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Review

Vitamin D Deficiency and Cardiovascular Disease: Potential Mechanisms and Novel Perspectives

Mohamed A. Abu el Maaty and Mohamed Z. Gad

Biochemistry Department, Faculty of Pharmacy and Biotechnology, German University in Cairo, Al Tagamoa Al Khames, New Cairo City 11835, Egypt

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Summary Interest in contemporary vitamin D research has been sparked in recent years, stemming from the identification of vitamin D receptors in virtually all cells as well as the enzymatic machinery necessary to produce its active form. Both epidemiological and in-vitro studies have linked vitamin D deficiency to enigmatic diseases including cardiovascular disease; however, a clear mechanistic link remains missing. This review highlights conclusions of observational studies, in-vitro experiments and randomized-controlled trials that aimed to link deficiency of the sunshine vitamin to one of the leading causes of death in the world, cardiovascular disease. Furthermore, putative mechanisms viewed from a novel perspective are also discussed.

Key Words vitamin D, cardiovascular disease, molecular mechanisms

Introduction

Vitamin D: more than just a vitamin

Although Adolf Windaus discovered vitamin D in the 1930s (*1*), it is by far the oldest "hormone" on the planet, from an evolutionary point of view. It is now clear that organisms known to have lived millions of years ago synthesized vitamin D when exposed to the sun's ultraviolet-B radiation (290–315 nm) (*2*). In view of this, one comes face-to-face with the question: *Why the sudden interest in vitamin D research?*

Discoveries in the second half of the twentieth century revealed that vitamin D receptors (VDRs), the means by which the vitamin exerts its actions, are present in an array of cells ranging from osteoblasts and chondrocytes of the musculoskeletal system, to T and B cells of the immune system, thus explaining its proposed skeletal and extra-skeletal activities (*3*). In terms of the cardiovascular system, VDRs have been identified in endothelial cells (*4*) and cardiomyocytes (*5*), as well as the presence of the enzymatic machinery needed to produce the appropriate ligand for the receptor (*6*, *7*), warranting the role of vitamin D in regulating cardiovascular health.

Vitamin D: D-fining the basics

Vitamin D exists in two forms, vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) (*8*). The human body acquires the former mainly through photosynthesis in the skin through exposure to the ultraviolet-B radiation of the sun as well as through the diet, notably from fatty fish (*8*). Ergocalciferol, on the other hand, is only obtained by humans through exogenous sources, whether from the diet through foods like mushrooms, or simply through supplementation (*8*). Both forms undergo the same metabolism in-vivo and are both equally prescribed to treat vitamin D deficiency. Herein, vitamin D represents both forms.

The first metabolizing step vitamin D undergoes occurs in the liver, which is catalyzed by the enzyme vitamin D-25-hydroxylase, encoded by the gene CYP2R1 (*9*). The resulting metabolite, 25-hydroxyvitamin D [25(OH)D], is the agreed upon biomarker for vitamin D status although it is not the biologically active form (*8*). Bound to vitamin D binding protein, the metabolite is then transported to the kidneys, which are considered the main, but not exclusive, site of the second metabolizing step, which yields the hormonally-active form, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], through actions of the enzyme 25-hydroxyvitamin D-1α-hydroxylase, encoded by the gene CYP27B1 (*8*) (Fig. 1).

Vitamin D's primary signaling pathway

The biological response elicited by vitamin D is the result of $1,25(OH)_2D$ binding to the nuclear VDR. This process may be endocrine or autocrine in nature. In other words, $1,25(OH)_2D$ may first be produced by the kidneys, prior to its transport to the target cell, or may be produced by the target cell expressing mitochondrial CYP27B1, which finally leads to the binding of the ligand to the receptor (*10*). The autocrine mechanism of action is illustrated in Fig. 2.

Guidelines and risk factors for vitamin D deficiency

Vitamin D deficiency is a term not easily defined. Guidelines obtained from both the US Endocrine Society and the US Institute of Medicine are significantly different (*11*). The former defines vitamin D deficiency as having a 25(OH)D concentration of less than 20 ng/mL whereas insufficiency lies between 21 and 29 ng/mL and finally sufficiency involves concentrations over 30 ng/mL while maintaining the safety margin set at 100 ng/mL to avoid the risk of hypercalcemia (*11*) (Table 1). On the other hand, the latter views concentrations equal to

E-mail: abu.el.maaty@gmail.com

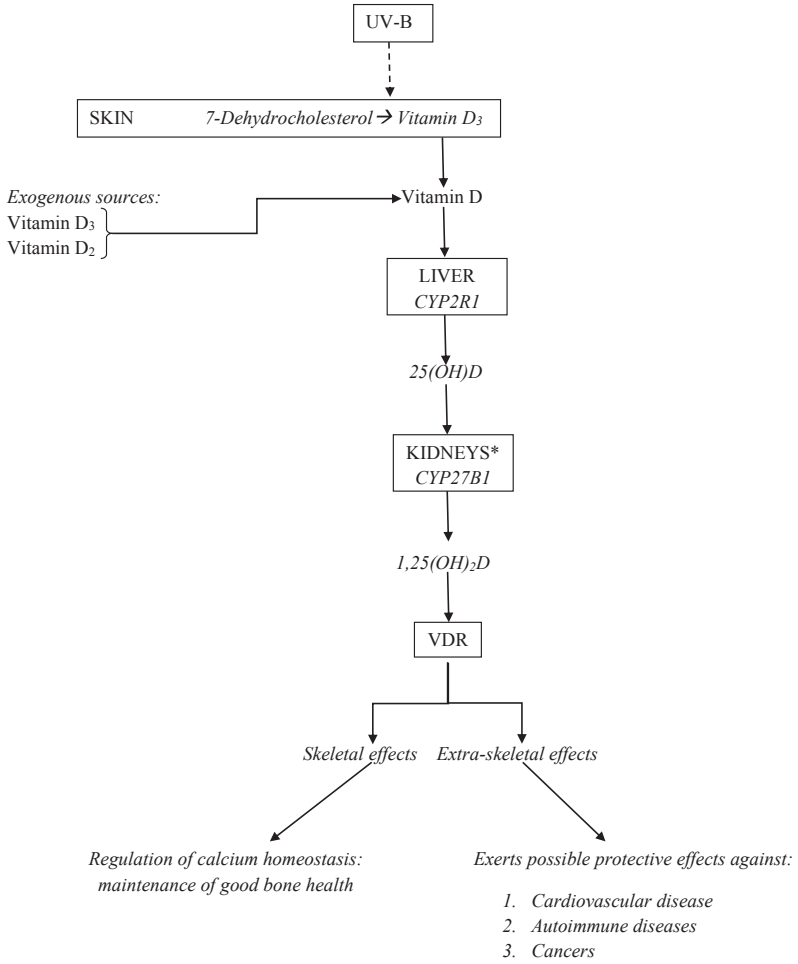

Fig. 1. Schematic representation of vitamin D photosynthesis, and metabolizing steps. * denotes the fact that although the kidneys are the main site of vitamin D activation, they are not exclusive in this role.

16 and 20 ng/mL as sufficient for approximately 50 and ≥97.5% of the population, respectively, while concentrations over 50 ng/mL are viewed as alarming (*11*).

Various risk factors may account for the fact that approximately 1 billion people worldwide are vitamin D deficient (*8*). While some of such factors may be modifiable such as obesity, use of certain medications and lack of exposure to sunlight, which may be due to physical inactivity, use of sunscreens or wearing customary body-covering attire mandated in various parts of the globe, others are not (*6*). Non-modifiable risk factors for vitamin D deficiency include, but are not limited to, genetic factors (described later on), aging (associated with a decrease in the skin's photosynthesizing power), race and latitude (*6*).

Vitamin D deficiency and cardiovascular disease: over two decades of research

The possibility of a relationship between vitamin D deficiency and cardiovascular disease was first struck by a case-control study, originating from New Zealand, that was published in 1990 (*12*). The finding was that individuals below the median 25(OH)D level had twice the risk of having a myocardial infarction compared to those above (*12*).

Since then, plentiful observational studies have linked insufficient 25(OH)D levels with various cardiovascular diseases including coronary heart disease (*13*), heart failure (*14*) and stroke (*15*), as well as with associated risk factors such as diabetes mellitus (*16*), hypertension (*17*, *18*), dyslipidemia (*19*) and endothelial dysfunction (*20*, *21*).

Surprisingly, with accumulating evidence supporting the association, vitamin D supplementation is still not indicated in the treatment or even as a prophylactic against cardiovascular disease, but the question remains: *Do we have enough data to strongly support vitamin D in the battle against cardiovascular disease, or is it simply a bystander?*

Discussion

Epidemiology: what we know from observational and interventional studies

I. Cardiovascular risk factors. A good point to start investigating the association at would be to highlight the results of studies linking vitamin D levels with the early marker of cardiovascular disease, endothelial dysfunction. Tarcin et al. (*20*) not only demonstrated that 25(OH)D deficiency is associated with a lower flow mediated dilatation compared to their controls, they also presented an improvement in the mentioned endothelial

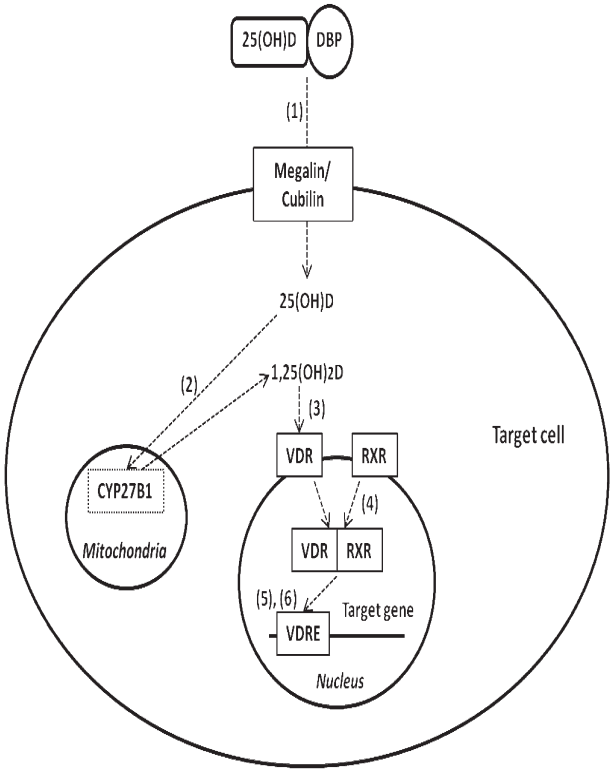

Fig. 2. Overview of vitamin D's molecular mechanism of action. The sequence of events leading to a biological effect starts with entry of the circulating form of the vitamin, 25(OH)D, into the cell via the plasma membrane-bound receptors megalin/cubilin (1). Upon entering the cell, 25(OH)D dissociates from the vitamin D binding protein (DBP) and is converted to the hormonally-active form via the mitochondrial CYP27B1 (2). $1,25(OH)_2D$ then binds to its nuclear VDR (3), which proceeds by hetero-dimerizing with retinoid X receptor (RXR) (4). This complex then binds to vitamin D response elements (VDRE) on target genes (5) and elicits a biological response by either increasing or decreasing transcription (6).

function parameter after supplementation with vitamin D_3. Complementing their results were those presented by Sugden et al. (*22*) who illustrated that supplementation with a single high dose of vitamin D_2 was capable of significantly improving type 2 diabetes patient's endothelial function, also assessed by flow mediated dilatation, compared to a placebo. Similarly, results from different laboratories supported the stated notion by demonstrating an association between optimal vitamin D levels and reactive hyperaemia index (*23*), as well as with circulating endothelial progenitor cells (*24*). In contrast, a study utilizing a relatively small cohort size, involving patients with peripheral artery disease, demonstrated that while a single large dose of vitamin D was capable of significantly raising 25(OH)D levels in the supplementation group, it was incapable of influencing endothelial function (*25*).

A highly investigated association is that of vitamin D deficiency with hypertension; however, it yields conflicting conclusions. Results of meta-analyses have reasonably contributed to the controversy where one illustrated that supplementation with vitamin D led to a minor, insignificant reduction (2 mmHg) in systolic blood pressure but not in the diastolic (*26*), whereas a different meta-analysis demonstrated a minor, yet significant reduction (4/3 mmHg) in blood pressure in subjects with elevated basal systolic blood pressure (*27*). These results bring forth a strongly conceivable notion that there is a possible protective effect of vitamin D against hypertension in certain groups. Further clarification would involve mentioning the result of a randomized-controlled trial involving a small number of diabetic patients, where the authors described administering 2 different large doses of vitamin D_3, as well as a placebo, to their subjects and monitoring changes in blood pressure at 8 and 16 wk. They concluded that a single, large dose of vitamin D was capable of significantly reducing systolic blood pressure compared to the placebo (*28*).

Table 1. Vitamin D status and corresponding 25(OH)D concentrations.

Vitamin D status	25(OH)D concentration (ng/mL)
Deficient	<20
Insufficient	$20 \leq x < 30$
Normal	≥ 30
Potentially toxic	>100

In terms of diabetes, a potential protective effect of vitamin D against both type 1 and 2 has been demonstrated by several studies (*21*). Since juvenile diabetes borders on the association of vitamin D with autoimmune diseases, reviewed elaborately elsewhere (*29*), only investigations involving type 2 diabetes are discussed in this review. A hallmark of both vitamin D deficiency and type 2 diabetes is obesity (*21*), which acts as an explanation for the compelling link between them, among other mechanistic explanations highlighted later on. The randomized-controlled trial conducted by von Hurst et al. (*30*) concluded that vitamin D supplementation improved insulin resistance and sensitivity in a small number of subjects who were insulin resistant. Similarly, a study involving a large number of subjects demonstrated that a larger dose of calcium and vitamin D may lead to a significant reduction in the risk of type 2 diabetes, compared to a lower dose, thus proposing a protective effect (*31*). Interestingly, Pittas et al. reviewed a large number of studies involving several cohorts and concluded that while the incidence of type 2 diabetes was lower in the highest vitamin D status group compared to the lowest; supplementation of vitamin D does not impact the incidence of type 2 diabetes (*26*).

Perhaps the least investigated association between vitamin D and a cardiovascular risk factor is that with dyslipidemia. While observational studies generally tend to highlight a beneficial correlation between vitamin D levels and various lipid profile parameters (*32–34*), for example, an inverse association between 25(OH)D

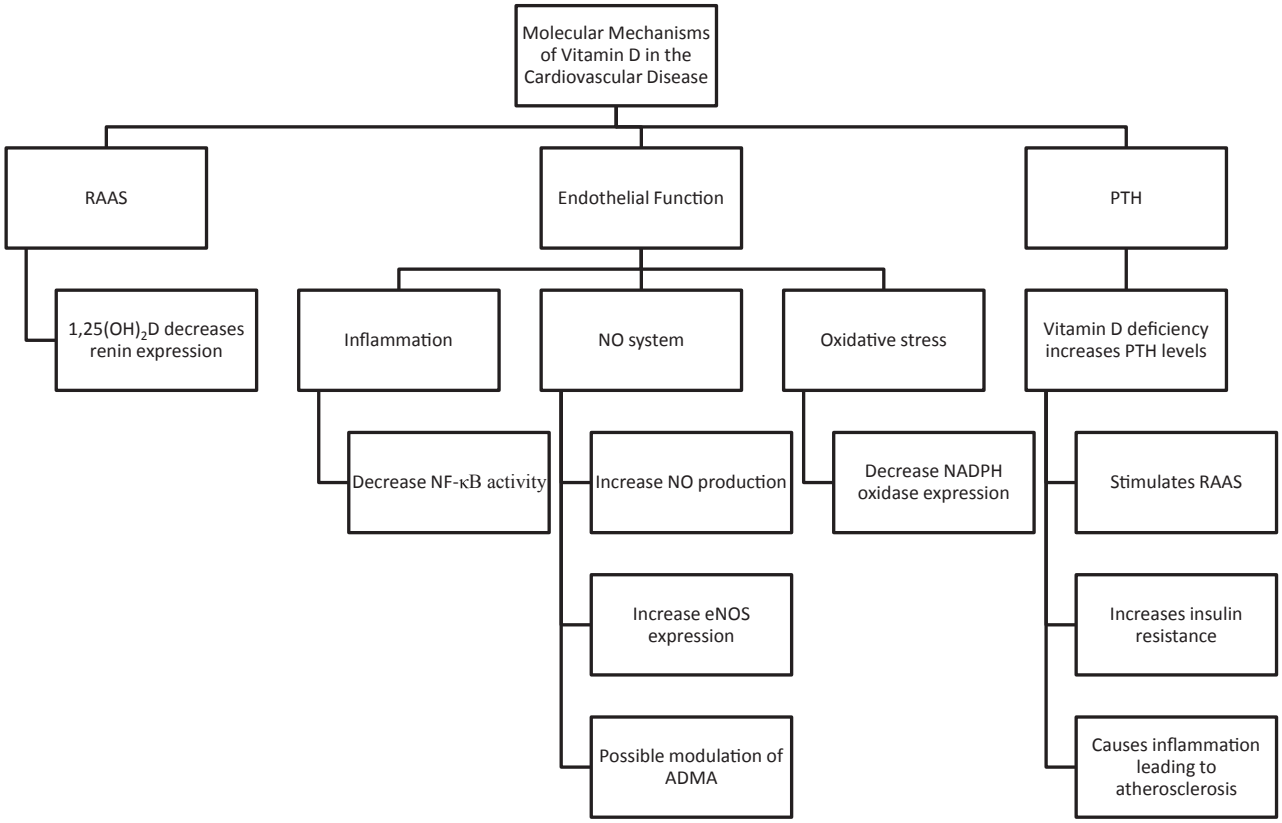

Fig. 3. Overall molecular mechanisms by which vitamin D modulates cardiovascular health. So far, potential mechanisms are classified as affecting the RAAS, endothelial function, or influencing PTH levels.

levels and triglycerides, numerous interventional studies propose the inability of vitamin D to influence lipid profile (*35–38*). Bearing in mind that most intervention studies were not specifically designed to investigate the association of vitamin D with lipid profile parameters, a recently published meta-analysis of the interventional studies conducted in this area, concluded that according to the data in hand, vitamin D supplementation leads to a significant increase in low density lipoprotein-cholesterol and total cholesterol but an insignificant decrease in high density lipoprotein-cholesterol and triglycerides (*19*). Large-scale interventional studies, specifically designed to test the association, are apparently needed, preferably with hyperlipidemia as their main inclusion criterion.

Chronic kidney disease patients tend to be at a higher risk for cardiovascular disease (*6*, *39*). Simultaneously, studies have shown that they also present with significant reductions in both 25(OH)D and $1,25(OH)_2D$ concentrations (*6*). Furthermore, vitamin D levels were shown to be associated with mortality in hemodialysis patients (*40*) and oral or injectable doses of vitamin D were also associated with improved survival in chronic kidney disease patients (*41*, *42*), rationalizing vitamin D's use in the disease.

II. Cardiovascular diseases. Similar to the scenario with cardiovascular risk factors, observational studies lean towards an association between suboptimal vitamin D levels and various cardiovascular diseases including coronary heart disease (*13*), heart failure (*14*), stroke (*43*), myocardial infarction (*44*) and total mortality (*45*, *46*). The meta-analysis conducted by Autier and Gandini (*47*) involving 18 randomized controlled trials concluded that vitamin D therapy led to a 7% reduction in all-cause mortality. Noteworthy is that the studies involved in the analysis used largely variable doses of vitamin D. Similarly, Wang et al. (*48*) reviewed 8 independent randomized controlled trials and concluded that an insignificant reduction in cardiovascular disease risk was observed upon supplementation of moderate to high doses of vitamin D.

Molecular mechanisms: raising the curtains

While several mechanisms have been suggested for the proposed cardio-protective properties of vitamin D, a definitive mechanism of action remains loosely defined. Possible explanations for the compelling role include influencing the Renin-Angiotensin-Aldosterone-System (RAAS) (*49*), endothelial function (*50*) and parathyroid hormone (PTH) levels (*51*) (Fig. 3).

I. Vitamin D as an effector in the RAAS. A brief description of the RAAS would be as follows: angiotensinogen is converted via renin to angiotensin-I which in turn is converted by angiotensin-converting enzyme (ACE) to angiotensin-II, which has widely known physiological and pathological effects (*49*). The notion that $1,25(OH)_2D$ is inversely associated with plasma renin activity was first presented by studies published over two decades ago involving hypertensive subjects (*52*,

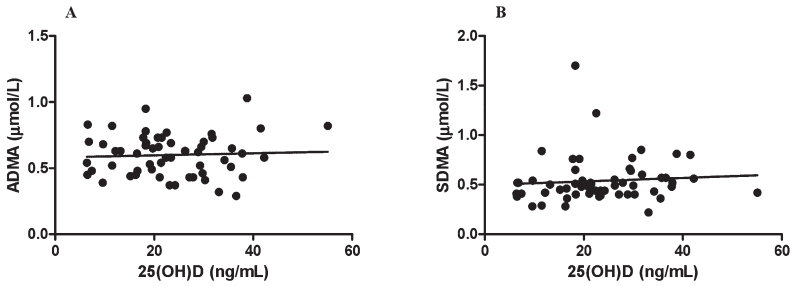

Fig. 4. Linear regression analyses investigating the correlation of 25(OH)D concentrations with ADMA (A) and SDMA (B) levels. An *R*-square of 0.002800 was obtained for (A) and 0.006479 for (B) demonstrating minimal correlation between the investigated parameters. *F*- and *p*-values were 0.049 and 0.783 respectively for (A), and 0.038 and 0.846 respectively for (B), thus illustrating a lack of significance in both cases.

53). The vital role of vitamin D as a negative regulator of renin activity was only significantly highlighted via valuable lessons learned from transgenic mice. Two vitamin D deficiency models were adopted, one utilizing VDR-null mice while the other utilized CYP27B1-null mice. Both models developed hyperreninemia mediated via the up-regulation of renin expression (54–56). Moreover, transgenic mice overexpressing human VDRs in the juxtaglomerular cells demonstrated a decrease in both renal renin mRNA and plasma renin activity (57).

The implications of these findings extend way beyond the importance of a scientific discovery. Current antihypertensive medications are associated with significant drawbacks. For instance, renin inhibitors, ACE inhibitors and angiotensin-II type 1 receptor blockers all act by decreasing angiotensin-II synthesis or block its biological effects. While this has been clinically proven to be an effective hypotensive mechanism, a major disadvantage remains. Homeostasis of the RAAS involves a negative feedback mechanism mediated via the angiotensin-II type 1 receptor (49). This mechanism is interrupted by the aforementioned classes of drugs and thus results in a compensatory increase in renin concentration, resulting in an increased production of angiotensin-II, hence counteracting their initial purpose (49). Vitamin D acts as a potential, potent alternative to these drugs as it has been shown to be able to down-regulate the expression of renin, or in other words, influence renin on a transcriptional level (49).

II. Vitamin D's effect on endothelial function. As described earlier, vitamin D status has been observationally linked to endothelial function. However, the complexity of the molecular basis of this association is currently being unraveled bit by bit. Whether via modulation of biochemical markers of endothelial function, influence on signaling pathways involved or even modulations of the expression of proteins implicated, vitamin D has been consistently portrayed as having a positive influence on endothelial function.

A considerable role for vitamin D in regulating the vascular endothelium is manifested in its ability to modulate the nitric oxide (NO) system. With the now appreciated roles of NO in various mammalian systems, its existence in the cardiovascular system is imperative for optimum cardiovascular health (58). NO is now recognized as a key player in regulating endothelial function and any disruption in its homeostasis will ultimately lead to endothelial dysfunction and hence, cardiovascular disease (59). A brief description of the NO system would be as follows: endothelial nitric oxide synthase (eNOS), the enzyme responsible for NO synthesis in endothelial cells, converts its substrate, L-arginine, into NO and citrulline (58). Other effectors in this system include the highly controversial asymmetric dimethylarginine (ADMA), which happens to be an endogenous inhibitor of eNOS (60). Noteworthy is that elevated levels of ADMA have been associated with various cardiovascular diseases (60, 61).

On the other hand, endothelial dysfunction may also be caused by oxidative stress and inflammation (59). The former may be substantially attributed to the enzyme NADPH oxidase, which has been recognized as a pro-atherogenic player via generating reactive oxygen species (62), whereas the latter may be mediated via nuclear factor-kappaB (NF-κB) signaling (63). It comes as no surprise that results of in-vitro studies have asserted vitamin D's role in influencing the aforementioned mechanisms of endothelial function.

In terms of the NO system, it was shown that the addition of $1,25(OH)_2D$ managed to normalize the activity and expression of eNOS after induction of chronic kidney disease-like conditions in human endothelial cells (64). Moreover, Molinari et al. (65) illustrated that $1,25(OH)_2D$, produces a dose-dependent increase in NO production in cultured endothelial cells, with the involvement of VDRs, by activation of eNOS. Additionally, Ngo et al. (66) highlighted an inverse correlation between 25(OH)D and ADMA levels in an ambulatory, healthy population. Worth mentioning is that our laboratory investigated the same association, as well as the association between 25(OH)D and ADMA's regioisomer symmetric dimethylarginine (SDMA), however in coronary artery disease patients, and found no significant correlation between the parameters (Fig. 4) (67).

Recently, Finch et al. (68) elucidated an increase in the expression of p22(phox) protein, a subunit of NADPH oxidase, in uremic rats compared to controls, which was counteracted by the addition of the VDR activator, pari-

calcitol. Similarly, Hirata et al. (*69*) observed a down-regulation in p22(phox) protein expression in femoral arteries of rats with type 2 diabetes upon administration of 22-oxacalcitriol as well as upon addition to cultured endothelial cells.

With regards to inflammation, several studies have demonstrated an inverse correlation between 25(OH)D and inflammation markers, including high-sensitivity C-reactive protein (*66*, *70*). Molecular studies aiming to clarify the underlying mechanisms linking vitamin D with inflammation include those proposing vitamin D exerts its anti-inflammatory property by decreasing the activity of NF-κB (*63*).

III. Interplay of vitamin D and PTH in cardiovascular disease. It is now known that vitamin D and PTH are active players in calcium homeostasis. Vitamin D deficiency leads to a decrease in calcium levels or hypocalcaemia, a condition which triggers the parathyroid gland to release PTH which stimulates the kidney's production of $1,25(OH)_2D$, which in turn increases calcium levels through increasing its intestinal absorption, mobilization from bones and tubular reabsorption in the kidney (*71*). Untreated vitamin D deficiency leads to a condition known as secondary hyperparathyroidism, which has been linked to adverse cardiovascular effects (*71*).

Studies have shown that elevated PTH levels are associated with an increased risk of mortality compared to subjects with normal PTH levels (*72*), as well as an increase in blood pressure (*73*) and myocardial contractility (*74*). Additionally, it is now realized that an increase in PTH levels and a decrease in 25(OH)D levels are associated with inflammation (*73*) which may lead to atherosclerosis. It is conceivable that the magnitude of vitamin D's role in cardiovascular disease through regulating PTH levels is quite vague at the moment.

Novel perspectives

I. Genetic variants in vitamin D homeostasis and cardiovascular disease. Recent advances in genetic testing have permitted, via genome-wide association studies (GWAS), the identification of various single nucleotide polymorphisms (SNPs) in genes encoding proteins involved in vitamin D synthesis, transport and metabolism, that are associated with circulating levels of 25(OH)D (*75*, *76*). Genes included in the results of the GWAS were 7-dehydrocholesterol reductase (DHCR7), CYP2R1, 1,25-dihydroxyvitamin D-24-hydroxylase (CYP24A1), and vitamin D binding protein (GC). Surprisingly, polymorphism of the VDR gene has not been identified, by GWAS, as having an effect on circulating vitamin D levels.

It is quite possible that since SNPs in the aforementioned genes significantly affect 25(OH)D status, they could also act as novel genetic markers for chronic diseases such as cancers and cardiovascular disease, which have been associated with vitamin D deficiency. Such notion has been investigated by a study published in 2012, with myocardial infarction as one of its main outcomes. Although Jorde et al. (*77*) found no association between their investigated SNPs and myocardial infarction (among other outcomes), Levin et al. (*78*) found that variations in the VDR gene may alter the association of 25(OH)D with major clinical outcomes, including myocardial infarction. While results of both studies were heavily supported by a large cohort size, their applicability to other populations is questionable on the basis that one study was confined to elderly white adults (*78*), whereas the other took place in Norway (*77*), the population of which does not possess a significantly lower 25(OH)D level compared to the rest of west Europe despite its latitude, and thus does not represent a vitamin D-depleted population. Nevertheless, it is abundantly clear that large-scale studies investigating different populations are warranted since SNPs in CYP2R1, CYP27B1 and DHCR7 have been associated with type 1 diabetes (*79*), warranting further investigations on diseases not covered by the previously conducted studies.

Our laboratory is currently undertaking several case-control studies investigating the association of selected SNPs in genes involved in the vitamin D pathway, with coronary artery disease. Expected results aim to elucidate novel vitamin D-related genetic markers for cardiovascular disease and thus contribute to the unmasking of the obscure relationship between vitamin D deficiency and cardiovascular disease.

II. Vitamin D and mammalian target of rapamycin (mTOR) signaling: discovery of the elixir of life. Calorie restriction (CR), also referred to as caloric restriction and dietary restriction, has been subject to immense research in recent years. The mere fact that CR enhances longevity and prevents age-related diseases including cardiovascular disease has been baffling scientists ever since its emergence in the early twentieth century (*80*). While the mechanisms by which CR leads to life-extension remain slightly understood, it seems that a key player implicated in the mechanism of CR is the nutrient-sensing mTOR (*80*), a kinase whose dysregulation has been implicated in diseases, notably cancer (*80*); however, its role in cardiovascular diseases in now being extensively studied (*81*). The agreed upon premise so far is that nutrients activate mTOR leading to ageing whereas CR activates sirtuins and AMP-activated protein kinase, which in turn inhibit the mTOR pathway, leading to longevity (*80*).

Concurrently, Sung and Dyck (*82*) recently reviewed the potentially beneficial effects of CR on the cardiovascular system, where they highlighted that these effects span from preventing age-related changes in gene expression to augmenting the cardio-protective signaling pathways, thereby decelerating cardiovascular disease risk factors.

On the other hand, the association of vitamin D with the seemingly complex process of mTOR signaling has been described by Lisse and Hewison (*83*), who illustrated that $1,25(OH)_2D$ causes the upregulation of DNA-damage-inducible transcript 4 mRNA and protein which in turn causes suppression of mTOR.

In view of this, one is intrigued by the notion that vitamin D may serve as the "elixir of life" by preventing debilitating, age-related diseases. Further exploration of

this finding would clarify how this fascinating hormone could act as a novel anti-ageing intervention strategy and how its use may inevitably lead to a healthier, disease-free life. Upon further clarification, this finding may also lead to a better understanding of how vitamin D exerts its cardio-protective properties.

Conclusion

The controversy surrounding the impact of vitamin D on cardiovascular health has reached a new high. Epidemiological data seems disparate, where observational studies from various parts of the globe point toward a strong affiliation between vitamin D deficiency and various cardiovascular diseases and risk factors associated whereas interventional studies seem conflicting. To our knowledge, two large-scale, randomized-controlled trials are currently underway with the purpose of testing the clinical efficacy of vitamin D against cardiovascular disease (*21*).

This review discusses the intricate association from a mechanistic perspective as well, which tends to more strongly support the alleged cardio-protective properties of the vitamin, in spite of inconsistent clinical results. Assessing the data in hand, it is very difficult to question the fact that VDRs are present in the cardiovascular system, affirming the advocated role.

Additional epidemiological and in-vitro studies are needed to shed light on the proposed relationship. Furthermore, with rising interest in the field of ageing biology, it is of paramount importance to explore the potential role of vitamin D as an mTOR inhibitor and thus as an anti-aging molecule.

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