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Assessment of the synergistic association of serum concentration of vitamin D, vitamin K and osteocalcin with coronary atherosclerosis in patients undergoing angiography

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HIGHLIGHTS

- Osteocalcin, Vitamin D level, was inversly associated with coronary atherosclerosis.
- Vitamin D level was associated with the severity of atherosclerosis.
- Deficiency of vitamin D not associated with K2 level.

ABSTRACT

Objective: The aim of this study was to assess the association between serum concentration of vitamin D, vitamin K, and osteocalcin with coronary atherosclerosis in patients undergoing angiography.
Design: In this case-control study, 138 people who referred to Bushehr Heart Center for routine examination participated. Demographic information of the participants was registered by using a standard questionnaire. Before undergoing the angiographic processes, venous blood samples were obtained via venipuncture from the antecubital vein into gel and clot activator tubes and stored at – 80 °C until the analysis. After the angiography process was over, the normal participants entered the control group and those with coronary atherosclerosis plaque placed in the case group. Finally, serum levels of vitamin K2 and Osteocalcin were measured in the samples, by using ELISA kits and the serum level of vitamin D was measured by using HPLC.
Result: serum levels of vitamin D in the case group were significantly lower than in the control ($p = 0.009$) and, serum levels of osteocalcin in the case group were significantly higher than in the control ($P = 0.019$). There was no difference in K2 level between the two groups ($P = 0.84$). By separating the three factors like age, sex and T2DM in the two groups, a significant synergistic was found between the concentration of osteocalcin, vitamin D (just in < 20 ng/ml) and coronary atherosclerosis ($p = 0.025$, $p = 0.029$ respectively). Further, an association was observed between vitamin D level and the severity of atherosclerosis ($P = 0.041$).).
Conclusion: the results of this study suggest that the increase in the level of osteocalcin and the deficiency of vitamin D is significantly associated with coronary atherosclerosis but, not with vitamin k2. Moreover, serum vitamin D concentration is associated with severity of coronary atherosclerosis.

1. Introduction

In recent years, a number of studies have pointed to vitamin D deficiency as a risk factor for cardiovascular diseases, and for the past two decades, the prevalence of vitamin D deficiency in the general population has been reported to be as 30–50% and, its consequences are beyond bone health [1]. Importantly, new studies demonstrated an association between vitamin D deficiency (< 20 ng/ml) and the metabolic syndrome, including hypertension, type2 diabetes and coronary artery disease (CAD) [2–4]. Osteocalcin (OC) is a non-collagenous protein containing Gama-carboxy glutamic acid expressed by osteoblasts [5–7]. Recent studies have suggested a synergistic between OC and atherosclerosis markers in humans but, there is inconsistency in the results [8,9]. A higher percentage of EPCs expresses OC in patients with coronary atherosclerosis as compared with the subjects having a normal endothelial function [10]. The data from other investigation showed that serum osteocalcin level was associated with decreased risk of CAD in Chinese adults [11]. Furthermore, observational studies have shown

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Abbreviations

MGP	Matrix gla protein
IL-6	Interleukin 6
TNF- α	Tumor necrosis factor - alpha
CRP	C-reactive protein
EPCs	Endothelial progenitor cells
CVD	Cardiovascular disease
HPLC	High Performance liquid Chromatography
ELISA	Enzyme Link Immunosorbent Assay
CAD	Coronary Artery Disease
T2DM	Type2 Diabetes Mellitus
BMI	Body mass Index
baPWV	Brachial-ankle pulse wave velocity

an inverse association between the intake of vitamin k2 and cardiovascular disease [12–14]. Further, vitamin k2 has a role in post-translational modifications of osteocalcin [15–17] and being a fat-soluble vitamin, it acts as a cofactor for γ-carboxylation of Matrix Gla family of Proteins (MGP). These proteins are identified to be related to coronary heart disease [14,18,and19]] and, play a role in the inhibition of coronary calcification [20,21]. Moreover, recent clinical research has shown that high dietary menaquinone (K2) intake are associated with reduced coronary calcification [14]. Interestingly, the results of one study suggested that vitamin D and K have a protective role in improving arterial stiffness and elastic properties of arteries, however, the benefits of supplementation of both the vitamins have not been validated in a randomized controlled trial [22]. Importantly, vitamin D induces the synthesis of osteocalcin by promoting the transcription of its gene [16]. Since, vitamin D, vitamin K, and osteocalcin in a different way have been related to coronary artery disease, therefore, we decided to investigate their synergistic involvement in affecting coronary atherosclerosis.

2. Methods and materials

2.1. Subjects and study design

In this case-control study, out of 143 participants who referred to Bushehr Heart Center for routine examination 138 were eligible for the study. By considering the confidence level as 90% and effect size of 0.5 based on the mean and SD of Vitamin D level in the control group as 65 ± 43 mmol/l and in the case group as 45 ± 35 mol/l (*), 64 participants were calculated for each group. Finally by considering %10 drop out, the sample size for each group considered to be as 70 and therefore the total samples will be 140 (recruitment detailed in Fig. 1). All the patients underwent routine examination including electrocardiography, echocardiography and angiography, therefore, those who found to be completely healthy entered the control group and those with coronary atherosclerosis plaque (> 30%) were placed in the case group. The study protocol was approved by human ethics committee of Bushehr University of Medical Sciences, Iran, (No: IR.bpums.rec.1395.13). The inclusion criteria were age between 30 and 70 years and nonsmokers, and the exclusion criteria were liver and bone diseases, kidney failure, and history of malignancy, consumption of warfarin, antibiotics, vitamin D and vitamin k2. A written informed consent was obtained from all the participants. Demographic and

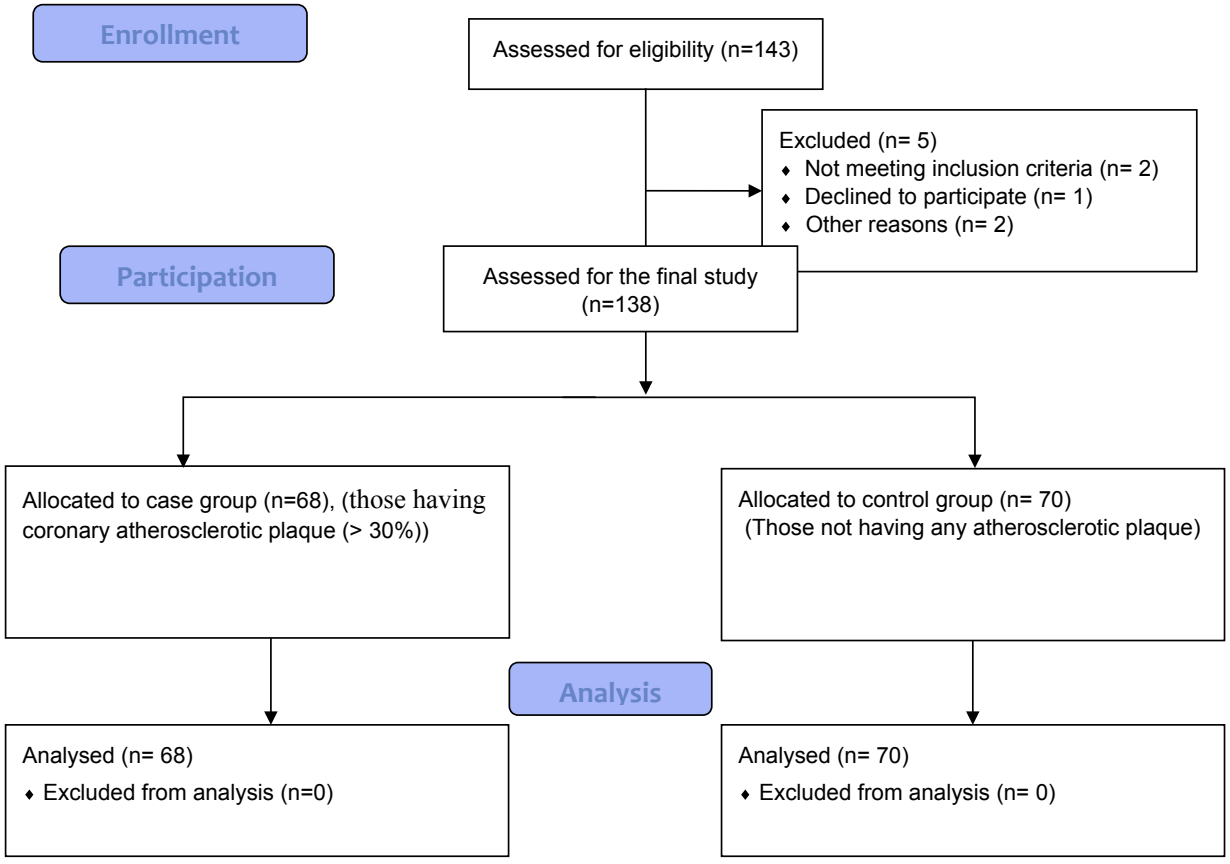

Fig. 1. The flow diagram of the recruitment and progression of subjects under the study.

hemodynamic properties including height and weight were measured according to standard procedures. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters using Global Database on BMI (World Health Organization, 2006). Blood pressure (BP) was measured two times in a 15-min interval using automated blood pressure device (Sazgan Gostar company model Vista). Ejection fraction was obtained from echocardiography records.

2.2. Angiography procedure

The participants were prepared to undergo angiography, local anesthesia was induced by using 1% Lidocaine (1 cc), followed by puncturing the radial artery and placing the arterial sheet into it. The radiocontrast agent (visipaque, 50 cc_s) was directed into the left and right coronary arteries by diagnostic catheters and at the same time using X-rays at different angles to get the images. Finally, the angiographic films obtained were assessed to identify the number of plaques formed, for the next plan, which is the treatment. The angiographic instrument used was from the Siemens Company [model 3800005, axiom artist.

2.3. Biochemical analysis

All the blood samples were collected in gel and clot activator tubes in a fasted state, the serums were separated and stored at – 80 °C until the analysis. Vitamin D (25(OH), vitamin k2 and osteocalcin concentrations were measured in the samples.

2.3.1. Measurement of serum vitamin D

Vitamin D (25-hydroxy) was measured in ng/ml by using high-performance liquid chromatography (Knauer platin blue c18 reversed column, the internal standard was from Sigma company and low & high controls were purchased from RECIPE Chemicals + Instruments GmbH. Since, sun exposure/dermal synthesis, season would have an influence on Vitamin D status, we chose the winter months to have less effect of these factors. Moreover, in the society under study, women and men were highly clothed; therefore, this could help us to reduce the environmental effect. Further, the detection limit of vitamin D was 2 ng/ml and the inter-assay variation coefficient was approximately < 5%.

2.3.2. Measurement of serum osteocalcin and vitamin k2

Serum osteocalcin and vitamin k2 were measured by using the method based on biotin double antibody sandwich technology (ELISA kit, Zell bio.Gmbh). The intro and interassay coefficient of variations (CV) were < 10% and 12%, respectively. The sensitivity of measuring osteocalcin and vitamin k2 were 0.1 and 2.5 ng/ml, respectively.

2.4. Statistical analyses

Descriptive statistics including mean and standard deviation, median and frequency were used to express continuous and categorical variables. Chi-square, Fisher's exact and independent t-tests were used for comparing categorical and continuous variables between the two groups, respectively. Kolmogorov-Smirnov test, as well as the ratio of skewness to its standard error, was used to check the normality of the distribution of serum levels of osteocalcin, vitamin K2 and vitamin D. In cases where the distribution was not normal, the logarithmic transformation of variables or Mann-Whitney test was applied. The independent *t*-test was used to check the differences in the serum levels of vitamin D, vitamin K2 and osteocalcin between the two groups. Logistic regression was used to check the association of vitamin D, vitamin K2 and osteocalcin with coronary artery disease adjusting for age, sex and diabetes that were significantly different between the two groups. The significance level was considered to be as 0.05, and SPSS 18 was used for the data analysis.

3. Results

3.1. Baseline characteristics of the participants

The baseline characteristics of the patients are shown in Table 1. Subjects in the case group had significantly, lower levels of 25(OH) D as compared with the control (p = 0.009). Mean Serum levels of osteocalcin were significantly higher in the case group as compared with the control (p = 0.019). There was no difference in mean serum levels of vitamin K2 between the two groups (p = 0.84) Table 1.

3.2. Association of 25(OH) D, vitamin K2 and osteocalcin with coronary artery atherosclerosis

By applying the logistic-regression analysis and after adjusting for age, sex and type 2 diabetes it was found that the deficiency of 25(OH) D (< 20 ng/ml) and the increase in the level of osteocalcin were significantly associated with coronary artery atherosclerosis (p = 0.025, p = 0.029, respectively), however, there was no association between vitamin K2 and atherosclerosis (p = 0.88) Table 2.

3.3. Association of 25(OH) D, vitamin K2 and osteocalcin with the severity of the atherosclerotic lesions

The results of this study showed that the serum levels of osteocalcin and vitamin K2 were not associated with the severity of atherosclerotic lesion (p = 0.09, p = 0.46, respectively). However, a significant

Table 1
Baseline characteristics of the participants.

Variable		Group		X^2	p-value
		Control Number (%)	Case Number (%)		
Sex	Male	22 (31.4)	36 (52.9)	6.55	0.01
	Female	48 (68.8)	32 (47.1)		
Hypertension	yes	37 (54.4)	36 (51.4)	0.12	0.72
	no	31 (45.6)	34 (48.6)		
Hyperlipidemia	yes	26 (38.2)	21 (30)	1.04	0.27
	no	42 (68.8)	49 (70)		
Type 2 diabetes	yes	10 (14.3)	25 (36.2)	9.20	0.002
	no	60 (84.2)	44 (63.8)		
Systolic BP[a] (mmHg))		126.69 ± 14.36	129.03 ± 8.12	- 0.87	0.38
Diastolic BP[a] (mmHg)		78.51 ± 9.11	81.81 ± 11.31	- 1.88	0.61
Heart rate[a] (bbm)		74.26 ± 9.42	75.12 ± 11.93	3.73	0.63
EF[a] (percent)		52.29 ± 7.45	47.29 ± 8.23	3.73	< 0.001
Age[a] (years)		52.46 ± 10.5	56.07 ± 9.73	- 2.9	0.038
BMI[a] (kg/m^2)		27.56 ± 5.02	27.08 ± 4.83	0.56	0.57

[a] Mean ± SD, the significance level was considered to be as 0.05.

Table 2
Association of 25(OH) D, vitamin K2 and osteocalcin with coronary artery atherosclerosis.

Variables		B	OR	95% CI	p-value
25(OH) D (ng/ml)	< 20	0.94	2.55	1.09–5.96	0.029
	20–30	1.16	3.07	0.99–9.54	0.052
	≥30	–	1	–	–
Vitamin K2 (ng/ml)		−0.001	0.99	0.98–1.01	0.88
Osteocalcin (ng/ml)		0.09	1.1	1.01–1.19	0.025

The significance level was considered to be as 0.05.

association was found between 25(OH) D and the severity of atherosclerotic lesion (p = 0.041) Fig. 2.

3.4. The concurrent association of 25(OH) D and osteocalcin with coronary atherosclerosis

The concurrent effect of serum level of 25(OH) D and osteocalcin on coronary atherosclerosis was assessed by performing logistic regression analysis and after adjusting for age, sex and type2 diabetes. The results showed that the odds of coronary atherosclerosis in people with the serum level of 25(OH) D below 30 ng/ml was 2.44 times more than those with 25(OH) D above 30 ng/ml (CI 95% = 1.1–5.4, p = 0.027).

4. Discussion

To our knowledge, this is the first study to show the synergistic association between vitamin D, K2 and osteocalcin status with coronary artery atherosclerosis. The result of this study showed an association between serum 25 (OH) D concentrations and coronary atherosclerosis, independently of standard cardiovascular risk factors. This is in line with the reports suggesting that vitamin D status is inversely associated with coronary artery disease after adjustment for cardiovascular risk factors [23–26]. Recent evidence suggested that vitamin D deficiency is related to many systemic diseases, including cardiovascular, cancers and immunologic disorders. Because, it is known that vitamin D receptors are involved in the expression of several human genes [1,27]. There are different mechanisms proposed to explain the involvement of vitamin D deficiency in the development of coronary atherosclerosis: Vitamin D receptors have been found in the vascular smooth muscle cells, macrophages, endothelium [28,29], and it is inversely associated with the expression of genes or activity of some white cells that are involved in atherosclerotic plaque formation (29). In addition, low levels of 25(OH) D is inversely related to the increase in the cytokines including Il-6, TNF-α and CRP, showing the formation of atherosclerotic plaques [28,30]. Further, vitamin D reduces uptake of cholesterol by macrophages, and suppresses the formation of foam cells [31]. Evidence indicates that 25(OH) D stimulates the production of prostacyclin by vascular smooth muscle cells which finally leads to the prevention of thrombus formation, cell adhesion and smooth muscle proliferation [32]. Furthermore, low levels of 25(OH) D is inversely associated with the increase in arterial stiffness and endothelial dysfunction [24].

Osteocalcin is secreted mostly by osteoblasts and has an important role in the body's metabolic regulation and bone-building, by nature [33]. Interestingly, the present study revealed a significant direct association between serum osteocalcin levels and coronary atherosclerosis, after adjusting for cardiovascular risk factors. The reports showing the association between osteocalcin levels and cardiovascular diseases are inconsistent: Reyes-Garcia has reported that coronary heart disease was associated with higher levels of osteoclcin in male and female patients [9] and, Holvick showed a direct association between serum osteocalcin levels and cardiovascular diseases in older-old women (R). However, the result from other studies has found an inverse association between osteocalcin levels and cardiovascular disease (R). Moreover, Holvick showed that serum osteocalcin concentration was inversely associated with cardiovascular diseases in old men [7]. Further, Bao reported that osteocalcin level was significantly lower in patients with coronary artery diseases [6]. In fact there are studies showing no association between osteocalcin levels and CADs [7]. The controversies over the results may be because of different population or ethnic groups under study, or the different in isoforms of osteocalcin. There is no specific mechanism available to show the synergistic between osteocalcin and CADs. However, several hypotheses have been proposed, in which osteocalcin is involved in glucose metabolism, therefore, it can be associated with type 2 diabetes which is considered to be a risk factor for CADs (8). Importantly, there are reports showing an association between low serum osteocalcin concentration and the increase in the level of CRP and LDL-C [34].

To our knowledge, this is the first study to investigate the association between serum vitamin k2 and coronary atherosclerosis. The potential role of vitamin K2 in cardiovascular health is mediated through carboxylation of the two proteins, osteocalcin and MGP which are known to be vitamin k-dependent proteins that act as arterial calcification inhibitors [35]. Also, studies have demonstrated a link between vitamin D deficiency and severity of atherothrombosis and vascular calcification [32,33].

The present study found no association between serum vitamin k2 concentration and coronary atherosclerosis. Importantly, our results are not consistent with the studies demonstrating the association of intake of vitamin k2 with the lower incidence of coronary heart disease [14,19and36]. In these studies vitamin k2 has been assessed by a standard Food Frequency Questionnaire (FFQ), as compared to our study in which K2 was measured in the serum samples of the participants. In fact, in the present study all the participants had normal range of vitamin K2 and this may be the reason why no association was found between serum K2 level and coronary atherosclerosis. Moreover, Ikari has reported that consumption of high dose supplementation of K2 may not protect against coronary artery calcification [33], however, it has been shown that Brachial-ankle pulse wave velocity (baPWV) for the measurement of arterial stiffness was not changed (33). There were some limitations in this study.

4.1. Limitations and strengths

The strength of this study is that, to our knowledge it is the first worldwide research to assess the synergistic association between serums 25(OH) D, vitamin K2 and osteocalcin concentration and coronary artery atherosclerosis. Further, this study might be the first one to measure the serum K2 and to assess its association with coronary

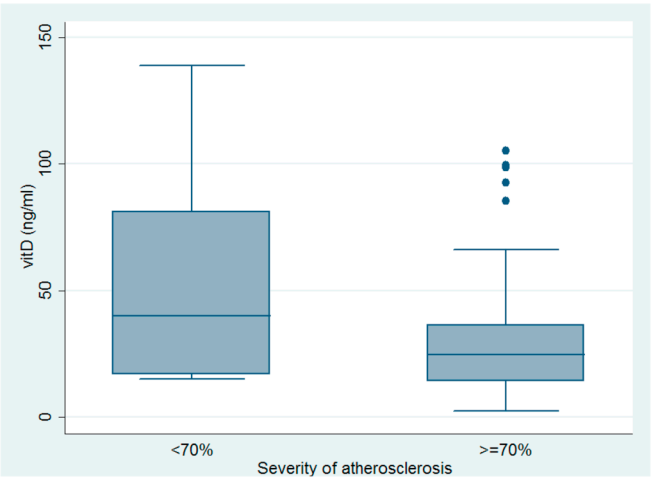

Fig. 2. Comparison of 25 (OH) D levels in patients (case group) with different level of atherosclerotic lesion.

atherosclerosis. Moreover, this study showed an inverse association of osteocalcin with coronary atherosclerosis. There were also few limitations. First, the study was single center with less number of participants. Second, since it was based on an observational study; therefore, some confounder factors may have been missed. Third, we measured the total OC; while there are two types: carboxylated and uncarboxylated; the high level of OC has been shown to be associated with the high risk of CAD [13]. On the other hand, serum carboxylated OC has been reported to have protective role. Fourth, the optimal cut-off point for vitamin D deficiency level in the Iranian population is still controversial and not fully understood. One more limitation was the lack of data on background diet.

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5. Conclusion

We conclude that the increase in the level of osteocalcin and the deficiency of vitamin D is significantly associated with coronary atherosclerosis. In addition, no relation was found between vitamin k2 and coronary atherosclerosis. The important finding in this study was to show an association between vitamin D and coronary atherosclerosis which depends on osteocalcin concentration. Further, serum vitamin D concentration is significantly associated with severity of coronary atherosclerosis.

Conflict of interest

No conflict of interest relevant to this article was reported.

CRediT authorship contribution statement

Zahra Elyaspour: Conceptualization, Writing - review & editing. **Daryoush Iranpour:** Data curation, Writing - review & editing. **Niloofar Motemed:** Formal analysis. **Ali Movahed:** Investigation, Conceptualization, Data curation, Writing - review & editing.

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