different levels of 25(OH)VD with risk of all-causes, cardiovascular diseases, and malignancy related mortalities in patients with HL.

		Serum 25(OH)VD (ng/mL)			
	>35.0	<17.3	17.3–23.1	23.2–28.2	28.3–35.0
All-cause mortality					
Model 1	1.00	**2.18 (1.62–2.95)**	**1.70 (1.28–2.25)**	1.25 (0.96–1.63)	0.98 (0.75–1.28)
Model 2	1.00	**2.06 (1.51–2.82)**	**1.65 (1.22–2.22)**	1.19 (0.91–1.56)	0.95 (0.72–1.25)
Cardiovascular mortality					
Model 1	1.00	**2.92 (1.77–4.84)**	1.26 (0.72–2.22)	1.43 (0.91–2.25)	1.14 (0.72–1.81)
Model 2	1.00	**2.52 (1.49–4.26)**	1.18 (0.67–2.10)	1.31 (0.82–2.10)	1.07 (0.67–1.71)
Model 3	1.00	**2.54 (1.50–4.24)**	1.09 (0.61–1.94)	1.32 (0.82–2.12)	1.11 (0.69–1.78)
Malignancies-specific mortality					
Model 1	1.00	1.28 (0.71–2.29)	1.16 (0.65–1.91)	0.69 (0.39–1.22)	0.91 (0.57–1.45)
Model 2	1.00	1.23 (0.64–2.38)	1.09 (0.62–1.94)	0.66 (0.36–1.19)	0.84 (0.53–1.36)
Model 3	1.00	1.16 (0.61–2.25)	1.02 (0.58–1.80)	0.63 (0.35–1.15)	0.86 (0.53–1.38)

Model 1: adjusted for age, race, gender and the length of follow-up by weighted COXPH model; Model 2: further adjusted from model 1 for BMI, CCI, HEI, medication usage, HDL-C, LDL-C, TG and TC status by weighted COXPH; Model 3: further adjusted from model 1 for BMI, CCI, HEI, medication usage, HDL-C, LDL-C, TG and TC status by weighted CRR model for cardiovascular/malignancies-specific mortality; Bold font means $p < 0.05$. CCI, Charlson Comorbidity Index; HEI, healthy eating index; BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; COXPH, Cox proportional hazard; CRR, competing risk regression.

association only approached statistical significance ($p = 0.068$) (**Figure 3**).

Discussion

Vitamin D (VD) plays an important role in pathophysiology of metabolic diseases, including diabetes, hyperuricemia, HL, obesity and metabolic syndrome (20–22). However, the relationship between serum 25(OH)VD and mortality of patients with HL are still unknown and may vary depending on the patients' clinical (23, 24), demographic (25), and lifestyle characteristics (26). To address this issue, we performed a large-scale cohort analysis of a representative adult population in the United States. Our findings indicate the serum 25(OH)VD status as an independent risk factor associated with all-cause and cardiovascular mortality in patients with HL. The findings were adjusted for known covariables, including the participants' demographics, dietary style, as well as clinical features such as BMI, lipids status, medication usage, and comorbidities. Moreover, our study also indicated non-linear relationships between serum 25(OH)VD and the HRs of all-cause and cardiovascular mortality in patients with HL, showing a threshold in serum 25(OH)VD below which the risk for mortality exponentially increases.

Several studies have shown that higher vitamin D level was associated with decreased mortality in patients with metabolic diseases, such as type II diabetes (5) and hypertension (27). However, it is still debatable whether VD supplementation could reduce the risk of mortality in patients with cardiometabolic diseases (28–30). Some studies have shown that low VD levels are associated with worse clinical presentations in several diseases, including asthma (31), COVID-19 infection (32), and hyperuricemia (33), and that VD supplementation can improve the clinical outcomes. However, many studies also highlighted that the benefits of VD may be overestimated (17, 34). Hence, these studies suggest that VD does not have an impact on the disease itself, but the elimination of VD deficiency is what actually improves survival. Our findings also supported this view. Serum 25(OH)VD levels below 25.6 and 25.2 ng/mL had a strong impact on the increased HRs for all-cause and cardiovascular mortalities, respectively. These findings were consistent with those reported by Wan (5), Zhao (4), and Al-khalidi (35). Moreover, Jani et al. (36) performed a meta-analysis, which showed that the lower level of circulating 25(OH)VD was dose-dependently associated with the higher risks of fatal, non-fatal and recurrent cardiovascular disease, while the thresholds varied for different outcomes. Heath et al. (37) made another meta-analysis that showed an inverse relationship between circulating 25(OH)VD status and cancer-specific mortality with moderate evidence, while for cardiovascular mortality, there was weak evidence showing its association with 25(OH)VD.

As for cancer mortality, in the present study we observed also an inverse association between lower serum 25(OH)VD (below 25.6 ng/mL) and cancer mortality, but this association only approached statistical significance, which might be due to the limited cases. Additionally, although several studies have shown that lower serum 25(OH)VD were related to increased cancer mortality, not all studies confirmed that, and the associations varied depending on the type of malignancy, race, sex, season/latitude and present co-morbidities (38–43). Furthermore, the U-shaped association between VD levels and overall and cancer-mortality have been shown in some studies

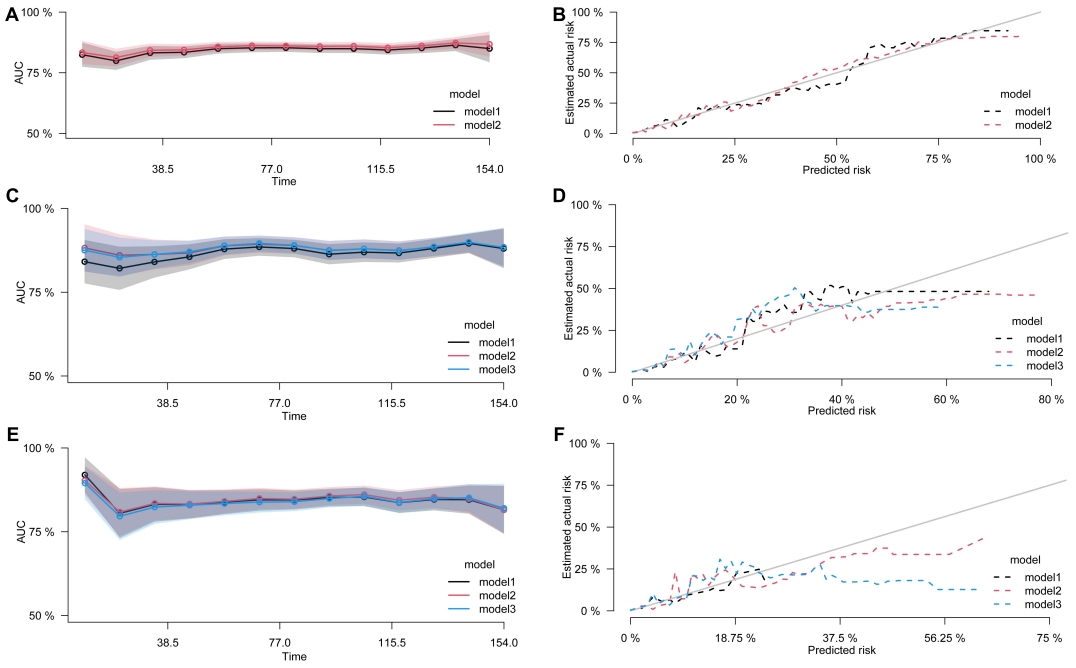

FIGURE 2
The discrimination and calibration curves of predict models. **(A,B)** All-cause mortality; **(C,D)** cardiovascular mortality; **(E,F)** malignancies-specific mortality. The ribbons indicate the 95% CIs of AUC.

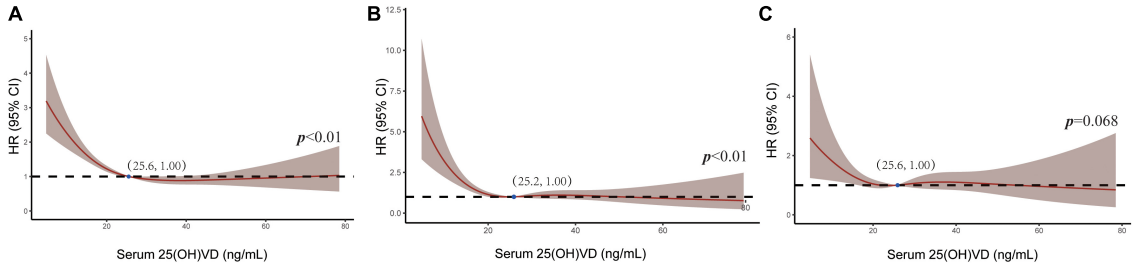

FIGURE 3
The RCS between 25(OH)VD and the mortality HRs for all-cause **(A)**, cardiovascular **(B)**, and malignancies-specific mortality **(C)**. The brown ribbon indicates the 95% CIs.

(44). In conclusion, more studies including different cancer types are needed to investigate the associations between serum 25(OH)VD and cancer-specific mortality.

A deficiency of VD is common in various populations due to inadequate dietary styles and lack of sunshine (45). The established cut-off values for deficiency, insufficiency, normal values, excess, and intoxication in sunny countries are <20, 20–32, 54–90, >100, and >150 ng/mL, respectively (46). However, these cut-off points are still debatable and may vary in different populations due to variations in exposure to sunshine, dietary styles, and disease incidence (47). Even though there is a general consensus is that serum 25(OH)VD below 20 ng/mL is classified as VD deficiency (48), more studies are needed to establish the optimal serum 25(OH)VD levels for individuals with various health conditions.

Considering the association between serum levels of lipid profiles and vitamin D, it is still debatable whether higher level of VD is related to decreased serum levels of LDL-C, TC, and TG and increased level of HDL-C. In the present study, elevated serum 25(OH)VD were associated with an increased level of HDL-C and TC, and lower serum 25(OH)VD were associated with higher TG and LDL-C levels. Many researchers have also reported similar results as our study (49, 50). However, elevated serum 25(OH)VD were also associated with higher level of TC in our study, which might be owing to the participants in our study were diagnosed with different types of HL, which could lead to a selection bias, since the relationship between VD levels

and serum levels of lipid profiles might be different according to various individual clinical statuses (5, 51, 52). Further research is needed for investigating these relationships.

The main strength of this study is that the large population was included. The data obtained from the NHANES survey allowed us to adjust the models for various variables, including the participants' baseline characteristics, dietary condition, comorbidities, and medication usage. However, the study has several limitations that have to be acknowledged. Although serum 25(OH)VD is a good biomarker and typically represents the VD status for nearly 2 months (46), these measurements may vary over time. However, in our study, the serum 25(OH)VD were only acquired at a single point in time. Moreover, we have not adjusted for season/altitude. Furthermore, the dietary style and sunshine exposure vary around the world, and therefore our findings cannot be generalized to other populations, and further studies are therefore recommended.

Conclusion

The serum 25(OH)VD were identified as an independent risk factor for all-cause and cardiovascular mortalities in patients with HL in the present study. Lower serum 25(OH)VD levels were associated with a significantly higher risk for all-cause and cardiovascular mortality, at the threshold of levels lower than 25.6 and 25.2 ng/ml, respectively. However, no significant associations were found between malignancy-specific mortality and serum 25(OH)VD, even the similar trend was noted under the threshold of 25.6 ng/mL.

Data availability statement

The raw data supporting the conclusions of this article are available in the NHANES repository (https://www.cdc.gov/nchs/nhanes/index.htm).

Ethics statement

The studies involving human participants were reviewed and approved by the National Health and Nutrition Examination Survey, ethical review and approval and written informed consents for participation were included in NHANES databases. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XC contributed to analyzing and interpreted data. XM contributed to designing the study. JC participated in data interpretation. HY, MZ, and YW assisted to depict figures and tables. All authors contributed to the article and approved the submitted version.

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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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Additive effects of obesity and vitamin D insufficiency on all-cause and cause-specific mortality

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Obesity and vitamin D deficiency are both considered risk factors for mortality, but the potential additive effects of vitamin D status and obesity on mortality has not been well-studied. We aimed to examine the possible additive effects of obesity and vitamin D status on all-cause and cause-specific mortality. The data from the NHANES III (1988–1994) and NHANES 2001–2014 surveys were used, and multivariate Cox regression models were performed to assess the additive effects of vitamin D status and overweight/obesity/abdominal obesity on the all-cause, cardiovascular and cancer mortality, by stratifying Cox Hazard Ratios (HRs) across different categories of vitamin D status and body mass index (BMI) and waist circumference (WC) categories. The models were adjusted for age, race/ethnicity, gender, educational level, family income to poverty ratio, leisure-time physical activity, smoking, and drinking. Across all BMI/WC categories, there was an additive effect of the vitamin D both insufficiency and deficiency on all mortality rates, with deficiency having much stronger effect than insufficiency. Interestingly, the effect of vitamin D deficiency overcame the effect of obesity on all mortality rates. The highest HRs for overall and cardiovascular mortality were observed among vitamin D deficient obese/abdominally obese subjects, while for cancer mortality among vitamin D deficient normal weight/non-abdominally obese subjects. In stratified analyses, regarding all-cause mortality, there was an additive effect of the vitamin D both insufficiency and deficiency in all BMI/WC categories. Regarding cardiovascular mortality, there was an additive effect of vitamin D deficiency in all BMI/WC categories, but the additive effect of vitamin D insufficiency reached significance only in normal weight subjects. Regarding cancer mortality, the effect did not reach significance among obese subjects for vitamin D deficiency, while for insufficiency, significance was reached only among non-abdominally obese subjects. Interestingly, vitamin D surplus was associated with increased risk for cancer mortality in obese subjects, but there was an inadequate number of subjects in this category to make proper judgment. In conclusion, vitamin D insufficiency and

deficiency gradually increase risk for mortality across all BMI/WC categories. In our analyses, vitamin D deficiency overcame the effect of obesity on mortality rates.

KEYWORDS

vitamin D, abdominal obesity, obesity, mortality, all-cause, cancer, cardiovascular

Introduction

Vitamin D, as an essential micronutrient, has pleiotropic skeletal and non-skeletal actions, including its anti-inflammatory, anti-proliferative, anti-oxidative, and immunomodulatory effects (1). An increasing number of studies have shown that vitamin D deficiency is associated with obesity and metabolic disorders related to obesity (2–4). Compared with normal weight subjects, obese subjects are more likely to have vitamin D deficiency, most probably due to volumetric dilution, even though other mechanisms also could have a role (5, 6). Currently, hypovitaminosis D is observed at high rates, most probably because of the modern lifestyle, but some argue that this may be also due to the high global obesity prevalence (5, 7).

Obesity and vitamin D deficiency are now considered major public health problems worldwide (2). Recent meta-analyses and systematic review studies proposed that vitamin D deficiency may be associated with mortality, including all-cause mortality and cause-specific mortality (8–10). According to previous studies, vitamin D deficiency may be more significantly associated with cardiovascular diseases (CVD) and was also closely related to some carcinoma types (11–13). Low levels of circulating 25-hydroxy-vitamin D [25(OH)D] may be associated with an increased risk of CVD, especially recurrent CVD events and CVD mortality (14). Meanwhile, the global prevalence of obesity is also rising, and CVD is one of the main causes of death among the obese (15). Additionally, obesity or serum vitamin D level may affect the severity or mortality of some diseases. For example, some research results showed that the incidence and mortality of novel coronavirus disease 2019 (COVID-19) were higher in several European countries with high latitude or high obesity prevalence (16). In some specific populations, such as menopausal women, it has been shown that the relationship between vitamin D levels and mortality may differ among participants with different body mass index (BMI) or waist circumference (WC), but there was no similar conclusion in the general population (17, 18). These studies all implied that vitamin D or obesity not only independently have an effect on mortality, but also there may be a potential additive effect between them. However, the potential additive effect of serum vitamin D status and obesity on mortality has not been well-studied.

We aimed to examine in detail the possible additive effect of obesity and vitamin D status [assessed through serum 25(OH)D concentration] with regard to all-cause and cause-specific mortality, using the data from National Health and Nutrition Examination Surveys (NHANES), large scale surveys conducted in the United States (19).

Materials and methods

The National Health and Nutrition Examination Survey (NHANES) is a nationally representative survey administered by the National Center for Health Statistics division of the Centers for Disease Control and Prevention and US Department of Agriculture. The NHANES utilizes a multistage, stratified area probability sampling design to select participants representative of the US population, and combines in-person interviews and physical examinations *via* a mobile examination center to collect data (20). NHANES was approved by the institutional review board of the National Center of Health Statistics. All participants provided written informed consent.

Data were analyzed from NHANES III (1988–1994) and NHANES 2001–2014. The average follow-up time was 11.9 years. Considering age-related reductions in lean muscle mass and subclinical disorders which may affect body weight, the analysis samples were limited to participants aged 20–79 years, without pregnancy and with complete data on BMI, WC, serum 25(OH)D concentrations and survival during follow-up (21).

Body measurements

In the current study, we classified participants as underweight (BMI < 18.5 kg/m^2), normal weight (BMI = 18.5–24.9 kg/m^2), overweight (BMI = 25.0–29.9 kg/m^2), and obese (BMI ≥ 30 kg/m^2) groups according to BMI (22). Abdominal obesity was defined as WC ≥ 102 cm for men and WC ≥ 88 cm for women (23).

Serum 25(OH)D concentration

Serum 25(OH)D concentrations are considered to be the most reliable index of vitamin D status. Serum 25(OH)D concentrations were measured by DiaSorin radioimmunoassay kit (Stillwater, MN) in the NHANES III (1988–1994) and NHANES 2001–2006, and then were determined by a standardized liquid chromatography–tandem mass spectrometry (LC-MS/MS) method (24). Serum [25(OH)D] data from NHANES III (1988–1994) and NHANES 2001–2006 have been converted by using regression to equivalent 25(OH)D measurements from a standardized LC-MS/MS method and we used the LC-MS/MS-equivalent data for all analyses as recommended by analytical guidelines (24). According to serum25(OH)D concentrations and the US Institute of Medicine (IOM) guidelines from 2011, the vitamin D status was categorized into deficiency (<12.0 ng/mL), insufficiency (12.0–19.9 ng/mL), sufficiency (20.0–50 ng/mL) and possibly harmful (>50 ng/ml) (25). Nevertheless, according to the Endocrine Society guidelines form 2011, vitamin D deficiency was defined as serum levels of 25(OH)D < 20 ng/mL, insufficiency as levels 20–30 ng/mL, while levels ≥ 30 ng/ml were characterized as the normal range (26). In our study we used the more strict IOM definitions (27).

Outcomes

Mortality related information for all causes and specific diseases was obtained by linking to the National Death Index as at December 31, 2015. Outcomes were classified using ICD-10 codes. CVD mortality codes include: ICD-10 codes I00–I09, I11, I13, I20–I51, or I60–I69, and cancer mortality codes include: ICD-10 codes C00–C97. Because the deaths due to CVD were not available on US National Death Index matched mortality dataset after December 31, 2011, we only included participants from NHANES III and NHANES 2001–2010 for CVD mortality.

Covariates

In the surveys, the self-reported data were collected on age, race/ethnicity, gender, educational level, ratio of family income to poverty, leisure-time physical activity, smoking, and drinking (3). Leisure-time physical activity was divided into three groups: inactive group (no leisure time physical activity), moderately active group (leisure time moderate physical activity 1–5 times per week or leisure-time vigorous physical activity 1–3 times per week), active group: more moderate or vigorous leisure time physical activity than above (28). The smoking status was categorized into "never," "former," or "current smoker." Never smoker was defined as smoking never or less than 100 cigarettes in life. Former smoker was defined as smoking more than 100 cigarettes in life but no smoking temporally, while current smokers were those who reported temporally smoking cigarettes "every day" or "some days" (29). Diet and alcohol consumption related data were from 24-h dietary recalls (only day 1 recall was included). Drinking was grouped according to the dietary guidelines for American residents (30).

Statistical analysis

For continuous and categorical variables, respectively, the analysis of variance and Pearson $\chi 2$ test were performed. In addition, the Bonferroni method *post-hoc* tests were made in analysis of variance, and *P*-value was corrected for multiple comparisons by the Bonferroni method. A *post-hoc* power analysis was also performed in our study and the result was satisfactory (power > 0.9). We used multiple imputation based on chained equations (MICE) to impute the missing data of covariates, but we also performed analyses with excluded all subjects who miss any of the main covariates (data presented in **Supplementary material**).

In our study, Cox proportional hazard regression models were used to estimate the hazard ratios (HR) and 95% confidence intervals (95% CI) for outcomes. Due to the rare number of underweight participants, we only performed descriptive statistics for them. The models were adjusted age, race/ethnicity, gender, educational level, family income to poverty ratio, leisure-time physical activity, smoking and drinking. To see the additive effects on mortality, we stratified Cox Hazard Ratios (HRs) across different categories of BMI/WC and vitamin D status. Reference groups were vitamin D sufficient, normal weight and not abdominally obese. Additionally, in supplementary multivariate models in **Supplementary Table 4** (**Supplementary Models 1–3**) we also included as covariates dietary supplement use, polyunsaturated fatty acid intake, calcium and magnesium intake (**Supplementary Table 4** in **Supplementary Model 1**), healthy eating index (HEI) (**Supplementary Table 4** in **Supplementary Model 2**) or specific foods intake in HEI scores (**Supplementary Table 4** in **Supplementary Model 3**) (HEI-1995 for NHANES 1988–1994 and HEI-2015 for NHANES 2001–2014), since those dietary factors have been shown to interact with associations of vitamin D status with mortality (31–34). Considering that vitamin D status is associated with renal function, we also further adjusted our main model for kidney function, estimated by the glomerular filtration rate (**Supplementary Table 4** in **Supplementary Model 4**) (35). We also repeated the main analyses after excluding the participants with missing data on any of the main covariates (age, gender, race, educational level, ratio of family income to poverty, leisure-time physical activity, smoking, and drinking). All analyses were performed using Stata software (version 16). Gpower software (version 3.1) was used for power analysis. Two-sided $P < 0.05$ was considered for statistical significance.

Results

Population characteristics

The baseline characteristics of the participants form NHANES III and NHANES 2001–2014 were shown in **Table 1**. The average follow-up time was 11.9 years. We included 40058 participants aged 20–79 who were not pregnant and had no missing data on BMI, WC, serum 25(OH)D concentrations and survival. The flow chart and the comparison of baseline characteristics between excluded and included participants can be seen in **Supplementary Figure 1** and **Supplementary Table 1**, respectively.

Among the 40,058 participants, just 1.6% were underweight, while other BMI categories were quite equally represented. However, there were significant gender differences regarding obesity and overweight prevalence: among females, the majority were obese (37.2%), and among males, the majority were overweight (39.9%). The highest proportion of obese was among non-Hispanic Blacks (40.2%), then Mexican American (33.8%), while the highest proportion of overweight was among Mexican American (39.2%), then non-Hispanic Whites (34.0%). In total, both Mexican Americans and non-Hispanic Blacks had the highest proportion of subjects with overweight/obesity. Interestingly, regarding education and family income-poverty ratio, there was no huge difference, but regarding leisure time physical activity, smoking and alcohol consumption, the lowest proportion of obese was among physically active, current smokers and those who consumed more that 14 g alcohol per day.

Abdominal obesity was present in half of the overweight subjects and in almost all of the obese subjects (95.7%).

In total, vitamin D deficiency [serum 25(OH)D < 12 ng/mL] was present in 6.0% of subjects, while insufficiency [serum 25(OH)D 12.0–19.9 ng/mL] was present in 22.9% of subjects. Among vitamin D deficient and insufficient subjects, there was the highest proportion of obese subjects (44.7 and 39.2%, respectively), then overweight subjects (27.3 and 33.4%, respectively), which means that among vitamin D deficient and insufficient subjects, respectively, 72.0 and 72.6% were with BMI ≥ 25 kg/m^2. Interestingly, in both obese and underweight subjects, the proportion of vitamin D deficiency or insufficiency was higher than in normal weight or overweight group.

Multivariate analysis of association between vitamin D status and all-cause and cause-specific mortality among different BMI/WC groups

First, we analyzed the association of obesity levels or vitamin D status with mortality in the total sample (**Tables 2**, **3**). The results indicated that obesity, abdominal obesity, vitamin D insufficiency and vitamin D deficiency were associated with increased risk for all-cause mortality and CVD mortality. Interestingly and unexpectedly, all-cause and CVD mortality HRs for vitamin D deficiency/insufficiency were higher than HRs for obesity/abdominal obesity. Vitamin D deficiency was associated with the highest risk for all-cause and CVD mortality. Interestingly, overweight had a protective effect on all-cause mortality. Regarding cancer-mortality, only vitamin D insufficiency and deficiency were associated with the increased risk. We also provide the HRs of all included covariates for all-cause and cause-specific mortality in the models which included both vitamin D status and BMI or WC categories (**Supplementary Tables 2**, **3**). Results were very similar, with again much higher HRs for vitamin D status vs. obesity status regarding all-cause and CVD mortality, with vitamin D deficiency bringing the highest risk. For cancer-mortality, only vitamin D status was a significant predictor, with both insufficiency and deficiency contributing to the higher risk (but deficiency contributed more). Adjusting for obesity level negligibly changed significance for vitamin D status HRs. Regarding other covariates, only leisure-time physical activity was not associated with mortality rates. Smoking brought the highest risk for all three mortalities (**Supplementary Tables 2**, **3**).

Next, we performed analyses to see the additive effects of vitamin D status and obesity levels, by comparing with the risk in "normal weight, vitamin D sufficient" subjects. In the whole sample analyses, there was the highest risk of all-cause and CVD mortality in participants with both obesity and vitamin D deficiency (**Table 4**). It is observable that through all BMI/WC categories, with deterioration of the vitamin D status, the risk for all-cause and CVD mortality gradually increases. The highest impact of joint vitamin D deficiency and obesity/abdominal obesity was on CVD mortality. Interestingly, the impact of vitamin D deficiency much overcame the effect of obesity for all-cause and CVD mortality. Actually, for all-cause mortality, among vitamin D sufficient subjects the effect of obesity was not significant, while overweight even had a protective effect. Regarding cancer mortality, only vitamin D status was associated with the increased risk, and on the highest risk were vitamin D deficient normal weight/not-abdominally obese subjects, then vitamin D deficient overweight subjects.

We further stratified the association between vitamin D status and mortality by different BMI/WC categories, with adjustments for age, race, gender, educational level, family income to poverty ratio, leisure-time physical activity, smoking, and drinking, to see in which BMI/WC category the effect of vitamin D deficiency/insufficiency will be the highest (**Tables 5**, **6** and **Figure 1**).

In all BMI- and WC-categories, both insufficiency and deficiency of vitamin D were additional risk factors for all-cause mortality. The participants with vitamin D deficiency had the most increased risk of all-cause mortality compared

TABLE 1 Baseline characteristics of participants from US National Health and Nutrition Examination Survey (US NHANES) III and 2001–2014.

Characteristics	Total *N* = 40058	Underweight *N* = 648 (1.6)	Normal weight *N* = 12309 (30.7)	Overweight *N* = 13731 (34.3)	Obese *N* = 13370 (33.4)
Mean age in years*	46.9 (46.8–47.1)	41.2 (39.8–42.6)	43.5 (43.2–43.8)	48.6 (48.3–48.9)	48.7 (48.4–48.9)
Gender*					
Male	19586 (48.9/100)	226 (34.9/1.2)	5794 (47.1/29.6)	7810 (56.9/39.9)	5756 (43.1/29.4)
Female	20472 (51.1/100)	422 (65.1/2.1)	6515 (52.9/31.8)	5921 (43.1/28.9)	7614 (57.0/37.2)
Race/ethnicity*					
Non-Hispanic white	17770 (44.4/100)	325 (74.0/1.8)	5883 (75.8/33.1)	6035 (73.5/34.0)	5527 (69.0/31.1)
Non-Hispanic black	9255 (23.1/100)	170 (11.4/1.8)	2514 (8.4/27.2)	2855 (9.6/30.9)	3716 (14.6/40.2)
Mexican American	8526 (21.3/100)	68 (2.9/0.8)	2240 (5.1/26.3)	3338 (7.8/39.4)	2880 (8.3/33.8)
Other	4507 (11.3/100)	85 (11.7/1.9)	1672 (10.7/37.1)	1503 (9.1/33.4)	1247 (8.2/27.7)
Education*					
Less than high school	13610 (34.0/100)	216 (33.3/1.6)	3724 (30.3/27.4)	4966 (36.2/36.5)	4704 (35.2/34.6)
High school or equivalent	12068 (30.1/100)	200 (30.9/1.7)	3758 (30.6/31.1)	4045 (29.5/33.5)	4065 (30.4/33.7)
College or above	14365 (35.9/100)	232 (35.8/1.6)	4821 (39.2/33.6)	4717 (34.4/32.8)	4595 (34.4/32.0)
Family income-poverty ratio*					
≤1.0	8916 (23.3/100)	192 (31.4/2.2)	2717 (22.9/30.5)	2914 (22.2/32.7)	3093 (24.3/34.7)
1.0–3.0	15707 (41.0/100)	259 (42.4/1.7)	4678 (39.4/29.8)	5330 (40.6/33.9)	5440 (42.6/34.6)
>3.0	13721 (35.8/100)	160 (26.2/1.2)	4468 (37.7/32.6)	4868 (37.1/35.5)	4225 (33.1/30.8)
Leisure-time physical activity*					
Inactive	16972 (47.0/100)	304 (50.1/1.8)	4790 (42.4/28.2)	5576 (45.1/32.9)	6302 (53.1/37.1)
Moderately active	12184 (33.8/100)	163 (28.0/1.3)	3902 (34.6/32.0)	4271 (34.5/35.1)	3848 (32.4/31.6)
Active	6945 (19.2/100)	116 (19.9/1.7)	2595 (23.0/37.4)	2518 (20.4/36.3)	1716 (14.5/24.7)
Smoking*					
Never	20630 (51.5/100)	287 (46.5/1.4)	6250 (49.0/30.3)	6890 (48.9/33.4)	7203 (50.9/34.9)
Former	9758 (24.4/100)	68 (10.3/0.7)	2402 (20.8/24.6)	3731 (27.9/38.2)	3557 (28.0/36.5)
Current	9657 (24.1/100)	293 (43.2/3.0)	3654 (30.2/37.8)	3107 (23.2/32.2)	2603 (21.2/27.0)
Alcohol, g/d*					
<14	32159 (81.3/100)	495 (76.6/1.5)	9473 (75.9/29.5)	10840 (76.7/33.7)	11351 (84.4/35.3)
14–28	2745 (6.9/100)	61 (10.8/2.2)	962 (8.3/35.1)	999 (8.2/36.4)	723 (5.9/26.3)
≥ 28	4670 (11.8/100)	83 (12.6/1.8)	1717 (15.8/36.8)	1734 (15.1/37.1)	1136 (9.7/24.3)
BMI, kg/m^2*	28.4 (28.3–28.5)	17.5 (17.4–17.6)	22.4 (22.4–22.5)	27.4 (27.3–27.4)	35.5 (35.4–35.6)
Waist circumference, cm*	96.9 (96.7–97.1)	70.5 (70.1–70.9)	82.4 (82.2–82.5)	95.8 (95.7–96.0)	112.7 (112.4–112.9)
Abdominal obesity*					
Not abdominally obese	19511 (48.7/100)	646 (99.7/3.3)	11354 (92.2/58.2)	6939 (50.5/35.6)	572 (4.3/2.9)
Abdominally obese	20547 (51.3/100)	2 (0.3/0.0)	955 (7.8/4.7)	6792 (49.5/33.1)	12798 (95.7/62.3)
Vitamin D status, ng/mL*	23.6 (23.5–23.7)	24.3 (23.4–25.2)	25.3 (25.1–25.4)	24.0 (23.9–24.2)	21.7 (21.5–21.8)
Vitamin D status*					
Sufficiency	24111 (60.2/100)	381 (67.4/1.6)$^{a, b}$	8074 (75.5/33.5)$^{a, c}$	8651 (71.8/35.9)$^{b, d}$	7005 (60.4/29.1)$^{c, d}$
Insufficiency	11662 (29.1/100)	161 (20.3/1.4) ^{a}	3040 (17.8/26.1)$^{b, c}$	3895 (22.1/33.4)$^{b, d}$	4566 (30.1/39.2)$^{a, c, d}$
Deficiency	3807 (9.5/100)	90 (9.6/2.4)$^{a, b}$	976 (4.7/25.6)$^{a, c, d}$	1041 (4.7/27.3)$^{c, e}$	1700 (8.7/44.7)$^{b, d, e}$
Possibly harmful	478 (1.2/100)	16 (2.7/3.4)$^{a, b}$	219 (2.0/45.8)$^{c, d}$	144 (1.3/30.1)$^{a, c, e}$	99 (0.8/20.7)$^{b, d, e}$
Death					
All-cause mortality*	6617 (16.5/100)	133 (20.5/2.0)$^{a, b}$	1949 (15.8/29.5)$^{a, c}$	2395 (17.4/36.2)$^{c, d}$	2140 (16.0/32.3)$^{b, d}$
CVD mortality	1320 (3.3/100)	17 (2.6/1.3)	365 (3.0/27.7)	476 (3.5/36.1)	462 (3.5/35.0)
Cancer mortality	1614 (4.0/100)	34 (5.3/2.1)	491 (4.0/30.4)	589 (4.3/36.5)	500 (3.7/31.0)

Data are N (% by column/% by row) or mean (95% CI). *There were statistical differences among different BMI categories ($P < 0.001$). *P*-values were calculated using analysis of variance with *post-hoc* Bonferroni test and $\chi 2$ test for continuous and categorical variables, respectively. The same superscript indicates statistically significant difference between the two groups ($P < 0.05$). *P*-values have been corrected by the Bonferroni method. CVD, cardiovascular disease; BMI, body mass index.

TABLE 2 The associations of different obesity levels with all-cause and cause-specific mortality in NHANES III and NHANES 2001–2014: HRs (95% CIs) for different BMI categories.

Death	HR (95% CI)	*P*-values
All-cause mortality		
Normal weight	Reference	
Overweight	0.91 (0.86–0.97)*	0.003
Obesity	1.08 (1.01–1.15)*	0.017
Non-abdominal obesity	Reference	
Abdominal obesity	1.09 (1.03–1.15)*	0.001
CVD mortality		
Normal weight	Reference	
Overweight	0.93 (0.81–1.07)	0.291
Obesity	1.25 (1.09–1.44)*	0.002
Non-abdominal obesity	Reference	
Abdominal obesity	1.28 (1.14–1.44)*	<0.001
Cancer mortality		
Normal weight	Reference	
Overweight	0.92 (0.81–1.04)	0.162
Obesity	1.02 (0.90–1.16)	0.779
Non-abdominal obesity	Reference	
Abdominal obesity	0.97 (0.88–1.08)	0.623

All models were adjusted for age, gender, race/ethnicity, educational level, family income to poverty ratio, leisure-time physical activity, smoking, and drinking. *$P < 0.05$. HR, hazard ratio; CI, confidence interval; BMI, body mass index; CVD, cardiovascular disease.

TABLE 3 The associations of serum vitamin D status with all-cause and cause-specific mortality in NHANES III and NHANES 2001–2014: HRs (95% CIs) for different vitamin D status categories.

Death	HR (95% CI)	*P*-values
All-cause mortality		
Sufficiency	Reference	
Insufficiency	1.18 (1.12–1.25)*	<0.001
Deficiency	1.48 (1.36–1.62)*	<0.001
Possibly harmful	1.00 (0.65–1.54)	1.000
CVD mortality		
Sufficiency	Reference	
Insufficiency	1.25 (1.10–1.42)*	0.001
Deficiency	1.65 (1.35–2.00)*	<0.001
Possibly harmful	–	–
Cancer mortality		
Sufficiency	Reference	
Insufficiency	1.18 (1.05–1.32)*	0.005
Deficiency	1.42 (1.19–1.69)*	<0.001
Possibly harmful	1.56 (0.80–3.01)	0.189

All models were adjusted for age, gender, race/ethnicity, educational level, family income to poverty ratio, leisure-time physical activity, smoking, and drinking. *$P < 0.05$. HR, hazard ratio; CI, confidence interval; CVD, cardiovascular disease.

with those with vitamin D sufficiency, across all categories of BMI and WC. The participants with vitamin D insufficiency had also the significantly higher risk of all-cause mortality compared with vitamin D sufficiency, but the risk was lower compared with vitamin D deficiency (Tables 5, 6). Across all BMI/WC categories, the risk vitamin D insufficiency/deficiency quite equally increased, but seems that highest effect was among normal weight and not abdominally obese subjects (Figure 1).

For CVD mortality, our data showed that the insufficient vitamin D status was an additional risk factor only in the normal weight and non-abdominally obese subjects, while in obese and abdominally obese subjects it only approached statistical

TABLE 4 The interaction effect of serum vitamin D status and obesity levels on all-cause and cause-specific mortality in NHANES III and NHANES 2001–2014: HRs (95% CIs) across different vitamin D status and obesity sub-categories.

	Vitamin D sufficiency HR (95% CI)	Vitamin D insufficiency HR (95% CI)	Vitamin D deficiency HR (95% CI)	Possibly harmful HR (95% CI)
All-cause mortality				
BMI				
Normal weight	Reference	1.19 (1.08–1.32)*	1.51 (1.30–1.76)*	0.80 (0.38–1.68)
Overweight	0.91 (0.84–0.99)*	1.08 (0.99–1.19)	1.33 (1.14–1.54)*	1.07 (0.51–2.25)
Obesity	1.08 (0.99–1.17)	1.24 (1.13–1.36)*	1.53 (1.33–1.75)*	1.25 (0.56–2.79)
WC				
Non-Abdominal obesity	Reference	1.22 (1.12–1.33)*	1.55 (1.36–1.77)*	0.95 (0.49–1.82)
Abdominal obesity	1.10 (1.03–1.18)*	1.26 (1.17–1.36)*	1.57 (1.41–1.76)*	1.16 (0.66–2.05)
CVD mortality				
BMI				
Normal weight	Reference	1.38 (1.10–1.74)*	1.59 (1.11–2.28)*	–
Overweight	0.98 (0.82–1.17)	1.08 (0.87–1.35)	1.70 (1.22–2.37)*	–
Obesity	1.25 (1.04–1.52)*	1.56 (1.27–1.92)*	2.07 (1.53–2.80)*	–
WC				
Non-Abdominal obesity	Reference	1.30 (1.07–1.58)*	1.78 (1.31–2.42)*	–
Abdominal obesity	1.31 (1.13–1.53)*	1.56 (1.31–1.85)*	2.00 (1.56–2.58)*	–
Cancer mortality				
BMI				
Normal weight	Reference	1.26 (1.03–1.54)*	1.60 (1.19–2.14)*	1.22 (0.39–3.83)
Overweight	0.94 (0.80–1.10)	1.09 (0.90–1.31)	1.49 (1.12–1.99)*	1.15 (0.29–4.62)
Obesity	1.06 (0.89–1.26)	1.22 (1.01–1.48)*	1.24 (0.93–1.66)	3.11 (1.16–8.37)*
WC				
Non-Abdominal obesity	Reference	1.25 (1.06–1.47)*	1.50 (1.16–1.93)*	1.07 (0.34–3.35)
Abdominal obesity	1.00 (0.87–1.14)	1.13 (0.96–1.31)	1.37 (1.09–1.72)*	1.94 (0.77–4.89)

All models adjusted for age, gender, race/ethnicity, educational level, family income to poverty ratio, leisure-time physical activity, smoking, and drinking. *$P < 0.05$. HR, hazard ratio; CI, confidence interval; BMI, body mass index; WC, waist circumference; CVD, cardiovascular disease.

significance. In contrast, vitamin D deficiency was an additional risk factor for CVD mortality in all BMI and WC-subgroups (Tables 5, 6 and Figure 1).

Regarding cancer mortality, the additive effect of vitamin D deficiency was seen in all BMI/WC categories, except in obese subjects, while the additive effect of vitamin D insufficiency was seen only in non-abdominally obese subjects. Interestingly, there was an additive effect of vitamin D surplus (levels above 50 ng/ml) and obesity on the risk for cancer mortality (Table 4). Actually, only in obese subjects the vitamin D surplus was associated with the increased risk for cancer mortality (Table 5). This observation is interesting and requires further exploration, but due to particularly small number of subjects in that category (only 4), it is not possible to draw proper concussion.

The results of our supplementary models were consistent with the results of the presented main models (Supplementary Table 4), with difference that the effect of vitamin D insufficiency on was also observed in obese and abdominally obese subjects. We also repeated analyses in the main models after excluding the participants with missing any of the main covariates, and the results were very similar, with only difference that the effect of vitamin D deficiency was no more seen in obese and abdominally-obese subjects for CVD mortality, nor was seen the effect of vitamin D surplus on cancer mortality (Supplementary Tables 5–8).

Discussion

Overall mortality

Our study, based on a large prospective cohort, indicated that lower levels of serum vitamin D were significantly associated with higher all-cause mortality. This was consistent with some data from previous research (36, 37). Additionally, there was an additive effect of the vitamin D both insufficiency and deficiency on all-cause mortality in all BMI/WC categories. It is well-known that obesity is one of the main risk factors for pre-mature death, and among examined 87 risk factors for pre-mature death, high BMI was on the fifth place, according to the Global burden of disease study report from 1990–2019 (38). Therefore, we expected that obesity will have much

TABLE 5 Stratified HRs (95% CIs) across different BMI categories and additive effect of serum 25(OH)D status on all-cause and cause-specific mortality in NHANES III and NHANES 2001–2014.

Vitamin D status	BMI Hazard ratio (95% CIs)								
	Normal weight			Overweight			Obese		
All-cause mortality	**n**		***P***	**n**		***P***	**n**		***P***
Number of deaths		1,949			2,395			2,140	
Sufficiency	1,160	1 (Reference)		1,403	1 (Reference)		1,037	1 (Reference)	
Insufficiency	577	1.19 (1.07–1.33)*	0.001	777	1.17 (1.07–1.29)*	0.001	817	1.15 (1.05–1.27)*	0.004
Deficiency	205	1.50 (1.28–1.76)*	<0.001	208	1.43 (1.22–1.67)*	<0.001	280	1.42 (1.23–1.65)*	<0.001
Possibly harmful	7	1.15 (0.38–3.54)	0.800	7	1.16 (0.55–2.45)	0.693	6	1.26 (0.56–2.82)	0.573
CVD mortality									
Number of deaths		362			468			450	
Sufficiency	210	1 (Reference)		286	1 (Reference)		219	1 (Reference)	
Insufficiency	116	1.42 (1.11–1.81)*	0.005	138	1.08 (0.87–1.34)	0.500	173	1.24 (1.00–1.53)	0.050
Deficiency	36	1.58 (1.09–2.30)*	0.017	44	1.68 (1.19–2.37)*	0.003	58	1.62 (1.17–2.23)*	0.003
Possibly harmful	0	–		0	–		0	–	
Cancer mortality									
Number of deaths		491			589			500	
Sufficiency	281	1 (Reference)		340	1 (Reference)		243	1 (Reference)	
Insufficiency	151	1.17 (0.95–1.45)	0.150	188	1.17 (0.97–1.41)	0.100	195	1.19 (0.98–1.45)	0.086
Deficiency	56	1.42 (1.04–1.93)*	0.027	59	1.63 (1.21–2.19)*	0.001	58	1.26 (0.93–1.73)	0.141
Possibly harmful	3	1.16 (0.37–3.64)	0.800	2	1.22 (0.30–4.90)	0.784	4	3.18 (1.18–8.61)*	0.023

All models were adjusted for age, race, gender, educational level, ratio of family income to poverty, leisure-time physical activity, smoking, and drinking. *$P < 0.05$. HR, hazard ratio; CI, confidence interval; BMI, body mass index; CVD, cardiovascular disease.

more significant impact on overall mortality, compared with vitamin D status. Nevertheless, the results from this study have shown quite the opposite: the impact of vitamin D status overcome the impact of overweight and obesity, since in our Cox regression models for overall mortality, HRs for vitamin D both insufficiency and deficiency were much higher, compared with HRs for overweight and obesity, which was an expected finding (actually, overweight had a protective effect). The explanations for the additive effects of obesity and vitamin D deficiency on overall mortality are not clear. Since CVD mortality was even more connected with additive effect of vitamin D status and obesity, it could be projected the effect on overall mortality was mostly conveyed through the effect on CVD mortality. In our study, CVD mortality represented only about one fifth of total mortality, but we did not include all surveys for assessing CVD mortality, and proportion of this mortality must be higher. Additionally, mortality of many other diseases and conditions can be associated with vitamin D status and obesity, e.g., mortality from respiratory diseases, infective diseases or some other chronic diseases, including endocrine (diabetes), neurological, kidney diseases, rheumatoid diseases, etc. (37). The mechanisms of the association between vitamin D and obesity can be multiple. Not only is there a simple dilution of vitamin D by increased fat deposits and plasma volume, but also some studies have indicated that in obesity the rate of uptake up vitamin D in adipose tissue may be increased, together with the increased rate of its inactivation/catabolism and elimination, and decreased rate of synthesis and absorption (6, 39, 40). Therefore, obese participants may need more vitamin D in order to maintain adequate status of serum vitamin D (5, 41, 42). With higher sequestration of vitamin D in adipose tissue, there will be much lower bioavailability of vitamin D for many other tissues, and it is known that vitamin D has numerous physiological effects (apart from the effect on calcium and phosphorus metabolism in bones and risk for fractures), including hormonal, metabolic, anti-oxidative, anti-proliferative, anti-infective, and immuno-modulatory actions. All these mechanisms may explain the higher risk for overall mortality in participants with both obesity and vitamin D deficiency.

Cardiovascular diseases mortality

With respect to CVD mortality, our results showed again that there was a strong additive effect of vitamin D deficiency and obesity, since participants with both obesity and vitamin D deficiency had the highest risk for CVD mortality, compared with other categories: almost 2.1 times higher than in vitamin D sufficient normal weight subjects. In contrast, obesity alone,

TABLE 6 Stratified HRs (95% CIs) across different WC categories and additive effect of serum 25(OH)D status on all-cause and cause-specific mortality in NHANES III and NHANES 2001–2014.

Vitamin D status	WC Hazard ratio (95% CIs)					
	Not abdominally obese			Abdominally obese		
All-cause mortality	**n**		***P***	**n**		***P***
Number of deaths		2,848			3,769	
Sufficiency	1,679	1 (Reference)		1,985	1 (Reference)	
Insufficiency	885	1.22 (1.11–1.33)*	<0.001	1,330	1.14 (1.06–1.23)*	0.001
Deficiency	275	1.53 (1.33–1.75)*	<0.001	442	1.42 (1.27–1.59)*	0.001
Possibly harmful	9	0.86 (0.45–1.66)	0.660	12	1.13 (0.64–1.99)	0.683
CVD mortality						
Number of deaths		509			788	
Sufficiency	298	1 (Reference)		426	1 (Reference)	
Insufficiency	160	1.32 (1.07–1.61)*	0.009	275	1.17 (1.00–1.37)	0.058
Deficiency	51	1.80 (1.31–2.48)*	<0.001	87	1.49 (1.16–1.92)*	0.002
Possibly harmful	0	–		0	–	
Cancer mortality						
Number of deaths		746			868	
Sufficiency	430	1 (Reference)		454	1 (Reference)	
Insufficiency	239	1.20 (1.01–1.42)*	0.033	304	1.15 (0.99–1.34)	0.075
Deficiency	74	1.41 (1.08–1.83)*	0.011	104	1.42 (1.12–1.79)*	0.003
Possibly harmful	3	1.01 (0.32–3.17)	0.980	6	2.10 (0.93–4.73)	0.072

All models were adjusted for age, race, gender, educational level, ratio of family income to poverty, leisure-time physical activity, smoking, and drinking. *$P < 0.05$. HR, hazard ratio; CI, confidence interval; CVD, cardiovascular disease; WC, waist circumference.

or vitamin D deficiency alone, had only 1.3 and 1.6 times increased risk, respectively. Interestingly and unexpectedly, the effect of overweight alone on CVD mortality was not observed. In stratified analyses, in all BMI/WC categories there was the additive effect of vitamin D deficiency, but for vitamin D insufficiency, the significant additive effect was only seen in normal weight and non-abdominally obese subjects. The possible reasons are that the effect of overweight/obesity and abdominal obesity overcomes the effect of vitamin D insufficiency on CVD mortality. Moreover, for CVD mortality we only included participants from NHANES III and NHANES 2001–2010, so the smaller number of participants probably did not allow to reach significance, even though the additive effect was still present. Additionally, there can be a cut-off level in vitamin D levels, under which an additive effect of vitamin D insufficiency on the risk for CVD mortality can be observed (37). Similarly, as for the all-cause mortality, with higher sequestration of vitamin D in adipose tissue, there will be much lower bioavailability of vitamin D for other tissues, which may have adverse effects on cardiovascular system, since vitamin D was shown as potent regulator of its components, despite some controversy on the particular mechanisms and outcomes (43–46). Our results on the effect of vitamin D status on CVD mortality are in accordance with the data from the recent systematic review by Heat et al. (47), which showed the evidence for the association in observational studies. However, the data from intervention studies did not show a significant effect, which indicates a probable confounding effect in observational studies (47).

Cancer mortality

It is worth noting that in our study, we observed the effect of vitamin D both deficiency and insufficiency on increased risk for cancer mortality, but the effect of obesity was not observed. In the whole sample, the highest increased risk was seen among vitamin D deficient normal weight/not-abdominally obese participants, then overweight participants. In the stratified analyses, the effect of vitamin D insufficiency/deficiency did not reach statistical significance in some weight categories, probably due to small numbers included. Interestingly, there was an additive effect of vitamin D surplus on the increased risk for cancer mortality among obese participants, both in whole sample and stratified analyses. However, because of small number of these participants (only 4), there could not be reliable conclusions and additional studies are needed. Our data are in accordance with the results from other studies, which show that vitamin D both deficiency and surplus may be associated with the increased risk for cancer mortality (37,

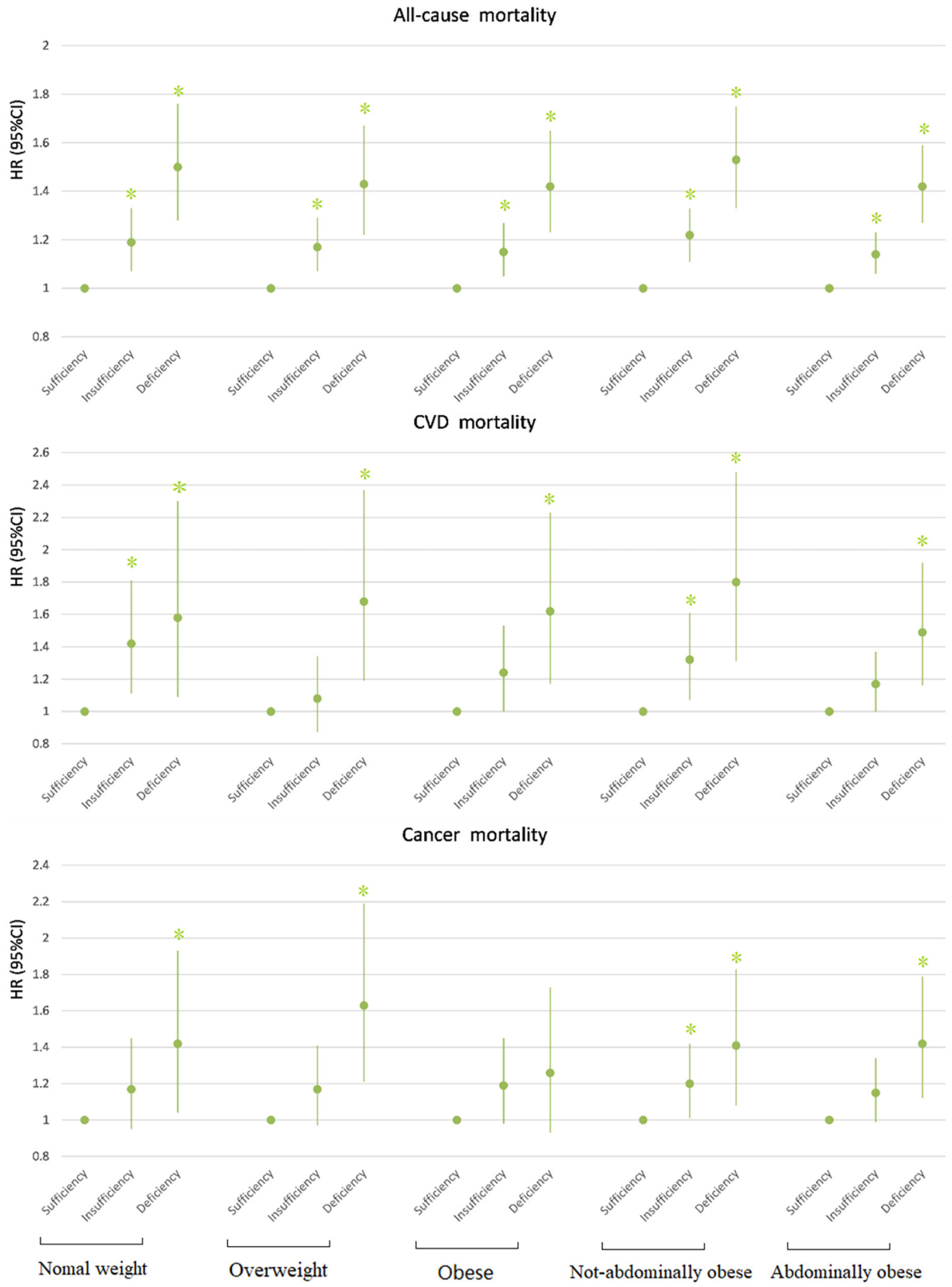

FIGURE 1
Association between 25(OH)D concentration and mortality by BMI/WC. All models adjusted for age, race, gender, educational level, ratio of family income to poverty, leisure-time physical activity, smoking, and drinking. *$P < 0.05$. HR, hazard ratio; CI, confidence interval; CVD, cardiovascular disease.

48–52). Better vitamin D status was associated with reduced mortality for breast cancer, colorectal cancer, prostate cancer, pancreatic cancer and hematological malignancies (37). Trials with vitamin D3 supplementation also show potential effect on reduced cancer mortality (53, 54). Interestingly, data show that vitamin D status was not associated with the increased risk for cancer incidence, indicating that vitamin D status might be more involved in cancer progression than initial cancerogenesis (37).

Study limitation

The main limitation of this study is that BMI, WC, and vitamin D concentrations were assessed only at one time point, at the baseline of quite long follow-up, so both vitamin D and obesity status could change in the meantime and we do not have insight into their dynamics, making reliability of our associations questionable. This is a common limitation of the long-term cohort prospective studies having only one time point measurement of the examined modifiable risk factors. Second, vitamin D levels may vary with season (55), and our data did not include the season of vitamin D measurement, as very important covariate, since relevant data were not available in NHANES III (3). Moreover, we also did not include data on sunlight exposure or latitude/altitude in the analyses, but surveys indicate that despite relatively sufficient sunshine, the prevalence of vitamin D deficiency is still high in Africa, India, Australia, Asia, South America, and even the Middle East (5). Additionally, work time physical activity and physical activity when commuting to work were not included when assessing physical activity level, because the related data was only available in NHANES 2001–2014. We used only one 24 h dietary recall to obtain data on dietary and alcohol intakes, which is probably not enough to adequately assese usual intakes. Even though we included multiple covariates, probably some other covariates could be also considered (e.g., existence of certain chronic diseases at baseline, medication usage, level of stress, professional, and environmental risk factors, etc.). Although we had a quite large number of included participants, for CVD mortality we had only data from NHANES III and NHANES 2001–2010, because data on CVD mortality were not available in the US National Death Index matched mortality datasets after December 31, 2011. Additionally, we used multiple imputation to deal with the missing covariates data, which may also affect the results. To avoid this issue, we repeated our analyses with omitting subjects with missing data, and results showed just a small difference, particularly in obesity subgroups. This may be because most of the excluded participants were obese, and smaller number of the participants remaining for analyses did not allow to reach statistical significance for some analyses. Finally, the data refer to average US population aged 20–79 years, the findings may not be generalizable to other populations and beyond this age range.

Conclusion

Our results showed that there was an additive effect of the vitamin D insufficiency/deficiency on the increased risk for all-cause and CVD mortality across all BMI/WC categories, with deficiency having much stronger effect than insufficiency. Regarding cancer mortality, vitamin D deficiency significantly increased the risk in all BMI/WC categories, except among obese subjects. The highest risk for all-cause and CVD mortality was observed among vitamin D deficient and obese/abdominally obese subjects, while for cancer mortality among vitamin D deficient and normal weight/non-abdominally obese subjects. Importantly, in our study the effect of vitamin D insufficiency overcame the effect of obesity/abdominal obesity on mortality. Maintaining sufficient serum vitamin D levels may help reduce mortality, particularly in populations at high risk. More long-term and large-scale prospective cohort studies and randomized controlled trials are required to test our findings.

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: https://www.cdc.gov/nchs/nhanes/index.htm. Data for this study NHANES subsample are available upon request.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of the National Center of Health Statistics. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SS and LF: conception and design. SS, YY, XW, and DZ: data collection. SS, YY, XW, QQ, and HW: data analyses and professional drafting. SS: manuscript writing. All authors were involved in writing the manuscript and had final approval of the submitted and published versions.

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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/fnut.2022.999489/full#supplementary-material

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The association between obesity and vitamin D deficiency modifies the progression of kidney disease after ischemia/reperfusion injury

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Acute kidney injury (AKI) alters renal hemodynamics, leading to tubular injury, activating pathways of inflammation, proliferation, and cell death. The initial damage caused to renal tissue after an ischemia/reperfusion (I/R) injury exerts an important role in the pathogenesis of the course of AKI, as well as in the predisposition to chronic kidney disease. Vitamin D deficiency has been considered a risk factor for kidney disease and it is associated with tubulointerstitial damage, contributing to the progression of kidney disease. Obesity is directly related to diabetes mellitus and hypertension, the main metabolic disorders responsible for the progression of kidney disease. Furthermore, the expansion of adipose tissue is described as an important factor for increased secretion of pro-inflammatory cytokines and their respective influence on the progression of kidney disease. We aimed to investigate the influence of vitamin D deficiency and obesity on the progression of renal disease in a murine model of renal I/R. Male Wistar rats underwent renal I/R surgery on day 45 and followed until day 90 of the protocol. We allocated the animals to four groups according to each diet received: standard (SD), vitamin D-depleted (VDD), high fat (HFD), or high fat vitamin D-depleted (HFDV). At the end of 90 days, we observed almost undetectable levels of vitamin D in the VDD and HFDV groups. In addition, HFD and HFDV groups presented alterations in the anthropometric and metabolic profile. The combination of vitamin D deficiency and obesity contributed to alterations of functional and hemodynamic parameters observed in the HFDV group. Moreover, this combination favored the exacerbation of the inflammatory process and the renal expression of extracellular matrix proteins and phenotypic alteration markers, resulting in an enlargement of the tubulointerstitial compartment. All these changes were associated with an increased renal expression of transforming growth factor β and

reduced expression of the vitamin D receptor. Our results show that the synergistic effect of obesity and vitamin D deficiency exacerbated the hemodynamic and morphological changes present in the evolution of renal disease induced by I/R.

KEYWORDS

chronic kidney disease, acute kidney injury, obesity, adipose tissue, vitamin D deficiency, inflammation, renal fibrosis

Introduction

Over the last few decades, there has been a growing interest in risk factors related to the progression of kidney disease. This fact is due to the high prevalence of chronic kidney disease (CKD) and its high costs, as well as the risk of progression to end-stage renal disease (1–3). In addition to advanced age, gender, and family history, there are other traditional and common risk factors related to the progression of kidney disease, including diabetes mellitus (DM), hypertension, obesity, and cardiovascular diseases (CVD) (3–5). Furthermore, non-traditional risk factors such as hypovitaminosis D has been considered as an aggravating feature regarding the evolution of kidney disease (2, 6–9). It is well known that kidney diseases are accompanied by decreased levels of vitamin D (9, 10), which impair the crucial role of the kidney in vitamin D metabolism. As a consequence, hypovitaminosis D disarranges the regulation of numerous physiological activities, including the renoprotection performed by that hormone (9, 10).

An increasing number of studies have been demonstrating a relation between hypovitaminosis D and anthropometric status (11–15). In 2015, a meta-analysis showed that the prevalence of vitamin D deficiency in obese and overweight individuals was, respectively, 35 and 24% higher when compared to lean subjects (16). This association between body mass index (BMI) and hypovitaminosis D is also described in sunny countries. Unger et al. observed a high prevalence of hypovitaminosis D in healthy Brazilian adults and showed a negative association between serum levels of vitamin D and BMI (17). Corroborating those data, Bolland et al. suggested a possible link involving the sequestration and deposition of vitamin D in the adipose tissue and the lower sun exposure by choice and lifestyle (17, 18). Moreover, it has been described that hypovitaminosis D in obesity occurs independently of factors, such as age, ethnicity, gender, and sun exposure (16, 19). In the 1970s, Rosenstreich et al. experimentally showed that adipose tissue had a greater storage capacity for the different forms of vitamin D compared to other organs and tissues (20). In 2000, Wortsman et al. demonstrated a lower bioavailability of vitamin D in obese patients compared to eutrophic individuals after acute ingestion of ergocalciferol or phototherapy session (21).

It is acknowledged that adipose tissue is not just a fat reservoir, but a dynamic tissue involved in the production of adipokines, including leptin, adiponectin, tumor necrosis factor α (TNF-α), chemotactic protein for monocytes 1 (MCP-1), transforming growth factor β (TGF-β), angiotensin II (Ang II), and endothelin-1 (22, 23). This endocrine action of adipose tissue generates oxidative stress, activates the renin-angiotensin-aldosterone system (RAAS), and promotes insulin resistance with subsequent abnormal production/accumulation of lipids (23–25). In addition, obesity has been linked to inflammation and is considered a risk factor for a decline in renal function (25, 26). Based on the information regarding the low levels of vitamin D in the course of renal diseases and the impaired bioavailability of this hormone in obese individuals, we aimed to study the influence of vitamin D deficiency and obesity in rats submitted to renal ischemia-reperfusion injury (IRI).

Materials and methods

Animals

Male Wistar rats (*Rattus novergicus*), weighing 180–200 g, were provided by the animal facility from the Institute of Biomedical Sciences–University of São Paulo. During the 90-day protocol, we kept our animals at a controlled temperature (23 ± 1°C) with a light/dark cycle of 12/12 h. All the experiments followed our institutional guidelines and were approved by the local Research Ethics Committee (CEUA, registration 1438/2020).

Diets

We used four different types of diet in this experimental protocol: (1) standard diet (20% protein, 70% carbohydrates, and 10% lipids); (2) standard diet depleted in vitamin D (20% protein, 70% carbohydrates, 10% lipids, and vitamin D-free); (3) high-fat diet (20% protein, 35% carbohydrates and 45% lipids); and (4) high-fat diet depleted in vitamin D (20% protein, 35% carbohydrates, 45% lipids, and vitamin D-free)—purchased

from PragSoluções Biociências (Jaú, São Paulo, Brazil). The animals were placed in cages according to their diet, with free access to water.

Experimental protocol

We allocated the rats to four groups according to each type of diet: Standard (SD, $n = 8$), fed the standard diet for 90 days; Vitamin D deficient (VDD, $n = 9$), fed the vitamin D-free diet for 90 days; High-fat (HFD, $n = 10$), fed the high-fat diet for 90 days; and High-fat vitamin D deficient (HFDV, $n = 10$), fed the high-fat vitamin D-free diet for 90 days. On day 45, all the rats were anesthetized with 2,2,2-tribromoethanol [250 mg/Kg body weight (BW)]. Subsequently, a suprapubic incision was made for induction of renal IRI by clamping both renal arteries for 45 min, followed by reperfusion.

Analysis of urine samples

Before the clearance studies, all the rats were placed in individual metabolic cages, on a 12/12-h light/dark cycle, with free access to drinking water. We collected 24-h urine to assess urinary output and then centrifuged the samples to remove suspended material. We evaluated urinary protein excretion by colorimetric assay (Labtest Diagnóstica, Minas Gerais, Brazil).

Anthropometry

On day 90, we evaluated anthropometric measurements in the animals under anesthesia just before the inulin clearance experiment. We used a sterile non-extensible measuring tape to assess: body length (cm), from the nostrils to the beginning of the tail (nose-to-anus); abdominal circumference (cm), taking the largest zone of the abdomen as the reference; and thoracic circumference (cm), immediately behind the foreleg (27, 28). We determined the BMI by dividing the body weight (g) by the body length squared (cm^2) (29).

Inulin clearance and hemodynamic studies

On day 90, we anesthetized the animals with sodium thiopental (50 mg/Kg BW) and then we cannulated the trachea with a PE-240 catheter for spontaneous breathing. The jugular vein was cannulated with a PE-60 catheter for infusion of inulin and fluids. To monitor mean arterial pressure (MAP) and collect blood samples, the right femoral artery was catheterized with a PE-50 catheter. We assessed MAP with a data acquisition system (MP100; Biopac Systems, Santa Barbara, CA). To collect urine samples, we cannulated the bladder with a PE-240 catheter by suprapubic incision. After the surgical procedure, a loading dose of inulin (100 mg/Kg BW diluted in 1 mL of 0.9% saline) was administered through the jugular vein. A constant infusion of inulin (10 mg/Kg BW) was started and continued at 0.04 mL/min throughout the whole experiment. We collected three urine samples at 30-min intervals. Blood samples were obtained at the beginning and at the end of the experiment. Inulin clearance values represent the mean of three periods. Plasma and urinary inulin were determined by the anthrone method, and the glomerular filtration rate (GFR) data were expressed as mL/min/100 g BW. To measure renal blood flow (RBF), we made a median incision and dissected the left renal pedicle for isolating the renal artery. An ultrasonic flow probe was placed around the exposed renal artery, and RBF was measured (mL/min) with an ultrasonic flow meter (T402; Transonic Systems, Bethesda, MD). We divided blood pressure by RBF to calculate renal vascular resistance (RVR, mmHg/mL/min).

Biochemical parameters

We collected blood samples after the clearance studies to assess plasma levels of 25-hydroxyvitamin D [25(OH)D], parathormone (PTH), aldosterone, Ang II, total cholesterol (cholesterol), triglycerides, glucose, leptin, phosphate (P_P), and calcium (P_{Ca}). We assessed 25(OH)D, PTH, aldosterone, Ang II, and leptin by enzyme-linked immunosorbent assay (ELISA) using commercial kits: 25-hydroxyvitamin D (ALPCO, Salem, NH, USA); Rat Intact PTH (Immutopics, Inc., San Clemente, CA, USA); Aldosterone (Enzo Life Sciences, Farmingdale, NY, USA); Rat angiotensin II (Elabscience, Houston, TX, USA); Leptin (EMD Millipore, St. Louis, MO, USA). We measured P_{Ca}, P_P, and glucose by colorimetric assay (Labtest Diagnóstica, Minas Gerais, Brazil). Plasma levels of cholesterol and triglycerides were determined by specific electrodes (ABL800Flex—Radiometer, Brønshøj, Denmark).

Tissue samples preparation

After the blood samples collection, we perfused the kidneys with a phosphate-buffered solution (PBS, pH 7.4). We froze the right kidneys in liquid nitrogen and stored them at –80°C for western blotting, ELISA, and real-time quantitative polymerase chain reaction (qPCR). The left kidneys were removed and a fragment of the renal tissue was fixed in methacarn solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for 24 h and replaced by 70% alcohol thereafter. The kidney blocks were embedded in paraffin and cut into 4-μm sections for histological and immunohistochemical (IHC) studies.

Total protein isolation

Kidney samples were homogenized in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, and 41 mM KOH, pH 7.5) containing a protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO, USA) with a homogenizer (Tissue Master TM125, Omni International, Kennesaw, GA, USA). Homogenates were centrifuged at 4,000 × rpm for 30 min at 4°C to remove nuclei and cell debris. Supernatants were isolated, and protein was quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot assay

For western blot analysis, 100 μg of total kidney protein was separated on SDS-polyacrylamide minigels by electrophoresis (30). After a transfer by electroelution to PVDF membranes (GE Healthcare Limited, Little Chalfont, UK), blots were blocked for 1 h with 5% non-fat milk in a Tris-buffered saline solution. Blots were then incubated overnight with a primary antibody for anti-VDR (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The labeling was visualized with a horseradish peroxidase-conjugated secondary antibody (anti-mouse, 1:2,000; Sigma Chemical, St. Louis, MO, USA) and enhanced chemiluminescence detection (GE Healthcare Limited, Little Chalfont, UK). Kidney protein levels were further analyzed with a gel documentation system (Alliance 4.2; Uvitec, Cambridge, UK) and the software Image J for *Windows* (Image J-NIH Image). We used densitometry to quantitatively analyze the protein levels, normalizing the bands to β-actin expression (anti-β-actin, Sigma Chemical, St. Louis, MO, USA).

Enzyme-linked immunosorbent assay in renal tissue

We assessed collagen type 3 (Col-3), Ang II, and MCP-1 in renal tissue by ELISA using commercial kits (Elabscience, Houston, TX, USA). The detection system and the quantification followed the protocols described by the manufacturer. The absorbances were obtained using the Epoch/2 device (Biotek Instruments, Winooski, VE, USA).

Light microscopy

Four-μm histological sections of kidney tissue were stained with Masson's trichrome and examined under light microscopy. We quantified the fractional interstitial area (FIA) by analyzing tubulointerstitial involvement and glomerular tuft area as well. For histomorphometry, the images obtained using microscopy were captured on a computer screen *via* an image analyzer software (ZEN, Carl Zeiss, Munich, Germany). For FIA evaluation, we analyzed 30–40 grid fields (0.09 mm^2 each) per kidney cortex. The interstitial areas were manually demarcated, and the proportion of the field was determined after excluding the glomeruli. For the glomerular area, we calculated the arithmetic mean after analyzing approximately 80 glomeruli per kidney section. The glomerular tuft area (μm^2) was manually circulated and automatically calculated by the software. We minimized bias during the morphometric analysis by keeping the observer blinded to the treatment groups.

Immunohistochemical analysis

Immunohistochemistry was performed on 4-μm-thick paraffinized kidney sections mounted on 2% silane-coated glass slides. We used the following antibodies: mouse monoclonal to CD68 (ED1, 1:100; BioRad, Hercules, CA, USA); rabbit polyclonal to mannose receptor (CD206, 1:2,000; Abcam, Cambridge, MA, USA); mouse monoclonal to CD3 (1:50; Dako, Glostrup, Denmark); mouse monoclonal to α-smooth muscle actin (α-SMA, 1:200; Millipore, Billerica, MA, USA); rabbit monoclonal to fibronectin (1:400; Abcam, Cambridge, MA, USA); mouse monoclonal to vimentin (1:100; Dako, Glostrup, Denmark); rabbit polyclonal to TGF-β1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal to proliferating nuclear cell antigen (PCNA, 1:50; Dako, Glostrup, Denmark); and mouse monoclonal to JG12, direct against to aminopeptidase P (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). We subjected the kidney tissue sections to IHC reaction according to the protocol for each primary antibody. Reaction products were detected by anti-rabbit or mouse EnVision+ System™ and the color reaction was developed in 3,3-diaminobenzidine (Dako North America, Carpinteria, CA, USA). Counterstaining was with Harris' hematoxylin. We analyzed 30–40 renal cortex fields (0.09 mm^2) to evaluate the immunoreactions. The volume ratios of positive areas of renal tissue (%), determined by the color limit, were obtained by ZEN image analyzer software (Carl Zeiss, Munich, Germany) on a computer coupled to a microscope (Carl Zeiss Axioskop 40) and a digital camera (2, 31). To minimize bias during the IHC analysis, the observer was blinded to the treatment groups.

Gene expression

We performed real-time qPCR in frozen adipose tissue assessing *VDR* gene (Rn00690616_m1). Firstly, we extracted and prepared total RNA by centrifugation technique using the commercial kit *SV Total RNA Isolation System* (Promega Corporation, Madison, WI, USA). Next, we determined the quantity and quality of RNA by Nanodrop™. We used 1 ul of RNA to prepare the cDNA following the manufacturer's

instructions of the *GoScript Reverse Transcription System* (Promega Corporation, Madison, WI, USA) and quantified again by Nanodrop™. We performed real-time PCR in 2 μl of cDNA (50 ng) using *GoTaq Probe qPCR Master Mix* (Promega Corporation, Madison, WI, USA) and *TaqMan on Step One Plus* (both from Applied Biosystems, Foster City, CA, USA). We evaluated relative gene expression with the $2^{-\Delta\Delta Ct}$ method (32) using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the housekeeping gene (Rn01775763_g1).

Statistical analysis

All data were expressed as mean ± SEM (standard error of the mean). Differences among groups were analyzed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA) by one-way analysis of variance followed by the Student–Newman–Keuls test. Values of $p < 0.05$ were considered statistically significant.

Results

Anthropometric parameters

We evaluated anthropometric data on day 90 of the experimental protocol. As expected, we observed a significant difference ($p < 0.001$) regarding the body weight of rats fed high-fat diets (HFD and HFDV) when compared to rats fed a standard diet (SD) or vitamin D-free diet (VDD) (Table 1). In addition, the body weight gain profile from HFD and HFDV groups reflected on the differences observed concerning the assessment of BMI (g/cm^2), AC (cm), and TC (cm), as shown in Table 1. These results demonstrate that high-fat diets were effective in the experimental development of obesity. We did not find any difference in the length (cm) of the animals, characterizing a homogeneous and adequate growth in relation to the age and period studied (Table 1).

TABLE 1 Anthropometric parameters evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV).

	SD	VDD	HFD	HFDV
Body weight (g)	425 ± 8	437 ± 10	531 ± 12^{ad}	538 ± 16^{ad}
BMI (g/cm^2)	0.61 ± 0.01	0.61 ± 0.01	0.72 ± 0.02^{ad}	0.72 ± 0.01^{ad}
AC (cm)	17.4 ± 0.2	17.4 ± 0.3	21.5 ± 0.4^{ad}	21.1 ± 0.4^{ad}
TC (cm)	14.1 ± 0.3	13.9 ± 0.2	15.4 ± 0.2^{ad}	15.4 ± 0.1^{ad}
Length (cm)	26.4 ± 0.1	26.4 ± 0.1	26.8 ± 0.2	26.9 ± 0.1

BMI, body mass index; AC, abdominal circumference; TC, thoracic circumference. Values are mean ± SEM. $^{a}p < 0.001$ *vs.* SD; $^{d}p < 0.001$ *vs.* VDD.

Vitamin D, parathormone, and metabolic profile

We evaluated plasma concentration of 25(OH)D at 45 and 90 days of the protocol. We observed significantly lower levels ($p < 0.001$) of that hormone on day 45 in the animals that received vitamin D-depleted diets (VDD and HFDV) when compared to SD and HFD animals. These results confirm that those animals were deficient in vitamin D at the time they were submitted to renal IRI. As expected, we found almost undetectable levels of vitamin D in VDD and HFDV groups on day 90 (Figure 1). In addition, we noted that the HFD group presented sufficient but lower levels of vitamin D compared to the SD group (Figure 1). Although without significant differences among the groups, we observed an evident upward tendency in plasma levels of PTH (pg/mL) on day 90 in the vitamin D deficient groups (VDD and HFDV) in relation to SD and HFD groups (Table 2). We did not find any difference regarding plasma phosphate and calcium levels (data not shown).

The analysis regarding lipid profile allowed us to observe isolated and synergistic actions of both high-fat and vitamin D-free diets on cholesterol and triglyceride levels. The evaluation of cholesterol levels (mg/dL) on day 90 showed a slight upward tendency in the VDD group in relation to the SD group. However, HFD group presented a significant increase concerning cholesterol levels compared to SD ($p < 0.001$) and VDD ($p < 0.05$) groups (Figure 2A). Furthermore, our results show that the vitamin D-free diet promoted a significant increase ($p < 0.05$) in triglyceride levels (mg/dL) in the VDD group compared to the SD group. We also observed higher and more significant triglyceride levels in the HFD group compared to the SD ($p < 0.001$) and VDD ($p < 0.01$) groups. Of note, the HFDV group presented higher levels of cholesterol and

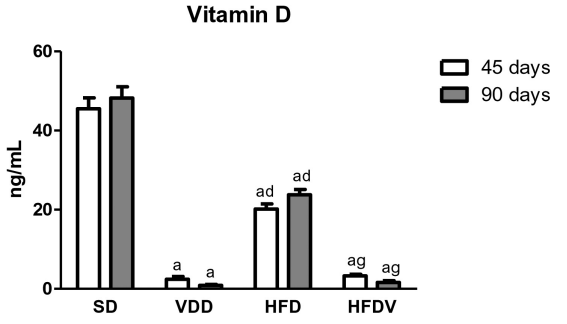

FIGURE 1
Plasma 25(OH)D levels evaluated at 45 and 90 days of protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV). Data are mean ± SEM. $^{a}p < 0.001$ *vs.* SD; $^{d}p < 0.001$ *vs.* VDD; $^{g}p < 0.001$ *vs.* HFD.

TABLE 2 Renal function, biochemical parameters, and glomerular tuft area evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV).

	SD	VDD	HFD	HFDV
P_{PTH} (pg/mL)	464 ± 73	933 ± 187	552 ± 52	964 ± 176
C_{in} (mL/min/100 gBW)	0.64 ± 0.11	0.41 ± 0.05^{c}	0.58 ± 0.02^{f}	0.36 ± 0.02^{bi}
UF (mL/24 h)	25 ± 2	16 ± 2^{c}	18 ± 3^{c}	15 ± 2^{c}
Proteinuria (mg/24 h)	8.51 ± 0.50	9.65 ± 0.41	8.18 ± 0.71	10.83 ± 0.77^{i}
RBF (mL/min)	6.06 ± 0.15	6.59 ± 0.15	5.20 ± 0.30^{ce}	4.82 ± 0.22^{bd}
RVR (mmHg/mL/min)	21.26 ± 1.20	21.80 ± 0.95	23.67 ± 1.43	26.68 ± 0.88^{cf}
GTA (μm^2)	8934 ± 215	8927 ± 128	9230 ± 210	8670 ± 166

P_{PTH}, plasma parathormone concentration; C_{in}, inulin clearance; UF, urinary flow; RBF, renal blood flow; RVR, renal vascular resistance; GTA, glomerular tuft area. Data are mean ± SEM. $^{b}p < 0.01$ and $^{c}p < 0.05$ *vs.* SD; $^{d}p < 0.001$, $^{e}p < 0.01$ and $^{f}p < 0.05$ *vs.* VDD; $^{i}p < 0.05$ *vs.* HFD.

triglycerides in relation to all the other groups, demonstrating an evident imbalance of the lipid profile in vitamin D deficiency associated with obesity (Figure 2B). In addition, the results of fasting blood glucose (mg/dL) showed an upward tendency for this parameter in the VDD and HFD groups in relation to the SD group (Figure 2C). The animals from the HFDV group showed a noteworthy and significant increase in fasting blood glucose compared to all the other groups (Figure 2C). As expected, we observed a significant increase ($p < 0.001$) in plasma leptin levels (ng/mL) in the HFD and HFDV groups when compared to the SD and VDD groups (Figure 2D). This alteration was even more remarkable in the HFDV group, with a significant increase in comparison to all the other groups.

Renal function and hemodynamic analysis

Our inulin clearance studies showed that the vitamin D-free diet associated or not with the high-fat diet modified the renal function. We observed a lower GFR (mL/min/100 g BW) in the VDD group ($p < 0.05$) compared to the SD group. This alteration was more evident in the HFDV group, which presented a lower GFR in comparison to the SD ($p < 0.01$) and HFD ($p < 0.05$) groups (Table 2). Regarding the urinary flow (mL/24 h), we found a lower urinary output ($p < 0.05$) in the VDD, HFD, and HFDV groups compared to the SD group (Table 2). In addition, we observed a slight upward tendency in proteinuria from VDD group in relation to SD and HFD groups and a significant increase ($p < 0.05$) of this parameter in the HFDV group compared to the HFD group (Table 2).

Our VDD, HFD and HFDV groups presented a higher MAP (mmHg) than the SD group. Supporting this data, we noticed a similar profile regarding plasma Ang II and aldosterone levels observed in the VDD, HFD, and HFDV groups in comparison to the SD group. Corroborating those findings, our results regarding the evaluation of renal expression of Ang II (pg/μg) showed an upward tendency in the amount of this polypeptide in the VDD, HFD, and HFDV groups in relation to the SD group. It is important to highlight that those alterations were more evident in the HFDV group (Figure 3).

We also observed the influence of high-fat diets regarding RBF and RVR. HFD and HFDV groups presented a lower RBF (mL/min) than SD and VDD groups (Table 2). In addition, the HFD group showed a slight upward tendency in the RVR (mmHg/mL/min), while the HFDV group presented a significant increase ($p < 0.05$) of this parameter in relation to SD and VDD groups (Table 2).

Vitamin D receptor expression and inflammation

We evaluated VDR protein expression in kidney tissue and VDR gene expression in the adipose tissue at the end of the 90-day protocol. The renal protein expression of VDR (%) was lower ($p < 0.001$) in the groups of animals that received the vitamin D-free diets (VDD and HFDV) when compared to the SD group (Figures 4A,B). Even with vitamin D deficiency, we observed a higher renal expression ($p < 0.01$) of VDR in the HFDV group than in the VDD group (Figures 4A,B). In addition, our results regarding real-time qPCR showed a downward tendency in VDR gene expression ($\Delta\Delta Ct$) in the adipose tissue from HFD and HFDV groups in relation to the SD group. Also, we noticed a significantly lower ($p < 0.001$) gene expression of VDR in the VDD group when compared to the SD group (Figure 4C).

It is well known that vitamin D and adipose tissue are closely related to inflammation status. Based on that, we firstly evaluated the renal amount of MCP-1 (ng/μg protein) by ELISA and the renal expression of CD3+ and CD68+ cells (T cells and macrophages, respectively) by IHC studies (%). As shown in Figure 5A, we found a higher MCP-1 amount ($p < 0.001$) in the renal tissue from VDD and HFD compared to the SD group. This alteration was more evident in the HFDV group, which showed a significant increase in the renal amount of MCP-1 when compared to all the other groups (Figure 5A). Similarly, Figures 5B,C show a higher renal expression of CD3+ cells ($p < 0.001$) in the HFDV compared to all the other groups. Our next step was focused on the macrophage infiltration in the renal cortex. By using an anti-CD68 antibody we immunolocalized the M1 and M2 macrophages, also known to express the glycoprotein ED1 on their lysosomal membrane (33). As illustrated in Figures 6A,D, we observed a higher expression of CD68+ cells ($p < 0.05$) in the renal cortex from the VDD, HFD, and HFDV groups when compared to the SD group. Furthermore, for knowledge and differentiation between the macrophage subtypes, we evaluated

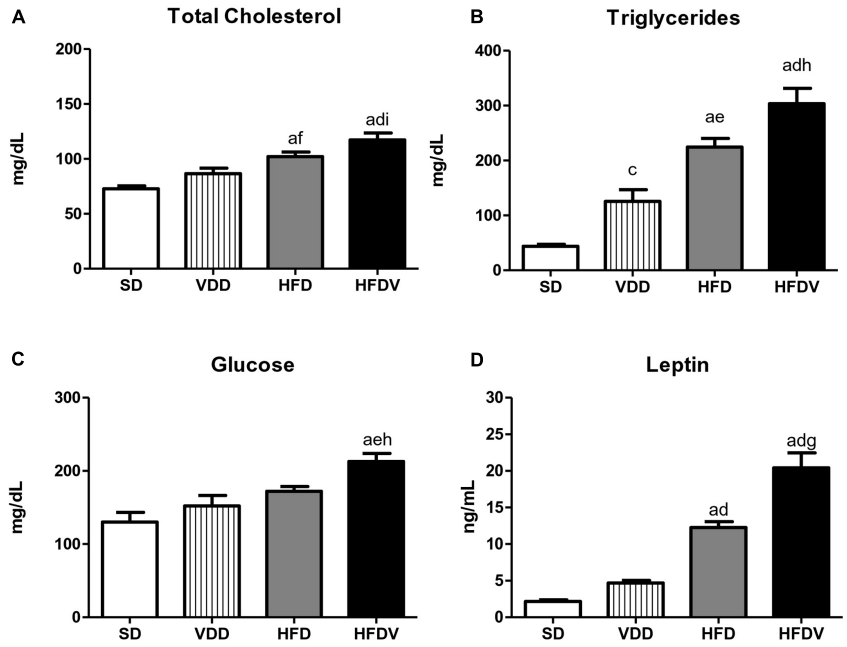

FIGURE 2
Plasma concentrations of **(A)** total cholesterol, **(B)** triglycerides, **(C)** glucose, and **(D)** leptin evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV). Data are mean ± SEM. $^{a}p < 0.001$ and $^{c}p < 0.05$ *vs.* SD; $^{d}p < 0.001$, $^{e}p < 0.01$ and $^{f}p < 0.05$ *vs.* VDD; $^{g}p < 0.001$, $^{h}p < 0.01$ and $^{i}p < 0.05$ *vs.* HFD.

the proportion of CD206+ cells (M2 macrophages) in relation to the whole amount of macrophages stained with CD68 (M1+M2 macrophages, **Figure 6C**). CD206 is also known as mannose receptor, which is an exclusive marker for M2 macrophages (1, 34). Although without significance among the groups, we observed a downward tendency in the expression of CD206+ cells in the VDD, HFD, and HFDV groups in relation to the SD group (**Figures 6B,E**), reinforcing the role of vitamin D and adipose tissue on the modulation of renal inflammation.

Synergistic effect of vitamin D deficiency and adipose tissue on the renal expression of transforming growth factor β1 and extracellular matrix proteins

High-fat diets and hypovitaminosis D seem to be associated with the susceptibility to renal fibrosis formation (RFF) through an increased expression of TGF-β and extracellular matrix (ECM) components (1, 2, 35–37). In this study, we could observe the isolated influence of vitamin D deficiency and obesity, which contributed to a higher renal expression of TGF-β1 (%) in the VDD and HFD groups than in the SD group ($p < 0.01$). Simultaneously, we noticed a synergistic influence of both risk factors, which promoted a significant increase ($p < 0.001$) in the expression of TGF-β1 in the HFDV group compared to all the other groups (**Figure 7**).

To assess the production and secretion of ECM components generated from fibroblast activation, we investigated the renal expression of two ECM proteins, including Col-3 and fibronectin. First, our results showed a slight upward tendency in the renal amount of Col-3 (ng/μg) and a higher expression of fibronectin (%) in the VDD group in relation to the SD group. Meanwhile, the HFD group presented a higher renal expression of Col-3 and fibronectin than the SD group. Of note, those alterations were even more pronounced in the HFDV group. This group not only presented a higher amount of Col-3 in relation to SD and VDD groups, but also a significant increase in fibronectin expression compared to SD and HFD groups (**Figure 8**).

Obesity and vitamin D deficiency increase the phenotypic change of renal cells

We studied the presence of markers of phenotypic change which included the renal expression of α-smooth muscle actin (α-SMA) and vimentin. We observed a mild upward tendency in the renal expression of α-SMA and a significant increase ($p < 0.05$) in vimentin expression in the VDD group in

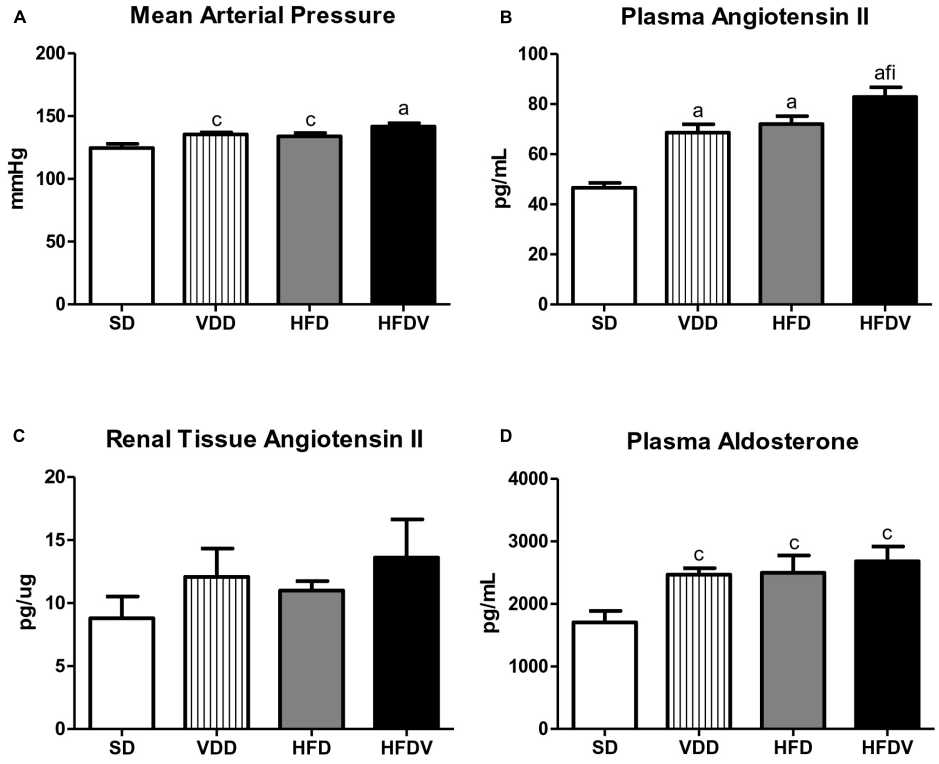

FIGURE 3
(A) Mean arterial pressure (MAP), **(B)** plasma angiotensin II concentration, **(C)** quantitative amount of angiotensin II in renal tissue, and **(D)** plasma aldosterone concentration evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV). Data are mean ± SEM. $^{a}p < 0.001$ and $^{c}p < 0.05$ *vs.* SD; $^{f}p < 0.05$ *vs.* VDD; $^{i}p < 0.05$ *vs.* HFD.

relation to the SD group. Simultaneously, we noticed a higher α-SMA expression ($p < 0.05$) and a slight upward tendency in vimentin expression in the HFD group compared to the SD group. Regarding the HFDV group, we found a higher vimentin expression than the SD group and a significant increase in renal expression of α-SMA compared to all the other groups (Figures 9, 10). Based on our results, it is plausible to suggest that the synergistic effect of vitamin D deficiency and obesity increased the phenotypical change of renal cells.

Adipose tissue and vitamin D roles on the cell proliferation, glomerular vascular endothelium, and tubulointerstitial alterations

The process of epithelial–mesenchymal transition (EMT) promotes greater cell division, allows cells to acquire a secretory phenotype, and contributes to greater deposition of ECM components. After our studies regarding ECM proteins and markers of phenotypical alterations, we proposed to investigate the proliferation of renal cells as well as the integrity of the glomerular vascular endothelium. Initially, we assessed the cell proliferation by evaluating the immunostaining for PCNA in the renal cortex. We verified a higher renal expression of PCNA (%) in the HFDV group ($p < 0.05$) compared to all the other groups (Figure 11). As a marker of glomerular vascular endothelium, we performed IHC studies using an antibody against JG12. Our data revealed a lower JG12 staining ($p < 0.001$) per glomerular tuft area (%) in the VDD and HFD groups compared to the SD group. This alteration was even more evident in the HFDV group, which showed a remarkable reduction in the expression of JG12 in comparison to all the other groups (Figure 12). We found no differences among the groups concerning the glomerular tuft area used to correct the expression of JG12 (Table 2).

Finally, we evaluated the tubulointerstitial involvement based on our dataset which included the results obtained from the experiments regarding the inflammatory infiltrate, RFF, and consequent interstitial expansion. By histomorphometry, we evaluated the FIA in the renal cortex. The histomorphometric studies revealed an upward tendency regarding FIA in the VDD group in relation to the SD group. In addition, we observed a larger FIA ($p < 0.05$) in the HFD group than in the SD group. Of note, the HFDV group showed a more evident increase ($p < 0.001$) in the FIA compared to all the other groups

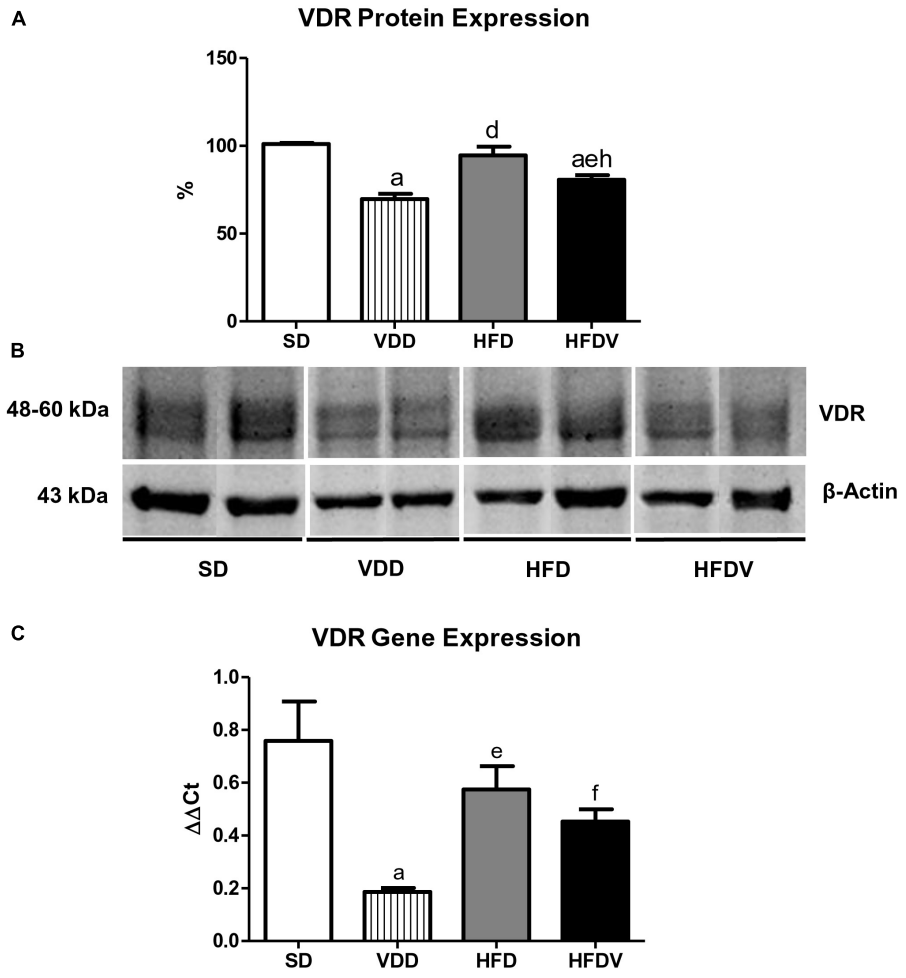

FIGURE 4
Semiquantitative immunoblotting for renal vitamin D receptor (VDR) protein expression and RT qPCR for adipose tissue VDR gene expression evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV). **(A)** Densitometric analysis of samples from an SD, VDD, HFD, and HFDV rat. **(B)** Representative immunoblots reacted with anti-VDR revealing a 51 kDa band. **(C)** Bar graph of VDR gene expression values. Data are mean ± SEM. $^{a}p < 0.001$ *vs.* SD; $^{d}p < 0.001$, $^{e}p < 0.01$ and $^{f}p < 0.05$ *vs.* VDD; $^{h}p < 0.01$ *vs.* HFD.

(Figure 13), which indicates a synergistic role of vitamin D and adipose tissue on the alterations of the tubulointerstitial compartment.

Discussion

Our results show that the animals fed a high-fat vitamin D-free diet and submitted to renal IRI presented almost undetectable levels of vitamin D and changes in the anthropometric and metabolic profile. The combination of vitamin D deficiency and obesity modified functional and hemodynamic parameters. Furthermore, we observed an increase in proteinuria, renal expression of MCP-1, infiltration of inflammatory cells, ECM proteins, and phenotypical markers in the HFDV. This group also presented a greater cell proliferation, impairment of the glomerular vascular endothelium, and expansion of the tubulointerstitial compartment. All those alterations were associated with a higher expression of TGF-β1 and a lower expression of VDR in the renal tissue from the HFDV group.

Plasma levels of vitamin D represent the sum of biological production from diet and sun exposure (38). It is important to state that our animals were kept without sun exposure and received diets depleted or not in vitamin D. The vitamin D-free diet groups (VDD and HFDV) had almost undetectable plasma levels of vitamin D at the end of the 90-day protocol, confirming the efficiency of the experimental model of vitamin D deficiency (2, 6, 8). Plasma levels of vitamin D have been inversely associated with obesity, insulin resistance, and type 2 diabetes (12, 15, 39). In addition to the HFDV rats, we observed lower plasma levels of vitamin D as well as a downward

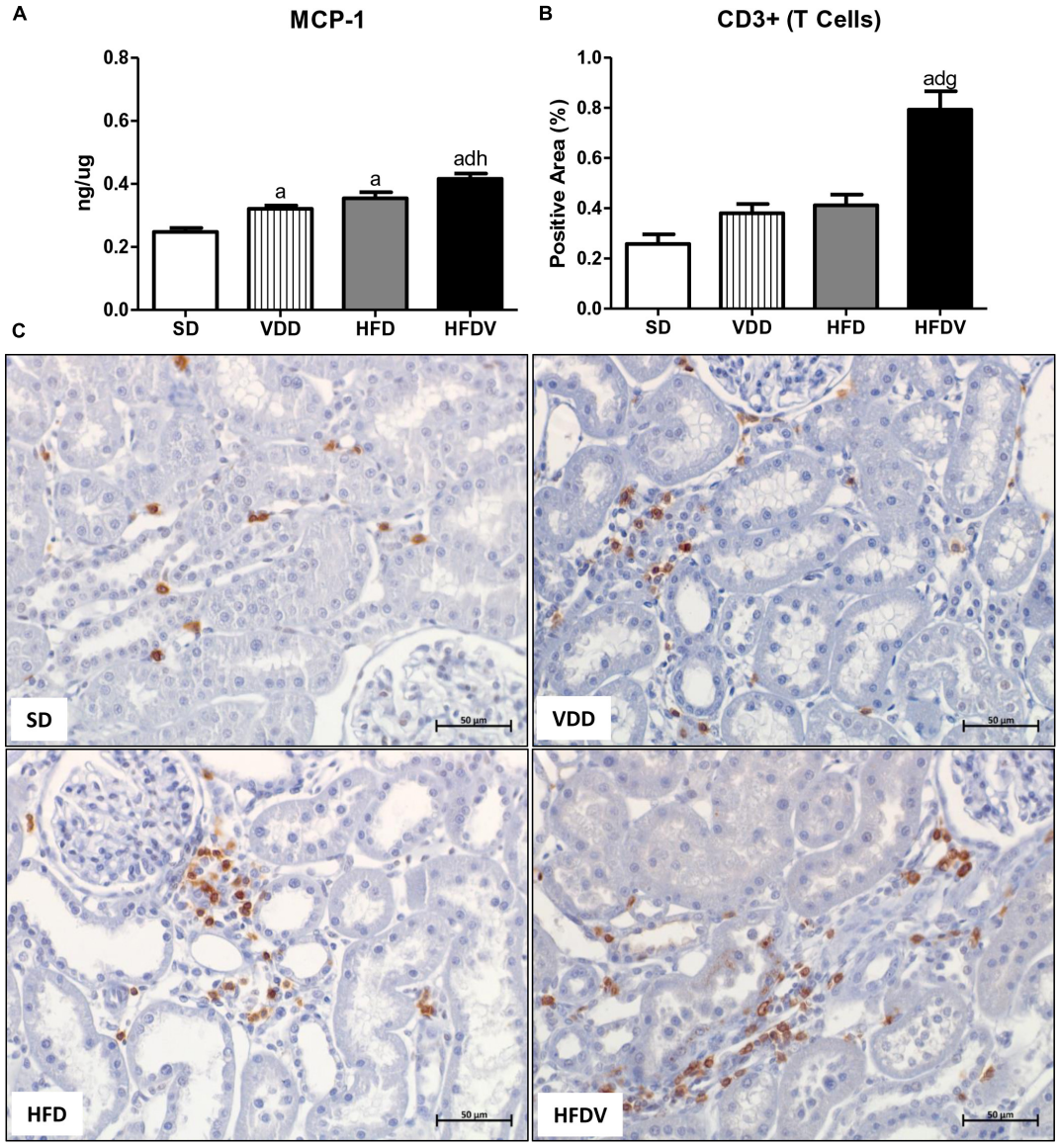

FIGURE 5
Quantitative amount of monocyte chemotactic protein 1 (MCP-1)—ELISA and immunohistochemical analysis for CD3+ cells (T cells) expression in renal tissue evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV). **(A)** Bar graphs of MCP-1 quantification and **(B)** CD3+ cells expression values. **(C)** Representative photomicrographs of immunostaining for CD3+ cells in the renal cortex from an SD, VDD, HFD, and HFDV rat (×400). Data are mean ± SEM. $^{a}p < 0.001$ *vs.* SD; $^{d}p < 0.001$ *vs.* VDD; $^{g}p < 0.001$ and $^{h}p < 0.01$ *vs.* HFD.

tendency regarding the renal expression of VDR in the HFD rats. Our results are consistent with the hypothesis of a lower and inadequate bioavailability of vitamin D in obesity (12, 15, 39).

Usually considered a storage organ for vitamin D, adipose tissue also seems to influence endocrine and paracrine actions, modulating the expression of enzymes responsible for the formation, activation, and degradation of vitamin D (12, 40, 41). Besides the renal protein expression, we evaluated VDR gene expression in the adipose tissue. Our VDD group showed a lower VDR gene expression, which primarily followed the deficient plasma levels of vitamin D. In 2015, Nguyen et al. described the association between plasma vitamin D levels and VDR gene expression in adipose tissue (39). However, we did not observe this relationship in our HFDV rats. Even receiving a vitamin D-depleted diet, HFDV rats showed a higher renal expression of VDR and a marked gene expression of this receptor in adipose tissue compared to VDD rats. In addition, our groups that received the high-fat diets (HFD and HFDV) showed a downward tendency in the VDR gene expression

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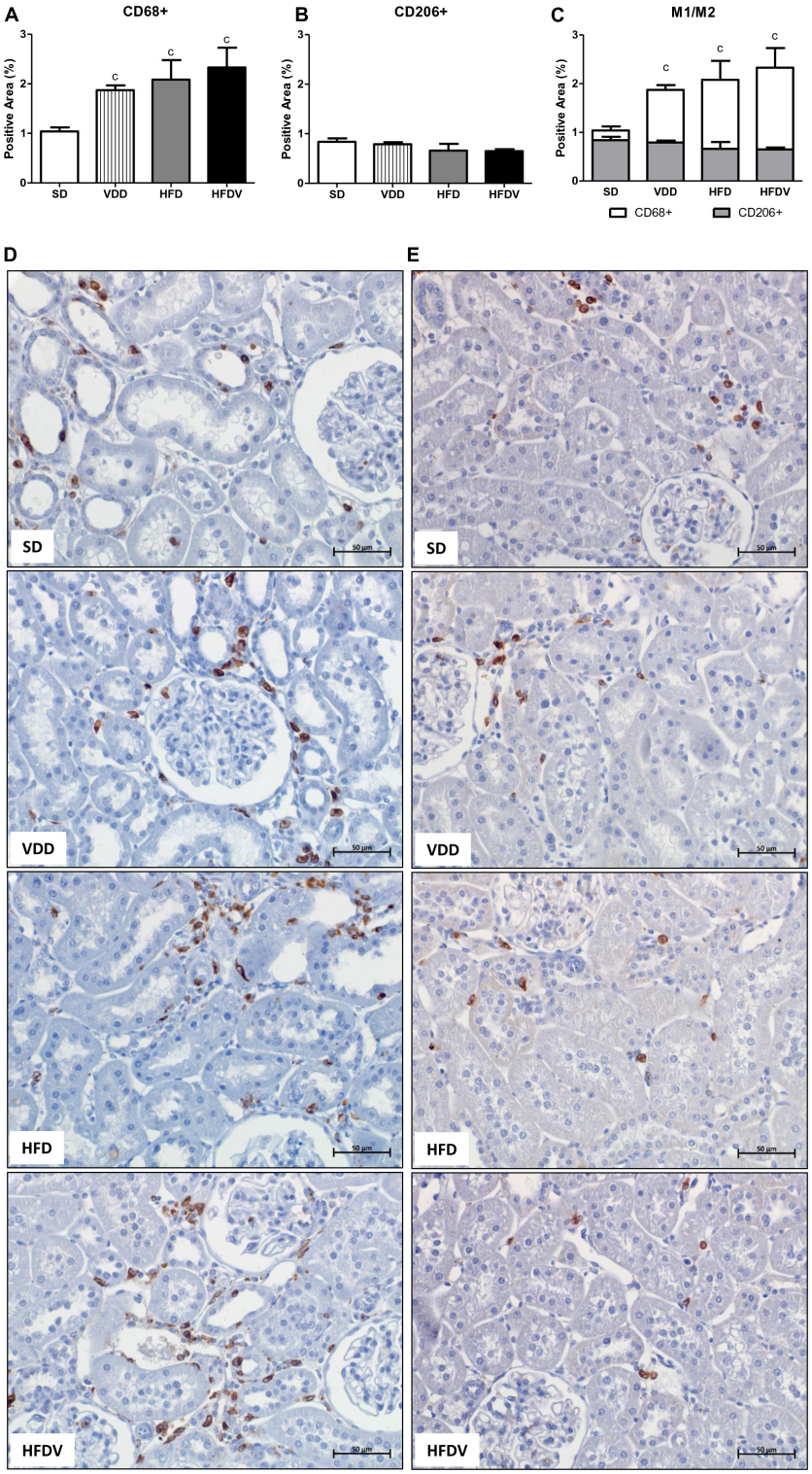

FIGURE 6
Immunohistochemical analysis for CD68+ cells (M1 + M2 macrophages) and CD206+ cells (M2 macrophages) expression in the renal cortex evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV). **(A)** Bar graph of CD68+ cells expression values. **(B)** Bar graph of CD206+ cells expression values. **(C)** Bar graph regarding the proportion of CD206+ cells in relation to the amount of CD68+ cells. Representative photomicrographs of immunostaining for CD68+ **(D)** and CD206+ **(E)** cells in the renal cortex from an SD, VDD, HFD, and HFDV rat (×400). Data are mean ± SEM. $^{c}p < 0.05$ *vs.* SD.

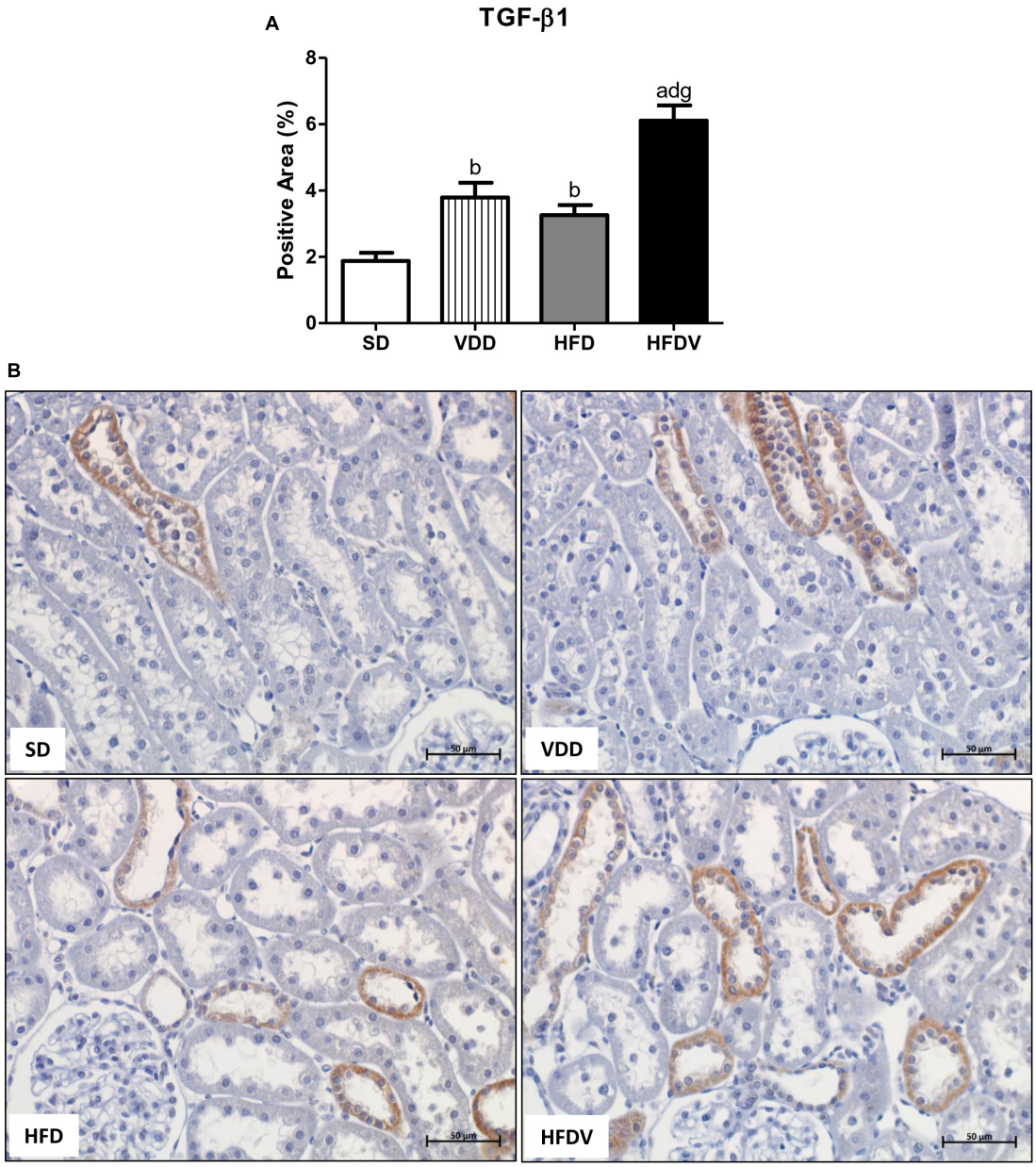

FIGURE 7
Immunohistochemical analysis for transforming growth factor β1 (TGF-β1) expression in the renal cortex evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD) or high-fat vitamin D-free diet (HFDV). **(A)** Bar graph of TGF-β1 expression values. **(B)** Representative photomicrographs of immunostaining for TGF-β1 in the renal cortex from an SD, VDD, HFD, and HFDV rat (×400). Data are mean ± SEM. $^{a}p < 0.001$ and $^{b}p < 0.01$ *vs.* SD; $^{d}p < 0.001$ *vs.* VDD; $^{g}p < 0.001$ *vs.* HFD.

in adipose tissue in relation to the SD group. Wamberg et al. also observed a reduced VDR gene expression in the subcutaneous adipose tissue of obese women in comparison to lean individuals. On the other hand, these authors demonstrated that there was a 33% higher expression of VDR in the visceral adipose tissue of obese women compared to lean women (41). Nevertheless, our results are not enough to understand the physiological actions of vitamin D on adipose tissue associated with the degree of obesity and future studies are needed to explain this relationship.

Hypercaloric and high-fat diets are described in the literature as being effective for the experimental induction of obesity in rodents (42–44). As a confirmation of the effectiveness of our experimental model, the anthropometric measurements used as markers of obesity demonstrated that HFD and HFDV rats presented higher body weight and

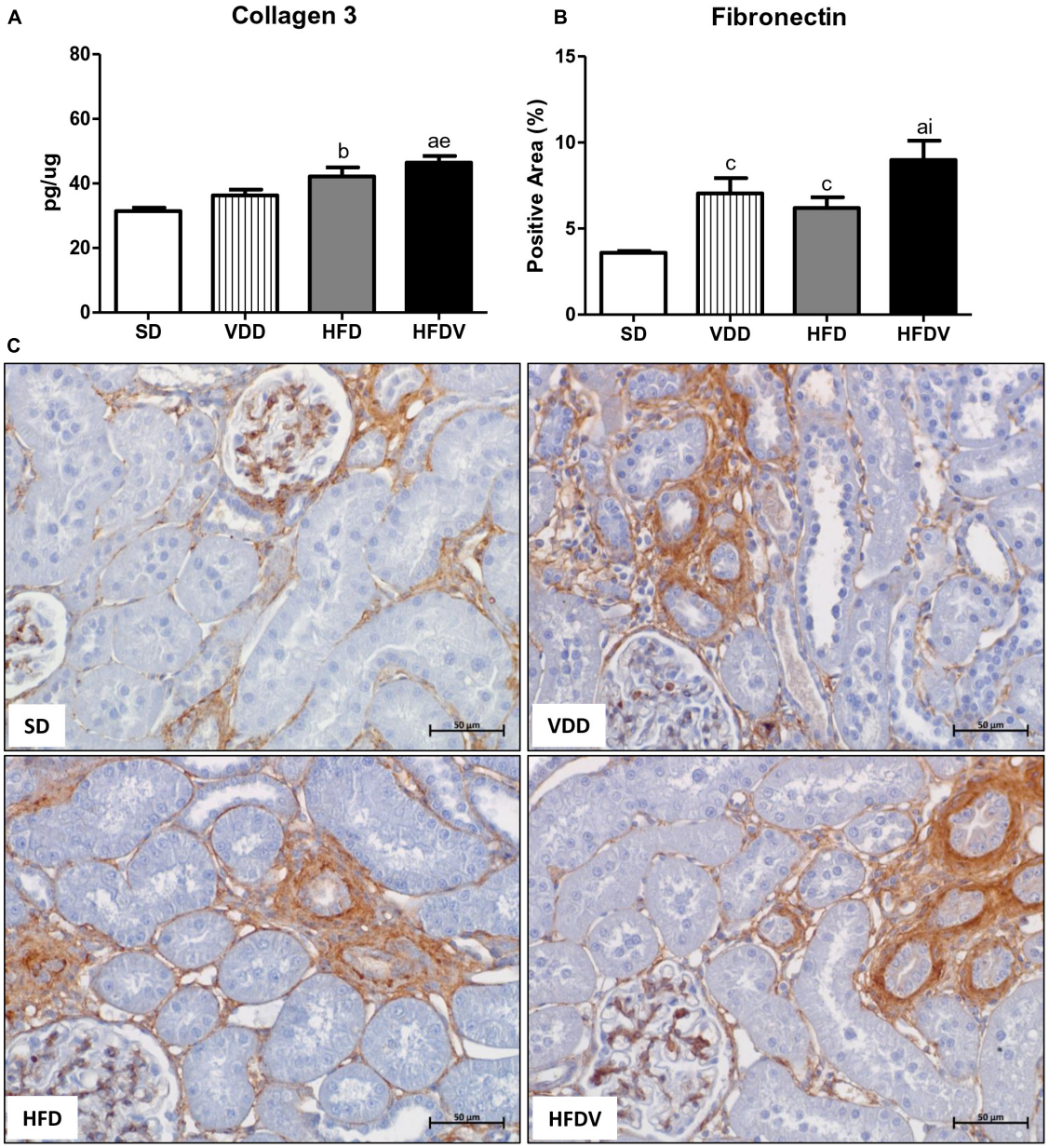

FIGURE 8
Quantitative amount of collagen 3—ELISA and immunohistochemical analysis for fibronectin expression in the kidney tissue evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV). Bar graphs of **(A)** collagen 3 and **(B)** fibronectin expression values. **(C)** Representative photomicrographs of immunostaining for fibronectin in the renal cortex from an SD, VDD, HFD, and HFDV rat (400×). Data are mean ± SEM. $^{a}p < 0.001$, $^{b}p < 0.01$ and $^{c}p < 0.05$ *vs.* SD; $^{e}p < 0.01$ *vs.* VDD; $^{i}p < 0.05$ *vs.* HFD.

BMI as well as a significant increase in their abdominal and thoracic circumferences.

Hyperlipidemia is commonly related to a high intake of diets rich in fatty acids and obesity (45, 46). It is described that the abnormal deposition of fat in adipose tissue and in other organs, such as the liver and kidneys, can be considered an important risk in the follow-up of pathologies, including CKD (45, 47, 48). The presence of dyslipidemia is reported in all stages of CKD, with impairment of the glomerular filtration barrier, tubular damage, and proteinuria (47). Our HFD and HFDV rats showed higher plasma levels of cholesterol and triglycerides than SD and VDD rats. Bhandari et al. also showed the adverse effects of obesity induced by a high-fat diet. Corroborating our data, these authors demonstrated alterations in the lipid profile of rodents, such as the presence of high levels of total cholesterol, triglycerides, and LDL, followed by low levels of HDL (43).

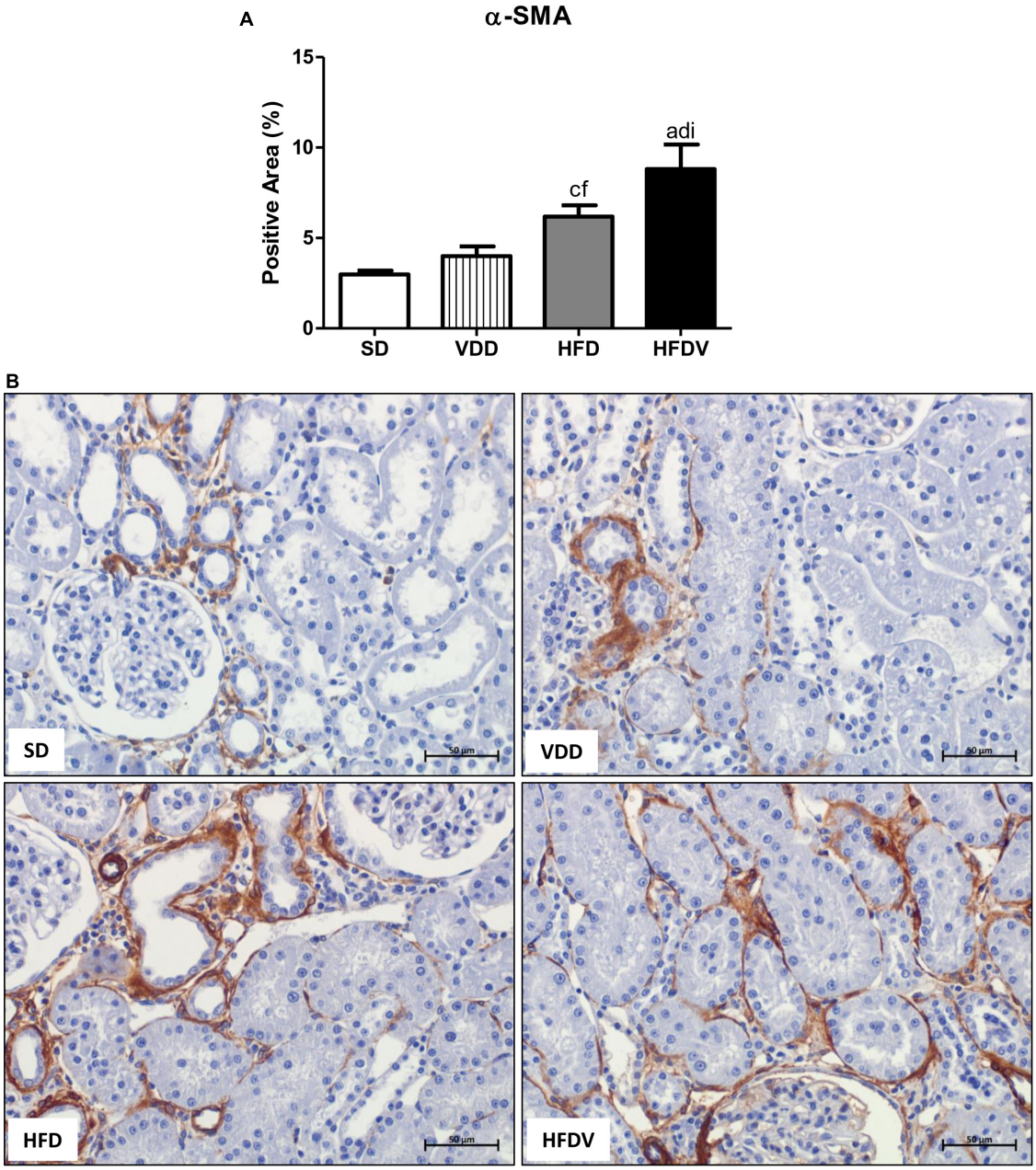

FIGURE 9
Immunohistochemical analysis for α-smooth muscle actin (α-SMA) expression in the renal cortex evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD) or high-fat vitamin D-free diet (HFDV). **(A)** Bar graph of α-SMA expression values. **(B)** Representative photomicrographs of immunostaining for α-SMA in the renal cortex from an SD, VDD, HFD, and HFDV rat (400×). Data are mean ± SEM. $^{a}p < 0.001$ and $^{c}p < 0.05$ *vs.* SD; $^{d}p < 0.001$ and $^{f}p < 0.05$ *vs.* VDD; $^{i}p < 0.05$ *vs.* HFD.

The relationship between lipid profile and vitamin D is also demonstrated in the literature (6, 49, 50). It has been reported that vitamin D and cholesterol share the same biosynthesis pathway, as they have 7-dehydroxycholesterol as a common precursor (46). In the present study, the vitamin D-deficient rats, particularly HFDV rats, had higher levels of cholesterol associated with remarkable triglyceride levels compared to SD animals. Vitamin D plays important roles in the regulation and absorption of calcium, thus reducing the absorption of fatty acids and exerting an influence on plasma cholesterol levels (51). In addition, low levels of vitamin D promote plasma PTH elevation. High levels of PTH increase lipogenesis, and bone remodeling, and reduce lipolytic activity, thus influencing lipid metabolism (46, 48, 51). As expected, our VDD and HFDV

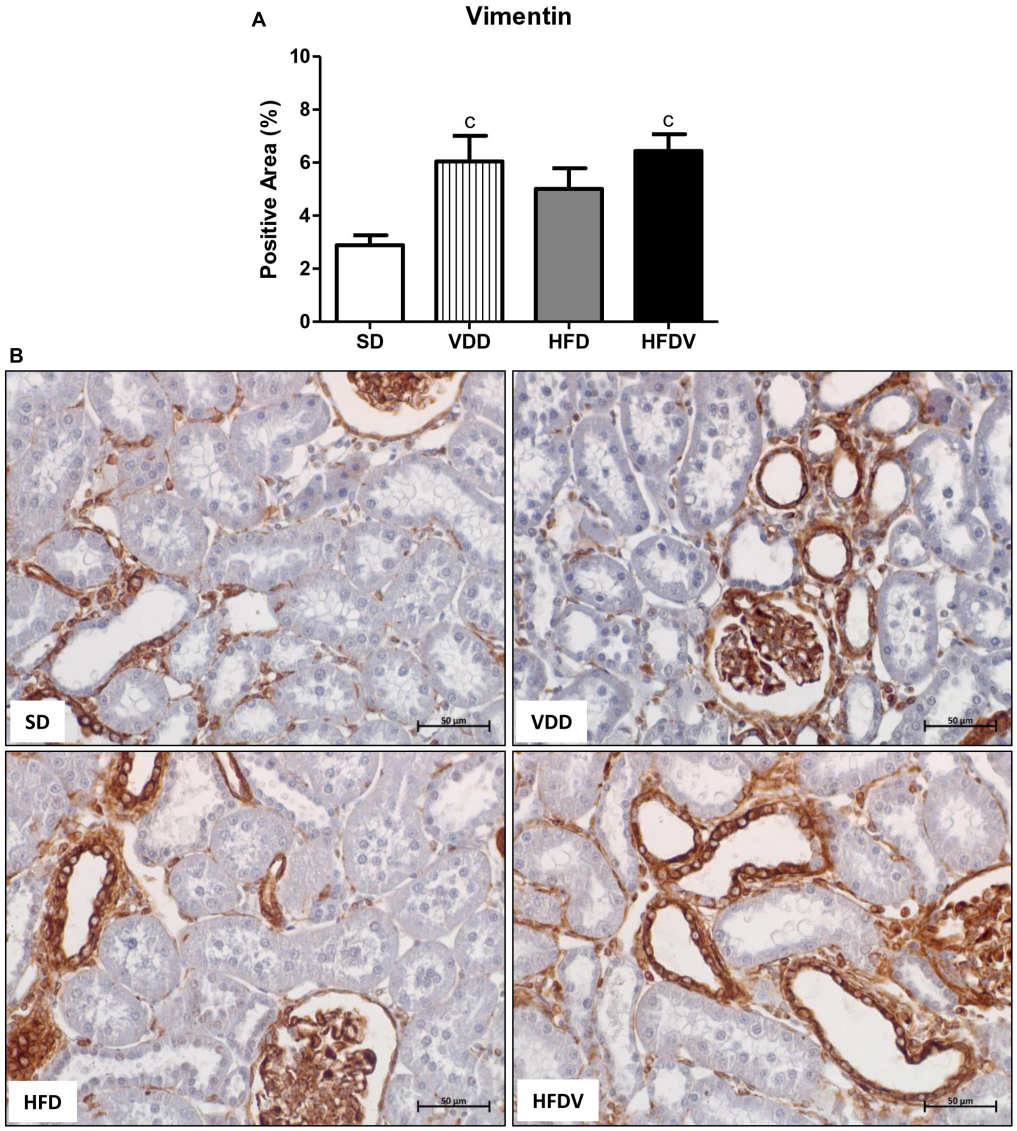

FIGURE 10
Immunohistochemical analysis for vimentin expression in the renal cortex evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD) or high-fat vitamin D-free diet (HFDV). **(A)** Bar graph of vimentin expression values. **(B)** Representative photomicrographs of immunostaining for vimentin in the renal cortex from an SD, VDD, HFD, and HFDV rat (400×). Data are mean ± SEM. $^{c}p < 0.05$ *vs*. SD.

groups presented elevated PTH levels in relation to SD and HFD groups, demonstrating the negative feedback caused by vitamin D deficiency.

Hyperglycemia is a typical sign of both reduced gluconeogenesis in the liver and reduced glucose uptake in skeletal muscle, liver, and adipose tissue, which feature the onset of insulin resistance (50, 52). In addition to overweight, increased visceral adipose tissue represents an important association with insulin resistance and changes in adipose tissue functionality (53). Vitamin D deficiency is also related to inadequate insulin secretion, altered blood glucose levels, and type 2 diabetes (54–57). One of the mechanisms by which hypovitaminosis D contributes to the installation of insulin resistance is *via* the regulation of intracellular calcium in pancreatic β-cells (50, 58). In the present study, we found an upward tendency in fasting plasma glucose in VDD and HDF groups. Simultaneously, the association between obesity and vitamin D deficiency promoted a higher glycemia in the HFDV group in comparison to all the other groups. Thereby our dataset regarding the metabolic parameters (cholesterol, triglycerides, and fasting blood glucose) allowed us to observe the influence of obesity and vitamin D deficiency, alone or in association, on the installation of metabolic syndrome, especially in the HFDV animals.

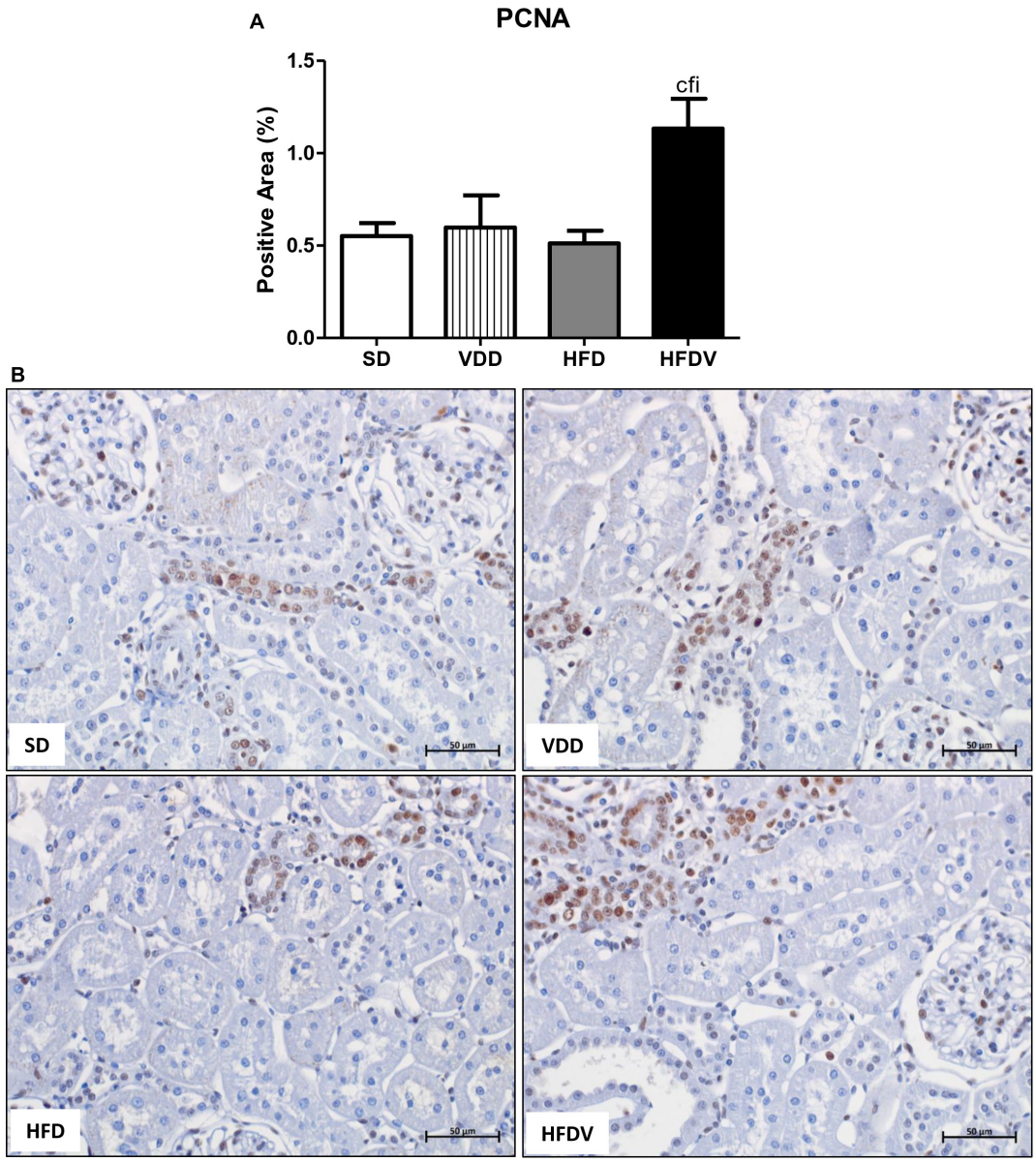

FIGURE 11
Immunohistochemical analysis for proliferating cell nuclear antigen (PCNA) expression in the renal cortex evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD), or high-fat vitamin D-free diet (HFDV). **(A)** Bar graph of PCNA expression values. **(B)** Representative photomicrographs of immunostaining for PCNA in the renal cortex from an SD, VDD, HFD, and HFDV rat (×400). Data are mean ± SEM. $^{c}p < 0.05$ *vs.* SD; $^{f}p < 0.05$ *vs.* VDD; $^{i}p < 0.05$ *vs.* HFD.

Adipose tissue is not only recognized for its capacity for energy storage and lipid mobilization, but also as an adipokine-secreting endocrine organ (22, 53). It is known that adipocytes, one of the main cell types of the adipose tissue, secrete a variety of factors and hormones, such as TNF-α, IL-6, PAI-1, MCP-1, adiponectin, resistin, and leptin (22, 23, 59). Physiologically, leptin plays an important role in the control of energy homeostasis and maintenance of body weight, with circulating levels proportional to food intake and body energy reserve from the suppression or activation of specific neurotransmitters (60, 61). The condition called hyperleptinemia in obesity induces leptin resistance, which is similar to insulin resistance in type 2 diabetes (61). In the present study, our HFD and HFDV groups showed higher plasma leptin levels than the SD group. Corroborating our results, previous studies also showed an increase in serum leptin levels in experimental models of obesity induced by high-fat diets (62–64). In addition to being directly related to the amount of adipose tissue, leptin concentration

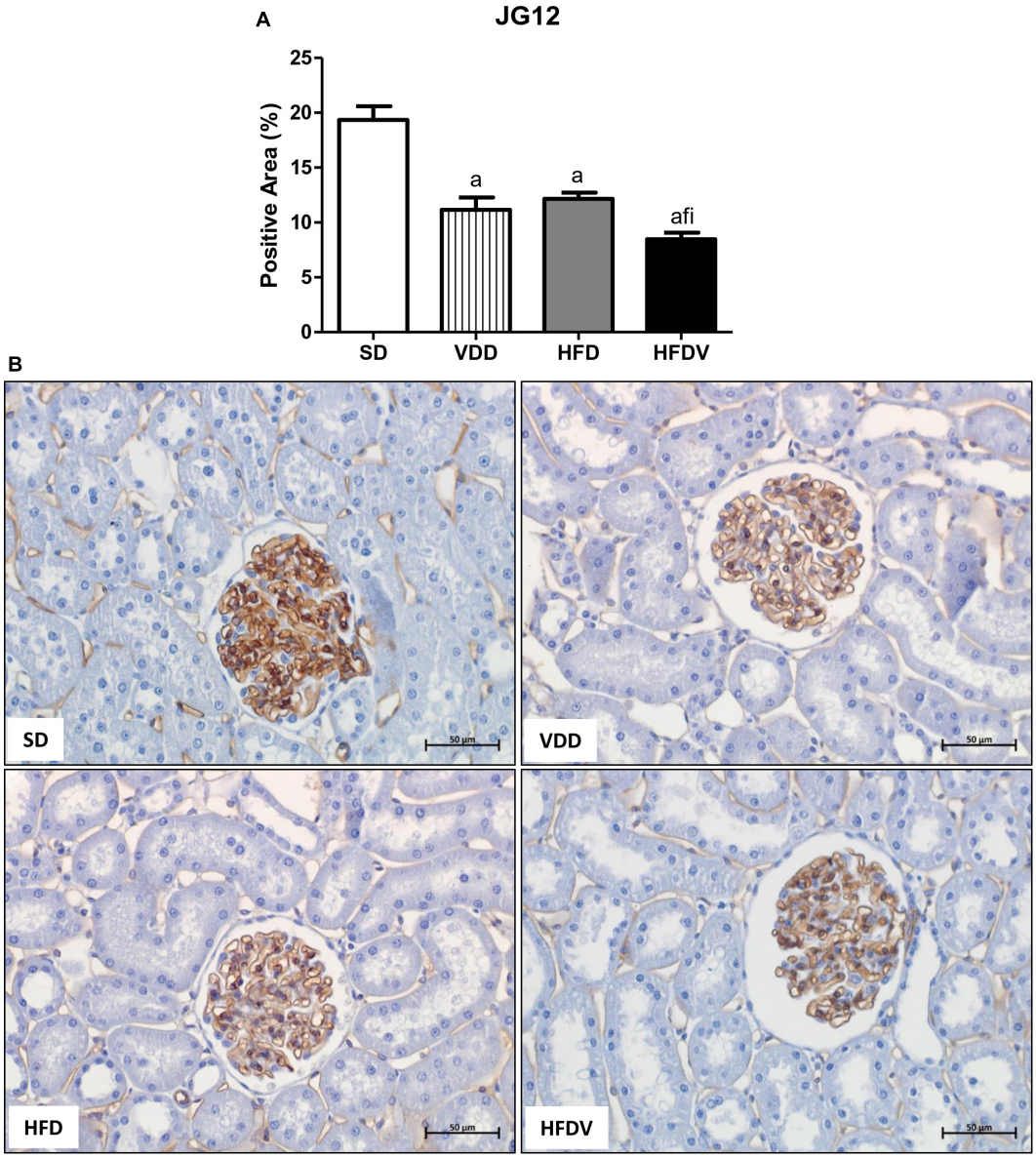

FIGURE 12
Immunohistochemical analysis for aminopeptidase P (JG12) expression by glomerular tuft area in the renal tissue evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD) or high-fat vitamin D-free diet (HFDV). **(A)** Bar graph of JG12 expression values. **(B)** Representative photomicrographs of immunostaining for JG12 in the renal cortex from an SD, VDD, HFD, and HFDV rat (×400). Data are mean ± SEM. $^{a}p < 0.001$ *vs.* SD; $^{f}p < 0.05$ *vs.* VDD; $^{i}p < 0.05$ *vs.* HFD.

seems to be also regulated by serum vitamin D levels (65, 66). Conversely, leptin appears to have an inhibitory effect on the conversion of 25(OH)D to 1,25$(OH)_2D_3$, by inhibiting 1-α-hydroxylase in renal and adipose tissue (66, 67). Those findings are in agreement with our data regarding the notorious high plasma leptin levels observed in the HFDV rats. Thus, our results allow us to infer that the synergistic effect of vitamin D deficiency and obesity could explain the significant increase in plasma leptin levels observed in the HFDV group.

It is well known that CKD, even in the early stages, is accompanied by a progressive decline in GFR and low levels of vitamin D (1, 7, 8, 10, 68). In 2011, de Boer et al. suggested that vitamin D deficiency may be a risk factor for the decline in GFR, especially when associated with diabetes and hypertension (69). Our VDD rats presented a lower GFR compared to SD and HFD rats, confirming previous results from our group regarding the role of vitamin D deficiency on the impairment of the renal function (1, 7, 8). In addition, obesity and dyslipidemia are

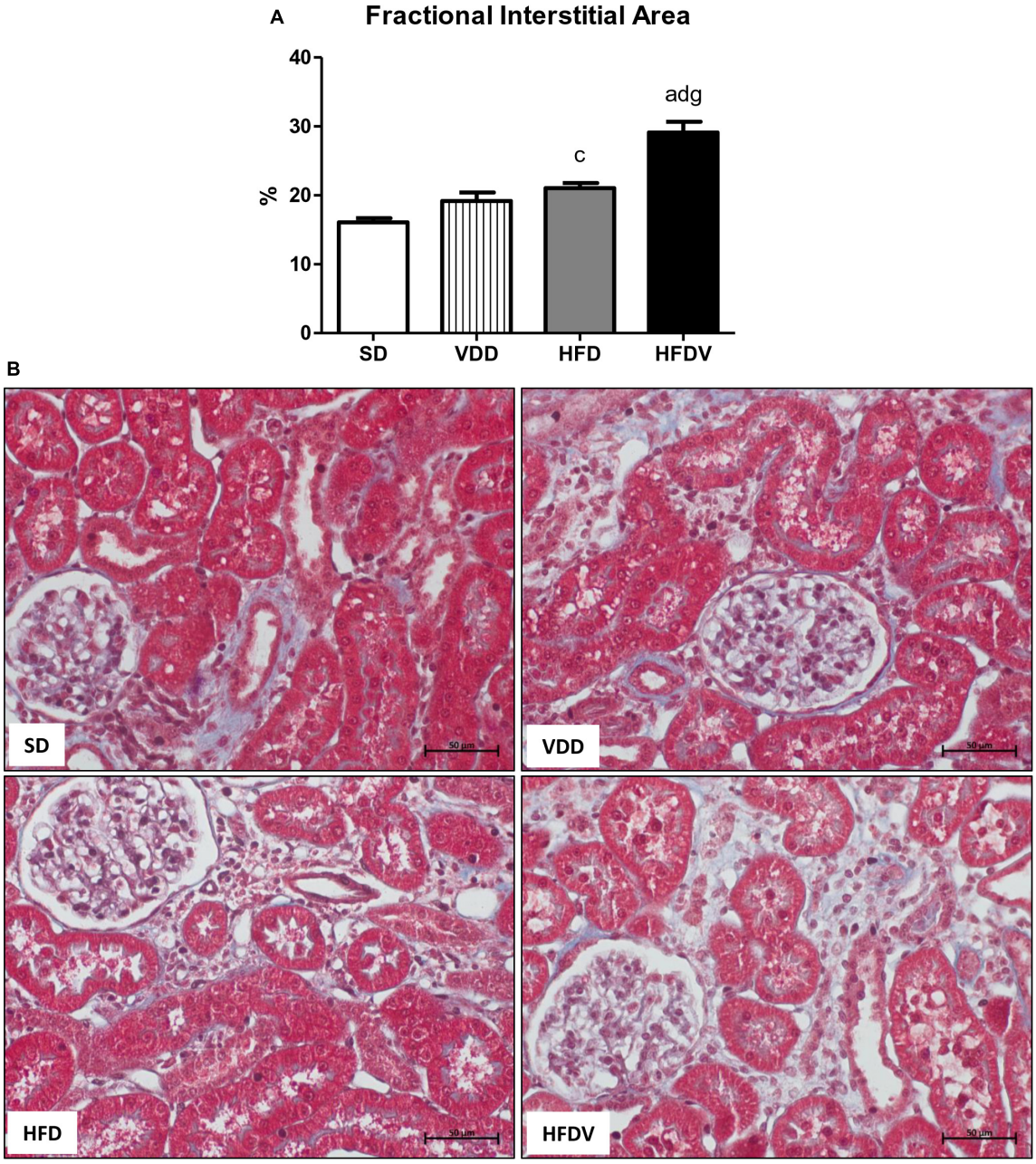

FIGURE 13
Fractional interstitial area (FIA) of the renal cortex evaluated after the 90-day protocol in rats submitted to renal ischemia-reperfusion insult on day 45 treated with standard diet (SD), vitamin D-free diet (VDD), high-fat diet (HFD) or high-fat vitamin D-free diet (HFDV). **(A)** Bar graph of FIA values. **(B)** Masson's trichrome representative photomicrographs of renal histological changes from an SD, VDD, HFD, and HFDV rat (×400). Data are mean ± SEM. $^{a}p < 0.001$ and $^{c}p < 0.05$ *vs.* SD; $^{d}p < 0.001$ *vs.* VDD; $^{g}p < 0.001$ *vs.* HFD.

described as aggravating factors in the progression of kidney disease, promoting damage to the glomerular filtration barrier and the subsequent presence of proteinuria (25, 47, 70, 71). Although not significant, the HFDV group presented a decrease of ∼13% in GFR in relation to VDD group and a greater proteinuria than the HFD group. Associated with these results, the HFDV group also presented a lower glomerular expression of JG12 compared to all the other groups. Corroborating these results, previous studies from our laboratory demonstrated a decreased renal JG12 expression in vitamin D-deficient rats in renal IRI and 5/6 nephrectomy model (7, 8). Since JG12 is an aminopeptidase responsible for anchoring the cells in the cell membrane, it is plausible to infer that the lower expression of this protein might have contributed to the impaired renal

function observed in the HFDV group. Taken together, those results suggest that either isolated or combined effects of obesity and vitamin D deficiency may impair renal function.

It is common knowledge that the RAAS is responsible for maintaining vascular resistance and extracellular fluid homeostasis (72). The negative endocrine regulation of the RAAS by vitamin D is demonstrated in the literature mainly by an inverse relation of this hormone levels and the expression of renin (72, 73). This impairment of systemic blood pressure control generated by hypovitaminosis D has also been demonstrated in previous studies from our group, which associated changes in the activation of the RAAS with alterations in endothelium and renal vasculature (1, 2, 7, 72–74). Furthermore, the association between obesity and increased MAP has been suggested as an important link in kidney injury (5). In the present study, we found a higher MAP as well as increased plasma levels of Ang II and aldosterone in the VDD, HFD, and HFDV groups, suggesting a greater activation of the RAAS. The main mechanisms involved in the elevation of MAP levels in the course of obesity are described as: (1) activation of the sympathetic nervous system by increasing intra-abdominal pressure and higher levels of leptin; (2) RAAS activation due to increased secretion of inflammatory cytokines such as TNF-α, IL-6, resistin, and leptin by adipocytes; (3) increased aldosterone levels by leptin stimulation to the adrenal gland, increasing Na+ retention and plasma volume expansion; and (4) reduced adiponectin expression, which appears to be one of the causes of increased inflammation in obesity (5, 26, 75, 76). Our data showed that the association between obesity and vitamin D deficiency potentiated hemodynamic impairment. Collectively, changes in MAP and plasma levels of Ang II/aldosterone as well as in RBF and RVR support the hemodynamic disturbance observed in the HFDV group.

Inflammatory cells including macrophages and T cells play a key role in tissue homeostasis and immune responses, especially in the course of kidney diseases (7, 77). Moreover, an exacerbated inflammatory response is usually associated with a growing RFF (7, 77). This process involves several steps, including the stimulation of cell division and the production of chemokines to recruit cells to the site of injury (78). In addition to a higher renal expression of MCP-1 and CD68+ cells, our HFD group presented an upward tendency regarding the expression of CD3+ cells in relation to the SD group, which demonstrates a possible influence of adipose tissue on the inflammatory response. Corroborating our results, Decleves et al. also observed increased renal and urinary expression of MCP-1 in mice fed a high-fat diet (35). In the present study, we also observed similar results concerning the renal expression of MCP-1, CD3+, and CD68+ cells in the VDD group, reinforcing the immunomodulatory effect of vitamin D (1, 7, 77). Of note, we found a higher renal expression of MCP-1, CD3+, and CD68+ cells in the HFDV group compared to all the other groups, demonstrating a synergistic effect of adipose tissue and vitamin D deficiency regarding the inflammatory process.

Macrophage activation and function are heterogeneous and regulated by the microenvironment and stage of tissue injury, reflecting in different phenotypes (1, 77, 79). In general, macrophages are classified into two subtypes: (a) M1 macrophages, classically activated and considered pro-inflammatory due to their ability to produce and release pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, IL- 23, and TNF-α; and (b) M2 macrophages, which are known to have anti-inflammatory and immunomodulatory functions (79, 80). In the present study, we observed a higher renal expression of CD68+ cells (M1+M2 macrophages) in the VDD, HFD, and HFDV groups compared to the SD group. In addition, we noted an upward tendency concerning the expression of those cells in the HFDV group. Although not significant, we noticed a lower renal expression of CD206+ cells (M2 macrophages) in relation to the total amount of CD68+ cells in the HFDV group. It is reported that the balance between M1/M2 macrophages is related to the renal microenvironment and may influence the progression of renal disease (1, 77, 80). In previous studies, we demonstrated that vitamin D deficiency contributed to the extension of the active state of inflammation, reinforcing the role of vitamin D in the modulation of inflammatory cells (1, 7, 77). Concurrently, macrophage infiltration is correlated with the degree of obesity, mainly with the M1 phenotype (79). The inflammatory cytokines produced by M1 macrophages neutralize the insulin-sensitizing actions of the hormones adiponectin and leptin, which eventually lead to insulin resistance (79). In contrast, macrophages in lean subjects express high levels of M2-specific genes, such as IL-10 and Arg-1 (79). Thus, our results show that the association between obesity and vitamin D deficiency contributed to an exacerbation of the inflammatory process observed in the HFDV group, which had higher expression of MCP-1 and CD3+ cells and a lower proportion of CD206+ cells in relation to the CD68+ cells.

As previously reported, AKI can result in incomplete tissue repair, persistent tubulointerstitial inflammation, fibroblast proliferation, and excessive deposition of ECM components (81). Furthermore, in response to kidney injury, cells that are normally stably differentiated to promote homeostasis can dedifferentiate into a new phenotype and redirect tissue repair in a process known as EMT (78). High-fat diets seem to be associated with susceptibility to RFF through increased expression of TGF-β and ECM components (36, 82). Furthermore, studies have shown that vitamin D can suppress the expression of TGF-β and its respective receptor and inhibit the EMT process, cell proliferation, and apoptosis as well (1, 2, 37). Corroborating those findings, we observed a higher renal expression of TGF-β1 in the VDD and HFD groups compared to the SD group. Importantly, the HFDV group presented a higher expression of TGF-β1 than all the other groups, which was associated with higher amounts of Col-3

and fibronectin in the renal tissue. These results suggest the interaction between obesity and vitamin D deficiency on the renal TGF-β1 expression and ECM production.

As aforementioned, EMT process promotes greater cell division, allows cells to acquire a secretory phenotype, and contributes to greater deposition of ECM components (78, 83). In 2012, Xiong et al. reported that the low expression of VDR in CKD would be involved in the relationship between inflammation and EMT (84). In the present study, we observed a higher renal expression of PCNA, α-SMA, and vimentin in the renal cortex from the HFDV group. Previous studies from our laboratory demonstrated the influence of vitamin D deficiency on increased phenotypic change (2) and cell proliferation (8). Corroborating our results regarding obesity, Coimbra et al. also observed a higher expression of vimentin in the renal tissue of obese Zucker rats (85). Furthermore, Amaral et al. showed a higher PCNA expression in the renal cortex of ovariectomized rats in obesity induced by a high-fat diet (86). Thus, similar to our data regarding ECM markers, our results suggest that the synergistic effect of obesity and vitamin D deficiency exacerbated cell proliferation and phenotype alteration of renal tubule cells in the HFDV group.

Fibrosis and inflammation are hallmarks in the course of kidney disease, in which unresolved kidney inflammation becomes an important driving force for the RFF (1, 87, 88). Some studies have been demonstrating that sufficient levels of vitamin D exert a protective effect in preserving cell integrity. Moreover, those reports show that a close relationship among vitamin D levels, VDR expression, and TGF-β could be involved in inflammation and EMT process (2, 37, 84). Previous data from our group showed that vitamin D deficiency caused a lower renal VDR expression and a higher renal TGF-β1 expression in rats submitted to renal IRI (2) and 5/6 nephrectomy (7, 77). Concomitantly, adipose tissue is recognized as an endocrine organ involved in the production and action of adipokines, including leptin, TNF-α, MCP-1, and TGF-β (23, 70, 89). In the present study, along with the increased expression of TGF-β1 and leptin as well as the decreased expression of the VDR, the HFDV group presented an exacerbated inflammatory infiltrate, greater cell proliferation, and phenotypic alteration of renal tubular cells. Taken together, these changes reflected a significant enlargement of the FIA in the HFDV group. Those results demonstrated a plausible synergistic effect of increased production and secretion of adipokines by adipose tissue and an impaired renoprotective action attributed to hypovitaminosis D. Hence, our data demonstrated that obesity associated with vitamin D deficiency led to a potentiation of the expression of inflammatory and pro-fibrotic factors in the progression following AKI induced by renal IRI.

Our results allow us to conclude that the association between vitamin D deficiency and obesity in the renal ischemia/reperfusion model modified functional, hemodynamic, and metabolic parameters and contributed to a greater expression of inflammatory and pro-fibrotic factors related to the progression of renal disease.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Research Ethics Committee of Faculty of Medicine, University of São Paulo (CEUA, registration 1438/2020).

Author contributions

DB, DC, MN, MS, AB, and RV: performed the experiments. DB, DC, AB, and RV: analyzed the data and contributed to the writing of the manuscript. All authors conceived and designed the experiments, reviewed the manuscript, and approved the submitted version.

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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 meta-analysis suggests the association of reduced serum level of vitamin D and T-allele of Fok1 (rs2228570) polymorphism in the vitamin D receptor gene with celiac disease

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Purpose: As an immune-modulator, vitamin D is known to regulate immune response and is implicated in disease pathogenesis. Celiac disease (CD) is a systemic autoimmune disease and susceptibility conferred by vitamin D metabolism is under investigation. Studies on the association of vitamin D metabolism and genetic polymorphisms are expected to explain CD pathogenesis. We performed a systematic review–based meta-analysis to investigate the 25(OH)D serum levels and susceptibility conferred by the genetic variants of VDR in CD.

Methods: Systematic review was conducted through a web-based literature search following stringent study inclusion–exclusion criteria. The Newcastle–Ottawa Scale and GRADE tools were used to assess the quality of evidence in studies and the study outcome. Cohen's κ value was estimated to access the reviewer's agreement. RevMan 5.4.1 was used to perform the meta-analyses. Weighted mean difference and Meta p-value was assessed for 25(OH)D serum levels. Meta-odds ratio and Z-test p-value were evaluated to estimate the allelic susceptibility of *VDR* variants.

Results: A total of 8 out of 12 studies were evaluated for "25(OH)D" serum level, while four studies were found eligible for SNPs (*Bsm1*, *Apa1*, *Fok1*, and *Taq1*) of *VDR*. Significantly higher levels [WMD = 5.49, $p < 0.00001$] of 25(OH)D were observed in healthy controls than in patients with CD. rs2228570-T (*Fok1*) [Meta-OR = 1.52, $p = 0.02$] was confirmed to be predisposing allele for CD.

Conclusion: Reduced serum level of 25(OH)D and association of *Fok1* T-allele of *VDR* confirmed in this study plays a critical role in immunomodulation and maintaining barrier integrity, which is majorly implicated in CD.

KEYWORDS

celiac disease, vitamin D deficiency, vitamin D receptor, Fok1 polymorphism, meta-analysis, autoimmunity

1. Introduction

Celiac disease (CD) is an immune-mediated gluten enteropathy affecting almost 1% of the population worldwide in individuals carrying HLA DQ2/8 susceptibility alleles, which are encoded by DQA1*0501-DQB1*02 and DQA1*0301-DQB1*0302 (1, 2). Almost 94.94% of the CD subjects are positive for this specific HLA-DQB1*02 allele (2). Though the etiology of CD is not well understood but is marked by the presence of inflammatory cytokines IL18, IL17, TNF-α, IL12, IL21, and IL15 (3). Vitamin D was found to be associated with reducing the effects of inflammatory molecules (4). Components of the immune system, such as B-lymphocytes, T-lymphocytes, and dendritic cells, are influenced by the regulatory effects of vitamin D and expressed vitamin D receptor (VDR), which is involved in the biological activity of $1,25(OH)_2D3$, and these cells also have the capability of locally synthesizing active $1,25(OH)_2D3$ (5). This active form of vitamin D exerts its effects by binding to the nuclear receptor VDR. The $1,25(OH)_2D3$-VDR complex dimerizes with the retinoid X receptor (*RXR*), and the $1,25(OH)_2D3$-VDR-RXR heterodimer translocates to the nucleus where it binds Vitamin D Response Element (VDRE) in the promoter regions of vitamin D responsive genes and induces the expression of vitamin D responsive genes (6). Some of the remarkable effects of vitamin D in immune system regulation were the suppression of Th1/Th17 CD4+ T cell proliferation and subsequent alteration of the cytokine responses (5). Thus, it is worth studying the association between vitamin D metabolism and various immune-mediated disorders.

Duodenal epithelial damage in CD is caused by the cytokines released by the activated T-cells upon exposure to gliadin peptides, and vitamin D is reported to suppress the proliferation of T-cells (7, 8). *In vitro* study performed on Caco-2 cell layers reported the protective role of $1,25(OH)_2D3$ on the damage of tight junction, which was induced by the pepsin–trypsin digested gliadin (PT–G). Vitamin D was observed to increase the expression of the tight junction–associated proteins and was also able to minimize epithelial permeability (9). Notably, vitamin D deficiency increases the risk of severe intestinal damage, which is a prominent symptom of CD (10). During the early infant stage, 25(OH)D concentrations between <30 and >75 nmol/L were associated with an increased risk of developing CD in genetically predisposed children. The non-linear relationship raises the need for more studies on the possible role of 25(OH)D in the onset of CD (11). *In vivo* studies have shown a positive response to vitamin D supplementation in the celiac mice model (12).

In the association study in the Spanish Basque population, four polymorphisms of *VDR* (*Bsm1*-rs1544410, *Apa1*-rs7975232, *Taq1*-rs731236, and *Fok1*-rs2228570) were genotyped. *Fok1* was reported to be a risk genotype in 25.64% of CD cases as compared to 9.89% of controls ($p = 0.01$, OR = 3.45) (13). Another association study on the Norwegian cohort did not find any association with the *VDR* marker (*Bsm1*) or serum vitamin D level (14). Two significant SNPs, *Fok1* and *Bsm1*, were reported in the Russian Tomsk group, $p = 0.009$ and $p = 0.001$, respectively (15). A total of 92 Viennese CD patients and controls were genotyped for *Apa1* and *Taq1* SNPs of the *VDR* gene; however, no significant risk association was observed (16). A recent meta-analysis study by Lu et al. (17) did not find any association with VDR genotype but reported lower levels of 25(OH)D in CD patients than in controls.

In this systematic review and meta-analysis, we intended to assess an association of serum level of 25(OH)D and *VDR* gene polymorphism with CD. Four SNPs (*Bsm1*-rs1544410, G>A; *Apa1*-rs7975232, C>A; *Taq1*-rs731236, T>C; and *Fok1*-rs2228570, C>T) of *VDR* were evaluated.

2. Methods

2.1. Search strategy

For the search and retrieval of relevant published literature, various web search engines for scientific databases such as Google scholar, NCBI (PubMed/MEDLINE), SCOPUS, EMBASE, and Web of Science were used. All the published literature until May 2022 on the association of vitamin D serum concentration and *VDR* gene polymorphism with celiac disease was searched. For this literature search, keywords were used as follows: "Celiac disease" AND "serum vitamin D concentration" OR "serum 25(OH)D concentration". For the genotype association study, the key terms used were as follows: "Celiac disease" AND "*VDR* polymorphism" OR "*VDR* genotype" OR "*VDR* variants" OR "*Bsm1* polymorphism" OR "*Apa1* polymorphism" OR "*Fok1* polymorphism" OR "*Taq1* polymorphism".

2.2. Inclusion and exclusion criteria

To limit the screening to relevant articles, the inclusion and exclusion criteria were defined. Articles were considered eligible for the study if they met the following inclusion criteria: (i) published full-text original research article, (ii) case–control study design, (iii) mean serum 25(OH)D concentration can be obtained, (iv) *VDR* gene polymorphism association study with celiac disease and healthy controls, and (v) Only articles written in the English language. Our study was not restricted to any specific population or ethnicity, and all the relevant articles available till May 2022 were considered.

The exclusion criteria for the study were as follows: (i) studies other than case–control, (ii) if the CD patients were

on a gluten-free diet or vitamin D supplementation, and (iii) review articles. Apart from this, duplicate publications were also excluded.

2.3. Data extraction and evaluation of confined studies

Two researchers independently performed a literature search checked the eligibility criteria, and extracted data from the shortlisted literature. From all the studies which we considered eligible according to our inclusion and exclusion criteria, relevant data for serum vitamin D concentration and *VDR* genotype in patients with CD and healthy controls were retrieved along with the name of the author and the year of publication. For the concentration of vitamin D, the serum levels of 25(OH)D [mean ± standard deviation (SD)] in patients with CD and healthy controls were extracted from every eligible article, and for the vitamin D concentration, the units considered were in ng/ml, data obtained in other units (such as nmol/L) were converted to ng/ml (1 ng/ml = 2.5 nmol/L). Few other information about the study population such as the mean age of the patients with CD and healthy controls and the male:female ratio in the study were also obtained.

For the *VDR* genotype association studies, *VDR* SNPs data of CD cases and controls were obtained. Four SNPs of the *VDR* gene: *Bsm1*-rs1544410, *Apa1*-rs7975232, *Fok1*-rs2228570, and *Taq1*-rs731236 were analyzed, and the genotype frequencies in CD cases and controls were obtained. After data extraction, to carry out a systematic review, standard checklists were used to analyze methodological quality and strength of association, which also included the risk of bias evaluation in observational studies as recommended by the Cochrane handbook (https://training.cochrane.org/handbook). The Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) statement was followed for reporting the findings (http://www.prisma-statement.org/) (18). The PRISMA statement is provided in Supplementary Table 1.

2.4. Quality assessment

Cohen's kappa (κ) value was calculated in order to estimate the extent of concordance between two reviewers, who performed a literature search, and checked eligibility and data extraction (19). Based on the percentage of agreement and Cohen's κ score, values were classified as poor, slight, fair, moderate, substantial, or almost perfect. Sensitivity analysis was

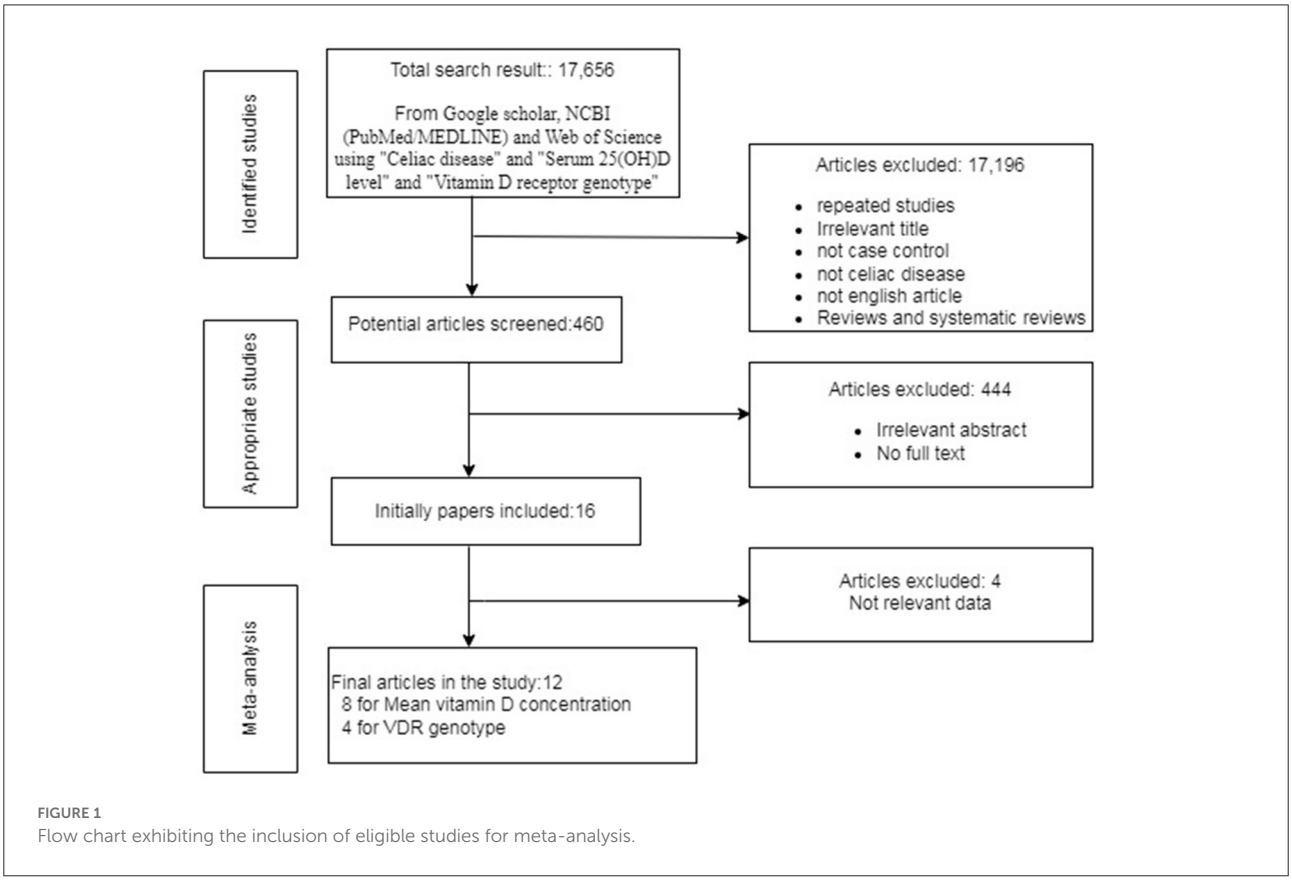

FIGURE 1
Flow chart exhibiting the inclusion of eligible studies for meta-analysis.

TABLE 1 Summary table of the alleles included in the meta-analysis and the quality of evidences as graded by the GRADE tool.

		N	Total CD cases	Total Healthy Controls	Ref Allele Meta-OR (95% CI)	I^2 (%) p-value	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	Quality of evidence	Importance
VDR	Bsm1 rs1544410	3	964	1468	0.98[0.83,1.16]	37% (p = 0.84)	Non-randomized observational case-control	Not serious	Not serious	Not serious	Not serious	None	Low	Important
	Apa1 rs7975232	2	262	398	1.21[0.61,2.38]	76% (p = 0.58)	Non-randomized observational case-control	Not serious	Not serious	Not serious	Not serious	None	Low	Important
	Fok1 rs2228570	2	176	402	1.52[1.06,2.18]	0% (p = 0.02)	Non-randomized observational case-control	Not serious	Not serious	Not serious	Not serious	None	Low	Important
	Taq1 rs731236	2	262	404	0.78[0.46,1.32]	57% (p = 0.35)	Non-randomized observational case-control	Not serious	Not serious	Not serious	Not serious	None	Low	Important

#, Quality of evidence was accessed using six parameters. QoE was downgraded (by 1 or 2 depending on the severity) for the following: study design- randomized controlled trials are preferred over non-randomized/observational/case-control studies; risk of bias-downgraded for weak study design, short follow-up, and not matched case controls; inconsistency-downgraded for considerable heterogeneity, direction of effect, and lack of replication; indirectness-downgraded when population and diagnostic criteria varies; imprecision-wide confidence of interval and optimal information size; publication bias-observation from funnel plots.

High quality, Further research unlikely change the study findings and effect estimates; Moderate quality, Further research is likely to change the study findings or the effect estimates; Low quality, Further research is very likely to have an impact on the confidence and effect estimates; Very low quality, Uncertain estimates; #, Primarily influenced by the study design alone; ∗, Beside study design, one or two factors are implicated.

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used to evaluate the impact of each study on the meta-analysis result by removing one study at a time from the combined dataset. Funnel plots were analyzed in order to detect any study biasness. If any of the studies fell outside the funnel plot or gave rise to an asymmetric funnel plot, then that study was considered to be biased. Irrelevant studies were ruled out after a thorough analysis. The Newcastle–Ottawa Scale (NOS) tool was used to assess the quality of the eligible studies (i.e., study participant selection, comparability, and outcome) (20). Studies were graded on a scale of 0 (lowest) to 9 (highest), with low (stars 7–9), moderate (stars 4–6), and high (stars 0–3) risk of bias. The analysis was limited to studies with low risk.

GRADEpro.v.3.6 was used to assess the quality of evidence (QoE) for each of the outcomes using The Grades of Research, Assessment, Development, and Evaluation (GRADE) tool (21). Based on the proposed criteria, the included study design, risk of bias, inconsistency, indirectness, imprecision, and publication bias, and evidence were grouped into four main categories: high, moderate, low, and very low.

2.5. Statistical analysis

For the meta-analysis of the serum 25(OH)D level, the mean difference was used to analyze the pooled continuous data of serum 25(OH)D concentration which was obtained as mean ± standard deviation (SD). To evaluate the combined MD, mean differences (MD) with 95% confidence intervals (CIs) were presented for all relevant studies on a forest plot. A collective study was carried out without dividing the study based on age group due to very few studies were obtained with relevant data in the adult age group. The dichotomous data of the alleles of the four SNPs of the *VDR* gene were utilized to calculate Meta-OR using Mantel–Haenszel (M–H) method with 95% CI). The statistical analysis for this study was carried out using the RevMan (version 5.4.1, The Cochrane Collaboration) software. For this meta-analysis, the assessment of study heterogeneity was done by chi-square *p*-value and I^2 value. The meta-analysis was carried out using the DerSimonian and Laird random effect model for $I^2 > 50\%$ and $p < 0.05$, and for $I^2 < 50\%$ and $p > 0.05$, the fixed effect model was used.

3. Results

3.1. Features of enclosed studies

After employing all the above-described literature retrieval strategies, we got a total of 17,656 studies. Overall, 17,196 studies were excluded based on the inclusion and exclusion criteria because of irrelevant titles or because they were review articles or were not in the English language. After screening the rest of the articles, 444 articles were excluded from our study because of

TABLE 2 Summary table of mean 25(OH)D concentration included in the meta-analysis and the quality of evidences as graded by the GRADE.

Overall study comparision							Assessment of quality of evidence (GRADE Tool)						
Serum concentration	N	Total CD cases	Total healthy controls	Mean-difference (95% CI)	I^2 (%) p-value	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	Quality of evidence	Importance
25(OH)D	8	592	754	5.49 [3.22, 7.76]	73% (p = 0.0001)	Non-randomized Observational Case-Control	Not serious	Not serious	Not serious	Not serious	None	Low	Important

#, Quality of evidence was accessed using six parameters. QoE was downgraded (by 1 or 2 depending on the severity) for the following: study design- randomized controlled trials are preferred over non-randomized/observational/case-control studies; risk of bias-downgraded for weak study design, short follow-up, and not matched case controls; inconsistency-downgraded for considerable heterogeneity, direction of effect, and lack of replication; indirectness-downgraded when population and diagnostic criteria varies; imprecision-wide confidence of interval and optimal information size; publication bias-observation from funnel plots. High quality, Further research unlikely change the study findings and effect estimates; Moderate quality, Further research is likely to change the study findings or the effect estimates; Low quality, Further research is very likely to have an impact on the confidence and effect estimates Very low quality, Uncertain estimates; #, Primarily influenced by the study design alone; *, Beside study design, one or two factors are implicated.

duplicate publications, irrelevant abstracts (*in silico*, *in vitro*, *in vivo* studies, case studies), and articles without full text. Initially, a total of 16 studies were considered for this study but due to the lack of relevant data, four articles were excluded. Finally, 12 studies that satisfied our inclusion and exclusion criteria and had the data of serum 25(OH)D level and *VDR* genotype in CD cases and control were included in this meta-analysis as shown in Figure 1. All these data of mean serum 25(OH)D concentration and the genotype of *VDR* SNPs *Bsm1* rs1544410, *Apa1* rs7975232, *Fok1* rs2228570, *Taq1* rs731236, and their summary statistics are provided in Tables 1, 2.

3.2. Publication bias evaluation and sensitivity analysis

In order to detect any study biasness in this meta-analysis, the funnel plots of all the studies were analyzed. Upon this analysis, the exclusion of any study was not done as study biasness was not detected. When it came to the inclusion and exclusion of relevant and irrelevant articles from this systematic review, reviewers were almost unanimous (Cohen's $\kappa = 0.96$; % agreement 98.28). All the 12 eligible studies included in this meta-analysis are given in Supplementary Figure 1.

3.3. Quality of the studies included and risk of biasness

Based on the evaluation of the quality of evidence, all 12 studies were identified to have a low risk of bias (NOS = 7–8) (Supplementary Table 2). Because of concise criteria for evaluation and homogeneous populations, GRADE's approach observation indicated that none of the 12 studies increased the possibility of bias, and the indirectness of the findings was not a concern. The evidence quality was insufficient to rule out any of the studies that were included. As a result, the entire study was deemed important and rated as low risk in Supplementary Table 2.

3.4. Concentration of 25(OH)D in CD patients and control

A total of eight studies were evaluated in this meta-analysis with mean serum 25(OH)D concentration of patients with CD and controls, the list of same is given in Supplementary Table 3 (22–29). It constitutes 592 patients with CD and 754 controls. This meta-analysis was carried out using the Random effect model. Significant associations were defined as Z-test *p*-values (i.e., Meta-*p*-values) of <0.05. The mean difference was evaluated for all the studies included, and a forest plot was plotted using the mean differences with 95% CI, of each of these studies to estimate the combined mean difference (Figure 2). The outcome of this meta-analysis exhibited that the mean 25(OH)D concentration in the healthy controls was 5.49 ng/ml higher than that of CD patients (WMD = 5.49, 95% CI = 3.22–7.76). The observed meta-*p*-value was significant ($p < 0.00001$) (Figure 2).

3.5. VDR allele association with CD patients and controls

For this meta-analysis of allelic association, four studies were included in which the allelic frequencies for the four *VDR* SNPs (*Bsm1* rs1544410, *Apa1* rs7975232, *Fok1* rs2228570, and *Taq1* rs731236) were given for patients with CD and healthy controls, basic information of which is provided in Supplementary Table 4. The meta-analysis for the SNPs *Bsm1* rs1544410 and *Fok1* rs2228570 was performed using the fixed effect model for insignificant heterogeneity ($I^2 < 50\%$ and p

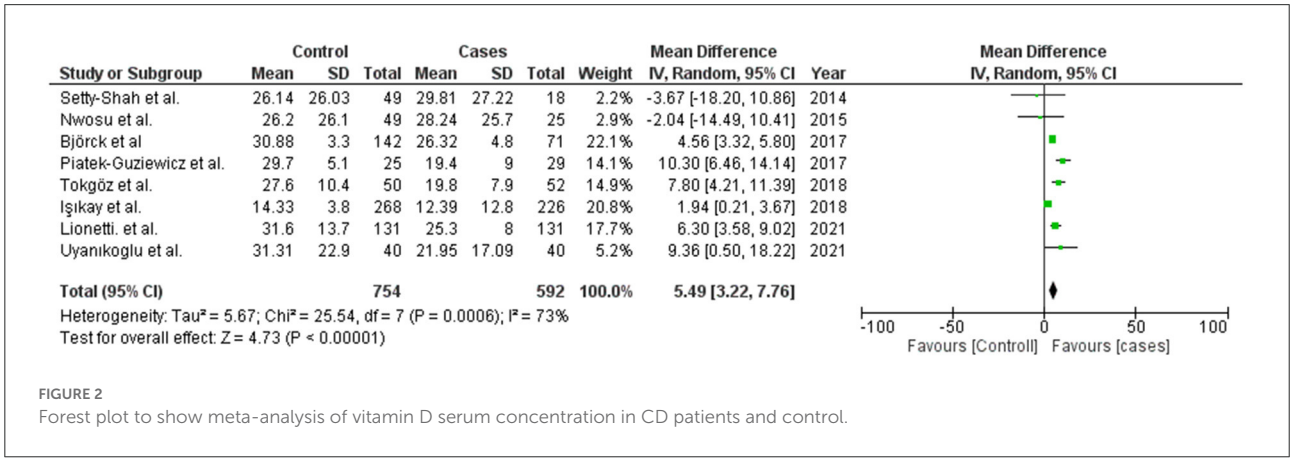

FIGURE 2
Forest plot to show meta-analysis of vitamin D serum concentration in CD patients and control.

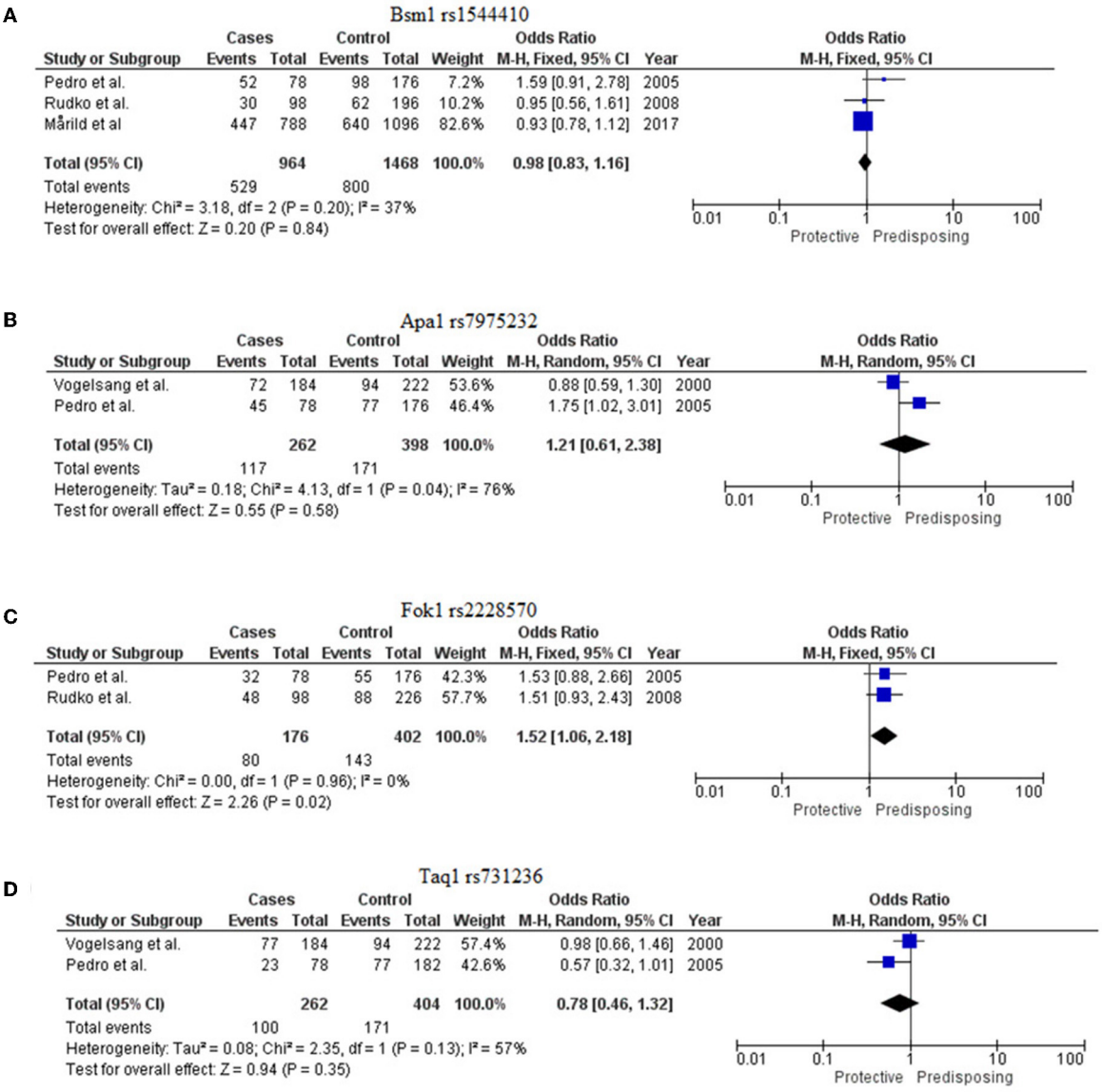

FIGURE 3
Forest plots to show meta-analysis of VDR SNPs [**(A)** *Bsm1 rs*1544410, **(B)** *Apa1 rs*7975232, **(C)** *Fok1 rs*2228570, and **(D)** *Taq1 rs*731236].

> 0.05), whereas for *Apa1* rs7975232 and *Taq1* rs731236, the random effect model was used because of a high degree of data heterogeneity. Significant associations were defined as Z-test *p*-values (i.e., Meta-*p*-values) <0.05. The Meta-OR was used to predict risk.

3.5.1. Bsm1

Three studies were evaluated (13–15). The G allele of this marker rs1544410 was found to be protective for CD [Meta-OR = 0.98 (0.83–1.16), $p = 0.20$]. The meta *p*-value was declared insignificant (Figure 3A).

3.5.2. Apa1

Two studies were considered for this *VDR* marker (13, 16). The C allele of this marker rs7975232 was found to confer risk for CD [Meta-OR = 1.21 (0.61–2.38), $p = 0.58$] but with insignificant *p*-value (Figure 3B).

3.5.3. Fok1

Two eligible studies were included for this marker (13, 14). The T allele of rs2228570 was identified to be significantly predisposing for the disease [Meta-OR = 1.52 (1.06–2.18), $p = 0.02$] (Figure 3C).

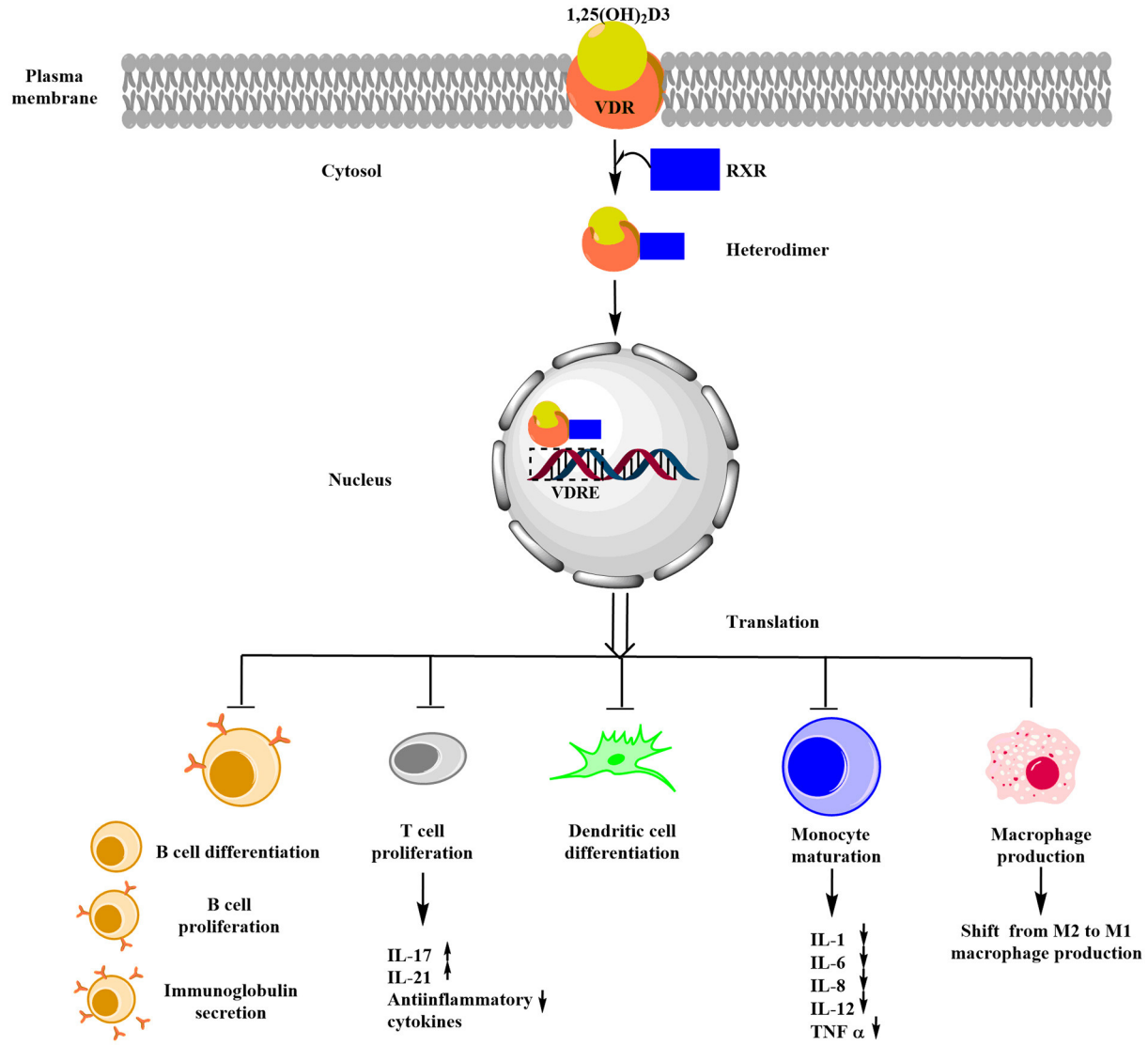

FIGURE 4
Schematic representation of the role of vitamin D in immune regulation in CD. Upon binding of vitamin D3 with VDR, RXR is recruited and results in the formation of a heterodimer known as VDR-RXR complex, whose translated product (formed by binding of the heterodimer with VDRE) exerts immune modulation by inhibiting differentiation and proliferation of B-cell, T-cell, and dendritic cells and also inhibits immunoglobulin secretion.

3.5.4. Taq1

Two studies were evaluated for this meta-analysis (13, 16). T allele of rs731236 was found to be protective [Meta-OR = 0.78 (0.46–1.32), $p = 0.13$]. But no significant association was found (Figure 3D).

4. Discussion

Multisystem CD is characterized by circulating innate lymphoid cells and increased levels of IL-18, IFN-γ, and innate lymphoid cell precursors were noted (3). A reduced vitamin D level has been reported to be correlated with higher IFN-γ and innate lymphoid cell precursor (4). Vitamin D deficiency is shown to induce T-cell-mediated pro-inflammatory immune responses that are pivotal in CD (30, 31). This gave an insight into dietary supplementation of vitamin D as a therapeutic approach to inhibit cytokine IFN-γ production (4). Several cross-section studies concluded the association of vitamin D deficiency with immune-related diseases (32–34). *In vitro* and *in vivo* studies on induced CD-like conditions reported vitamin D supplementation rescue from cellular and tissue damage, which directly indicated the protective role of vitamin D in CD (8, 35, 36).

This meta-analysis suggests the association of the reduced serum level of 25(OH)D [MD = 5.49; $P < 0.00001$] and rs2228570-T (*Fok1*) [Meta-OR = 1.52, $p = 0.02$] with CD. All the studies were performed in the last decade and on a modest sample size (Figure 2). Cross-section studies with case-control study design, which was included in this meta-analysis, are however unable to comment on the cause-effect relationship between VDD and CD. Nevertheless, this finding suggests vitamin D supplements to subjects with CD to restore the normal duodenal mucosal barrier and suppress inflammatory immune responses as illustrated in Figure 4. 25(OH)D is the most stable form of vitamin D, and its transport and stability are determined by the availability of vitamin D binding protein (VDBP) in the serum. To date, no reports are available on the association of serum VDBP with CD.

The extra-calcium role of vitamin D and its involvement in immune modulation suggested that in genetically predisposed individuals, vitamin D deficiency can be an underlying cause for the onset of CD in children. Moreover, vitamin D deficiency can lead to dysregulated immune responses that result in abnormal intestinal mucosa and a greater risk of developing acute gastrointestinal infection (37). Several reports are available to suggest significant improvements in subjects with CD following vitamin D supplementation alongside gluten-free diet (GFD) (38).

Very limited genetic association studies were performed on CD to determine the contribution of vitamin D metabolism to the disease. Only four research articles were available on VDR, and all the studies were performed in populations with European ancestry (Supplementary Table 4). Four polymorphisms namely, *Bsm1* (rs154441, G>A), *Apa1* (rs7975232, C>A), *Fok1* (rs2228570, C>T) and *Taq1* (rs731236, T>C) were considered where at two studies were available for the meta-analysis. The association of the *Fok1*-T allele with CD (OR = 1.52, $p = 0.02$) suggested the putative role of this gene in the disease pathogenesis (Figure 3C).

The *Fok1* polymorphism of *VDR* is associated with several other immune-mediated disorders such as type 2 diabetes (T2DM) (39). The *Fok1* polymorphism also known as the start codon polymorphism (SCP), in exon 2 of the *VDR* has been shown to alter the structure of the VDR. The change in C > T also represented as F > f leads to a threonine to methionine substitution and provides two possible sites for the initiation of translation (40). The shorter VDR form, that is, 424 amino acid protein (encoded by the common allele C) in the FF genotype appears to be more effective in binding $1,25(OH)_2D_3$ and has a higher binding capacity (41), while rs2228570-T (in ff genotype) leads to the production of 427 amino acid protein product, which is comparatively 1.7 times less efficient at the binding of $1,25(OH)_2D_3$. Reduced binding efficiency with $1,25(OH)_2D_3$ thus restricts VDR activation, and therefore, may limit regulating the expression of specific genes that are implicated in immune regulation.

5. Conclusion

In this meta-analysis, lower levels of serum 25(OH)D were observed in patients with CD, which indicates that deficiency of vitamin D may play a significant role in the pathogenesis of CD. The SNPs of the *VDR* gene (*Bsm1*-rs1544410, *Apa1*-rs7975232, and *Taq1*-rs731236) did not show any significant association with CD, but *Fok1* (rs2228570-T) was identified to be providing significant risk for CD. However, due to limitations in the number of studies performed on the association of *VDR* gene polymorphism and CD, strong evidence to support this association is still lacking.

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

SS conceptualized the study. SS and TS designed the study and performed the initial literature search. PB and TS performed the systematic review. SS and PB performed the analysis and wrote the manuscript. All the authors read the final manuscript and approved it for publication.

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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/fnut.2022.996450/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Funnel plot analysis of all eligible studies for serum vitamin D concentration and VDR gene SNPs.

SUPPLEMENTARY TABLE 1
PRISMA checklist.

SUPPLEMENTARY TABLE 2
The Newcastle-Ottawa Scale (NOS) assessment for all the twelve studies found eligible for this study ensuring the quality of evidence.

SUPPLEMENTARY TABLE 3
Mean difference of vitamin D [25(OH)D] concentration in serum.

SUPPLEMENTARY TABLE 4
Allele frequencies of four VDR SNPs those were included in the study of healthy controls and CD patients.

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Effects of vitamin D supplementation on the regulation of blood lipid levels in prediabetic subjects: A meta-analysis

Yixue Yang[1], Shoumeng Yan[2], Nan Yao[1], Yinpei Guo[1], Han Wang[1], Mengzi Sun[1], Wenyu Hu[1], Xiaotong Li[1], Ling Wang[1] and Bo Li[1]*

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This meta-analysis aimed to systematically investigate whether vitamin D supplementation reduces blood lipid—total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and triglyceride (TG)—levels in prediabetic individuals. Pubmed, Web of Science, Cochrane Library, Embase, CNKI, and WANFANG databases were searched for studies published before 13 February 2022 (including 13 February 2022). Five articles were included. The results showed that vitamin D intervention led to a significant reduction in TG compared with control or placebo treatment (−0.42 [−0.59, −0.25], $P < 0.001$). Subgroup analyses showed that this effect was particularly significant among the studies that included obese subjects (−0.46 [−0.65, −0.28], $P < 0.001$), the studies that also included men (not only women) (−0.56 [−0.78, −0.34], $P < 0.001$), and the studies with intervention durations longer than 1 year (−0.46 [−0.65, −0.28], $P < 0.001$). Both relatively low doses of 2,857 IU/day (−0.65 [−0.92, −0.38], $P < 0.001$) and relatively high doses of 8,571 IU/day (−0.28 [−0.54, −0.02] $P = 0.04$) of vitamin D supplementation reduced TG levels, and the effect was observed both in Northern Europe (−0.65 [−0.92, −0.38], $P < 0.001$) and Asian (−0.25 [−0.48, −0.03], $P = 0.03$) country subgroups. No significant effects on TC, HDL-C, and LDL-C were shown. In conclusion, vitamin D supplementation might beneficially affect TG levels in individuals with prediabetes. Particularly longer durations of treatment, more than 1 year, with doses that correct vitamin deficiency/insufficiency, can have a beneficial effect. This meta-analysis was registered at www.crd.york.ac.uk/prospero (CRD42020160780).

KEYWORDS

vitamin D, meta-analysis, prediabetes, cholesterol, LDL cholesterol, HDL cholesterol, triglycerides

1. Introduction

The global burden of diabetes mellitus cannot be ignored. In the last several decades, the prevalence of diabetes mellitus has extensively increased worldwide (1). It has been estimated that in 2011, there were approximately 366 million patients with diabetes and that the number is expected to reach 552 million by 2030 (2). Additionally, diabetes is associated with a high risk of cardiovascular diseases, mortality, and high economic costs related to the treatment and associated working disability (3, 4).

Diabetes can be preceded by prediabetes, and timely intervention during the prediabetic state is important for preventing the progression of diabetes (1, 5, 6). Prediabetes is defined as a state with a blood glucose level beyond the normal value but not reaching the diagnostic criteria for diabetes, including impaired fasting glucose (IFG, defined as fasting plasma glucose of 6.1–6.9 mmol/L or 5.6–6.9 mmol/L), impaired glucose tolerance (IGT, defined as 2h OGTT plasma glucose of 7.8–11.1 mmol/L), or glycated hemoglobin A1c (HbA1c) levels between 39 and 47 mmol/mol (7). Dyslipidemia is an important characteristic of both prediabetes and diabetes and may aggravate diabetic complications (8–11).

Vitamin D deficiency is another growing health concern in many parts of the world, affecting more than 50% of the general population worldwide (12). At the same time, it has been observed that people with lower 25(OH)D levels tend to have higher blood glucose (9), insulin resistance (10), and a higher risk of type 2 diabetes mellitus (T2DM) (11). In addition, some studies have shown that vitamin D supplementation may ameliorate dyslipidemia in subjects with T2DM (13, 14). Potential mechanisms included reduced intestinal cholesterol absorption, decreased low-density lipoprotein deposition in macrophages and foam cell formation, increased lipoprotein lipase gene expression in muscles and adipose tissue, etc. (15–17). However, there is still controversy over whether vitamin D supplementation can improve lipid levels in subjects with prediabetes since such studies are rare and more equivocal.

Therefore, we performed a meta-analysis evaluating the effect of vitamin D supplementation on blood lipid levels in subjects with prediabetes.

2. Materials and methods

2.1. Data sources and searches

We comprehensively searched the PubMed, Web of Science, Cochrane Library, Embase, CNKI, and WANFANG databases for all studies with human subjects in any language published before 13 February 2022 (including 13 February 2022) (Figure 1). We explored changes in serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) levels before and after intervention with vitamin D supplementation in comparison with changes in blood lipids on a control treatment without vitamin D supplementation. The control treatment was defined as no supplementation, placebo supplementation, or another treatment without vitamin D supplementation that was also present in the vitamin D supplementation group (e.g., lifestyle intervention, calcium carbonate supplementation, and omega-3 fatty acid supplementation). Two investigators independently reviewed the literature, discussed the inconsistencies, and worked independently during the selection process, data collection process, and study risk of bias assessment (Figure 2).

2.2. Inclusion and exclusion criteria

The included studies met the following criteria. Subjects should meet the diagnostic criteria for prediabetes (International Diabetes Federation (IDF)/World Health Organization (WHO) from 2006: fasting plasma glucose value in the range of 6.1–6.9 mmol/L or 110–125 mg/dl, or 2h oral glucose tolerance test (OGTT) plasma glucose value in the range of 7.8–11.0 mmol/L or 140–200 mg/dl (18) and American Diabetes Association (ADA) from 2004: fasting plasma glucose value in the range of 5.6–6.9 mmol/L or 100–125 mg/dl, or 2h OGTT plasma glucose value in the range of 7.8–11.0 mmol/L or 140–200 mg/dl; or HbA1c in the range of 39–47 mmol/mol or 5.7–6.4% (19). We restricted included studies to prospective intervention studies; studies included at least one vitamin D intervention group and one control group receiving no vitamin D supplementation, with the only difference between the intervention group and control group being vitamin D intervention; studies included at least one of the blood lipid indicators (TC, LDL-C, HDL-C, or TG); studies provided quantitative data before and after vitamin D intervention or quantitative changes after vitamin D intervention compared with baseline data of blood lipid indicators.

Duplicate articles in databases, studies that did not meet the above inclusion criteria, animal experiments, *in vitro* studies, reviews, and conference papers were excluded.

2.3. Data extraction

We read all included articles and then abstracted the following data: primary authors, nationality, and publication year; average age, gender, BMI, region, and the number of participants in each group; vitamin D supplement dose and time; criteria for prediabetes definition; TC, LDL-C, HDL-C, and TG alterations in intervention groups and control groups. If the included original article had more than one intervention group or control group, we chose the most suitable group for further analysis.

2.4. Quality assessment

Two investigators independently assessed the risk of bias using RevMan 5.3, including selection bias, performance bias, detection bias, attrition bias, reporting bias, and other bias, which were classified into three levels as high, low, or unclear, along with discussion and negotiation with respect to inconsistency. The

Abbreviations: HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; SMD, standardized mean difference; CI, confidence interval; BMI, body mass index; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; FEM, fixed effect model; REM, random effect model; SD, standard deviation; PPARγ, peroxisome proliferator-activated receptor-gamma; C/EBPα, CCAAT enhancer binding protein-alpha; IDF, International Diabetes Federation; ADA, American Diabetes Association; WHO, World Health Organization; HbA1c, glycated hemoglobin A1c; T2DM, type 2 diabetes mellitus; PTH, parathyroid hormone; RAAS, renin-angiotensin-aldosterone system; SREBP1c, sterol regulatory element-binding protein 1c; LPL, lipoprotein lipase; AP2, adipocyte-binding protein 2.

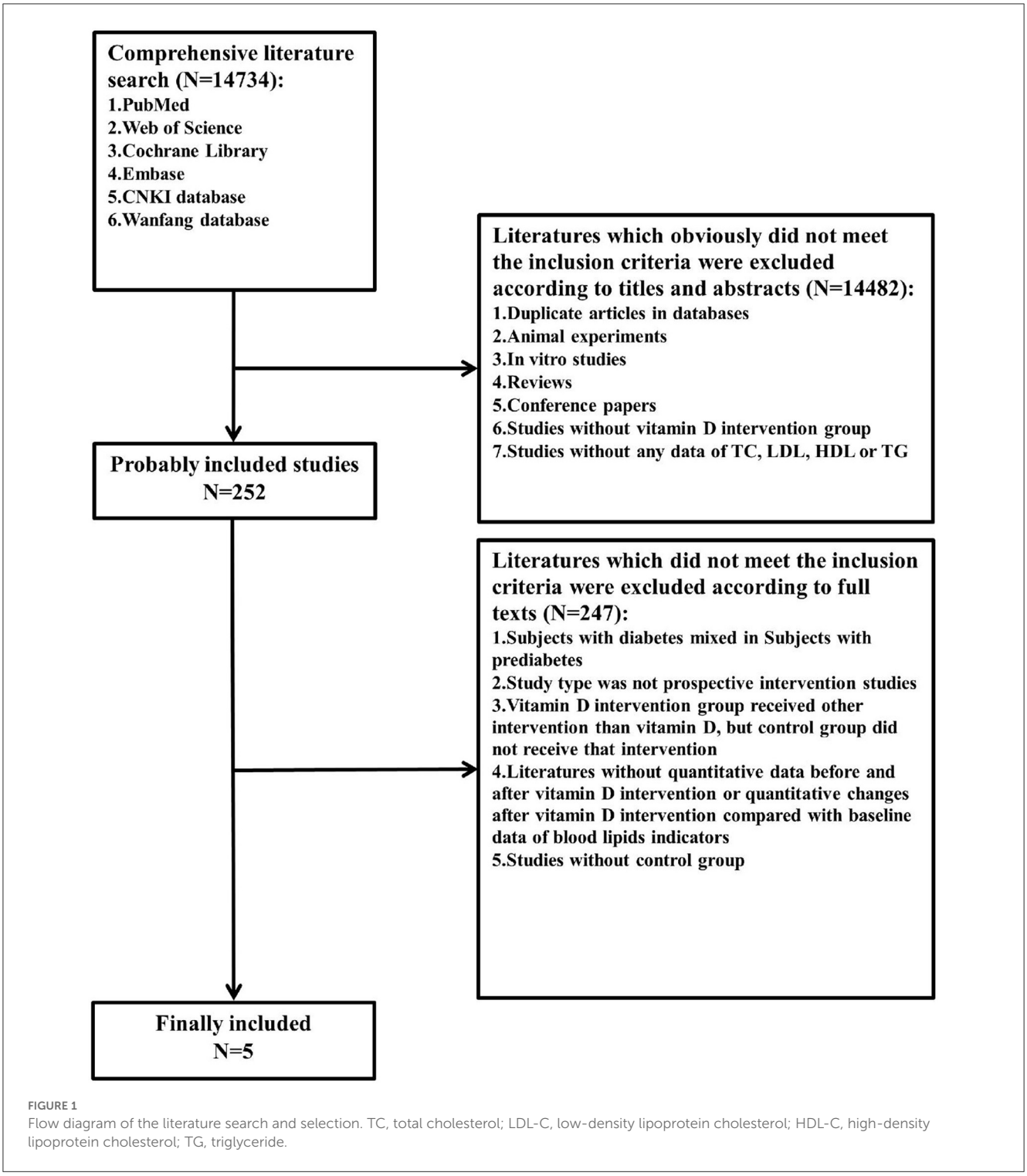

FIGURE 1
Flow diagram of the literature search and selection. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

judgment standards were established from Cochrane Handbook for Systematic Reviews of Interventions (20).

2.5. Statistical analysis

In this study, the RevMan 5.3 and Stata 12.0 software were used for statistical analysis. The average differences between the intervention group and the control group were calculated by the average changes in blood TC, LDL-C, HDL-C, and TG levels compared to baseline values (mean ± SD) (SD: standard deviation). When the original studies did not provide changes in SD, the formula in the Cochrane handbook was used to calculate (20).

$$SD_{change} = \sqrt{SD^2_{baseline} + SD^2_{end} - (2 \times R \times SD_{baseline} \times SD_{end})}$$

The correlation coefficient R of the equation was estimated using the baseline value, endpoint, and change values of blood

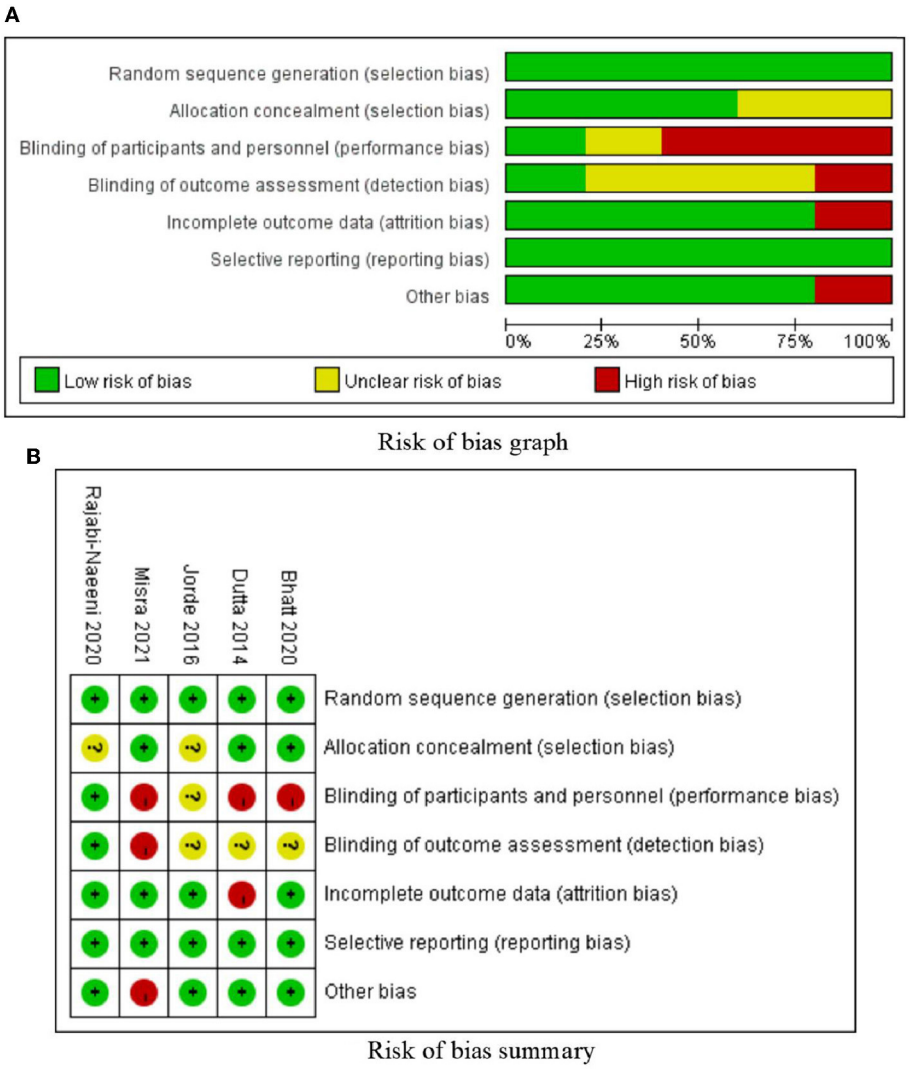

FIGURE 2
Risk of bias.

lipids from other studies with vitamin D supplementation. Finally, the estimated R-value of this study was 0.84. The 95% confidence interval (CI), interquartile range (IQR), and 5th and 95th percentiles could also be transformed into SD (20) (1–0.95/2 = 0.025, then the value x was found using the formula = tinv(1–0.95, N-1) in Excel, where N means the population of this group).

$$SD = [\sqrt{N} \times (95\%CI_{\text{Upper limit}} - 95\%CI_{\text{Lower limit}})] \div 2x$$

$$SD = IQR \div 1.35 = (95\text{th percentiles} - 5\text{th percentiles}) \div 3.29$$

The mean and SD of serum TC, LDL-C, HDL-C, and TG concentrations changes in the intervention group and the control group were compared by standardized mean difference (SMD). Cochran's Q-statistics and I^2-statistics were used to evaluate the statistical heterogeneity in the meta-analysis. In a meta-analysis, the random effect model (REM) was used when data were heterogeneous, and the fixed effect model (FEM) was used when data were not heterogeneous (20), but model-using in subgroup analyses of TC, LDL-C, HDL-C, and TG remained consistent with the total meta-analysis of TC, LDL-C, HDL-C, and TG separately. In this study, SMD and 95% CI of TC and HDL-C changes were measured by REM; SMD and 95% CI of LDL-C and TG changes were measured by FEM; and data were compared between the vitamin D group and the control group.

In the Q-test, a p-value of <0.05 was indicative of heterogeneity, and the I^2-value was used to evaluate the degree of heterogeneity. Influence analysis and Egger's test were performed using the Stata software to determine the stability and possible sources of heterogeneity. Combining the opinions of two investigators, the RevMan software was used for risk assessment. In addition, subgroup analysis was conducted according to BMI [overweight defined as 23–24.9, obesity as 25 or over 25 in Indian studies (21); overweight defined as 25–29.9, obesity as 30 or over 30 in other studies (22)], region (Northern Europe and Asia), vitamin D supplement dose (relatively low dose, relatively medium dose, and relatively high dose; according to included studies, we have found that doses of included studies were 2,857 IU/day, 3,571 IU/day, and 8,571 IU/day, therefore, we defined 2,857 IU/day as relatively low

TABLE 1 Basic information of included studies.

References	Region	Intervention Dose (IU/D)	Intervention Duration (Day)	Study participants Total (intervention /control)	Sex [Female *N* (%)/Male *N* (%)]		Baseline BMI (kg/m^2)		Baseline age	
					Intervention	Control	Intervention	Control	Intervention	Control
(A)										
Bhatt et al. (21)	India	8571 or 200[a]	546	121 (61/60)	61 (100.0)/0 (0.0)	60 (100.0)/0 (0.0)	31.10 ± 6.20	28.80 ± 3.90	20-60	20-60
Dutta et al. (5)	India	8571[b]	56	104 (55/49)	33 (60.0)/22 (40.0)	26 (53.1)/23 (46.9)	26.32 ± 4.52	26.83 ± 4.63	48.37 ± 10.47	47.40 ± 11.51
		2000[b]	≥309							
Jorde et al. (23)	Norway	2857	365-1825	227 (116/111)	43 (37.1)/73 (62.9)	39 (35.1)/72 (64.9)	30.10 ± 4.10	29.80 ± 4.40	62.30 ± 8.10	61.90 ± 9.20
Rajabi-Naeeni et al. (30)	Iran	3571	56	84 (42/42)	42 (100.0)/0 (0.0)	42 (100.0)/0 (0.0)	27.01 ± 2.91	27.28 ± 2.74	39.92 ± 6.04	41.85 ± 7.48
Misra et al. (31)	India	8571 or 200[a]	720	65 (37/28)	37 (100.0)/0 (0.0)	28 (100.0)/0 (0.0)	–	–	48.10 ± 6.70	46.10 ± 8.10

BMI data are shown as mean ± SD; age data are shown as mean ± SD or age range; original doses were converted into doses per day and were rounded if they were not integers; 1 month was converted into 30 days. –Data were unavailable or could not be calculated.
[a]Intervention dose: gave 60 000 IU/week for the first 8 weeks, adjusted doses every 24 weeks according to blood 25(OH)D levels, gave 60,000 IU/week for 8 weeks to subjects with vitamin D deficient, gave 200 IU/day to subjects with normal blood 25(OH)D level.
[b]Intervention dose: 60,000 U/W for the first 8 weeks, then 60,000 U/M, subjects were followed up for at least 12 months. BMI, body mass index; SD, standard deviation.

References	TC change (mmol/L)		LDL-C change (mmol/L)		HDL-C change (mmol/L)		TG change (mmol/L)	
	Intervention	Control	Intervention	Control	Intervention	Control	Intervention	Control
(B)								
Bhatt et al. (21)	−0.18 ± 0.60	0.26 ± 0.61	0.12 ± 0.49	0.07 ± 0.44	0.08 ± 0.16	0.03 ± 0.17	−0.10 ± 0.48	−0.01 ± 0.37
Dutta et al. (5)	–	–	−0.25 ± 0.46	−0.18 ± 0.36	−0.12 ± 0.15	−0.03 ± 0.16	−0.06 ± 0.40	0.09 ± 0.41
Jorde et al. (23)	−0.41 ± 0.59	−0.50 ± 0.59	−0.14 ± 0.52	−0.18 ± 0.52	0.09 ± 0.21	0.04 ± 0.21	−0.20 ± 0.43	0.10 ± 0.49
Rajabi-Naeeni et al. (30)	−0.27 ± 0.53	−0.11 ± 0.49	−0.27 ± 0.46	−0.11 ± 0.44	0.01 ± 0.16	−0.01 ± 0.13	0.04 ± 0.49	0.12 ± 0.39
Misra et al. (31)	−0.85 ± 0.66	−0.86 ± 0.87	–	–	–	–	–	–

Data are shown as mean ± SD. – Data were unavailable or could not be calculated. HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation.

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dose, 3,571 IU/day as relatively medium dose, and 8,571 IU/day as a relatively high dose in this meta-analysis), intervention time [short term defined as <365 days and long term defined as ≥365 days according to some articles (6, 23–29)], sex (only female or and male), and criteria for prediabetes definition (according to IDF/WHO or ADA criteria).

3. Results

3.1. Literature search

A total of 14,734 citations were retrieved, and only 5 papers fully met the inclusion criteria (5, 21, 23, 30, 31). These five articles included a sample of 601 subjects, with 311 in the vitamin D intervention group and 290 in the control group. Basic characteristics and details are shown in Table 1. The duration of treatment in the included studies ranged ≥56 days, and the dose of vitamin D ranged from 200 to 8,571 IU/day. In two studies, the control treatment involved only a placebo (23, 30); in the other two studies, placebo plus calcium carbonate supplementation (21, 31); and in one study, placebo plus calcium carbonate supplementation and lifestyle intervention (5) (note: also in the vitamin D intervention group, the same control treatments were applied).

As shown in Table 1, four studies were conducted in Asia and one in Northern Europe; one study excluded obese subjects (i.e., only normal weight and overweight subjects were included), three studies included obese subjects, and one did not provide BMI information. The experimental group in one study was supplemented with relatively low-dose vitamin D, one was supplemented with relatively medium-dose vitamin D, and three were supplemented with relatively high-dose vitamin D; one study carried out short-term interventions, and four carried out long-term interventions; three studies only had female subjects, and two had both male and female subjects; Four studies used ADA criteria for prediabetes; one used IDF/WHO criteria.

3.2. Risk of bias assessment

The risk of bias is shown in Figures 2A, B. Systematically speaking of this meta-analysis, the risks of random sequence generation bias and reporting bias in the included studies were very low; the risks of allocation concealment bias, attrition bias, and other biases were low; the risk of detection bias was relatively low; and the risk of performance bias was relatively high.

3.3. Meta-analysis

The differences in TG change ($P < 0.001$; Figure 3D, Table 2) between the intervention group and the control group were statistically significant. Compared to the control group, TG in the blood in the intervention group decreased more after vitamin D supplementation. But there were no significant differences in blood TC change ($P = 0.33$; Figure 3A, Table 2), blood LDL-C change ($P = 0.73$; Figure 3B, Table 2), or blood HDL-C change ($P = 0.86$; Figure 3C, Table 2) between the intervention group and the control group.

3.4. Subgroup analysis results

Only in the subgroup of studies that included obese subjects (not only normal weight and overweight), vitamin D supplementation led to more reductions in TG levels compared to the control treatments ($P < 0.001$; Figure 4). The effect of vitamin D supplementation on TG levels was observed both in the subgroup of Asian countries ($P = 0.03$; Figure 5) and the subgroup of Northern European counties ($P < 0.001$; Figure 5), and both in the relatively high-dose subgroup ($P = 0.04$; Figure 6) and in the relatively low-dose subgroup ($P < 0.001$; Figure 6). Only in the long-term intervention subgroup (more than 1 year of vitamin D supplementation) ($P < 0.001$; Figure 7) and only in the subgroup with both female and male subjects included (not only females) ($P < 0.001$; Figure 8), the effect of vitamin D supplementation on TG levels was observed. Both in the IDF/WHO subgroup ($P < 0.001$; Figure 9) and the ADA subgroup ($P = 0.03$; Figure 9), vitamin D supplementation led to more reductions in TG levels compared to the control treatments. It was noteworthy to emphasize that studies with obese subjects included were all long-term interventions (more than 1 year of vitamin D supplementation), while the only study (30) with non-obese subjects included was at the same time a short-term intervention (only 56 days), and the vitamin D levels in that study were not corrected at the end of the study (they remained insufficient). Additionally, this short-term intervention study was the only study with a relatively medium dose included, where the effect was not observed ($P = 0.41$; Figures 4, 6, 7).

The effects on TC in obese, relatively high-dose, and long-time subgroups and on HDL-C in obese, relatively high-dose, Asian, long-time, female and male, and ADA subgroups showed heterogeneity (Table 3).

3.5. Influence analysis and Egger's test

The impact of every single article on heterogeneity was observed in Figure 10. The study by Jorde et al. (23) seemed different from the others in this meta-analysis of TC. In addition, according to Egger's test, there was no significant publication bias in any of the included articles for TC ($P = 0.540$), LDL-C ($P = 0.213$), HDL-C ($P = 0.529$), or TG ($P = 0.096$).

4. Discussion

The results of our meta-analysis showed that vitamin D supplementation could decrease circulating TG levels in subjects with prediabetes, especially in certain situations, but failed to confirm the effects on TC, HDL-C, and LDL-C levels.

Many studies have shown that low serum 25(OH)D concentration was associated with adverse lipid status (32), and some studies indicated that vitamin D supplementation could improve serum TC, TG, and LDL-C levels also in patients with T2DM (13, 14) and in subjects with metabolic syndrome (33).

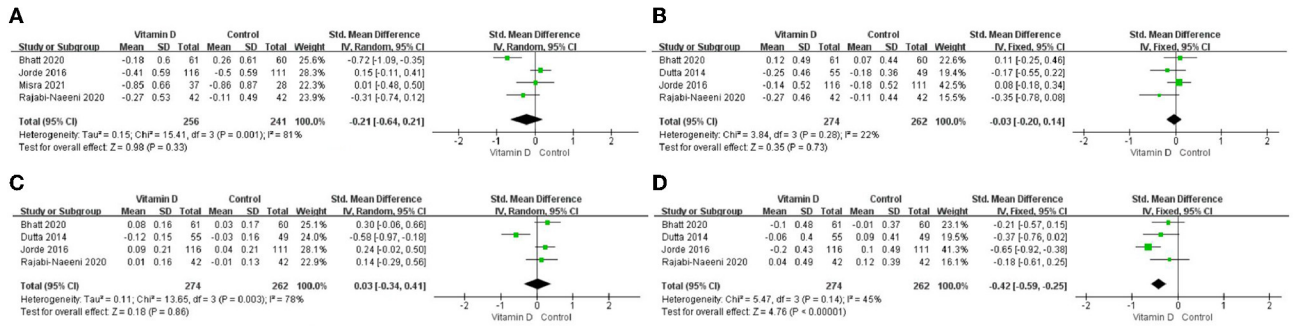

FIGURE 3
Forest map of lipid changes between two groups. **(A)** TC, **(B)** LDL–C, **(C)** HDL–C, and **(D)** TG. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

TABLE 2 Results of meta-analysis in five included articles.

Index	Number of Studies	SMD [95%CI]	I^2
TC	4	−0.21 [−0.64,0.21]	**81%**‡
LDL-C	4	−0.03[−0.20, 0.14]	22%
HDL-C	4	0.03 [−0.34, 0.41]	**78%**‡
TG	4	**−0.42 [−0.59, −0.25]**†	45%

†P < 0.05 of the test for overall effect; ‡P < 0.05 of the test for heterogeneity. SMD, standardized mean difference; CI, confidence interval.

The effect of vitamin D supplementation on TG levels can be mediated through (1) increased calcium levels; (2) suppression of parathyroid hormone (PTH) secretion; (3) inhibition of lipolysis; (4) suppression of inflammation; (5) suppression of renin-angiotensin-aldosterone system (RAAS) activity; (6) its interaction with glucocorticoids and sex hormones; (7) upregulation of adiponectin; (8) improvement in insulin resistance and insulin levels; (9) its direct inhibition of the expression of nuclear factor sterol regulatory element-binding protein 1c (SREBP1c) involved in hepatic TG synthesis; (10) increased TG clearance by upregulation of lipoprotein lipase (LPL), neutral sphingomyelinases, PPARγ, and adipocyte-binding protein 2 (AP2); or by (11) upregulation of mitochondrial oxidation (34–41).

Since adipose tissue can sequestrate and metabolize vitamin D and consequently lower its circulating and bioavailable levels for other metabolically active tissues involved in lipid metabolism (including muscle, liver, and pancreas) (42–45), we conducted an additional stratified analysis according to BMI categories. Our stratified analysis has shown that, particularly in the studies (5, 21, 23) that included obese subjects with prediabetes (not only normal weight and overweight subjects), the effect on TG levels was more marked compared with the study that excluded obese subjects (30). This result might be affected by the fact that in some of the studies that included obese subjects, men were also included (not only women). Our sex-subgroup analysis showed that the effect on TG was more marked in the mixed-sex studies (5, 23) than in the studies that included only women (21, 30) where the effect was not significant. Additionally, the durations of interventions in the studies (5, 21, 23) that also included obese subjects were over 1 year, while the duration of the treatment in the study that excluded obese subjects (30) was 8 weeks. Those factors can be significant confounders, which need to be taken into consideration when making conclusions. Nevertheless, there might be a more direct association. For example, obese subjects can have much higher TG levels compared with non-obese subjects, and therefore the effect can be more observable, especially during prolonged treatment. An additional explanation could be that obese subjects are more prone to vitamin D deficiency, while improvements in insulin resistance and related metabolic features can be observed after its correction. However, the later explanation failed to be confirmed in this study. In the study by Rajabi-Naeeni et al. (30) (where the effect on TG was not shown), the included normal weight and overweight subjects were with vitamin D insufficient status, in the study by Bhatt et al. (21) (where the effect was also not shown) the included overweight and obese subjects were with vitamin D deficient status, while in the studies by Dutta et al. (5) and Jorde et al. (23) (the ones which have found the significant effect on TG), they were with vitamin D insufficient status and no BMI restrictions. Therefore, the effect was neither associated with the baseline vitamin D status nor the BMI status, which is in agreement with a recent pooled meta-analysis (46). However, it is important to say that in the study by Bhatt et al. (21), the BMI-cut off for obesity was set at a much lower level (>25 kg/m^2) according to Indian references, whereas in the other studies, it was set at the BMI-cut off for overweight. Finally, the effect cannot be explained by the rationale that vitamin D supplementation could affect body weight since not enough evidence exists on the effect of vitamin D on body weight reduction (42, 43, 47, 48), and no significant reductions in BMI were shown in the analyzed studies by vitamin D supplementation in comparison with the control treatments (5, 21, 23). Therefore, the finding of the more pronounced effect on TG in the studies which also included obese subjects was probably confounded by the influence of duration of treatment and/or possible gender differences in the response to supplementation (49).

As there are huge ethical/regional differences in vitamin D levels and responses to supplementation (50–56), we also conducted a region-subgroup analysis. The results showed that the effect on TG was not region-specific and was observed in both region-subgroups (Asia and Northern Europe). However, since this meta-analysis only included studies from India (three studies), Iran (one study), and Norway (one study), the results probably cannot

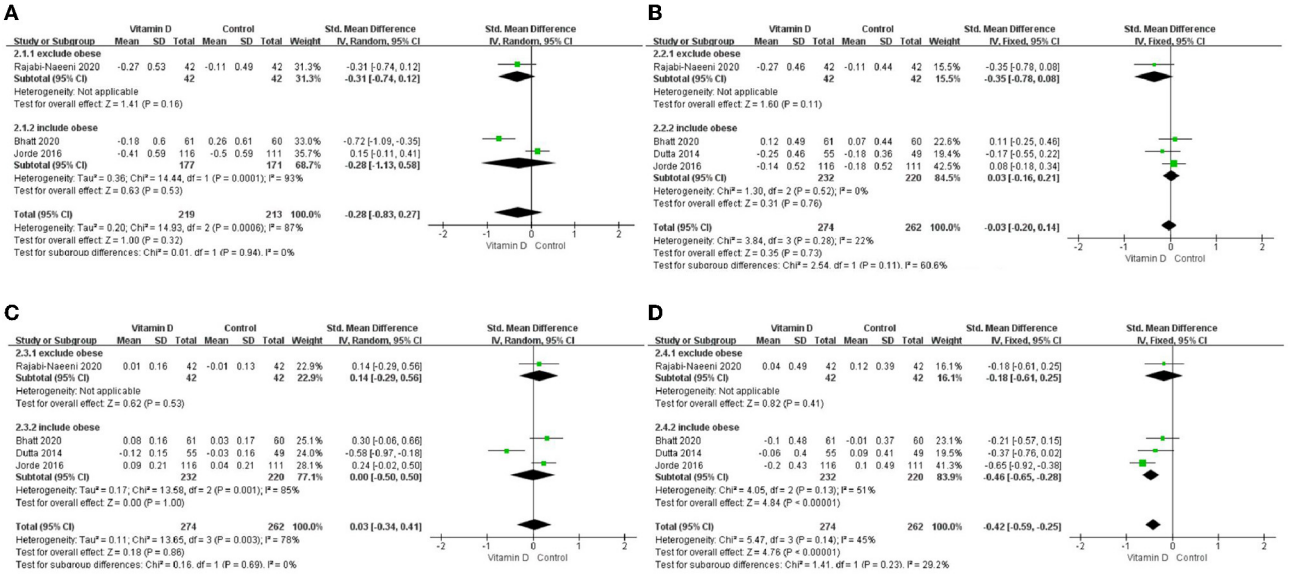

FIGURE 4
Subgroup analysis by BMI. **(A)** TC, **(B)** LDL–C, **(C)** HDL–C, and **(D)** TG. BMI, body mass index; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

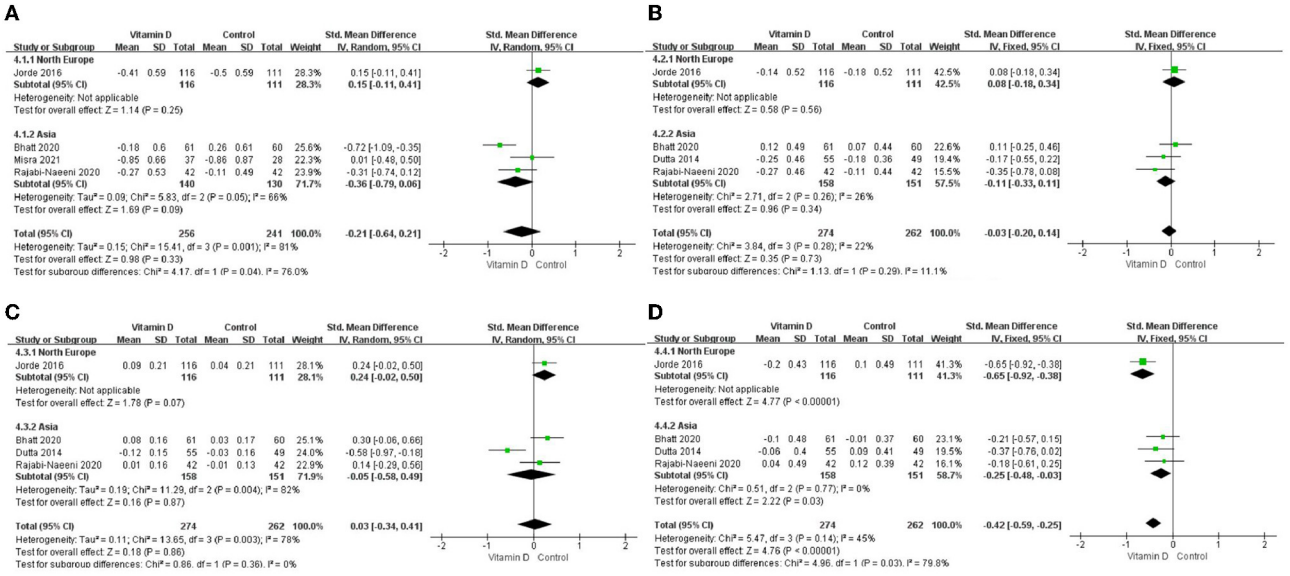

FIGURE 5
Subgroup analysis by region. **(A)** TC, **(B)** LDL–C, **(C)** HDL–C, and **(D)** TG. TG. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

be extrapolated to other regions or ethnicity subgroups, and relative trials in more regions are needed.

We conducted a subgroup analysis of intervention dose and time according to some studies with the idea that insufficient vitamin D dose and intervention time might affect the research results (24). The results showed that in our relatively low-dose (2,857 IU/day) and relatively high-dose (8,571 IU/day) subgroups, vitamin D intervention provided significantly larger reductions in TG, but such an effect was not observed in the relatively medium-dose subgroup. However, the relatively medium-dose group only included one study by Rajabi-Naeeni et al. (30), which was at the same time the only study in the short-duration group. Changes in glucose tolerance and blood lipid levels are usually a slow and gradual process, and previous research suggests that interventions lasting only a few months may be a too short time frame to evaluate the benefits of vitamin D, implying that even 1 year is not enough for a long-term intervention (6, 24, 25, 33). In our analysis, significantly larger reductions in TG levels in the

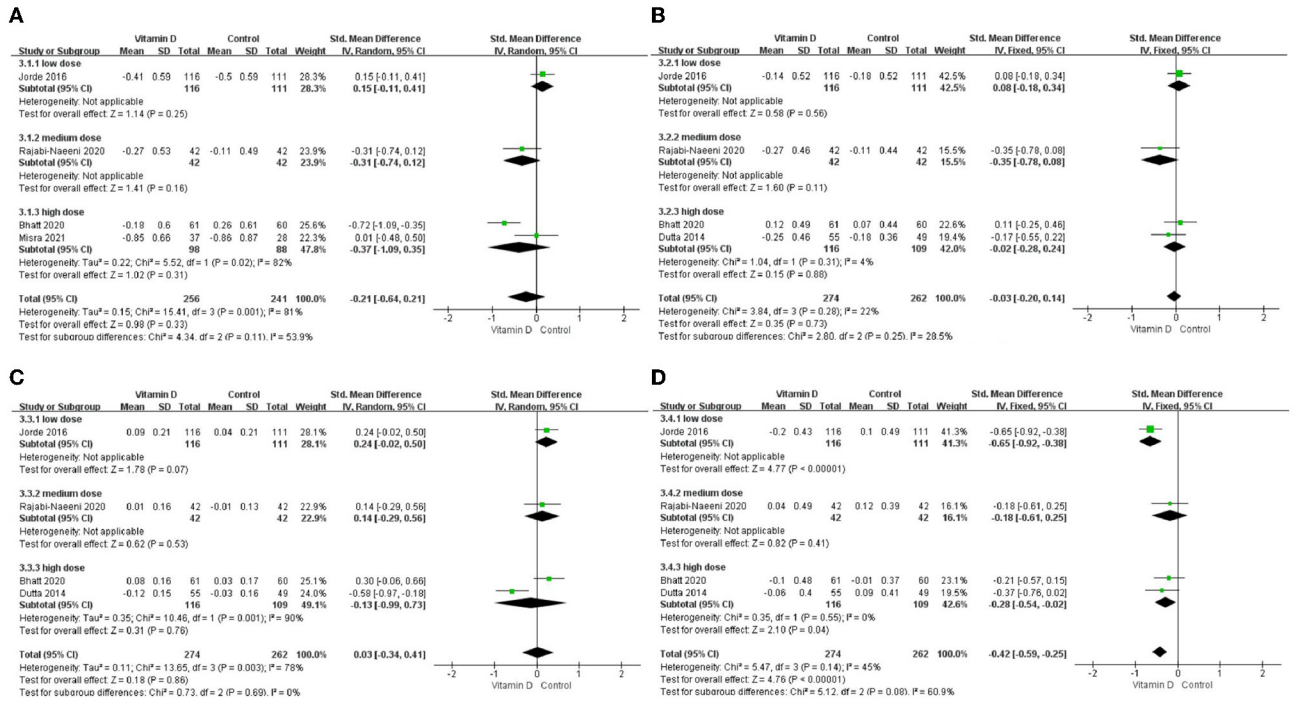

FIGURE 6
Subgroup analysis by intervention dose. **(A)** TC, **(B)** LDL-C, **(C)** HDL-C, and **(D)** TG. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

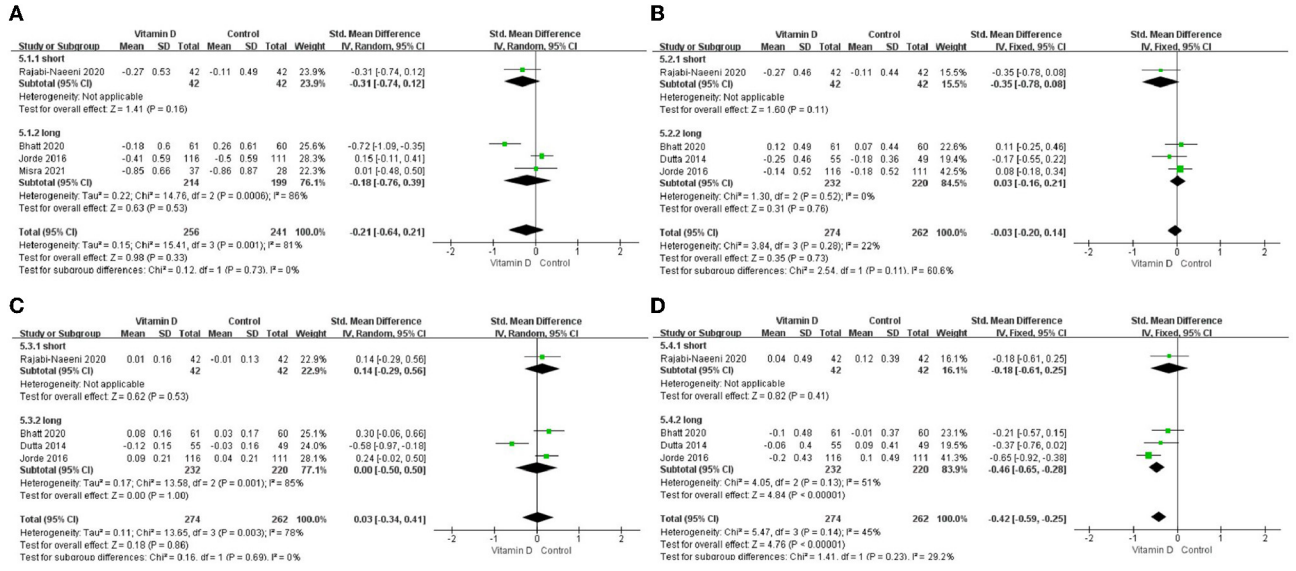

FIGURE 7
Subgroup analysis by intervention duration. **(A)** TC, **(B)** LDL-C, **(C)** HDL-C, and **(D)** TG. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

intervention group had been observed only in the long-duration subgroup but not in the short-duration subgroup. Additionally, the vitamin D levels in the short-duration subgroup of the study (30) did not change to achieve vitamin D sufficiency after only 8 weeks of treatment. This implied that the relatively medium-dose group in our analysis was probably affected by the short duration of the study by Rajabi-Naeeni et al. (30). This is in agreement with a recent meta-analysis in subjects with metabolic syndrome, where the effects on TG levels were not shown in studies lasting <1 year (33). More short-term interventions are needed for further verification. Additionally, lower vitamin D doses than 2,857 IU/day or higher doses than 8,571 IU/day need to be tested in the future.

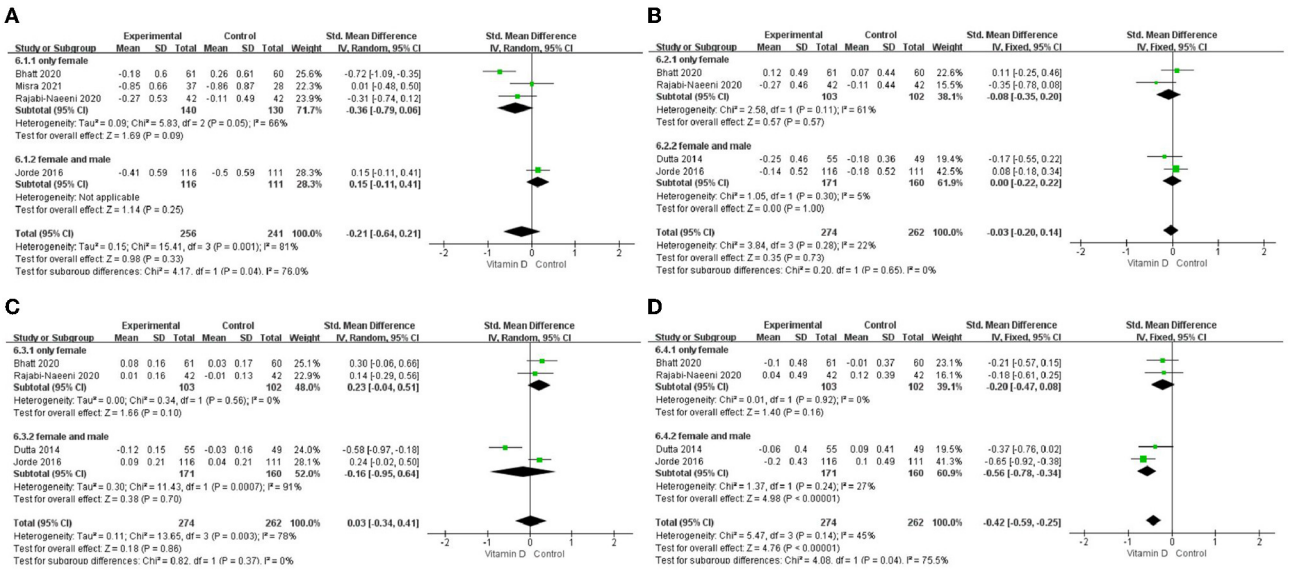

FIGURE 8
Subgroup analysis by sex. **(A)** TC, **(B)** LDL-C, **(C)**, HDL-C, and **(D)** TG. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

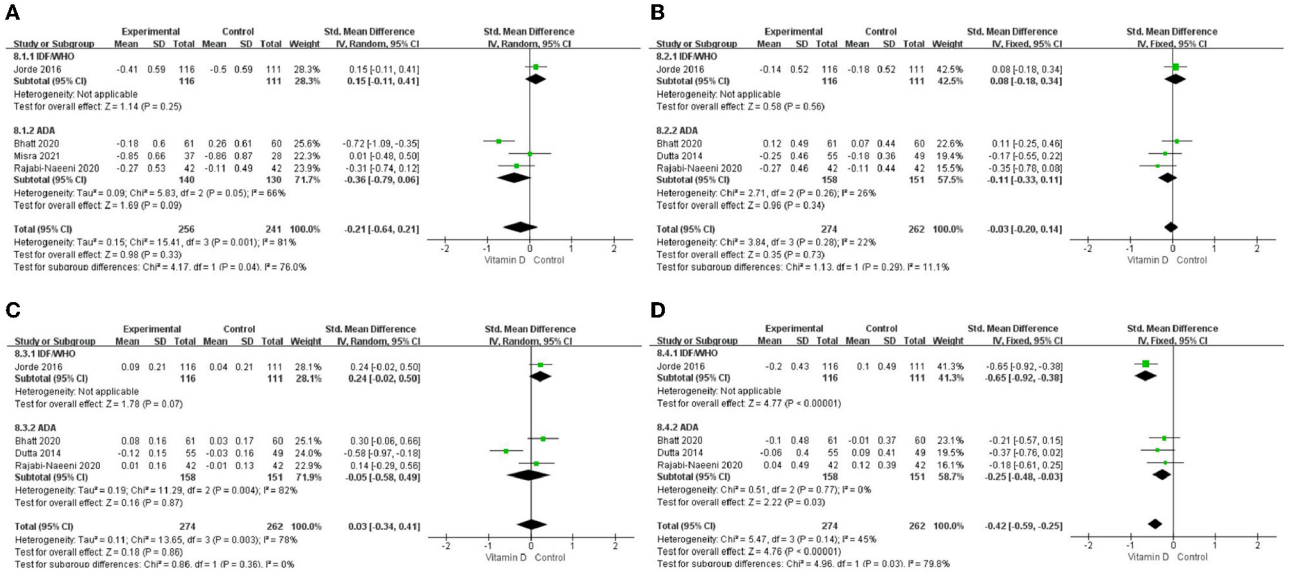

FIGURE 9
Subgroup analysis by criteria for prediabetes definition. **(A)** TC, **(B)** LDL-C, **(C)** HDL-C, and **(D)** TG. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; IDF, International Diabetes Federation; ADA, American Diabetes Association; WHO, World Health Organization; Criteria of IDF/ WHO from 2006: fasting plasma glucose value in the range of 6.1–6.9 mmol/L or 110–125 mg/dl, or 2h oral glucose tolerance test (OGTT) plasma glucose value in the range of 7.8–11.0 mmol/L or 140–200 mg/dl; Criteria of ADA from 2004: fasting plasma glucose value in the range of 5.6–6.9 mmol/L or 100–125 mg/dl, or 2 h OGTT plasma glucose value in the range of 7.8–11.0 mmol/L or 140–200 mg/dl; or HbA1c in the range of 39–47 mmol/mol or 5.7–6.4%.

We have also conducted subgroup analyses of the criteria for prediabetes. The results showed that the effect on TG was probably not affected by different criteria for prediabetes since it was observed in both criteria subgroups (IDF/WHO and ADA).

Our heterogeneity might come from studies with different study designs (different control interventions, doses, durations, different inclusion criteria, baseline vitamin D status and corrections achieved, BMI, sex, and ethnicity of the subjects included). On the one hand, the significant heterogeneity disappeared after region, sex, and criteria for prediabetes subgroup analysis of TC, showing that region, gender, or criteria for prediabetes could affect the heterogeneity of our meta-analysis

TABLE 3 Results of subgroup meta-analysis.

	Index	BMI Subgroup		Dose Subgroup			Region Subgroup	
		Exclude Obese	Include Obese	Low Dose	Medium Dose	High Dose	Northern Europe	Asia
(A)								
Number of Studies	TC	1	2	1	1	2	1	3
	LDL-C	1	3	1	1	2	1	3
	HDL-C	1	3	1	1	2	1	3
	TG	1	3	1	1	2	1	3
SMD [95%CI]	TC	−0.31 [−0.74, 0.12]	−0.28 [−1.13, 0.58]	0.15 [−0.11, 0.41]	−0.31 [−0.74, 0.12]	−0.37 [−1.09, 0.35]	0.15 [−0.11, 0.41]	−0.36 [−0.79, 0.06]
	LDL–C	−0.35 [−0.78, 0.08]	0.03 [−0.16, 0.21]	0.08 [−0.18, 0.34]	−0.35 [−0.78, 0.08]	−0.02 [−0.28, 0.24]	0.08 [−0.18, 0.34]	−0.11 [−0.33, 0.11]
	HDL–C	0.14 [−0.29, 0.56]	0.00 [−0.50, 0.50]	0.24 [−0.02, 0.50]	0.14 [−0.29, 0.56]	−0.13 [−0.99, 0.73]	0.24 [−0.02, 0.50]	−0.05 [−0.58, 0.49]
	TG	−0.18 [−0.61, 0.25]	**−0.46 [−0.65, −0.28]**†	**−0.65 [−0.92, −0.38]**†	−0.18 [−0.61, 0.25]	**−0.28 [−0.54, −0.02]**†	**−0.65 [−0.92, −0.38]**†	**−0.25 [−0.48, −0.03]**†
I^2	TC	–	**93%**‡	–	–	**82%**‡	–	66%
	LDL-C	–	0%	–	–	4%	–	26%
	HDL-C	–	**85%**‡	–	–	**90%**‡	–	**82%**‡
	TG	–	51%	–	–	0%	–	0%

	Index	Duration subgroup		Sex subgroup		Prediabetes criteria subgroup	
		Short	Long	Only female	Female and male	IDF/WHO	ADA
(B)							
Number of Studies	TC	1	3	3	1	1	3
	LDL-C	1	3	2	2	1	3
	HDL-C	1	3	2	2	1	3
	TG	1	3	2	2	1	3
SMD [95%CI]	TC	−0.31 [−0.74, 0.12]	−0.18 [−0.76, 0.39]	−0.36 [−0.79, 0.06]	0.15 [−0.11, 0.41]	0.15 [−0.11, 0.41]	−0.36 [−0.79, 0.06]
	LDL-C	−0.35 [−0.78, 0.08]	0.03 [−0.16, 0.21]	−0.08 [−0.35, 0.20]	0.00 [−0.22, 0.22]	0.08 [−0.18, 0.34]	−0.11 [−0.33, 0.11]
	HDL-C	0.14 [−0.29, 0.56]	0.00 [−0.50, 0.50]	0.23 [−0.04, 0.51]	−0.16 [−0.95, 0.64]	0.24 [−0.02, 0.50]	−0.05 [−0.58, 0.49]
	TG	−0.18 [−0.61, 0.25]	**−0.46 [−0.65, −0.28]**†	−0.20 [−0.47, 0.08]	**−0.56 [−0.78, −0.34]**†	**−0.65 [−0.92, −0.38]**†	**−0.25 [−0.48, −0.03]**†
I^2	TC	–	**86%**‡	66%	–	–	66%
	LDL-C	–	0%	61%	5%	–	26%
	HDL-C	–	**85%**‡	0%	**91%**‡	–	**82%**‡
	TG	–	51%	0%	27%	–	0%

†P < 0.05 of the test for overall effect; ‡P < 0.05 of the test for heterogeneity. BMI, body mass index; SMD, standardized mean difference; CI, confidence interval. BMI definition: in Indian studies, overweight = 23–24.9, obesity≥25; in other studies, overweight = 25–29.9, obesity≥30. Dose definition: relatively low-dose = 2,857 IU/day; relatively medium dose = 3,571 IU/day; relatively hig IU/day. Duration definition: short-term <365 days; long-term≥365 days. Bold values: P < 0.05.

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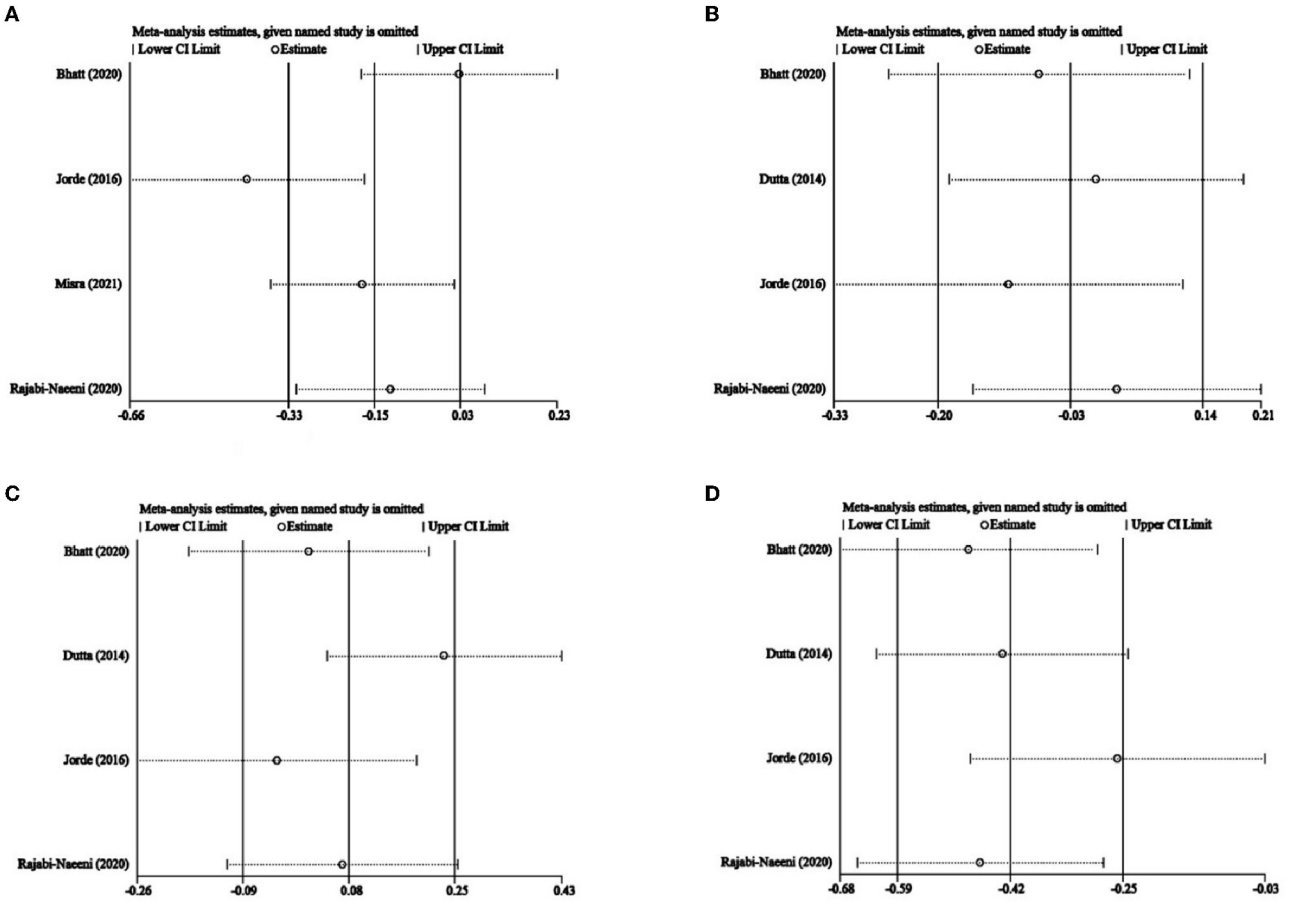

FIGURE 10
Influence analysis. **(A)** TC, **(B)** LDL-C, **(C)**, and HDL-C, and **(D)** TG. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

of TC. On the other hand, Figure 10 shows that the included article of Jorde et al. (23) was quite different from the others in this meta-analysis of TC. In the present study, in the subgroups with a North European population and relatively low dose and IDF/WHO prediabetic inclusion criteria, only the study of Jorde et al. (23) was included while regarding sex subgroups, only this study had a higher percentage of male participants. This suggested that the heterogeneity of this meta-analysis might come from the region, intervention dose, criteria of prediabetes, and sex of participants in the article by Jorde et al. (23). Besides, although it was not observed in Figure 10, the article by Rajabi-Naeeni et al. (30) was exceptional as well, with a relatively medium dose, a short duration, and the exclusion of obese subjects. Therefore, significant heterogeneity might also come from BMI, intervention dose, and intervention duration of the article by Rajabi-Naeeni et al. (30).

To make our analysis an all-around study as far as possible, we restricted our selection to prospective intervention trials based on the Cochrane Handbook for Systematic Reviews of Interventions and relative references (20, 57). Limitations of this meta-analysis were that there were not many studies (especially with normal BMI, more other regions, lower vitamin D doses than 2,857 IU/day and higher doses than 8,571 IU/day, and a low intervention duration of vitamin D supplementation) that could be included in this meta-analysis. More studies are needed in the future. Several included studies did not state clearly if they collected blood lipid data when participants were fasting, and only few of the included studies controlled for the usage of lipid lowering medications in the study by Bhatt et al. (21), no information on lipid-lowering drugs usage was provided; in the study by Dutta et al. (5), participants using metformin, fever/active oral hypoglycaemic agents, oral contraceptive pills, steroids, and anti-epileptics were excluded; in the study by Jorde et al. (23), participants with use of statins were included, but there was no significant difference in use of statins between groups and those who changed their use of statins during the course of study, were excluded; in the study by Misra et al. (31), participants on medications within last 1 month which could potentially influence insulin secretion, insulin sensitivity, vitamin D, or calcium metabolism, including metformin, thiazolidinediones, steroids, and calcitonin were excluded; and in the study by Rajabi-Naeeni et al. (30), participants using herbal or chemical medications affecting lipids were excluded). Besides, there was significant heterogeneity in the studies included, related to different durations and doses of treatment, different BMI and gender of the subjects included, different regions and ethnic populations, different criteria for

prediabetes, different control interventions, baseline vitamin D status, and corrections achieved.

However, despite all these limitations, our results may provide a basis for the implementation of regular assessment of vitamin D status among patients with prediabetes and consecutive supplementation in vitamin D deficient/insufficient patients to prevent an increase in blood lipids.

5. Conclusion

Vitamin D supplementation might beneficially affect TG levels in individuals with prediabetes. Particularly longer durations of treatment, more than 1 year, with doses that correct vitamin deficiency/insufficiency, can have a beneficial effect. Considering that there were not many studies that could be included in this meta-analysis, more studies are needed in the future.

Author contributions

YY and SY designed the study and analyzed the data. YY drafted the first manuscript and conducted the visualization. NY, YG, HW, MS, WH, XL, and LW validated the results. YY, SY, and BL participated in amending the manuscript. All authors contributed to the article and approved the submitted version.

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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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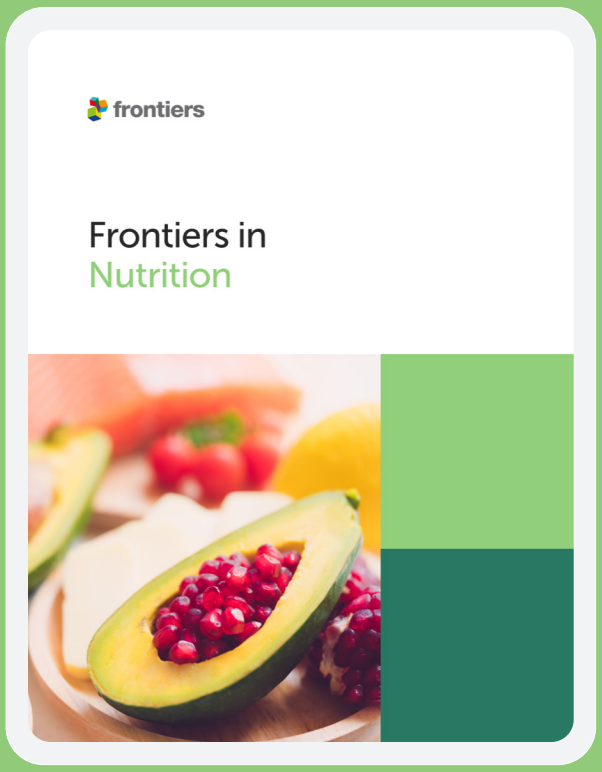

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