

## Vitamin D concentrations, endothelial progenitor cells, and cardiovascular risk factors

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**Our study aimed to establish the association of vitamin D status with the level of circulating endothelial progenitor cells (EPCs) and circulating angiogenic cells (CACs) and to demonstrate the effect of vitamin D on the level of lipoproteins responsible for increased cardiovascular risk and high blood pressure. 41 healthy adults were selected. EPCs were defined as CD34+/KDR+ cells, and CACs were defined as cells that expressed endothelial markers after incubation of mononuclear blood cells with endothelial growth factors during 5 days. We found a positive association between EPCs, CACs and the level of vitamin D and an inverse correlation between several subclasses of lipoproteins. The level of vitamin D higher than 40 ng/ml demonstrated a positive effect on regulation of blood pressure, and there was significant difference in cholesterol/HDL ratio, very low-density lipoproteins, and triglycerides for groups of subjects with varying levels of vitamin D.**

**KEY WORDS:** Endothelial progenitor cells, Vitamin D, cardiovascular risk factors.

The purpose of this study was to establish the association of vitamin D status with the level of circulating endothelial progenitor cells (EPCs). Vitamin D deficiency is a common condition, present in approximately 30% to 50% of the general population. Low 25-hydroxyvitamin D levels may have an effect on cardiovascular health, cancer, and diabetes.<sup>1-9</sup>

Studies on the connection between ischemic heart disease, hypertension and vitamin D are conflicting.

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Some studies found an inverse relationship between levels of the active form of vitamin D, blood pressure, and plasma renin activity.<sup>10, 11</sup> Another study reported a positive association.<sup>12</sup>

Because the vitamin D receptor (VDR) is present in most cells, vitamin D has a wide range of therapeutic and health-related benefits. The active form of vitamin D<sub>3</sub> is a steroid hormone shown to regulate more than 60 genes.<sup>13,14</sup> The translocation of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> into cells, where it binds with high affinity to vitamin D nuclear receptors, results in altering rates of gene expression. By this pathway, the active form of vitamin D influences a number of genes relevant to arterial wall functions. These include VEGF, matrix metalloproteinase, myosin, and structural proteins. Vitamin D receptors are densely distributed in the endothelium, and vitamin D<sub>3</sub> modulates vascular tone by reducing calcium influx into the endothelial cells. 1,25(OH)<sub>2</sub>D is a very effective modulator of the immune system. In animal models, it has been demonstrated that pretreatment with 1,25(OH)<sub>2</sub>D is effective in preventing the onset of type 1 diabetes, multiple sclerosis, rheumatoid arthritis, and Crohn's disease.<sup>15</sup>

Our goal was to prove that optimal levels of vitamin D could be correlated with the increased number of circulating EPCs and angiogenic cells. The number of

circulating EPCs in an individual's blood may be an indicator of overall vascular health. In atherosclerosis, the endothelial layers may become damaged, and nearby endothelial cells are recruited to help repair vessels or form new ones. EPCs are generated from bone marrow and contribute to repair of the endothelium. A lack of EPCs can lead to vascular dysfunction and a progression of atherosclerosis. There are no systematic studies regarding the physiological variation in the number or the lifetime of EPCs in physiological or pathological conditions.<sup>16</sup> The effect of cardiovascular risk factors, diabetes, acute myocardial infarction and vascular trauma on the number of EPCs has been described in several studies.<sup>17-21</sup> In a large population-based study it was shown that there was a positive relation between EPC numbers and vascular risk factors and a positive association of EPCs with Framingham risk factors.<sup>22</sup>

Our focus was to evaluate if the population of EPCs could be correlated with vitamin D status. The levels of EPCs and the level of angiogenic cells developed from PBMSc in vitro were measured and compared with the level of vitamin D in serum.

## Materials and methods

### *Study population and methods*

Forty-one adults ages 23-74 were included. Exclusion criteria were hypertension (use of anti-hypertensive therapy), diabetes mellitus, and a history of neoplasm or active cancer. The study was approved by the Institutional Review Board committee, and informed consent was obtained from all subjects. Fasting serum and blood samples were taken and analyzed within 4 hours of collection. The serum was used for measurements of the lipid profile (total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoproteins (LDL), triglycerides, very low-density lipoproteins [VLDL]), glucose, and C-reactive protein by established clinical laboratory tests. Blood pressure was measured for each participant on the same day. Serum level of vitamin D (25(OH)D) was determined by radioimmunoassay (RIA kit, DiaSorin, Stillwater, MN). Blood circulating progenitor cells were phenotyped and quantified by flow cytometric analysis. Blood collection was taken at the same time (8-10 a.m.), as there are indications that the number of EPCs exhibit diurnal variations with an increase

between 3 pm and 10 p.m.<sup>22</sup> All participants were asked to stop taking vitamin D at least one week before blood drawing.

### *Method of measurements of endothelial progenitor cells*

EPCs have been identified as a circulating cell population in peripheral blood that co-expresses hematopoietic stem/progenitor cell markers (CD34 or AC133) as well as endothelial markers (VE-Cadherin or VEGFR-2). These cells may augment the injury-repair process and promote angiogenesis.<sup>23, 24</sup>

The level of peripheral blood progenitor cells was analyzed by the expression of cell-surface antigens with direct 2-color cytometric analysis using fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs). To measure EPCs, the peripheral blood mononuclear cells were isolated from peripheral blood using density-gradient centrifugation. Separated mononuclear cells were labeled for 20 to 30 min at 4°C using manufacturer-recommended concentrations with antihuman-KDR-PE (Becton Dickinson, San Diego, California) and antihuman-CD34-FITC (Miltenyi Biotec, Auburn, CA, USA). Fluorescent isotype-matched antibodies were used as controls. Cell fluorescence was measured immediately after staining. A morphological gate included lymphocytes and monocytes. Circulating EPCs were defined as CD34+/KDR+ cells.

### *Method of measurement of angiogenic cells*

In addition to the population of circulating EPCs defined by a specific stem/progenitor cells marker, we measured the level of angiogenic cells after culturing mononuclear cells on fibronectin in an endothelial medium. This cell population was defined as circulating angiogenic cells (CACs)<sup>25, 26</sup> and expresses monocyte/macrophage markers and markers of endothelial cells. The population includes endothelial cells, endothelial progenitor cells and cells-expressed monocyte/macrophage markers. To find the number of CACs, mononuclear cells were plated in an EBM-2 medium supplemented with endothelial growth factors on fibronectin-coated 6-well plates for 5 days. Adherent cells were detached and stained for markers of endothelial cells: KDR, CD144, CD105, and CD62E. In addition, a portion of the cells was incu-

TABLE I.—Participants' characteristics.

	Average	Min	Max	Units
Age	49.65	20	76	
Cholesterol	205.56	141	277	mg/dL
Cholesterol/HDL Ratio	3.58	2	6.3	
LDL	114.05	47	164	mg/dL
CRP	3.15	0.11	16.63	mg/L
Glucose	100.73	79	198	mg/dL
HDL Cholesterol	62.12	35	124	mg/dL
LDL/HDL Ratio	2.00	0.9	3.6	
Triglycerides	143.26	48	457	mg/dL
Vitamin D	29.82	6	60	ng/mL
VLDL	27.1	10	60	mg/dL
Systolic pressure	123.90	92	158	mmHg
Diastolic pressure	73.85	58	96	mmHg

bated with Dil-labeled acLDL (Molecular probe) and with FITC-labeled *Ulex europaeus* agglutinin I (ulexlectin, Sigma). Morphological analysis of these cells after 5 days of culture of the mononuclear cells demonstrated that some cells developed elongated, spindle-shaped and fibroblast-like morphology after adhesion to the plate.

#### Statistical analysis

The data were analyzed by Systat software (Systat, Inc) and Kaleidagraph software. Variables were presented as mean values  $\pm$  SD, or as medians with corresponding 25th percentiles. Association of EPCs with the level of vitamin D and vascular risk factors was assessed using linear models. Statistical significance was accepted if the null hypothesis could be rejected at  $P \leq 0.05$ .

## Results

### Positive correlation of EPCs, angiogenic cells, and vitamin D

The study sample was a representation of the healthy general population. Baseline characteristics of participants are shown in Table I (average, minimal, and maximal values of the age and clinical tests). The mean 25(OH)D concentration for all participants was 29.8 ng/mL. The percentage of participants with a level of vitamin D less than 20 ng/mL, which is considered deficient, was 36%. A plasma level of vitamin D in the range 20-30 ng/mL was considered as insufficient, and sufficient levels were greater than 30-40 ng/mL. Optimal levels are between 40-80 ng/mL. Other para-

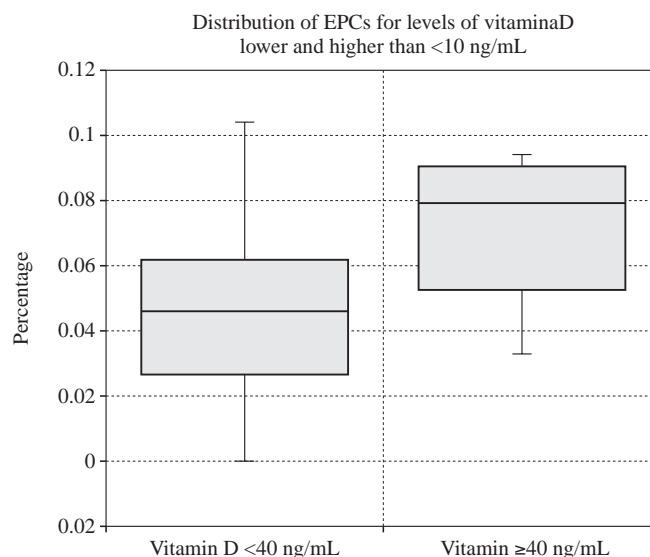


Figure 1.—Distribution of EPCs for the levels of vitamin D lower and higher than 40 ng/mL. Each box encloses 50% of data with the median value of the variable.

meters included in Table I are conventional cardiovascular risk factors and the level of glucose.

The effect of vitamin D status and risk factors on the number of circulating EPCs and cultured angiogenic cells was analyzed for all study subjects. The number of EPCs measured in circulation was determined as CD34+/KDR+ cells. The mean number of EPCs in PBMCs in the gating area that excluded granulocytes was  $(0.05 \pm 0.03\%)$ . To prove that vitamin D had an effect on the level of endothelial progenitor cells in circulation, the values of measured CD34/KDR positive cells were compared for subjects with a level of vitamin D higher and lower than sufficient levels.

The distributions of EPCs for the levels of vitamin D higher and lower than 40 ng/mL are shown in Figure 1. The mean percentage of EPCs for subjects with a level of vitamin D lower than 40 ng/mL was significantly different than the mean percentage of EPCs for subjects with a level of vitamin D higher than 40 ng/mL (0.045 versus 0.068,  $P$  value for trend  $< 0.01$ ). Mean values of EPCs for the levels of vitamin D less or higher than 30 ng/mL were 0.046 and 0.060 respectively ( $P < 0.05$ ).

A positive correlation was found between CACs and the level of vitamin D. For development of CACs, mononuclear cells were placed on fibronectin-coated plates in a medium with several growth factors: vas-

TABLE II.—Average values of EPCs for the levels of test parameters higher and lower than the risk levels.

Parameter	Test values lower than borderline	Percentage of EPCs	Test values higher than borderline	Percentage of EPCs	P for trend (one-sided)
Glucose	<100mg/dL	0.061	>100 mg/dL	0.044	0.02
VLDL	<30 mg/dL	0.061	>30 mg/dL	0.035	0.001
Systolic pressure	<140 mmHg	0.06	>140 mmHg	0.04	0.01
Diastolic pressure	<80 mmHg	0.058	>80 mmHg	0.043	0.02
C-reactive protein	<5 mg/L	0.055	>5 mg/L	0.047	0.2 (NS)
Cholesterol/HDL	< 4.5	0.059	>4.5	0.049	0.2 (NS)

TABLE III.—The average values of clinical tests for subjects with sufficient and insufficient or deficient levels of vitamin D.

Clinical tests	Serum 25-OH vitamin D levels		P for trend (one-sided)
	<40 ng/mL	≥40 ng/mL	
VLDL	31.2	18.7	0.002
Systolic blood pressure	127	115.8	0.03
Diastolic blood pressure	76.6	66.4	0.003
Cholesterol/HDL ratio	3.7	3.0	0.02
Triglycerides	162	110	0.05
C-reactive protein	2.7	1.7	0.08 (NS)

cular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF). After 5 days of exposure to growth factors, attached cells were analyzed for the presence of endothelial markers. The angiogenic origin of cultured cells was demonstrated by expression of VEGF R2 (KDR), CD105, CD62E and CD144. The expression of these endothelial marker proteins on adherent cells was measured by flow cytometry. In addition, cells were double stained by Dil-ac-LDL and lectin. Most of the cells from the population of attached cells were positive for endothelial markers. The percentage of KDR, CD105, and CD62E –positive cells was 50% -100% in the gated region, which included large elongated cells, and 15%-50% in all attached cells. The expression of CD144 was lower than 10% for all measured samples.

Because these types of cells participate in vascular repair and angiogenesis, we further characterized the effect of vitamin D on the ability of cultured mononuclear cells to differentiate to angiogenic cells. The percentage of cells differentiated in angiogenic cells in a medium with endothelial growth factors was compared with the measured levels of vitamin D in the blood for all participants. The number of CACs demon-

strated a positive association with the level of vitamin D. Percentage of the total attached cells expressing KDR was 21% and 34% for the levels of vitamin D less and higher than 40 ng/mL ( $P < 0.05$ ). For the cells counted in the gated region of 10-14  $\mu$ M size cells, these values were 74% (vitamin D < 40 ng/mL) and 89% (vitamin D ≥ 40ng/ml) with  $P < 0.02$ .

The same results were found for the population of cells expressing CD105 and CD62E. The percentage of cells stained positive for CD62E was 72% for levels of vitamin D less than 40ng/mL, and 89% for levels of vitamin D higher than 40ng/ml. Mean percentage of CD105 positive cells from attached cell population was increased from 77% to 95% for subjects with an insufficient or deficient level of vitamin D in comparison to the subjects with a sufficient level of vitamin D. In addition, cultured cells were double stained by Dil-Ac-LDL and lectin and showed that  $50\% \pm 24\%$  of attached cells were stained positive.

#### *Factors that have an inverse correlation with the number of circulating endothelial progenitor cells*

After characterization of the endothelial progenitor cells by expression of CD34 and VEGF receptor (KDR), the percentage of these cells in circulation was compared with all other measured parameters: lipid profile, C-reactive protein, level of fasting glucose, and blood pressure. We first investigated whether there was a correlation between the level of EPCs in blood and the level of several subclasses of lipoproteins. For many subclasses of lipoproteins, clinical tests generally focus on the following types: high-density lipoproteins (HDL), which transport cholesterol away from arteries and are protective; low-density lipoproteins (LDL), which can penetrate the arterial wall and deposit cholesterol within the artery, thus contributing to heart disease; and very-low-density lipoproteins (VLDL),

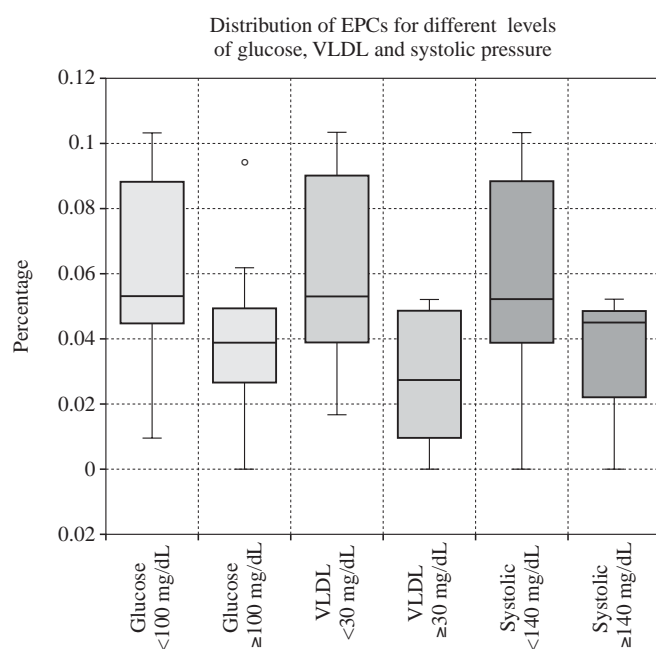


Figure 2.—Percentage of circulating EPCs in subjects with higher and lower than high borderline of normal range levels of glucose, VLDL, and systolic pressure.

which are similar to LDLs but can more easily penetrate the artery wall. Data of inverse correlation of risk factors with EPCs are shown in Table II.

Data in Table II show the mean percentage of EPCs for levels of risk factors higher and lower than the upper border of the normal range and p-values for trend. For all parameters except C reactive protein, borderlines for risk factors are chosen as the highest level of normal range (mean + 2SD) previously estimated in our clinical laboratory. For CRP the level of borderline equals 5 mg/L and marks the level of high risk of cardiovascular disease. The average percentages of EPCs for levels of risk factors higher and lower than the highest level of normal range are presented in Table II and show a significant difference in the level of EPCs for the level of fasting glucose, VLDL, and blood pressure.

Distributions of the percentage of endothelial progenitor cells in groups of subjects with levels of glucose, VLDL, and systolic pressure higher or lower than upper borderline of normal range are presented in Figure 2.

According to our data, there was an inverse correlation between indicated levels of risk factors and the

number of endothelial progenitor cells in circulation, and a trend towards a lower level of EPCs for subjects with an increased level of fasting glucose, VLDL, and blood pressure. For a VLDL level less than 30 mg/dL, the average value for the percentage of EPCs in circulation was 0.061%, and for VLDL higher than 30 mg/dL the mean value of EPCs was 0.035% ( $P < 0.001$ ). The inverse association was also found between the fasting level of glucose and the level of EPCs (0.060% for the level of glucose less than 100 mg/dL, and 0.044% for the level of glucose higher than 100 mg/dL,  $P < 0.02$ ). According to the linear correlation analysis, there was statistically significant inverse correlation between the level of EPCs and the level of glucose ( $P < 0.05$ ) and VLDL ( $P < 0.01$ ).

The mean level of EPCs was decreased from 0.06% for the levels of systolic pressure less than 140 mmHg to 0.044% for systolic pressure greater than 140 mmHg ( $P < 0.01$ ). Diastolic pressure mean values of EPCs were decreased from 0.058% to 0.043% for borderline pressure 80 mmHg ( $P < 0.02$ ). According to these results, increased blood pressure was related to a decreased number of endothelial progenitor cells, and participants with increased blood pressure had the reduced number of EPCs in circulation.

The comparison of the levels of C-reactive protein with the measured level of EPCs for all participants demonstrated that for a level of CRP higher than 5, the mean number of endothelial progenitor cells was decreased from 0.055% to 0.047%, but the difference was not statistically significant.

Finally, there was an inverse association between the levels of several risk factors and the EPC number, and the percentage of circulating CD34+KDR+ endothelial progenitor cells was decreased with increased values of blood tests, which are indicators of the occurrence of cardiovascular events or diabetes.

#### *Serum vitamin D inversely correlated with the level of lipoproteins responsible for the increased cardiovascular risk and the level of blood pressure*

Because vitamin D may play a role in blood pressure regulation and vitamin D deficiency may alter the effects of risk factors, we examined the association between vitamin D status and cardiovascular risk factors. The values of cholesterol/HDL ratio, triglycerides, very-low density lipoproteins, C-reactive protein, and blood pressure averaged for subjects with a level of vitamin D higher and lower than 40ng/mL are

presented in Table III. For a vitamin D level higher than 40ng/mL, there was a decrease in diastolic pressure (76.6 for vitamin D levels less than 40 ng/mL and 66.4 for vitamin D levels higher than 40 ng/mL,  $P < 0.003$ ). For systolic pressure, there was also a decrease in values from 127 to 115.8 with  $P$  for trend 0.03 for subjects with a sufficient level of vitamin D in comparison with subjects with an insufficient or deficient level of vitamin D.

Vitamin D levels were inversely correlated with the level of lipoproteins responsible for the increased cardiovascular risk. There was an inverse relationship between the levels of vitamin D and cholesterol to HDL ratio. This factor is considered as a marker of cardiovascular disease with the average risk factor in the range between 3.4 and 5.0. Subjects with sufficient vitamin D status ( $\geq 40$  ng/mL) showed reduction of this parameter from average value 3.7 to 3.0 ( $P < 0.02$ ). Vitamin D deficiency was associated with increased level of VLDL (31.2 mg/dL versus 18.7 mg/dL,  $P < 0.002$ ).

The increased level of CRP is considered a risk factor of cardiovascular disease. Mean values of CRP for the ranges of vitamin D higher and lower than 40 ng/mL were 1.7 and 2.7 ( $P$  value for trend 0.08).

## Discussion

This study indicates that vitamin D status has an effect on the number of EPCs in circulation and on the ability of peripheral mononuclear cells to differentiate in angiogenic cells. Mean values of EPCs for subjects with a sufficient level of vitamin D (30-40 ng/mL) were higher than for subjects with an insufficient or deficient level of vitamin D (0.069 versus 0.045,  $P < 0.01$ ). The number of circulating angiogenic cells developed from the peripheral mononuclear cells was higher in subjects with a higher level of vitamin D in the blood.

The possible explanation for these results may be that the vitamin D hormone is a developmental hormone. A higher level of vitamin D in circulation and genetic variations in response to vitamin D may have an impact on the ability of stem cells in circulation to differentiate in endothelial phenotype. This possibility is supported by a study in which it was shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> may regulate phospholipase C production by the cells, which in turn may modulate signal transduction by receptors with tyrosine kinase activi-

ty, including VEGF-R1 and VEGF-R2.<sup>27</sup> Another study has shown that vitamin D has an effect on the proliferation of stem cells (human bone-marrow derived CD34+ and human peripheral blood-derived CD133+ cells).<sup>28</sup> Our data of increased level of circulating EPCs in subjects with a sufficient level of vitamin D may be explained by the ability of vitamin D to modulate the number of stem cells and differentiate these cells in progenitor phenotype.<sup>27-29</sup>

An increased number of CACs in subjects with a sufficient level of vitamin D may be explained by the fact that during angiogenic cell development growth factors presented in medium could modulate the expression of the nuclear vitamin D receptors, presented in several subpopulations of mononuclear cells, which will contribute to the development of angiogenic types of cells.

Our study supported results of other studies that demonstrated that risk factors of cardiovascular disease and diabetes have an inverse correlation with the number of circulating EPCs.<sup>16, 17, 30</sup> Cardiovascular risk factors induce endothelial injury and a cascade of pro-inflammatory events, resulting in the infiltration of monocytic cells and smooth muscle cells proliferation, which leads to the formation of atherosclerotic lesions. EPCs incorporate into the sites of neovascularization and provide endothelial repair. However, continued exposure to cardiovascular risk factors not only damages the endothelial layer but may also lead to depletion of EPCs.

In this study, we compared the levels of EPCs with risk factors. Our data demonstrated that there is an association between increased blood pressure and the level of circulating EPCs. The percentage of EPCs was decreased from 0.06 to 0.04 for populations with a level of systolic pressure higher than 140 mmHg ( $P < 0.01$ ). An increased level of diastolic pressure had an inverse association with the number of EPCs (0.058 for diastolic pressure less than 80 mm Hg and 0.043 for diastolic pressure higher than 80 mmHg,  $P < 0.02$ ). The same tendency was found for the cholesterol/HDL ratio, concentration of very-low density lipoproteins, and increased level of fasting glucose. The mechanism by which risk factors may affect EPCs has been suggested in studies in which it was hypothesized that progenitor cells are more sensitive to apoptosis induction.<sup>17, 31</sup>

The difference between two groups of subjects with sufficient and insufficient or deficient levels of vitamin

D was significant for cholesterol/HDL ratio, VLDL, triglycerides, and blood pressure. A level of vitamin D higher than borderline 40 ng/mL demonstrated a positive effect on blood pressure. While it is clear that vitamin D plays some role in the regulation of blood pressure, the mechanism of these complex relationships must be evaluated.

Blood vessels and the heart have large numbers of vitamin D receptors, which means that vitamin D is providing some function in regulating these tissues. Laboratory studies have found that vitamin D suppresses the activity of the hormone renin, high levels of which can cause raised blood pressure.<sup>32</sup>

To maximize health and reduce the risk of common diseases, it is important to pay attention to the 25(OH)D concentrations. Maintaining a healthy 25(OH)D concentration may be important to prevent coronary disease. According to our results, the minimum concentration of 25(OH)D should be 40 ng/mL; and for maximum bone health and prevention of many chronic diseases, the vitamin D concentration should be higher than 40 ng/mL. Larger clinical trials evaluating nutritional, environmental and population factors may better define the possible roles of vitamin D levels in cardiovascular prevention.

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