Chapter 7

Non-linear associations between serum 25(OH) vitamin D and indices of arterial stiffness and arteriosclerosis in an older population

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ABSTRACT

Background

Several studies have been pointing towards a non-linear relationship between serum 25(OH)D and cardiovascular disease. Next to vitamin D deficiency, also higher levels of 25(OH)D have been reported to be associated with increased cardiovascular risk. We aimed to investigate the nature of the relationship between serum 25(OH)D and measures of arterial stiffness and arteriosclerosis in an elderly population.

Design

Cross-sectional

Setting/subjects

A subgroup of the B-PROOF study was included to determine associations between serum 25(OH)D and arterial stiffness and atherosclerosis (N = 567, 57% male, age 72.6 ± 5.6 yrs, mean serum 25(OH)D 54.6 ± 24.1 nmol/l).
Methods: Carotid intima media thickness (IMT) was assessed using ultrasonography and pulse wave velocity (PWV) was determined with applanation tonometry. Associations were tested using multivariable restricted cubic spline functions and stratified linear regression analysis.

Results

The associations between serum 25(OH)D and carotid IMT or PWV were non-linear. Spline functions demonstrated a difference between 25(OH)D deficient and sufficient individuals. In serum 25(OH)D sufficient participants (≥ 50 nmol/l; N = 287) a positive association with IMT and serum 25(OH)D was present (beta 1.24 95%CI [0.002 ; 2.473]). PWV levels were slightly lower in vitamin D deficient individuals, but the association with 25(OH)D was not significant.

Conclusion

Our study demonstrates that associations of serum 25(OH)D and PWV and IMT in an elderly population are not linear. In particular from serum 25(OH)D levels of 50 nmol/l and up, there is a slight increase of IMT with increasing 25(OH)D levels.

INTRODUCTION

Cardiovascular disease is more common among individuals with low serum 25(OH)D levels compared with 25(OH)D sufficient individuals [1-6]. However, not only vitamin D deficiency has reported to be associated with increased cardiovascular risk, but high serum 25(OH)D levels have been as well. Taken these findings together, evidence of the recent years has been pointing towards a non-linear association between serum 25(OH)D level and cardiovascular disease [7]. High serum 25(OH)D levels could affect the vascular wall, either indirectly or via a direct effect of serum 25(OH)D. These processes may also have a role in the arterial stiffening process [8;9].

Up till now, only inverse linear associations between serum 25(OH)D level and arterial stiffness have been reported. Earlier studies have been performed in younger subjects and disease specific populations, like diabetics [8-12]. Very recently, the Baltimore Longitudinal Study of Ageing confirmed such an inverse linear association in an older population [13]. An inverse association between serum 25(OH)D and arteriosclerosis has only been described in serum 25(OH)D deficient individuals [14-16]. Because both arterial stiffness and atherosclerosis are important pre-clinical stages of cardiovascular disease, we aimed to investigate the nature of the relationship (e.g. linear, monotone or other) between serum 25(OH)D levels and indices of arterial stiffness and arteriosclerosis in older individuals.

METHODS

Study participants

The present study was conducted as a cross-sectional baseline analysis within the framework of the vascular subgroup of the B-PROOF (B vitamins for the prevention of osteoporotic fractures) study (N = 567). A detailed description of this randomized controlled trial has been reported elsewhere [17]. In short, B-PROOF is a multi-center, randomized, placebo controlled, double-blinded trial including 2919 participants from three areas in the Netherlands. Main inclusion criteria were age 65 years and older, and an elevated homocysteine level (12 – 50 µmol/l). Main exclusion criteria were renal insufficiency (creatinine level > 150 µmol/l) and presence of a malignancy. All participants gave written informed consent before the start of the study. The Wageningen Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Rotterdam and Amsterdam gave approval for local feasibility. At the Erasmus Medical

Center (Rotterdam) and VU University Medical Center (Amsterdam), a random subsample of participants underwent vascular measurements (N = 567).

Clinical characteristics

Height was measured in duplicate to the nearest 0.1 cm while standing erect and wearing no shoes, using a stadiometer [17]. Weight was measured with the participant wearing light garments without shoes and empty pockets to the nearest 0.5 kg using a calibrated weighing device (SECA 761) [17]. Body Mass Index (BMI) was calculated as weight divided by squared height and expressed as kg/m^2.
Self-reported medication use, alcohol intake and smoking habits were determined using a questionnaire [17]. Alcohol intake was assessed using the Garret structure classifying alcohol use into four categories (very excessive, excessive, moderate, light) [18].
Questions regarding cardiovascular disease history, like angina pectoris, myocardial infarction, transient ischemic attack and/or stroke were also included in this questionnaire [17]. Furthermore, cardiovascular risk factors such as hypertension, hypercholesterolemia and diabetes mellitus were assessed with this questionnaire [17].

Serum 25(OH) vitamin D

Morning venous blood samples were obtained when participants were in a fasted state, or after a restricted breakfast [17]. Samples were stored at -80˚C until determination in 2012. In short, measurement of serum 25(OH)D occurred by releasing it from its binding protein(s) and by adding a denaturized internal standard (IS: $25(OH)D_3$-d6. Samples were extracted and analyzed by XLC-MS/MS (a Symbiosis online SPE system (Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). The inter-assay coefficient of variation was 9% at a level of 10 ng/mL (= 24.9 nmol/l) and 6% at a level of 25 ng/mL (= 62.4 nmol/l). All analyses were performed in the Endocrine Laboratory of the VU University Medical Center. Vitamin D deficiency was defined as a serum 25(OH)D level < 50 nmol/L according to literature since 25(OH)D levels < 50 nmol/l have reported to be related to several clinical disorders such as osteoporosis [19].

Serum creatinine

Serum creatinine was measured with the enzymatic colorimetric Roche CREA plus assay (CV = 2%). The estimated glomerular filtration rate (eGFR) was estimated with the Modification of Diet in Renal Disease (MDRD) and was calculated in ml/min/1.73 m^2 with the formula: 186 * (serum creatinine (µmol/l) / 88.4) – $^{1.154}$ * age (years) – $^{0.203}$ * 0.742 (for females) [20].

Vascular measurements

Only a random subsample of the B-PROOF study underwent vascular measurements (n = 567). Participants with cardiac rhythmic disturbances were excluded from these additional measurements. During the measurements, participants were situated in supine position on a flat examination couch in a quiet laboratory room for at least 5-10 minutes prior to the measurements. They were not allowed to speak during the measurements. Use of alcohol or coffee during 12 hours before the measurements was prohibited [21].

Blood pressure measurement

Peripheral blood pressure at the time of vascular function tests was measured once with a semi-automatic oscillometric device (Datascope Accurator Plus device, Datascope Corp., NJ, USA) after at least five minutes of supine rest. Blood pressure measurements were conducted at the right arm and measured in mmHg. The mean arterial pressure (MAP) was measured and pulse pressure was calculated as systolic minus diastolic blood pressure.

Applanation tonometry

Arterial tonometry was obtained from the right carotid and right femoral artery using the Sphygmocor device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia). Aortic pulse wave velocity (PWV) was measured with a three channel ECG recording and simultaneously recording of the right carotid artery pulse wave form and subsequently the femoral artery pulse waveform. The PWV was calculated as the delay between the femoral pulse wave and the carotid pulse wave, divided by the transit distance (intra-CV = 5 %, inter-CV = 8%). Transit distance was assessed with body surface measurement from the carotid artery to the femoral artery [21].

Increased arterial stiffness was defined as a PWV > 12 m/s, as this level is associated with increased cardiovascular risk [22].

Carotid Intima Media Thickness

For carotid B-mode ultrasonography, the L105 40 mm 7.5 MHz array transducer was used (Picus, Pie Medical Equipment, Maastricht, the Netherlands) on the right carotid artery. Intima media thickness (IMT) is evaluated as the distance luminal - intimal interference and the media - adventitial interface (Art.Lab, Esoate Europe, Maastricht, the Netherlands) at ~ 1 cm proximal from the carotid bifurcation. In order to obtain accurate measurements, the standard deviation of the mean values of 6 independent measurements had to be < 10% of the mean (intra-CV = 8%, inter-CV = 15%).

Statistical analysis

We have included all participants who had data available regarding serum 25(OH)D and carotid IMT and PWV measures in order to do a complete case analysis. For missing covariates, the statistical program automatically uses the mean for missing cases.

First, normal distribution was tested in all continuous variables and subsequently curve estimation models were created in order to investigate whether the associations between serum 25(OH)D levels and arterial stiffness parameters and carotid IMT were linear. Curve estimation models were tested by analysis of variance of the linear and non-linear component of the association between serum 25(OH)D and variables of interest. Statistics were performed using the statistical package SPSS 20.0 (SPSS Inc., Chicago, IL, USA). In case the associations were not linear, cubic spline analysis was performed to investigate non-linear associations between serum 25(OH)D and arterial stiffness measures and carotid IMT using R version 3.0.0. Spline regression models are piecewise polynomial functions that join smoothly at points called knots. In contrast to categorical models that assume a constant association within categories, in spline models all data points are used, providing a better estimate of dose-effect relationships [23]. Spline models were visually tested with 3 to 5 knots. In the final analyses we used 3 knots based on the size of our study sample and because this gave the best fit, measured with the likelihood ratio. We adjusted for covariates that were considered clinically relevant or gave a considerable change of the likelihood ratio.

After this, we performed a multivariable linear regression analysis using SPSS 20.0, stratified for serum 25(OH)D levels based on the depicted spline plots. We accounted for confounders that were considered clinically relevant or contributed to a more than 10% change of the point estimate. Potential confounders were age, gender, study center, BMI, eGFR, MAP, heart rate, smoking, alcohol use, CRP and physical performance. Smoking was added to the model as a binary variable: yes or no and this was also done for alcohol use: yes (moderate – very excessive) and no use (light).

Furthermore, we tested whether age, gender, cardiovascular disease history, diabetes, hypercholesterolemia and use of calcium and/or vitamin D supplementation use significantly interacted with the association between serum 25(OH)D and preclinical measures of cardiovascular disease. If a variable interacted, stratified analysis was performed. Concerning all analyses, two-sided *P*-values of < 0.05 were considered statistically significant.

RESULTS

Characteristics of the study population (N = 567) are shown in Table 1. Mean age was 72.5 ± 5.6 years and gender was equally distributed. The mean serum 25(OH)D level in our population was 54.6 ± 24.1 nmol/l and 50.3% of the participants were vitamin D deficient (<50 nmol/l).

Curve estimation modelling showed that the relationship between carotid IMT and serum 25(OH)D was not linear, and that a cubic model had the best fit (Table 2). The association between serum 25(OH)D and PWV was also not linear, but rather quadratic or cubic because of the explained variability, although neither of the models fitted the data well.

In Figure 1 the spline curves created for each variable are depicted. As demonstrated, high levels of serum 25(OH)D were associated with higher measures of carotid IMT. Furthermore, both low and high levels of serum 25(OH)D were associated with lower levels of PWV.

Since the association between serum 25(OH)D and carotid IMT visually appeared to be linear above a level of serum 25(OH)D of 50 nmol/l, we performed a stratified linear regression analysis for vitamin D deficient versus vitamin D sufficient participants. This analysis showed a positive association between serum 25(OH)D and carotid IMT (beta 1.24 95% CI [0.002; 2.473]) in participants with serum 25(OH)D levels ≥ 50 nmol/l. In vitamin D sufficient older persons, an increase of 1 nmol/L of serum 25(OH)D corresponded to an increase of 1.24 µm carotid IMT (Table 3).

Table 1. Clinical and hemodynamic characteristics total population (n = 567)

Parameter	Value
Age (years)	72.5 ± 5.6
80+	61 (10.8)
Gender, males	315 (55.6)
BMI (kg/m^2)	27.3 ± 3.7
Smoking	
Never	198 (34,9)
Current	53 (9,3)
Former	316 (55,7)
Alcohol use	
Light	358 (63,1)
Moderate	179 (31,6)
Excessive	25 (4,4)
Very excessive	5 (0,9)
Self-reported medical history of	
Cardiac disease	61 (10.8)
TIA or stroke	45 (7.9)
Diabetes	65 (11.5)
Hypertension	211 (38.1)
Hypercholesterolemia	151 (26.6)
Self-reported use of	
Vitamin D supplementation	9 (1.6)
Calcium supplementation	20 (3.5)
Calcium + vitamin D supplementation	18 (3.2)
Mean dosage of used vitamin D (IE)	39.1 ± 144.8
Serum Vitamin D level (nmol/l)	54.6 ± 24.1
Vitamin D deficiency (< 50 nmol/l)	274 (48.3)
Serum Creatinine level (µmol/l)	83.2 ± 17.2
eGFR (ml/min/1.73m^2)	91.3 ± 35.9
Blood pressure	
SBP (mmHg)	137.7 ± 18.1
DBP (mmHg)	77.3 ± 9.6
Hypertension during measurement*	339 (59.8)
PWV (m/s)	14.3 ± 4.5
PWV > 12 m/s	313 (55.2)
Carotid IMT (µm)	717.0 ± 163.2

Data are presented as number (percentage) or as mean ± SD. Abbreviations: eGFR: estimated glomerular filtration rate; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: Pulse Pressure; PWV: aortic pulse wave velocity; IMT: intima media thickness. *Hypertension defined as SBP > 140 and/or DBP > 90 mmHg.

Table 2. Curve estimation models for both carotid IMT and PWV

	Carotid IMT		IMT	
	R^2	*P*-value	R^2	*P*-value
Linear	0.001	0.65	$0.23 \cdot 10^{-3}$	0.73
Logarithmic	$0.46 \cdot 10^{-4}$	0.89	$0.66 \cdot 10^{-4}$	0.85
Quadratic	0.028	0.004	0.003	0.41
Cubic	0.045	$0.40 \cdot 10^{-3}$	0.009	0.20

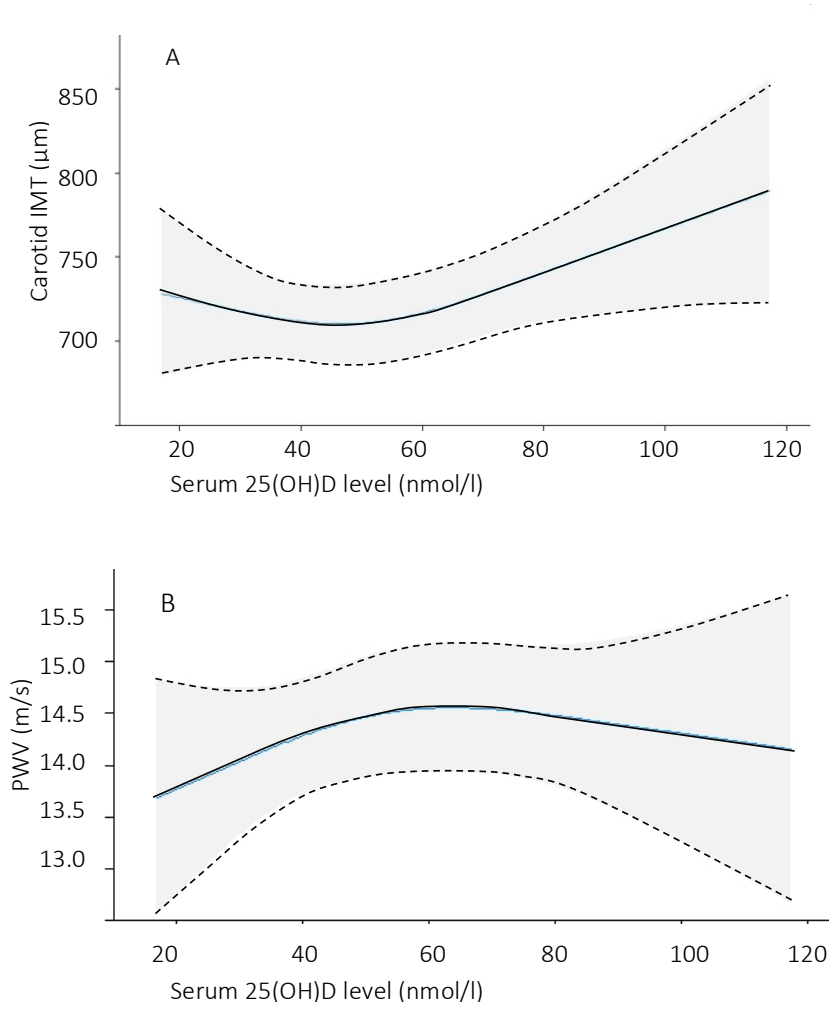

Figure 1. Spline plots of serum 25(OH)D level with carotid IMT and PWV
A. Carotid IMT. B. PWV. Carotid IMT data were adjusted for age, gender and eGFR. PWV data were adjusted for age, gender, study center, eGFR, MAP and heart rate. Abbreviations: as in table 1.

Table 3. Linear regression analysis of the associations between serum 25(OH)D level and IMT and PWV stratified per vitamin D category

	Serum 25(OH)D < 50 nmol/l N = 274	Serum 25(OH)D ≥ 50 nmol/l N = 287
Carotid IMT	-0.79 [-3.217 ; 1.644]	1.24 [0.002 ; 2.473]*
PWV	0.04 [-0.020 ; 0.090]	$-0.31 \cdot 10^{-3}$ [-0.030 ; 0.029]

Values are presented as B ± 95% CI and according to carotid IMT adjusted for age, gender and eGFR. Adjustments made for the analysis with PWV were age, gender, study center, eGFR, MAP and heart rate. * = P <0.05.
Abbreviations: as in Table 1.

Age was a significant effect modifier in this association (*P* = 0.01), showing that the association was only present in participants under the age of 80 (B 1.51 95% CI [0.25 ; 2.77]) (data not shown). In vitamin D insufficient participants, calcium use was a significant effect modifier (*P* = 0.02), however stratified analysis did not demonstrate any differences between users and non-users. Gender, cardiovascular disease history, diabetes and hypercholesterolemia did not interact with the association between serum 25(OH)D and carotid IMT.

Furthermore, the direction of the association between serum 25(OH)D and PWV also changed at the level of 50 nmol/l, showing an inverse association between PWV levels and 25(OH)D below the concentration of serum 25(OH)D level of 50 nmol/l. In order to further investigate this association we performed a stratified linear regression analysis for vitamin D deficient versus vitamin D sufficient individuals, however we found no significant associations between serum 25(OH)D level and PWV for the separate groups (Table 4). Age, gender, cardiovascular disease history, diabetes, hypercholesterolemia or calcium use was not interacting within the association between serum 25(OH)D and PWV.

Table 4. Covariate adjustment in the linear regression analysis of the associations between serum 25(OH)D level and IMT and PWV stratified per vitamin D category

	Serum 25(OH)D < 50 nmol/l N = 274	Serum 25(OH)D ≥ 50 nmol/l N = 287
Carotid IMT	-0.79 [-3.217 ; 1.644]	1.24 [0.002 ; 2.473]*
Age	9.12 [5.14 ; 13.09]	6.36 [2.37 ; 10.90]
Gender	-3.86 [-57.28 ; 49.57]	14.01 [-38.50 ; 66.51]
eGFR	0.59 [0.19 ; 1.37]	0.52 [-0.24 ; 1.27]
PWV	0.04 [-0.020 ; 0.090]	$-0.31 \cdot 10^{-3}$ [-0.030 ; 0.029]
Age	0.35 [-0.02 ; 0.09]	0.27 [0.17 ; 0.38]
Gender	-1.17 [-2.43 ; 0.09]	-0.17 [-1.46 ; 1.13]
Study center	-0.54 [-1.26 ; 0.19]	0.01 [-0.56 ; 0.58]
eGFR	-0.01 [-0.02 ; 0.02]	0.01 [-0.02 ; 0.02]
MAP	0.05 [0.01 ; 0.09]	0.03 [-0.01 ; 0.07]
Heart rate	0.06 [0.01 ; 0.10]	0.05 [-0.01 ; 0.10]

Values are expressed as B [95% CI].

DISCUSSION

Our study shows that the association between serum 25(OH)D and pre-clinical stages of cardiovascular disease in elderly subjects is non-linear. In particular, we have shown that high levels of serum 25(OH)D, starting from serum 25(OH)D levels of ≥ 50 nmol/l, were associated with higher values of carotid IMT in vitamin D sufficient individuals. Also, the association between serum 25(OH)D and PWV was non-linear, potentially monotone,

however in contrast with the effect on IMT the effect size for PWV was small and not within clinically relevant ranges.

In contrast to other studies, we did not find linear associations between serum 25(OH)D and indices of arterial stiffness or arteriosclerosis [10;12;13]. Our study supports the abovementioned non-linear association with cardiovascular morbidity and mortality [7] because in particular for vitamin D sufficient individuals (serum 25(OH)D ≥ 50 nmol/l) an increase in carotid IMT per point increase of serum 25(OH)D level was present. Although this association was clear, one may argue about the clinical relevance, because the carotid IMT only increased 1.24 µm per 1 nmol/l of serum 25(OH)D. Nevertheless, our finding gives an insight into potential mechanisms in which serum 25(OH)D might lead to increased cardiovascular risk.

Although it has been reported that vitamin D deficiency is associated with cardiovascular disease and with higher IMT levels [1;5;6;14-16;24], we were not able to confirm the latter in our study. The spline plot of carotid IMT did however show a significant positive association between serum 25(OH)D levels with carotid IMT in high-normal ranges of serum 25(OH)D. Several mechanisms may explain why high serum 25(OH)D levels are associated with an elevated carotid IMT. First, an increase of vitamin D levels will lead to an increased calcium absorption in the gastrointestinal tract, which in turn may result in higher circulating calcium concentrations. These higher calcium levels could contribute to vascular calcification, in particular in atherosclerotic plaques in the vessel wall. This may be further aggravated because high levels of vitamin D are known to up-regulate vitamin-D-receptors in vascular smooth muscle cells, reducing the activity of matrix metalloproteinases, which also contributes to calcium deposition in the vessel wall [25]. Furthermore, extra-renal activated macrophages express 1,α-hydroxylase, converting 25(OH)D into the hormonally active 1.25(OH)D form and activate pro-calcification in the vessel wall, which also contributes to arterial stiffness [8;9].

In comparison with the recently published Baltimore study [13], we were not able to confirm a linear association between serum 25(OH)D and PWV. Visually the association between 25(OH)D and PWV even appeared to be opposed compared to the association of carotid IMT with serum 25(OH)D. However, the differences in PWV levels between vitamin D sufficient and insufficient were very small and not likely to be clinically relevant. The small difference in PWV levels may also explain why the serum 25(OH)D stratified analysis with continuous PWV measures did not demonstrate a significant association with serum 25(OH)D. Therefore, we may conclude that an association between serum 25(OH)D and PWV level is absent in our study, indicating that serum 25(OH)D within relatively large ranges does not directly affect the arterial stiffening process.

Calcium supplement use was significantly interacting in the association between serum 25(OH)D and carotid IMT in vitamin D deficient individuals. A meta-analysis demonstrated that calcium usage is potentially unsafe in terms of higher cardiovascular risk [26], however in our study we could not confirm calcium use being harmful for arteriosclerotic processes. The lack of interaction in vitamin D sufficient individuals potentially has to do with the lower number of calcium users within the vitamin D sufficient group compared to the group with low levels of serum 25(OH)D.

Our study has several limitations. First, this study has a cross-sectional design. Therefore causality cannot be ascertained. In order to investigate the causal pathway of the association between serum 25(OH)D and indices of arterial stiffness and arteriosclerosis in older individuals, a prospective, preferably randomized controlled trial, comparing vitamin D supplementation with placebo, within this age group is needed. A second limitation is that our findings cannot be easily translated to the general population, because our study population consists of mildly hyperhomocysteinemic elderly, which is an inclusion criteria of the B-PROOF study. Third, one might argue about the sample size of our study. Although a sample size calculation is not appropriate because of the non-linearity of the models, other studies investigating this relation had equal population sizes, making a power issue less likely. Furthermore, the number of participants was quite equal between the groups and can therefore not explain the lack of an association in participants with low serum 25(OH)D levels. Also, with regard to the vascular measurements, we followed the restrictions as provided by the manufacturer. Since detailed restrictions were not clearly included in guidelines at the time of the measurements as they are today, our measures may not be fully comparable to other, more recent studies. However, we tried to minimise the potential influence for example of smoking and physical exercise by considering these factors as potential covariates. The main strengths of our study are our accurately measured cardiovascular parameters and multiple measures of preclinical cardiovascular disease.

Overall, the association between serum 25(OH)D and parameters of arterial stiffness and arteriosclerosis in elderly was non-linear. Caution is therefore warranted when investigating this association. In particular, we demonstrated that vitamin D sufficient participants have higher carotid IMT levels compared to vitamin D deficient individuals. This finding suggests that also normal ranged vitamin D levels might potentially increase cardiovascular risk because of its association with preclinical cardiovascular disease.

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Conflict of interest

None declared

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