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Role of Vitamin D in Atherosclerosis

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Atherosclerosis, the principal cause of cardiovascular diseases (CVDs), is a process that involves a complex interplay among different factors and cell types, including cells of the immune system (T cells, B cells, natural killer cells, monocytes/macrophages, dendritic cells) and cells of the vessel wall (endothelial cells [ECs], vascular smooth muscle cells [VSMCs]). The atherogenic process evolves in different stages, starting from inflammatory endothelial activation/dysfunction and resulting in plaque vulnerability and rupture.¹

Several cardiovascular risk factors have been recognized. Among them, vitamin D deficiency [25(OH)D <20 ng/mL] is emerging as a new one. In addition to its well-defined role in bone and calcium metabolism, vitamin D has been identified as an important factor in cardiovascular health.²⁻⁸

Vitamin D deficiency affects almost 50% of the population worldwide. It has been suggested that this pandemic might contribute to the worldwide increased prevalence of CVD.⁹⁻¹¹

Several mechanisms have been proposed to account for this inverse relationship. In addition to its effects exerted on numerous tissues and organs that indirectly participate in the atherosclerosis, vitamin D is directly involved in this systemic inflammatory process.^{12,13} Vitamin D receptors (VDRs) are present in all cells implicated in atherosclerosis, including ECs, VSMCs, and immune cells. Vitamin D appears to regulate a wide range of physiological and pathological processes like vascular cell growth, migration, and differentiation; immune response modulation; cytokine expression; and inflammatory and fibrotic pathways, all of which play a crucial role, starting from the early stage of endothelial activation/dysfunction to the later stages of the plaque vulnerability and rupture.

In this review, we provide current data on the effects of vitamin D on cells directly implicated in atherosclerosis such as ECs, VSMCs, and immune cells (lymphocytes, monocytes, macrophages, etc) with a focus on the underlying molecular mechanisms, which are still largely unknown. We also summarize reports related to the favorable (anti-atherogenic) actions of vitamin D in tissues and organs that indirectly participate in the atherogenic process. Finally, we critically discuss clinical studies to assess the protective role of vitamin D and the efficacy of vitamin D and VDR agonists in CVD. Because a comprehensive background is a prerequisite for further discussions of vitamin D-induced effects,

we provide a brief description of vitamin D metabolism and mechanism of action.

Vitamin D Metabolism and Mechanism of Action

Vitamin D is a steroid hormone that comes in 2 forms that differ chemically in their side chain, D₂ and D₃ (Figure 1). Either produced in the skin (D₃) from 7-dehydrocholesterol by exposure to ultraviolet-B light or ingested with foods of plant or animal origin (D₂ and D₃, respectively), vitamin D is biologically inert and requires 2 hydroxylations to form its active metabolite.¹⁰ The first hydroxylation is constitutive and takes place in the liver by vitamin D-25-hydroxylase to form 25(OH)D. The second hydroxylation is catalyzed by 25(OH)D-1αOHase (CYP27B1) to form the biologically active form of vitamin D, 1,25(OH)₂D (calcitriol; see Figure 1). This latter 1α-hydroxylation of 25(OH)D takes place in most tissues and cells of the body; however, serum levels of 1,25(OH)₂D are determined mainly by renal 1α-hydroxylase activity. This activity is regulated by serum calcium, phosphate, parathormone, and fibroblast growth factor 23.

It is important to know that circulating 25(OH)D levels are a main determinant of extrarenal tissue levels of 1,25(OH)₂D and thus are the best indicator of whole-body vitamin D status. Actually, 25(OH)D is used for the classification of the vitamin D status as deficient or sufficient.¹¹

The biological responses to the 1,25(OH)₂D₃ hormone and its analogs are mediated by the VDR, which is a DNA-binding transcription factor. However, VDR is also localized to the plasma membrane caveolae and may result in activation of signal transduction pathways that generate rapid nongenomic responses.¹⁴

Interestingly, genome-wide associations studies revealed a genetic influence on 25(OH)D levels by 25-hydroxylase, 24-hydroxylase, and 7-dehydrocholesterol reductase, the enzyme that catalyzes the last step in cholesterol biosynthesis.¹⁵ Moreover, the genes *CYP2R1*, *CYP24A1*, and *DCHR7* encoding the aforementioned enzymes, respectively, were also significant at the methylation level according to a genome-wide methylation study.¹⁶

Details on vitamin D metabolism and mechanism of action are given in the online-only Data Supplement.

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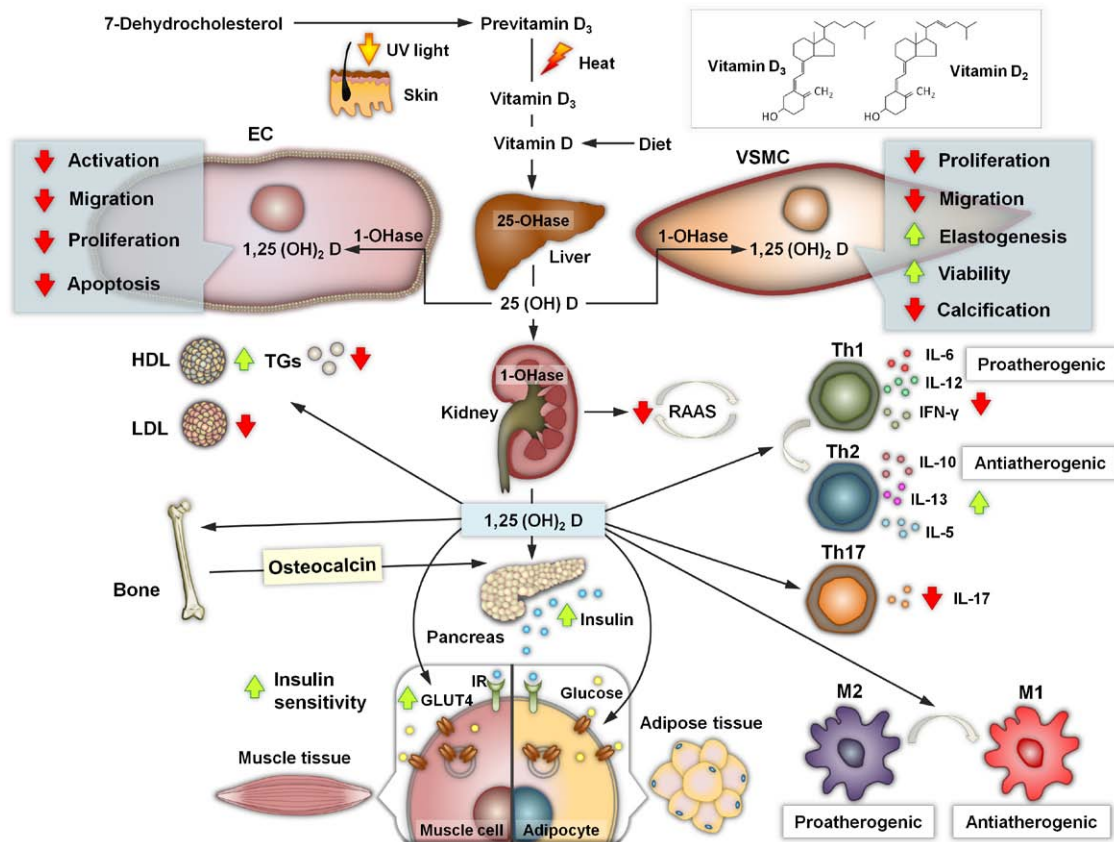


Figure 1. Schematic representation illustrating the metabolism and actions of vitamin D in cells and tissues that are implicated directly and indirectly in the atherogenic process. EC indicates endothelial cell; Glut-4, glucose transporter 4; HDL, high-density lipoprotein; IL, interleukin; IR, insulin receptor; LDL, low-density lipoprotein; M1, macrophage/monocyte 1; M2, macrophage/monocyte 2; RAAS, renin-angiotensin-aldosterone system; TGs, triglycerides; Th, T helper; and VSMC, vascular smooth muscle cell.

Direct Effects of Vitamin D on the Atherogenic Process

Effects of Vitamin D on ECs

It is well known that endothelium is the key vessel wall component in the initiation of the atherogenic process. Its possible role in the later stages has been strongly suggested.^{17,18}

Studies revealed that ECs express VDRs and have the ability to synthesize calcitriol [$1\alpha,25(\text{OH})_2\text{D}_3$] because they express 1 α -hydroxylase.¹⁹ The coexistence of these 2 crucial elements of vitamin D metabolism strengthened the hypothesis of an autocrine/intracrine mechanism of vitamin D action as a modulator of endothelial functions.²⁰

Vitamin D exerts protective effects on endothelial activation/dysfunction, an inflammatory process that precedes atherosclerosis, through several mechanisms both genomic and nongenomic (Figure 2). Among the main alterations ascribable to endothelial dysfunction are the reduced availability of nitric oxide (NO) and increased production of reactive oxygen species.¹⁸

Vitamin D found to stimulate NO production in human umbilical vein ECs cultures through endothelial NO synthase activation. This effect was dose dependent, VDR mediated, and accompanied by a significant increase in the level of phosphorylation of intracellular kinases such as p38, protein kinase B (AKT), and extracellular signal-regulated kinases.^{21,22}

The multimeric enzyme complex NADPH oxidase is the major enzyme system that generates superoxide (the main reactive oxygen species) in the vasculature. In vivo and in vitro experiments have recently demonstrated that a vitamin D analog (22-oxacalcitriol) significantly suppressed the elevated expression of p22(phox) and NADPH oxidase subunit and improved endothelial NO synthase coupling, thus reducing oxidative stress in endothelium.²³

Moreover, vitamin D protected ECs against H_2O_2 oxidative stress, counteracting superoxide anion generation and apoptosis and blocking the extrinsic caspase cascade by positively controlling the level of phospho-active extracellular signal-regulated kinases. Exploring in vitro the $1\alpha,25(\text{OH})_2\text{D}$ downstream effector, Polidoro et al²⁴ found that $1\alpha,25(\text{OH})_2\text{D}$ upregulated SirT-1 expression in human umbilical vein ECs and reverted the SirT-1 downregulation induced by H_2O_2 .

The above currently identified antioxidative stress mechanisms of vitamin D are in line with the observed reduction of the basal reactive oxygen species level in the ECs of spontaneously hypertensive rat treated long term with $1\alpha,25(\text{OH})_2\text{D}_3$.²⁵

Vitamin D appears also to be implicated in the modulation of the vascular tone via regulation of the release of vasoconstrictor metabolites of arachidonic acid called endothelium-derived contracting factors.²⁶ The release of endothelium-derived contracting factors occurs via a calcium-dependent process in which the calcium influx

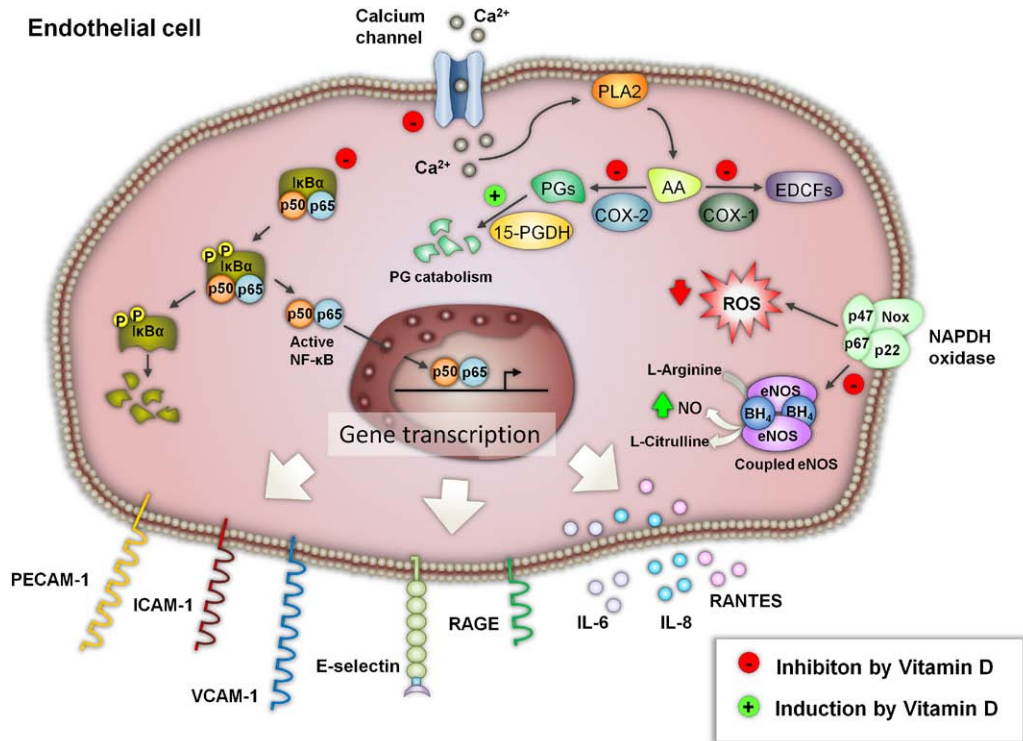


Figure 2. Vitamin D exerts protective effects on endothelial activation/dysfunction. Vitamin D suppresses the NADPH oxidase subunit p22(phox) and improves the endothelial nitric oxide (NO) synthase (eNOS) coupling, thus increasing NO production while decreasing reactive oxygen species (ROS) production. $1\alpha,25(\text{OH})_2\text{D}$ inhibits the expression of interleukin (IL)-6, IL-8, RANTES (regulated on activation, normal T cell expressed, and secreted), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), receptor of advanced glycation end products (RAGE), and E-selectin through a nuclear factor- κB (NF- κB)–mediated mechanism. Vitamin D represses the expression of cyclooxygenase (COX)-2 and upregulates the expression of 15-hydroxyprostaglandin dehydrogenase (15PGDH), the enzyme initiating prostaglandin (PG) catabolism, thereby reducing the levels of prostaglandins and suppressing the production of several proinflammatory cytokines. It can also acutely modulate vascular tone by reducing calcium influx into the endothelial cells (ECs) and hence decreasing the production of endothelium-derived contracting factors (EDCFs). However, a recent study also recognized a calcium-independent mechanism through a direct effect of vitamin D downregulating the expression of COX-1, which is the major source of endothelium-derived contracting factors, in ECs. AA indicates arachidonic acid.

activates phospholipase A2, which in turn converts the membrane phospholipids to arachidonic acid. Of note, vitamin D acutely modulated vascular tone by reducing calcium influx into the ECs and hence decreasing the production of endothelium-derived contracting factors.²⁷ However, a recent study also recognized calcium-independent mechanism through a direct effect of vitamin D downregulating the expression of cyclooxygenase-1, the major source of endothelium-derived contracting factors, in ECs.²⁸

In addition to their effects on vascular tone, prostaglandin metabolism and signaling appear to play important role in the inflammatory component of atherosclerosis. It is well known that cyclooxygenase catalyzes the conversion of arachidonic acid to prostaglandin endoperoxides, which in turn are converted enzymatically into prostaglandins and thromboxane A2, both of which play pathological roles in vascular function as proinflammatory molecules. Cyclooxygenase-2, although absent from most normal tissues, is expressed in atherosclerotic lesions by ECs and VSMCs, probably in response to proliferative and inflammatory stimuli.²⁹

It is noteworthy that calcitriol significantly repressed the mRNA and protein expression of cyclooxygenase-2 but upregulated the expression of 15-hydroxyprostaglandin dehydrogenase, the enzyme initiating prostaglandin catabolism,

thereby reducing the level of prostaglandins and suppressing the production of several proinflammatory cytokines. This could successfully prevent the immune-mediated changes in the ECs.³⁰

Equils et al³¹ also demonstrated an effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on microbial antigen-induced EC activation. According to their data, lipopolysaccharide stimulation led to the release of proinflammatory cytokines, which induced $1\alpha\text{-OHase}$ activity and consequently calcitriol expression. Calcitriol in turn inhibited interleukin (IL)-6, IL-8, and RANTES (regulated on activation, normal T cell expressed, and secreted) expression through inhibition of nuclear factor- κB (NF- κB) in an auto-crine/paracrine fashion to “switch off” the immune activation.

The subendothelial migration of the monocytes and subsequent transformation into foam cells depend on the interaction between adhesion molecules such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin expressed by ECs, and integrins (eg, LFA-1, VLA-4) expressed by leucocytes.³² Recent studies showed that the tumor necrosis factor- α (TNF- α)–induced increase in protein levels of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 was significantly abolished after incubation of human ECs with $1\alpha,25(\text{OH})_2\text{D}_3$, an effect independent of NF- κB activation.^{33,34}

Talmor et al³⁵ demonstrated inhibitory effects of calcitriol on the expression of intercellular adhesion molecule-1, platelet EC adhesion molecule-1, and IL-6 in ECs, effects mediated through NF- κ B and p38 mitogen-activated protein kinase. Moreover, recent studies have revealed that pretreatment with 1 α ,25(OH)₂D₃ significantly inhibited the TNF- α -induced expression of E-selectin, vascular cell adhesion molecule-1, and IL-8 on human coronary arterial ECs via inhibition of NF- κ B.^{36,37}

Finally, calcitriol may also act as a vascular protective agent counteracting the deleterious effects of advanced glycation end products on endothelial function.³⁸ Increased concentrations of glucose and advanced glycation end products acting through its receptor of advanced glycation end products can induce the expression of proinflammatory molecules by ECs. Calcitriol acting via VDR has been found to decrease the expression of genes involved in advanced glycation end products-activated inflammatory pathway such as *RAGE*, *IL-6*, and *IL-8* through an NF- κ B-mediated mechanism.³⁹

Interestingly, by acting on ECs, vitamin D may also affect the later stages of atherosclerosis. It is believed that angiogenesis is mainly responsible for the rupture of an unstable atherosclerotic plaque. Vitamin D inhibited angiogenesis, which is greatly influenced by the proliferation and migration of ECs.⁴⁰ It is of interest that Mantell et al⁴⁰ showed that vitamin D inhibits vascular endothelial growth factor-induced EC sprouting and the formation of EC networks within 3-dimensional collagen gels. They further showed that 1 α ,25(OH)₂D₃ promoted cellular regression as a result of apoptosis, specifically within the sprouting cell population.

Moreover, in vitro experiments in human ECs showed that 1 α ,25(OH)₂D₃ attenuates the expression of membrane type 1 matrix metalloproteinase and platelet activation through a reduction of the CD62p platelet adhesion molecule, further highlighting its possible therapeutic effects against destabilization of atherosclerotic plaques and thrombosis.³⁴

Confirming the in vitro data, several clinical studies showed both an inverse relationship between vitamin D levels and endothelial dysfunction and favorable effects of the vitamin D replacement on endothelial function and indicated the important role of NF- κ B in mediating most of the beneficial action of vitamin D on endothelial dysfunction.⁴¹⁻⁴³ Indeed, Jablonski et al⁴² found that vascular EC expression of NF- κ B was greater in 25(OH)D-deficient than -sufficient subjects, whereas inhibition of NF- κ B improved flow-mediated dilation to a greater extent in subjects with lower versus higher 25(OH)D.

Effects of Vitamin D on VSMCs

Studies have shown that the antiatherogenic effects of vitamin D extend beyond the endothelium to the VSMCs. VSMCs, which constitute the most common cell type in the medial layer of the arterial wall, play a pivotal role in the pathogenesis of atherosclerosis through proliferation and migration from the media to the intima layer, through morphological changes, or via secreting inflammatory molecules.⁴⁴

The presence of VDRs in the VSMCs, which also express an enzymatically active 1 α -hydroxylase, highlights the

biological importance of 1 α ,25(OH)₂D in the regulation of VSMC homeostasis and function^{45,46} (Figure 1).

Antiproliferative effects of 1 α ,25(OH)₂D₃ on VSMCs have been demonstrated by several studies, but little is known about the molecular mechanisms involved.⁴⁷⁻⁴⁹ It has been shown that 1 α ,25(OH)₂D₃ inhibits the proliferative effects of both epidermal growth factor and endothelin on VSMCs, the latter via decreasing cyclin-dependent kinase 2 activity, that actually regulate the cell cycle machinery.^{47,50} Additionally, calcitriol inhibited proliferation by an acute influx of Ca²⁺ into the VSMCs⁵¹ (Figure 1).

The effects of 1 α ,25(OH)₂D₃ on VSMC migration appear to be divergent. At high doses, calcitriol can induce VSMC migration, an effect that involved both nongenomic (through activation of PI3K pathway) and genomic (through reducing the expression of β_1 -integrin receptors on VSMC surface) actions.^{52,53} However, in physiological doses, 25(OH)D and calcitriol inhibited VSMC migration and proliferation by reducing the activity of vitamin D-binding protein, an effect mediated through the attenuation of extracellular signal-regulated kinase 1/2 phosphorylation.⁵⁴

Other aspects of VSMC biology besides proliferation and migration are affected by vitamin D. Vitamin D appears to exert morphological effects, including increased elastogenesis and stabilization of musculoelastic multilayer of VSMCs, via regulation of the production of proteins that are related to the vascular wall, including myosin, collagen type 1, matrix metalloproteinase-9, and elastin.⁵⁵ Furthermore, 1 α ,25(OH)₂D has been shown to enhance prostacyclin production, a prostanoid that inhibits the aggravation of atherosclerosis, in VSMCs via the cyclooxygenase pathway.⁵⁶

1 α ,25(OH)₂D₃ increased the superoxide dismutase activity in VSMCs via stimulation of the expression of I κ B- α , thus reducing vascular cell-mediated oxidation of low-density lipoprotein.⁵⁷ Moreover, it improved the recovery of stressed VSMCs and their viability via regulating heat-shock protein 70 and possibly via increasing telomerase activity. It has recently been shown that vitamin D₃ supplementation improved telomerase maintenance and prevented peripheral blood mononuclear cells senescence.⁵⁸

In accordance with the aforementioned in vitro data, a 16-week clinical trial showed that 2000 IU daily vitamin D₃ supplement counterbalanced the progression of arterial stiffness, as estimated by the carotid-femoral pulsed-wave velocity.⁵⁹

Vitamin D also regulates the expression of profibrotic and antifibrotic factors.⁶⁰ Plasminogen activator inhibitor-1 is produced by a broad range of cells, among them vascular ECs and VSMCs. It is considered an inflammatory response gene that is associated with increased risk for thrombosis and atherosclerosis.^{61,62} Activated vitamin D analogs (calcitriol and paricalcitol) have been reported to suppress plasminogen activator inhibitor-1 in human coronary artery smooth muscle cells.⁴⁸ Of interest, Chen et al⁶³ found that 1 α ,25(OH)₂D₃ suppressed the upregulation of plasminogen activator inhibitor-1 induced by TNF- α and lipopolysaccharide by blocking NF- κ B activation.

Additionally, vitamin D has been found to regulate the expression of antifibrotic factors. It was demonstrated that exposing mesenchymal multipotent cells to 1 α ,25(OH)₂D

increased through VDR-mediated genomic effects the expression of bone morphogenetic protein-2 and BMP7 (transforming growth factor- β 1 antagonists), matrix metalloproteinase-8 (a collagen breakdown inducer), and follistatin (an inhibitor of the profibrotic factor myostatin) and decreased the expression of collagen I and III, which define a fibrotic process.⁶⁰

Vascular calcification is nearly a universal feature of atherosclerosis; hence, it can be considered synonymous in this perspective. In particular, intima calcification is gaining interest in the context of its association with atherosclerosis and its prognostic significance of coronary artery disease.⁶⁴ Studies have shown that osteogenic transformation of VSMCs enables them to secrete an osteoid-like extracellular matrix that calcifies over a defined time course.⁶⁵ Various proteins involved in osteogenesis have been detected in VSMCs and atherosclerotic lesions such as osteopontin, bone morphogenetic protein-2 and matrix Gla protein, fetuin-A, osteoprotegerin, and receptor activator of NF- κ B ligand, playing either a stimulatory or an inhibitory role in the calcification process.⁶⁶ Data on the role of vitamin D in the vascular calcification are conflicting. In vitro experiments support that VSMCs undergo calcification when treated with 1 α ,25(OH)₂D through several mechanisms. Among them, a VDR-mediated stimulation of calcium influx in VSMCs, a VDR-dependent suppression of parathormone-related peptide and parathormone receptor signaling, and downregulation of fetuin-A expression have been proposed.^{67–69} Of note, a recent study by Shalhoub et al⁷⁰ identified an altered gene expression pattern that was representative of multiple pathways implicated in the calcitriol-induced vascular calcification such as osteochondrogenesis, mineralization, and apoptosis. However, it should be noted that the inductive effects of 1 α ,25(OH)₂D on vascular calcification are exerted under specific culture conditions (ie, in the presence of high phosphate medium).

On the other hand, protective effects of calcitriol and other VDR agonist on vascular calcification have also been recognized in vitro. Among the proposed mechanisms is upregulation of the expression of matrix Gla protein and osteopontin by VSMCs, which are both potent inhibitors of vascular calcification.⁹ Interestingly, Zitman-Gal et al,⁷¹ using microarray technology to detect gene expression profiles in VSMCs exposed to diabetic-like conditions, found that the addition of physiological concentration of calcitriol caused a decrease in the receptor activator of NF- κ B ligand/osteoprotegerin mRNA ratio and in receptor activator of NF- κ B expression, as well as in NF- κ B p50/p65 protein expression, an outcome that might attenuate the calcification process.

Moreover, a recent study showed that VDR agonists (both calcitriol and maxacalcitol) suppressed the TNF- α -induced expression of the *runx2*, *osteocalcin*, and *MMP-2* genes in VSMCs.⁷²

Clinical studies have shown either a positive or an inverse relationship between vitamin D levels and vascular calcification. Studies conducted in subjects with chronic kidney disease (CKD) reported an inverse relationship between serum 1 α ,25(OH)₂D₃ levels and total (intimal and medial) coronary artery calcification.^{73–75} On the other hand, studies conducted in patients with CKD found a significant positive correlation between the extent of vascular calcification and 1 α ,25(OH)₂D₃

concentration.^{76–78} However, in CKD, particular factors may contribute to vascular calcification, among them elevated levels of phosphorus, calcium, parathormone, and other potential uremic toxins that may influence the transformation of VSMCs into osteoblast-like cells. Of note, elevated phosphate was found to directly induce the expression of Runx2 and Osterix in VSMCs.⁷⁹

These contradictory data probably reflect the complex, vascular-renal-endocrine-bone axis implicated in this process. Interestingly, Shroff et al⁸⁰ reported that in CKD patients both low (<20 pg/mL) and high (>60 pg/mL) levels of 1 α ,25(OH)₂D are related to increased carotid intima-media thickness and vascular calcification, possibly through a dual effect on parameters implicated in the calcification process such as calcium/phosphorus metabolism and inflammation. It is important to note that the protective or harmful effect of active vitamin D on vascular calcification remains a contentious issue, presumably because of the differences in the experimental model or the dose or type of active vitamin D used. In terms of dose, there is probably a fine balance between the protective and vascular calcifying effects of vitamin D; the latter may itself occur with either high or low doses of vitamin D analog. In terms of the type of active vitamin D used, clinical observations demonstrated that vitamin D analogs such as paricalcitol provide benefit for patients with CKD not only because of their less calcemic and phosphatemic effects but also by exerting direct protective effects on vascular calcification.⁸¹

Recently, observations clearly suggest the emerging roles of the fibroblast growth factor 23–Klotho axis in atherosclerosis. Current data on the role of vitamin D regulation of the fibroblast growth factor–Klotho axis in the pathogenesis of vascular calcification in CKD and in non-CKD subjects are provided in the online-only Data Supplement.

Effects of Vitamin D on Immune Cells

Both innate and adaptive immune responses are involved in the atherogenic process. Several subsets of T cells such as naive, T helper (Th) 1, Th2, T regulatory cells, Th17, and B cells, along with leucocytes and transformed monocytes, are present within atherosclerotic lesions.¹¹ Interestingly, the Th1 subtype primarily drives the inflammatory response in atherogenesis process by producing cytokines such as interferon- γ , TNF- α , IL-6, and IL-12, which are known to be proatherogenic.^{82,83}

On the other hand, Th2, by secreting antiatherogenic cytokines such as IL-5, IL-10, and IL-13, neutralizes the Th1 effect.⁸⁴ It has been demonstrated that 1,25(OH)₂D shifts the immune response away from a Th1 and toward a Th2 profile, thus promoting an antiatherogenic immune profile⁸⁵ (Figure 1).

Although the exact role of IL-17 and its producing Th17 cells has not been fully addressed, recent studies have demonstrated the existence of IL-17A⁺ cells within atherosclerotic lesions, suggesting a potential role in atherosclerosis.⁸⁶ Actually, the IL-17A-dependent response was found to occur in parallel with the Th1-dominant and unfavorable immune response during the atherogenic process. Treatment of T cells in vitro with 1 α ,25(OH)₂D₃ suppressed the development of Th17 and inhibited IL-17 production via a posttranscriptional mechanism⁸⁷ (Figure 1).

It has been shown that vitamin D is carried by low-density lipoprotein particles and is internalized by various cells, including monocytes, through low-density lipoprotein receptor either in its active form or as 25(OH)D. Once monocytes migrate transendothelially and are transformed into foam cells, 25(OH)D is accumulated in subendothelial space or in the atherosclerotic plaque and may be converted to its active form by 1 α -hydroxylase, which expressed by monocyte-derived cells.^{88,89} In turn, 1,25(OH)₂D may alter macrophage gene expression, influencing the expression of a variety of factors that are implicated in the atherosclerosis process (eg, type I collagen, vascular endothelial growth factor, matrix metalloproteinases, elastin) by ECs and VSMCs.⁵²

Riek et al⁹⁰ found that 1 α ,25(OH)₂D₃ affects macrophages by lowering acetylated and oxidized low-density lipoprotein uptake, thus reducing foam-cell formation. Interestingly, 1 α ,25(OH)₂D supplementation suppressed cholesteryl ester formation and enhanced cholesterol efflux in macrophages compared with vitamin D–depleted cells, suggesting facilitation of cholesterol egress in the presence of 1 α ,25(OH)₂D.⁹⁰ Moreover, 1 α ,25(OH)₂D₃ acting as a natural endoplasmic reticulum stress reliever was found to induce an M1 antiatherogenic macrophage/monocyte phenotype over M2 (with increased cholesterol uptake and deposition) and to decrease mRNA expression of the monocyte adhesion molecules PSGL-1, β (1)-integrin, and β (2)-integrin, resulting in an increase of monocyte adhesion to activated endothelium.⁹¹

Moreover, 1 α ,25(OH)₂D₃ dose-dependently inhibited the production of IL-6 and TNF- α by monocytes, an effect exerted at a posttranscriptional level, and possibly contributed to the aforementioned suppression of Th1 proatherogenic immune response.⁹² In addition to their immunoregulatory effects, monocytes are implicated in the development of atherothrombotic lesions by regulating coagulant and anticoagulant factors. It has been demonstrated that vitamin D, via VDR, upregulates the expression of the anticoagulant glycoprotein thrombomodulin and downregulates the expression of a critical coagulation factor, tissue factor, in human peripheral monocytes.⁹³ The physiological role of vitamin D–VDR in the maintenance of antithrombotic homeostasis has been confirmed with VDR knockout mice.⁹⁴

Supporting the in vitro and animal studies, ex vivo experiments showed that 1 α ,25(OH)₂D₃ modulates inflammation in monocytes isolated from diabetic patients through downregulating the expression of TNF- α , IL-6, IL-1, and IL-8.⁹⁵ Additionally, in 2 cohort studies, 25(OH)D levels were inversely associated with C-reactive protein and IL-6, whereas supplementation with vitamin D was found to suppress serum TNF- α , IL-6, and C-reactive protein levels and to increase IL-10 levels.^{96–98}

In summary, the beneficial action of vitamin D on endothelial dysfunction, VSMC proliferation and migration, and calcification, as well as on the inflammatory/immune process of atherosclerosis, is well documented in the literature, even though the signal mechanisms by which these responses are elicited need to be fully characterized. NF- κ B appears to play a crucial role in mediating many of the benefit effects of vitamin D. However, other signaling pathways, either genomic or nongenomic, appear to be implicated (Figure 2).

Indirect Effects of Vitamin D on the Atherogenic Process

In addition to the direct effects on cells implicated in the atherogenic process, vitamin D exerts protective effects against systemic conditions that promote atherosclerosis such as insulin resistance, β -cell dysfunction, dyslipidemia, the renin-angiotensin-aldosterone system (RAAS), and consequent hypertension (Figure 1).

Insulin Secretion/Insulin Resistance

Vitamin D deficiency has been reported to exert negative effects on β -cell function and insulin sensitivity, whereas it may be causally related to the development of diabetes mellitus type 2.^{99–101}

Although the exact mechanism for such a pathogenic role has not yet been fully established, it was found that the promoter region of the *insulin* gene contains vitamin D–responsive elements, postulating a direct regulation of its transcription by 1 α ,25(OH)₂D₃.¹⁰² Additionally, insulin secretion is a calcium-dependent process, and vitamin D is known to regulate the calcium flux and intracellular cytosolic calcium [Ca]²⁺_i pool in the pancreatic β cell. Intriguingly, pancreatic β cell expresses 1 α -hydroxylase and 24-hydroxylase, indicating a possible autocrine link between vitamin D status and pancreatic function.¹⁰⁰

Current data demonstrated a significant downregulation of several genes related to islet growth and function such as *FOXO1*, *TCF7L2*, and *IRS-1* in vitamin D–deficient mouse islets.¹⁰² Furthermore, hypovitaminosis D decreased the islet renin-angiotensin system activity, which in turn induces β -cell dysfunction in mice.¹⁰³

Along with β -cell function, experimental data suggest effects of vitamin D against insulin resistance, which per se is associated with endothelial dysfunction, and increased carotid intima-media thickening. VDR is expressed in adipose tissue and skeletal muscle, tissues that play a central role in peripheral insulin sensitivity. Adipocytes also express the *CYP27B1* (1 α -hydroxylase) gene.¹⁰⁴ Vitamin D directly activated the transcription factor peroxisome proliferator-activated receptor- γ , which is implicated in the regulation of fatty acid metabolism in muscle and adipose tissue.¹⁰⁵ Moreover, vitamin D contributed to normalization of extracellular calcium, ensuring normal calcium influx through cell membranes and adequate [Ca]²⁺_i pool to promote proper insulin-mediated intracellular processes such as phosphorylation of insulin receptor and translocation of glucose transporter-4¹⁰⁶ (Figure 1).

Finally, *osteocalcin*, which is another gene classically induced by 1 α ,25(OH)₂D₃ in osteoblasts, has been identified as a bone-secreted hormone that both improves insulin release from β cells and increases insulin metabolic responsiveness¹⁰⁷ (Figure 1).

Lipid Profile

An association between vitamin D deficiency and atherogenic dyslipidemia has also been suggested. In a cross-sectional study that included 107 811 patients, vitamin D was associated with a significant increase in total cholesterol and high-density lipoprotein cholesterol.¹⁰⁸ Recently, using the filaggrin

genotype as an instrumental variable to estimate the causal effect of vitamin D on serum lipids, Skaaby et al¹⁰⁹ showed a 23.8% higher high-density lipoprotein cholesterol level and a 30.5% lower serum level of triglycerides per doubling of vitamin D.

The mechanisms by which vitamin D may affect the lipid metabolism are largely unknown. In vitro studies showed that incubation with calcitriol increases lipoprotein lipase expression and activity in cultured adipocytes.¹¹⁰

Recently, serum 25(OH)D was found to be positively associated with lipoprotein lipase concentration, a finding that could explain the inverse association between serum 25(OH)D and triglycerides.¹¹¹

Moreover, a 25(OH)D receptor binding site modifying the *APOA5* promoter polymorphism was found to be associated with lower high-density lipoprotein in 25(OH)D-deficient individuals.¹¹²

The RAAS

The RAAS is the major regulator of blood pressure. Its components, angiotensin II and aldosterone, appear to be directly involved in the pathogenesis of atherosclerosis (see the online-only Data Supplement).¹¹³

A growing body of evidence supports that vitamin D is a potent endocrine suppressor of the RAAS.¹¹⁴ In vitro studies using a juxtaglomerular cell model showed that vitamin D suppresses both *renin* gene expression via a vitamin D-responsive element in the promoter of the *renin* gene and the expression of the *angiotensinogen* gene by blocking the NF- κ B pathway.^{115,116} Interestingly, VDR knockout mice have elevated circulating levels of renin and angiotensin II and develop hypertension.

Corroborating the in vitro data, clinical studies revealed that vitamin D is inversely associated with plasma renin activity, whereas both 25(OH)D and 1 α ,25(OH)₂D emerged as independent predictors of plasma renin and angiotensin II in a large cohort of patients referred for coronary angiography.^{117,118}

In summary, it could be argued that vitamin D, acting either directly or indirectly, has a variety of favorable effects on the function and pathology of cells and tissues involved in the atherogenic process, therefore suggesting a potential role in the management of disorders associated with dysfunctional vascular wall such as atherosclerosis (Figure 1).

Vitamin D Deficiency and CVD Risk: Epidemiological and Clinical Studies

Ecological studies have indicated a higher incidence of CVD disease with increasing distance from the equator, suggesting an association with vitamin D insufficiency in regions with less sun exposure.¹¹⁹

In addition, the majority of observational studies suggested an inverse association between 25(OH)D levels and clinical CVD events (Table).^{4-6,96,120-124} Notably, Martins et al,³ analyzing data from >13 000 adults in the Third National Health and Nutrition Examination Survey (NHANES III), demonstrated a strong association between hypovitaminosis D and key CVD risk factors (diabetes mellitus, blood pressure, overweight, hypertriglyceridemia) that was independent of multiple variables. Furthermore, concentrations of 25(OH)D

were inversely associated with CVD mortality among adults with hypertension in the United States.¹²³ The weight of the evidence in support of a strong inverse relationship between vitamin D levels and CVD events has become stronger with the Framingham Offspring Study and the Health Professionals Follow-up Study, showing an approximately doubled risk for cardiovascular events in vitamin D-deficient subjects.^{4,5}

Along those lines, the Intermountain Heart Collaborative (IHC) Study Group analysis of a retrospectively collected database of 27 686 patients reported that vitamin D levels were highly associated with coronary artery disease and myocardial infarction (Table).⁶

In a prospective cohort study, cardiovascular mortality was higher for patients in the lower two 25(OH)D quartiles (median, 7.6 and 13.3 ng/mL) compared with patients in the highest 25(OH)D quartile (median, 28.4 ng/mL).⁹⁶ In accordance, Semba et al¹²² reported a strong increased risk (2.64-fold) in the lowest quartile (<10.5 ng/mL). Moreover, a recent case cohort study by Karakas et al¹²⁴ showed that the hazard ratios comparing the tertile extremes of serum levels of 25(OH)D were 0.42 (95% confidence interval, 0.19–0.93) for women and 0.84 (95% confidence interval, 0.52–1.35) for men after adjustment for potential confounders (Table).

When this evidence was analyzed in recent meta-analyses of observational studies, an inverse association between 25(OH)D and cardiovascular risk emerged, although the strengths of associations in longitudinal studies were attenuated compared with cross-sectional studies.^{127,128} However, it should be noted that in the Framingham Offspring Study the association appeared to be nonlinear (U shaped) with the suggestion of a slightly increased risk in CVD events at 25(OH)D levels >30 ng/mL.⁴ A similar U-shaped relationship for 25(OH)D, with increased total mortality not only at low (<20 ng/mL) but also at high (>50 ng/mL) levels, has also been suggested in an NHANES III analysis.¹²⁰

Notably, although several studies have evaluated the prognostic value of 25(OH)D for CVD incidence and mortality, population-based studies specifically looking at 25(OH)D and atherosclerosis are scarce. Lower 25(OH)D concentration was associated with an increased risk for incident coronary artery calcification, a measure of coronary atherosclerosis, in the Multi-Ethnic Study of Atherosclerosis (Table).¹²⁵ In addition, Lim et al¹²⁶ recently demonstrated that serum 25(OH)D levels were negatively associated with significant coronary artery stenosis evaluated with a multidetector-row cardiac computed tomography scanner. Interestingly, the odds ratios were 2.08 for a 25(OH)D concentration of 15 to 29.9 ng/mL versus at least 30 ng/mL and 3.12 for 25(OH)D concentration <15 ng/mL versus at least 30 ng/mL (both $P < 0.05$; the Table).

Although observational studies suggest that vitamin D deficiency is related to a higher risk for CVD, numerous interventional studies that have recently been conducted showed conflicting results. However, it should be noted that randomized, controlled studies with CVD and, more specifically, atherosclerosis as the primary prespecified outcome are lacking.

The recently published Randomised Evaluation of Calcium or Vitamin D (RECORD) trial with a secondary outcome of CVD events showed no effects of vitamin D₃

Table. Summary of the Most Important Population-Based Studies Evaluating Atherosclerosis and CVD Risk in Patients With Vitamin D Insufficiency/Deficiency

Author	Study Design	Study Population			Setting	Follow-Up Length, y	25(OH)D Measurement	Type of Outcome
		Total (Cases), n	Mean Age, y	Male, %				
Giovannucci et al ¹⁵	NCCS (prospective)	1354 (454)	63.8	100	Health care professionals, initially healthy	10	RIA	Fatal CHD and non-fatal MI
Wang et al ⁴	CS (prospective)	1739 (120)	59	45	Population-based, initially healthy	7.6 (max) 5.4 (mean)	RIA (DiaSorin)	Cardiovascular events (MI, stroke, angina, transient ischemic attack, peripheral claudication)
Bolland et al ¹²¹	CS	1471 (52)	74	0	Healthy postmenopausal women who participated in an RCT for calcium supplementation	5	RIA (DiaSorin)	MI
Dobnig et al ⁹⁶	CS (prospective)	3217 (463)	62	70	Symptomatic patients scheduled for coronary angiography at a single tertiary center	7.7 (median)	RIA* (DiaSorin)	Fatal cardiovascular events
Melamed et al ¹²⁰	CS (prospective)	13 331 (777)	44.8	45	Population-based	8.7 (median)	RIAI (DiaSorin)	Fatal cardiovascular events
Anderson et al ⁶	CS (retrospective)#	27 686 (763)	66.6 (≥50)	25	General healthcare population**	1.3 (mean) 6.6 (max)	RIA (DiaSorin)	Coronary artery disease/MI
Zhao et al ¹²³	Cohort study (prospective)	2609 (68)	57.5	49	Hypertensive adults selected from a nationally representative survey sample	3.7 (mean)	RIA (DiaSorin)	Fatal cardiovascular events
Semba et al ¹²²	CS (prospective)	1006 (107)	74 (median) ≥65	75	Population-based	6.5	RIA (DiaSorin)	Fatal cardiovascular events
De Boer et al ¹²⁵	CS (prospective)	647 (135)	45-84 (range)	37	Population-based	3 (median)	RIA (DiaSorin)	Coronary artery calcification‡‡
Lim et al ¹²⁶	CS	921 (156)	76	44	Population-based	...	UPLC-MS/MS	Coronary artery stenosis >50%‡‡‡

Confounders Adjusted in Analysis	Relative Risk (95% CI) According to 25(OH)D Levels (Range or Median), ng/mL	Limitations
Adjustment for age, month, and year of blood sampling, smoking status, family history of MI before 60 y of age, history of DM, history of hypertension, alcohol intake, BMI, physical activity, region, race, multivitamin use, marine omega-3 intake, and fasting status. HDL-C-, LDL-C, and triglyceride levels	<p>≥30 1.00</p> <p>22.6–29.9 1.60 (1.1–2.32)</p> <p>15.1–22.5 1.43 (0.96–2.13)</p> <p>≤15 2.09 (1.24–3.54)</p>	The number of fatal CHD cases was too small to make definitive conclusions on the association between 25(OH)D levels and fatal CHD
Adjustment for age, sex, systolic BP, antihypertensive treatment, DM, serum creatinine, total-to-HDL-C ratio, cigarette smoking, BMI, and CRP	<p>≥15 1.00</p> <p><15 1.59 (1.03–2.45)</p> <p><10 1.81 (1.03–3.18)</p>	Lack of adjustment for season of blood sampling. Inadequate statistical power to evaluate the effect of milder degrees of vitamin D deficiency (15–30 ng/mL) and vitamin D sufficiency given the low proportion of individuals in the cohort with levels >30 ng/mL (10%)
Adjustment for treatment allocation (calcium or placebo) and baseline age, body weight, smoking status, systolic BP, and history of ischemic heart disease, stroke or transient ischemic attack, dyslipidemia, DM, and adjustment for seasonal variation in 25(OH)D concentrations	<p>≥20 1.00</p> <p><20 1.2 (0.7–2.2)</p>	Participants in the study were volunteers for an RCT for calcium supplementation. It is possible that volunteer bias occurred so that these women were healthier than their peers; therefore, findings are not generalizable to the general population
Adjustment for age, sex, BMI, and physical activity level, active smokers, DM, albumin level, cystatin C level, triglyceride level, N-terminal pro-BNP level, systolic and diastolic BP, LDL-C and HDL-C levels, and the use of statins, aspirin, β-blockers, bronchodilators, and angiotensin-converting enzyme inhibitors	<p>Q4†: 28.4 1.00</p> <p>Q3: 18.9 1.4 (0.9–2.1)‡</p> <p>Q2: 13.3 1.82 (1.29–2.58)</p> <p>Q1: 7.6 2.22 (1.57–3.13)</p>	Limitations of the study are related to 25(OH)D level determination and possibly race/ethnicity and changes of serum 25(OH) D levels over time§
Adjustment for age, sex, race, season of blood sampling, BP, history of CVD, DM, smoking, HDL-C, triglycerides, use of cholesterol-lowering medications, estimated GFR categories, serum albumin level, log albumin to creatinine ratio, log CRP, BMI, physical activity level, use of vitamin D supplementation, and low socioeconomic status	<p>Q4: >32.1 1.00</p> <p>Q3: 24.4–32.1 0.83 (0.65–1.07)</p> <p>Q2: 17.8–24.3 0.88 (0.69–1.14)</p> <p>Q1: <17.8 1.2 (0.87–1.64)</p>	Northern states were only sampled in the summer in NHANES III; therefore, the full extent of 25(OH)D deficiency in the population is probably underestimated
Adjustment for age, sex, BP, hyperlipidemia, DM, and peripheral vascular disease	<p>>30 1.00</p> <p>16–30 1.15 (1–1.35)††</p> <p>≤15 1.45 (1.2–1.78)</p>	The incident events were limited to those identified within the Intermountain Healthcare system. Both recognized (eg, female sex, risk of osteoporosis) and unrecognized selection biases could exist relating to the reasons that providers performed the vitamin D measurements.
Adjustment for age, examination period, sex, race/ethnicity, education, BMI, smoking, physical activity, heavy alcohol drinking, concentrations of serum non-HDL, HDL, calcium, elevated CRP, estimated GFR, use of dietary and vitamin supplements, and history of DM, CAD, stroke, and cancer	<p>Q4: >29 1.00</p> <p>Q3: 23–28 2.33 (0.88–6.12)</p> <p>Q2: 17–22 2.42 (0.85–6.9)</p> <p>Q1: <17 3.2 (1.14–8.99)</p>	Lack of adjustment for season of blood sampling The follow-up period of mortality (3.7 y) was relatively short, resulting in a relatively small number of deaths, especially deaths resulting from CVD. Inability to distinguish those adults with severe conditions at baseline who may contribute to early death
Adjustment for age, sex, education, season of blood sampling, BMI, smoking, aspirin use, physical activity, total cholesterol, HDL-C, MMSE score, and renal insufficiency	<p>Q4: >25.6 1.00</p> <p>Q3: 16.1–25.6 2.19 (1.05–4.6)</p> <p>Q2: 10.5–16 1.68 (0.76–3.62)</p> <p>Q1: <10.5 2.64 (1.14–4.79)</p>	The present study may possibly underestimate the relationship between low 25(OH)D and mortality because the reference quartile of 25(OH)D consisted of many participants who were still in the insufficient range of 25(OH)D. There are other unmeasured confounding factors: traditional risk factors for CVD such as DM, BP
Adjustment for age, sex, race/ethnicity, season, physical activity, BMI, DM, BP, smoking, CRP, total-C, HDL-C, triglycerides, and measurement batch§§	<p>≥15 1.00</p> <p><15 1.38 (0.95–1.99)</p>	It cannot be determined whether lower vitamin D concentrations are associated with the development of atherosclerotic plaque per se or with its calcification
Adjustment for age, sex, BMI, BP, alcohol intake, smoking status, exercise habit, HbA1C, and triglycerides HDL-C, LDL-C, ALT, creatinine, hsCRP, HOMA-IR, phosphorus, iPTH	<p>≥30 1.00</p> <p>15–29.9 2.08 (1.16–4.68)</p> <p><15 3.12 (1.89–5.59)</p>	No data on hard end points such as the incidence or mortality of CVD. Data that can affect vitamin D levels such as seasonal variation were not captured

(Continued)

Table. Continued

Author	Study Design	Study Population			Setting	Follow-Up Length, y	25(OH)D Measurement	Type of Outcome
		Total (Cases), n	Mean Age, y	Male, %				
Karakas et al ¹²⁴	CCS (prospective)	964 (225)	35-74##	100	Population-based, initially healthy	11	EIA (IDS)	Fatal and nonfatal MI, SCD
		819 (73)	35-74***	0				

25(OH)D has a relatively long circulating half-life (≈ 3 weeks) and is considered the best indicator of whole-body vitamin D status, but a single measurement cannot fully capture cumulative vitamin D exposure. ALT indicates alanine aminotransferase; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; CCS, case cohort study; CHD, coronary heart disease; CI, confidence interval; CLIA, chemiluminescence immunoassay; CS, cohort study; CVD, cardiovascular disease; DM, diabetes mellitus; EIA, enzyme immunoassay; GFR, glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment-insulin resistance; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; IP-10, interferon- γ -inducible protein 10; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LDL-C, low-density lipoprotein cholesterol; MI, myocardial infarction; MMSE, Mini-Mental State Examination; N/A, not available; NCCS, nested case-control study; Q, quartile; RCT, randomized, controlled trial; RIA, radioimmunoassay; SCD, sudden cardiac death; sICAM-1, soluble intercellular adhesion molecule 1; T, tertile; and UPLC-MS/MS, ultraperformance liquid chromatography-tandem mass spectrometry.

*In a random sample of 100 study participants, 25-hydroxyvitamin D level was also determined using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), and a highly significant correlation was noted between the RIA and LC-MS/MS ($R=0.87$).

†Monthly quartiles.

‡Graphically determined.

§A retrospective classification of all individual 25 (OH)D levels into quartiles was performed on the basis of 202 to 358 measurements of 25 (OH)D levels each month.

||The RIA kit was calibrated using high-performance liquid chromatography-purified 25(OH)D every 6 months.

#Prospectively designed analysis of a retrospectively collected and observational medical records database.

**25(OH)D levels were drawn at the providers' discretion for clinical indications (eg, osteoporosis risk).

††The 95% confidence intervals are graphically determined.

‡‡Coronary artery calcification was quantified using an electron-beam computed tomography (CT) scanner or a multidetector CT system. Any Agatston score >0 defining the presence of coronary artery calcification.

§§Measurements were completed using the same assay in three batches by estimated GFR. Because measurement batch was determined by estimated GFR, adjustment for batch also adjusts for chronic kidney disease.

|||Coronary artery stenosis was evaluated by CT angiography was performed with a 64-slice multidetector-row cardiac CT scanner.

##Weighted means 56.8 y (0.53) for cases and 51.9 y (0.42) for noncases.

***Weighted means 57.7 y (0.76) for cases and 52.5 y (0.39) for noncases.

supplementation on vascular disease mortality and all-cause mortality.¹²⁹ The Women's Health Initiative (WHI) reported a nearly statistically significant harmful effect with combined vitamin D₃ and calcium supplementation on a composite cardiac outcome that included nonfatal myocardial infarction, coronary heart disease death, or need for revascularization (relative risk, 1.08; 95% confidence interval, 0.99–1.19).¹³⁰ In both studies, participants were followed up for 2 to 7 years but were tested only for 800 and 400 IU of vitamin D₃ daily, respectively, a dose that can increase the serum 25(OH)D concentration only by 2 to 4 ng/mL, which is unlikely to be associated with any change in risk of CVD outcomes. Another trial that tested 100 000 IU vitamin D₃ or placebo every 4 months for up to 5 years, with CVD as a secondary outcome, did not show statistically significant results. However, the authors noted that although the concentration of 25(OH)D in the treatment group was 40% higher than placebo, it still did not achieve physiological concentrations.¹³¹ The recently completed PRIMO (Paricalcitol Capsules Benefits Renal Failure Induced Cardiac Morbidity in Subjects With Chronic Kidney Disease Stage 3/4) trial found no change in left ventricular mass index with 48 weeks of paricalcitol treatment (2 μ g/d) in patients with CKD and mild to moderate left ventricular hypertrophy, although in post hoc analysis paricalcitol reduced left atrial volume, a measure linked to adverse cardiovascular events.¹³²

Elamin et al¹³³ conducted a systematic review of the literature to summarize the available evidence of the possible cardiovascular benefits of vitamin D supplementation. According to their meta-analysis of 51 trials, vitamin D-raising interventions were associated with an insignificant effect on myocardial infarction (relative risk, 1.02; 95% confidence interval, 0.93–1.13; $P=0.64$; $I^2=0\%$, representing low inconsistency), stroke (relative risk, 1.05; 95% confidence interval, 0.88–1.25; $P=0.59$; $I^2=15\%$, representing low inconsistency), lipid fractions, glucose, or blood pressure.

Of note, the long-term effects of high-dose vitamin D supplementation on CVD events (myocardial infarction, stroke, and CVD mortality) are expected to be evaluated by the ongoing Vitamin D and Omega-3 Trial (VITAL), which is a large, randomized, double-blind, placebo-controlled, 2 \times 2 factorial trial of vitamin D (vitamin D₃, 2000 IU/d) and omega-3 fatty acid supplements in the primary prevention of CVD in a multiethnic population of 20 000 US men ≥ 50 years of age and women ≥ 55 years of age. The mean treatment period will be 5 years.¹³⁴

Several potential reasons could explain the lack of apparent concordance between the various cross-sectional, longitudinal observational, and randomized studies. The substantial heterogeneity of studies with respect to definition of vitamin D status, age structures, definition and determination of cardiovascular outcome, and exposure definitions and the different

Confounders Adjusted in Analysis	Relative Risk (95% CI) According to 25(OH)D Levels (Range or Median), ng/mL		Limitations
Adjustment for age, sex, season, survey, BMI, smoking, physical activity, alcohol, systolic BP, total cholesterol/HDL, or parental history of MI, CRP, IL-6, sICAM-1, and IP-10	T1: 10.81	1.00	The number of female cases was considerably lower than male cases. No data on comedication influencing bone metabolism and thereby altering vitamin D levels in serum. Lack of adjustment for DM (traditional risk factor for CVD)
	T2: 17.42	0.66 (0.43–1.02)	
	T3: 0.84	0.84 (0.52–1.35)	
	T1: 10.57	1.00	
	T2: 15.86	0.67 (0.35–1.29)	
	T3: 23.43	0.42 (0.19–0.93)	

adjustments for confounders such as the seasonality of 25(OH)D or even the existence of residual confounding are identified as a significant shortcomings in the literature. Finally, the use of single baseline vitamin D measurements, which may not reflect long-term vitamin D status, and the duration, dosing regimen, intervention formulations, use of concomitant therapies, and methodology used for vitamin D assessment may affect the quality of the intervention studies.

Evaluation and Treatment of Vitamin D Deficiency

The Institute of Medicine and the Endocrine Society Task Force both published recently guidelines for the evaluation, treatment, and prevention of vitamin D deficiency.^{11,135} On the basis of their data, the Endocrine Society Task Force defined vitamin D deficiency as a 25(OH)D level <20 ng/mL, vitamin D insufficiency as a 25(OH)D level of 21 to 29 ng/mL, and vitamin D sufficiency as a 25(OH)D level ≥30 ng/mL.¹¹ However, the Institute of Medicine committee found optimal bone health outcomes with 25(OH)D levels between 16 and 20 ng/mL.¹³⁵

In any case, both committees used skeletal health as an end point, and they both concluded that the available scientific evidence supports a causal role for vitamin D only in skeletal health but is not yet compelling that either confers benefits for or is causally related to non-skeletal health outcomes like CVD. Thus, prescribing vitamin D supplementation for the purpose of preventing CVD beyond recommended daily needs cannot be recommended.

The Endocrine Society Task Force suggests using either vitamin D₂ or vitamin D₃ for the prevention and treatment of vitamin D deficiency. However, there is currently a lack of clarity in the literature as to whether there is a definitive difference in the potency between D₂ and D₃ as modulators of bone metabolism factors, and it is more unclear whether any differences translate to different effects of these 2 forms (D₂/D₃) on cardiovascular risk factors.

Of interest, total 25(OH)D levels have been shown to be correlated with various adverse cardiometabolic risk factors in adolescents, whereas recently published studies aiming to compare prospective distinct associations of 25(OH)D₂ and 25(OH)D₃ with cardiovascular risk factors in younger ages have shown that higher 25(OH)D₃ concentrations are associated with higher levels of high-density lipoprotein cholesterol in childhood and lower fasting insulin in adolescence.^{136,137} Moreover, 25(OH)D₂ was inversely associated

with apolipoprotein A1 and triglycerides and positively associated with C-reactive protein and IL-6.¹³⁸

Taking into account that pharmaceutical preparations in some countries (ie, the United States) contain only vitamin D₂ whereas multivitamin preparations contain either D₂ or D₃, the identification of possible differences is of great importance in suggesting the use of one or the other form for achieving favorable effects on cardiovascular risk factors. Details on the treatment of vitamin D deficiency in special conditions and the re-evaluation of the subjects supplemented with vitamin D are provided in the online-only Data Supplement.

Conclusions and Future Perspectives

Vitamin D exerts a variety of favorable effects on endothelial dysfunction, VSMC proliferation and migration, and calcification, as well as on the inflammatory/immune process of atherosclerosis; moreover, it exerts beneficial effects against systemic conditions that promote atherosclerosis such as insulin resistance, β-cell dysfunction, dyslipidemia, RAAS, and consequent hypertension, therefore suggesting a potential therapeutic role. Elucidating the molecular foundation of these diverse effects and the possible mediating effects of genetic factors on the relationship between vitamin D and CVD is also of great importance.

Mendelian randomization studies, which are believed to overcome unmeasured confounding, should be conducted to estimate the causal effect of vitamin D status on atherosclerosis and CVD risk.

In addition, rigorous large-scale, randomized, clinical trials to test the effects of vitamin D (D₃ and D₂) on atherosclerosis and CVD as the primary outcome emerge as a particular priority to firmly establish the role of vitamin D supplementation on CVD risk and mortality. Dose-response randomized trials are important to identify threshold effects and possible adverse effects.

Disclosures

None.

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SUPPLEMENTAL MATERIAL

Contemporary Review in Cardiovascular Medicine

The Role of Vitamin D in Atherosclerosis

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Vitamin D Metabolism and Mechanism of Action

Vitamin D is a steroid hormone that comes in two forms which differ chemically in their side chain, D₂ and D₃ (see Figure 1 in main text). These structural differences alter their metabolism, but in general the biologic activity of their active metabolites is comparable.¹ Vitamin D₂ or ergocalciferol is obtained from the UV irradiation of the yeast sterol ergosterol and is found naturally in foods of plant origin such as sun-exposed mushrooms.

On the other hand, vitamin D₃ or cholecalciferol is mainly synthesized in the skin from 7-dehydrocholesterol by exposure to ultraviolet-B light (290-315 nm) while is also abundant in foods of animal origin e.g., oil-rich fishes or cod-liver oil. The major source of vitamin D comes from exposure of the human skin to sunlight between 10am and 15pm in the spring, summer, and fall.

Apart from its natural sources vitamin D₃ and D₂ are commercially available and used for food fortification and in vitamin D supplements.^{2,3}

Vitamin D (either D₂ or D₃) is absorbed by the small intestine passively and is incorporated into chylomicrons entering thus the venous blood through the lymphatic system.

Either produced in the skin or ingested, vitamin D is biologically inert and requires two hydroxylations to form its active metabolite. The first hydroxylation is constitutive and takes place in the liver by the vitamin D-25-hydroxylase (25-OHase) to form the 25(OH)D. The second hydroxylation is catalyzed by the 25(OH)D-1 α Oase (CYP27B1) to form the biologically active form of vitamin D 1,25(OH)₂D (calcitriol) (see Figure 1 in main text). This latter 1 α -hydroxylation of 25(OH)D, takes place in most tissues and cells of the body, however, serum levels of 1,25(OH)₂D are mainly determined by renal 1 α -hydroxylase activity; this activity is regulated by serum calcium, phosphate, parathormone (PTH) and fibroblast growth factor 23 (FGF23).

It is important to know that circulating 25(OH)D levels are a main determinant of extrarenal tissue levels of 1 α ,25(OH)₂D and are thus the best indicator of whole body vitamin D status. Actually, 25(OH)D is used for the classification of the vitamin D status into deficient or sufficient.⁴

The vitamin D metabolites are transported in blood bound to vitamin D-binding protein (DBP) while a very small amount circulates as the free form.

The biological responses to the 1 α ,25(OH)₂D₃ hormone and its analogues are mediated by the VDR. VDR is a DNA-binding transcription factor which gives rise to an active signal transduction complex consisting of a heterodimer of the 1 α ,25(OH)₂D-liganded VDR and retinoid X receptor (RXR). The VDR–RXR heterodimer recruits cofactors to form a transcriptional complex that recognizes and binds vitamin D responsive elements (VDREs) in the promoter region of vitamin D-

regulated genes. VDR is widely distributed in over 38 tissues while the vitamin D endocrine system regulates about 3% of the human genome exerting widespread effects.⁵

It should be noted that the activation of the VDR by vitamin D analogues - such as paricalcitol or 22-oxacalcitriol- can recruit different cofactors from those recruited by $1\alpha,25(\text{OH})_2\text{D}_3$.⁶ Different cell events after activation of the same VDR by the different analogs may offer an explanation for the differences observed between calcitriol and analogues in their effects on -among others- atherosclerotic process.

VDR is also localized to the plasma membrane caveolae and may result in activation of signal transduction pathways that generate rapid responses. These rapid non-genomic effects include the activation of G protein-coupled receptors, phosphatidylinositol-3-kinase (PI3K), phospholipase C, or protein kinase C (PKC) which in turn may upregulate the mitogen-activated protein kinase (MAPK) cascade resulting in a number of possible outcomes including opening of the voltage-gated calcium channels, rapid pancreas insulin secretion etc. The biological effects of calcitriol are curtailed by the $1\alpha,25(\text{OH})_2\text{D}_3$ 24-hydroxylase which catalyzes the degradation of $1\alpha,25(\text{OH})_2\text{D}_3$, providing thus an "off" signal right after the hormone has exerted its physiologic regulation of gene expression.⁷ Mice with ablation of the *CYP24A1* gene die early because of $1\alpha,25(\text{OH})_2\text{D}_3$ toxicity.

It is noteworthy that genome wide associations studies (GWAS) revealed a genetic influence on 25(OH)D levels by DBP, 25-hydroxylase, 24-hydroxylase as well as 7-dehydrocholesterol reductase, the enzyme that catalyzes the last step in cholesterol biosynthesis.⁸

Regulation of FGF23–Klotho Axis by Vitamin D

FGF23 is a recently discovered glycoprotein, synthesized primarily in osteocytes and osteoblasts.⁹ Klotho is synthesized in the distal tubular cells of the kidney, in the parathyroid glands, and in the choroid plexus. It is well known that circulating Klotho acts as a mandatory cofactor for FGF23 and mediates the effects of FGF23 to suppress renal production of $1\alpha,25(\text{OH})_2\text{D}_3$, induce urinary phosphate excretion, and inhibit synthesis and secretion of parathyroid hormone.⁹

There is increasing evidence for a possible role for FGF23–Klotho axis in the atherosclerosis process. The clinical and laboratory findings in Klotho-null mice and FGF23-deficient mice which exhibit among others abnormal vitamin D homeostasis, atherosclerosis, vascular calcifications, suggest that this axis may have a large impact on the pathophysiology of CV disorders in CKD and even beyond.¹⁰

Klotho appears to directly regulate both human endothelial and VSMCs functions. Studies in animal models showed that Klotho protein can protect against endothelial dysfunction through the regulation of NO release.¹¹ Moreover, Klotho was associated with increased resistance against oxidative stress involved in the pathogenesis of vascular diseases. It was demonstrated that Klotho via inhibition of insulin/IGF-1/PI3K/Akt signaling cascade, induces expression of manganese superoxide dismutase facilitating thus the removal of ROS.¹²

Moreover, Hu et al. showed that circulating Klotho can suppress VSMC calcification through inhibition of Na^+ -dependent uptake of phosphate.¹³

In addition to the role of circulating Klotho, recent data provide evidence for functional Klotho protein expressed in human VSMCs which may play a critical role in maintaining smooth muscle cell integrity. Of note, suppression of Klotho in VSMCs, under procalcific stress conditions, was associated with upregulation of Runx2 and myocardin–serum response factor-dependent pathway, which in turn

orchestrated VSMCs phenotype transformation in osteoblast-like cells by regulating downstream bone-related proteins, including alkaline phosphatase and osteocalcin.¹⁴

FGF23, the second partner of the FGF23–Klotho axis, was found to induce proliferation and inhibit VSMC-mediated extracellular calcifying matrix deposition via p-ERK and p-AKT pathway. Interestingly, these effects were abolished following Klotho knock-down implying a Klotho-dependent function of FGF23 in VSMCs. Recently, it was demonstrated that suppressed Klotho levels under the influence of procalcific conditions can be restored by VDR activator. Notably, apart from the active form of vitamin D [1 α ,25(OH) $_2$ D $_3$], the inactive calcidiol [25(OH)D] also restored Klotho expression suggesting that the VSMC 1 α -hydroxylase (CYP27B1) enzyme is involved in mediating supportive autocrine/paracrine effects in the regulation of Klotho.¹⁴ Furthermore the restoration of Klotho with VDR activator rendered VSMCs again FGF23 responsive, reactivating the proliferation and calcification inhibitory effects, confirming that both the endocrine and autocrine/paracrine vitamin D systems are involved in regulating local vascular Klotho.¹⁴

Using a uremic mouse model Lau et al. recently examined the effect of calcitriol or its analog paricalcitol on vascular calcification. They revealed that active vitamin D, in doses equivalent to those given to patients with CKD, increases serum and urinary levels of secreted Klotho and expression of the calcification inhibitor osteopontin in aortic medial cells, and ameliorates aortic medial calcification.¹⁵

However, it still remains to be determined the exact role of vitamin D regulation of FGF23–Klotho axis in the pathogenesis of vascular calcification in CKD as well as in non-CKD subjects and whether the *in vitro* and animal model data can be translated in clinical practice.

Role of RAAS in Atherogenic Process

It is widely accepted that angiotensin II (Ang II) plays a central role in the pathophysiology of vascular remodeling through smooth muscle growth and collagen deposition.^{16,17} Moreover, Ang II can activate PLA2 and induce expression of COX-2 in VSMCs enhancing the synthesis of PGs, which are important modulators of vascular inflammatory response.¹⁸

Recently, Ang II has been recognized as a physiological activator of oxidative stress and endothelium dysfunction. It can stimulate NADPH oxidase via acting on AT1 receptors and regulating PKC in human VSMCs.¹⁹ Apart from Ang II, aldosterone also causes directly structural and functional changes in ECs and VSMCs, promoting the atherogenic process. Indeed, VCAM-1, E-selectin, ICAM-1 gene expression in HUVEC were significantly increased after incubation with aldosterone.²⁰ In addition, microarray and quantitative RT-PCR experiments showed that aldosterone activates expression of endogenous human coronary VSMC genes, including several involved in vascular fibrosis, inflammation, and calcification.^{21,22}

Evaluation and Treatment of Vitamin D Deficiency

Serum 25(OH)D is the best indicator of whole body vitamin D status while 1,25(OH)₂D₃ assay is indicated for monitoring certain conditions, such as acquired and inherited disorders of vitamin D and phosphate metabolism (i.e., CKD, granulomatous diseases, etc).²³

The level of 25(OH)D which is defined as vitamin D deficiency or sufficiency still remains somewhat controversial. Using PTH levels as an indicator of vitamin D sufficiency, several studies showed that PTH begins to plateau in adults who have

blood levels of 25(OH)D between 30 and 40ng/mL and this is in accordance with the threshold for bone fracture prevention from a recent meta-analysis of double-blind randomized controlled trials.²⁴

The recommended daily needs seem to differ according to the age as well as to special conditions such as lactation, pregnancy.²³

In adults, according to IOM an intake of 600IU/d for subjects aged 19-70 years and 800IU/d of vitamin D for subjects aged >70 years has been supported relative to maximize bone health and muscle function in general population.²⁵ However, it seems that 600-800IU/day of vitamin D are not enough to provide all of the potential nonskeletal health benefits (such as cardiovascular) associated with vitamin D.

According to Endocrine Society Task Force vitamin D deficient adults should be treated with 50,000IU of vitamin D once a week for 8 weeks or its equivalent of 6000IU of vitamin D daily to achieve a blood level of 25(OH)D above 30ng/mL, while it may require at least 1500-2000IU/day of supplemental vitamin D to maintain these levels.²³

Obese, dark skin pigmented people as well as patients on multiple anticonvulsant medications, glucocorticoids, or antifungals such as ketoconazole, elderly etc. are at higher risk for vitamin D deficiency and need much higher dose to correct deficiency.^{23,26}

The first re-evaluation is suggested to be performed not earlier than three months after beginning supplementation. Special attention must be given to patients with extrarenal production of 1 α ,25(OH)₂D₃ and primary hyperparathyroidism, where a close monitoring of serum calcium is needed during treatment with vitamin D to prevent hypercalcemia. Moreover, when a vitamin D₂ is used for supplementation, it

is recommended against using RIA methodology for re-evaluation since it underestimates D_2 .²⁷

For upper levels of serum 25(OH)D, sparse data are available, particularly regarding long-term effects of chronically high concentrations. Thus, serum 25(OH)D levels chronically more than 50ng/mL should cause concern about potential adverse effects.²⁸

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