SUPPLEMENTAL MATERIAL

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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, D$_2$ and D$_3$ (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 D$_2$ 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 D$_3$ 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 D$_3$ and D$_2$ are commercially available and used for food fortification and in vitamin D supplements.$^{2,3}$
Vitamin D (either D$_2$ or D$_3$) 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-1aOHase (CYP27B1) to form the biologically active form of vitamin D 1,25(OH)$_2$D (calcitriol) (see Figure 1 in main text). This latter 1a-hydroxylation of 25(OH)D, takes place in most tissues and cells of the body, however, serum levels of 1,25(OH)D are mainly determined by renal 1a-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 1a,25(OH)$_2$D 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 1a,25(OH)$_2$D$_3$ 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 1a,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.\textsuperscript{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\textsubscript{a},25(OH)\textsubscript{2}D\textsubscript{3}.\textsuperscript{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\textsubscript{a},25(OH)\textsubscript{2}D\textsubscript{3} 24-hydroxylase which catalyzes the degradation of 1\textsubscript{a},25(OH)2D3, providing thus an "off" signal right after the hormone has exerted its physiologic regulation of gene expression.\textsuperscript{7} Mice with ablation of the \textit{CYP24A1} gene die early because of 1\textsubscript{a},25(OH)\textsubscript{2}D\textsubscript{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.\textsuperscript{8}

\textbf{Regulation of FGF23–Klotho Axis by Vitamin D}
FGF23 is a recently discovered glycoprotein, synthesized primarily in osteocytes and osteoblasts. 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 an mandatory cofactor for FGF23 and mediates the effects of FGF23 to suppress renal production of 1α,25(OH)₂D₃, induce urinary phosphate excretion, and inhibit synthesis and secretion of parathyroid hormone.

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.

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. 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.

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

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.\textsuperscript{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 [1a,25(OH)\textsubscript{2}D\textsubscript{3}], the inactive calcidiol [25(OH)D] also restored Klotho expression suggesting that the VSMC 1a-hydroxylase (CYP27B1) enzyme is involved in mediating supportive autocrine/paracrine effects in the regulation of Klotho.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 \textit{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{21,22}

Evaluation and Treatment of Vitamin D Deficiency

Serum 25(OH)D is the best indicator of whole body vitamin D status while 1a,25(OH)\textsubscript{2}D\textsubscript{3} 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).\textsuperscript{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.\textsuperscript{24}

The recommended daily needs seem to differ according to the age as well as to special conditions such as lactation, pregnancy.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 1a,25(OH)\textsubscript{2}D\textsubscript{3} and primary hyperparathyroidism, where a close monitoring of serum calcium is needed during treatment with vitamin D to prevent hypercalcemia. Moreover, when a vitamin D\textsubscript{2} is used for supplementation, it
is recommended against using RIA methodology for re-evaluation since it underestimates D$_2$.\textsuperscript{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.\textsuperscript{28}
References


