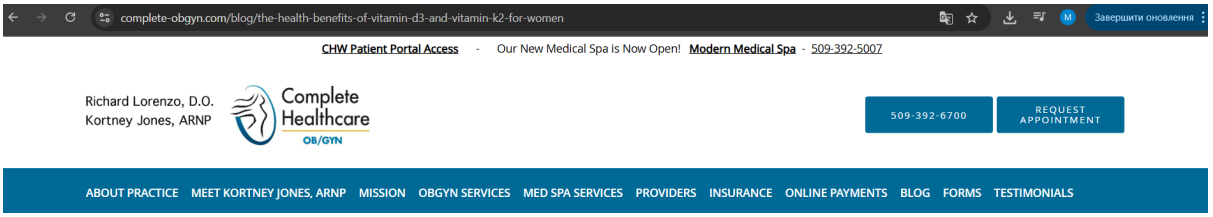


# The Health Benefits of Vitamin D3 and Vitamin K2 for Women

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Vitamin D3, chemically known as cholecalciferol, is an essential fat-soluble vitamin that has taken center stage in recent health discussions. For women, maintaining optimal levels of this vitamin is crucial for a myriad of physiological processes. The interplay of Vitamin D3 and Vitamin K2 presents a fascinating case of how two nutrients can synergistically influence women's health.

Here's why:

1. Bone Health and Osteoporosis Prevention:
One of the most celebrated benefits of vitamin D3 lies in its role in calcium metabolism. Vitamin D3 aids the intestines in absorbing calcium, which is vital for bone mineralization. For women, particularly post-menopausal women, there's a heightened risk of osteoporosis. Adequate levels of vitamin D3 can help mitigate this risk, ensuring bones remain dense and robust.

2. Mood Regulation and Depression Prevention:
Studies have suggested a relationship between vitamin D3 deficiency and mood disorders, including depression. While the mechanisms aren't fully understood, it's believed that vitamin D3 might play a role in the synthesis of serotonin, a neurotransmitter associated with mood regulation.

3. Immune System Support:
Vitamin D3 has been found to modulate both innate and adaptive immune responses. In simple terms, it helps keep the immune system balanced, which is crucial for fighting off infections and warding off illnesses.

4. Heart Health:
Vitamin D3 may play a protective role in heart health, especially hypertension, atherosclerosis, and chronic inflammation — all significant risk factors for heart disease in women.

5. Support in Pregnancy:
Adequate vitamin D3 levels are crucial during pregnancy. A deficiency might be linked to preeclampsia, gestational diabetes, and even bacterial vaginosis. Ensuring optimal levels can support both maternal and fetal health.

6. Alzheimer's Prevention and Cognitive Health:
Emerging research indicates a potential link between vitamin D3 and cognitive health. Here's why:

- Neuroprotection: Vitamin D3 possesses neuroprotective properties, possibly reducing the risk of neurodegenerative diseases.
- Amyloid Plaque Reduction: A hallmark of Alzheimer's is the buildup of amyloid plaques in the brain. Some studies suggest that vitamin D3 might help in reducing the formation of these plaques.
- Inflammatory Response Modulation: Chronic inflammation is believed to play a role in Alzheimer's progression. Vitamin D3 can modulate the body's

inflammatory response, potentially offering protection against inflammation-driven cognitive decline.

Recommended Dosages for Women:

The recommended dosage of vitamin D3 varies based on several factors such as age, skin type, geographical location, and dietary habits. Here's a general guideline:

Adults 800 IU - 5,000 IU per day.

However, many experts believe that these recommended dosages are conservative and that higher doses might be required for optimal health, especially in regions with limited sunlight.

In Conclusion:

Vitamin D3 is undoubtedly a vital component of women's health. From maintaining bone density to supporting mood and immunity, its benefits are manifold. Women, especially those living in areas with limited sun exposure or those with specific dietary restrictions, should consider their vitamin D3 levels and consult with Complete Healthcare for Women for personalized advice.

Remember, while supplements can be beneficial, natural sunlight remains one of the best sources of vitamin D3. Aim for 10-15 minutes of direct sunlight exposure several times a week to harness its benefits.

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# The Impact of Vitamin D on Skin Aging

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**Abstract:** The active metabolites of vitamin $D_3$ ($D_3$) and lumisterol ($L_3$) exert a variety of antiaging and photoprotective effects on the skin. These are achieved through immunomodulation and include anti-inflammatory actions, regulation of keratinocytes proliferation, and differentiation programs to build the epidermal barrier necessary for maintaining skin homeostasis. In addition, they induce antioxidative responses, inhibit DNA damage and induce DNA repair mechanisms to attenuate premature skin aging and cancerogenesis. The mechanism of action would involve interaction with multiple nuclear receptors including VDR, AhR, LXR, reverse agonism on RORα and -γ, and nongenomic actions through 1,25$D_3$-MARRS receptor and interaction with the nongenomic binding site of the VDR. Therefore, active forms of vitamin $D_3$ including its canonical (1,25$(OH)_2D_3$) and noncanonical (CYP11A1-intitated) $D_3$ derivatives as well as $L_3$ derivatives are promising agents for the prevention, attenuation, or treatment of premature skin aging. They could be administrated orally and/or topically. Other forms of parenteral application of vitamin $D_3$ precursor should be considered to avoid its predominant metabolism to 25$(OH)D_3$ that is not recognized by CYP11A1 enzyme. The efficacy of topically applied vitamin $D_3$ and $L_3$ derivatives needs further clinical evaluation in future trials.

**Keywords:** skin aging; photoaging; skin immune responses; vitamin D; vitamin D metabolites; photoprotection



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## 1. Introduction

Skin, like any other organs, undergoes progressive decline in its physiological, morphological, and functional features during aging [1–4]. The phenomenon of aging is natural and genetically predisposed. The functions of the skin are crucial for the homeostasis and survival. As the largest organ in the human body, the skin, together with the hypodermis (subcutaneous fat), is both the source and the target for several hormones and neuromediators [5–17], making it an independent peripheral endocrine organ [5,18]. The skin has also the capacity of producing the prohormone vitamin D and transforming it to active metabolites [19–25], which can exert several different effects on the main skin cells (keratinocytes and fibroblasts) [20,25–29] and immune cells [4,28,30,31] via the activation of the nuclear vitamin D receptor (VDR) [29,32–35]. Vitamin D plays a pivotal role in skin homeostasis contributing to its barrier function [20,29,36–38]. Moreover, as an essential part of a functioning immune system, active forms of vitamin D modulate the cutaneous immunity [8,30,39].

The gold standard of analyzing a vitamin D status is by measuring its major circulating metabolite, 25-hydroxyvitammin $D_3$ (25$(OH)D_3$), via high-performance liquid chromatography (HPLC) or liquid chromatography tandem–mass spectrometry (LC-MS/MS) [40–42].

Recently, a novel sensitive and specific LC-MS/MS method of the simultaneous measurement of 13 circulating metabolites of vitamin $D_3$ and $D_2$ was presented [43].

Importantly, subclinical (30–50 nmol/L) and clinical vitamin D deficiency (<30 nmol/L) in the general population has become a global problem worldwide [44–48]. Several physiological factors may influence vitamin D status, such as age, body mass index (BMI), skin type, pregnancy, and exclusive breastfeeding [49–53]. The genetic polymorphisms of some genes involved in skin pigmentation (*TYR*, *TYRP1*, *EXOC2*, and *DCT*) are also associated with 25(OH)$D_3$ serum concentration [54]. Many environmental factors contribute to vitamin D deficiency, such as the winter season, inadequate sun exposure, and high latitude location [55,56]. Sun avoidance and air pollution are the main factors leading to insufficient UVB exposure. Moreover, ozone and particulate matter (PM) can directly affect the cutaneous production of vitamin D [57]. Additionally, air pollutants, persistent organic pollutants, and heavy metals can behave like endocrine-disrupting chemicals (EDCs), which may cause vitamin D deficiency directly or indirectly. The latter would be secondary to a weight gain, the dysregulation of parathyroid hormone and calcium homeostasis, and a thyroid dysfunction [57,58]. Increasing evidence suggests that smoking can also decrease the serum levels of 25(OH)$D_3$ [57].

With advancing age, the capacity of the skin to produce vitamin $D_3$ decreases (irrespective of the season), and the degradation of its active forms increases [59,60]. It was found that the concentration of the precursor of vitamin $D_3$ in the skin, 7-dehydrocholesterol (7-DHC), declines approximately by 50% from age 20 to age 80 years [59]. Several other factors contribute to the vitamin D deficiency state in accelerated age, including limited sun exposure, insufficient dietary intake of vitamin D, or diseases causing malabsorption. The vitamin D deficiency, which is common in advanced age, can decrease the important physiological functions of the skin such as protection from the environment and prevention of cancer development [25,39,61–66].

In this review, we aimed to discuss the significance of vitamin D in the skin aging process.

## 2. Skin Aging—Your Skin Can Reveal Stories

Skin aging is a complex process that is influenced by the total exposure of both intrinsic and extrinsic factors over the human lifespan (skin exposome), which is responsible for the progressive morphological and functional alterations of the aged skin [1,67–69]. The main internal factors affecting the chronological (physiological) skin aging include a hormonal decline and changes in gene expression with advancing age [1]. In addition, the cutaneous regenerative potential decreases with the age due to the excessive senescence of keratinocytes, fibroblasts, and melanocytes over time, which contributes to skin aging [70–72]. The aged skin is characterized by fine wrinkles and atrophy with reduced elasticity. This chronological aging affects all skin areas but shows phenotypic differences among different anatomical regions, and it varies significantly within different populations [67,73]. The single nucleotide polymorphisms (SNPs) of the *MC1R* gene (main regulator of melanin pigmentation [74]) are significantly linked to a perceived facial age, providing a new molecular base of youthful looks [75].

The most prominent external stressors affecting skin and causing its premature aging include ultraviolet (UV) radiation [76,77], ambient pollutants [78–82], and smoking [57,83]. The continuous exposure of the skin to these environmental insults stimulates the production of reactive oxygen species (ROS) and generate oxidative stress [84,85]. The environmental factors can also cause an impairment of the epidermal barrier function [69] and alterations in skin microflora [86,87], leading to significant morbidity [2,88].

Ultraviolet radiation (UVR) is the most harmful external factor contributing to the cutaneous photodamage on the sun-exposed areas. Photoaged skin is presented as a dry, deep-wrinkled skin with rough texture, dyschromia and senile lentigines, vascular complications, etc. [68,89]. UVR decreases the expression of filaggrin that contributes to epidermal hydration, so its downregulation from UVR could explain the skin xerosis in photoaging [90]. Both UVA (315–400 nm) and UVB (280–315 nm) have been shown

to contribute to photoaging, either by imbalanced ROS production or by direct DNA damage [83]. However, UVA is considered to play a major role in the aging process. UVA represents more than 80% of total daily UV irradiation and can penetrate 5–10 times deeper into the reticular dermis, where it can damage the extracellular matrix (ECM) more significantly than UVB [91]. Moreover, UVA exposure increases the expression of matrix metalloproteinases (MMPs), especially the expression of the collagenolytic enzyme MMP-1 in dermal fibroblasts, which acts as an important regulator in photoaging [92,93]. Additionally, the chronic UVA irradiation inhibits hyaluronan synthesis, thus altering the composition of proteoglycans in the dermis [94]. A long-term exposure of UVA is related to photoaging and photocancer due to an overproduction of ROS and reactive nitrogen species (RNS), which can disrupt both the nuclear and mitochondrial DNA [95,96]. UVB can penetrate only through the epidermis but is biologically more active. It also induces the transformation of 7-DHC to vitamin $D_3$ [97,98]. UVB absorbed by DNA and RNA induces a formation of cyclobutane pyrimidine dimers (CPDs) and other photoproducts [99], thus inducing various solar signature mutations in specific genes, including the tumor suppressor gene p53 [100,101]. UVR induces an accumulation of p53 protein in the nucleus that in turn activates the transcription of genes responsible for cell cycle arrest allowing DNA repair, as well as causing an induction of apoptosis of the cells with unrepaired DNA damage [102,103]. Specific p53 mutations can be found in high rates, not only in actinic keratosis (precancerous state) and squamous cell carcinomas (60–90%) with typical UV signature, but also in the normal appearance of UV-exposed skin (about 75%), compared with a much lower rate of such mutations in healthy sun-protected skin (5% of all cases) [104].

Chronic sunlight exposure, together with the persistence of cellular senescence, can drive an impaired regenerative capacity of the skin, chronic inflammation, and photoaging, which correlates with enhanced cancer risk [77,105–107]. Thereby, photoaging results in premature skin aging. Although some aging mechanisms share several similarities or overlapping, photoaged skin differs from physiologically aged skin in the ECM changes. Photoaged skin is characterized by degraded collagen and accumulated aberrant elastin fibers and glycosaminoglycans, whereas physiologically aged skin is presented by the atrophy of dermal structures [108].

The negative impact of ambient pollutants on human health and the human skin is of growing concern [109]. Ozone ($O_3$) from the smog and PM, primarily contacting with the skin, is capable to stimulate ROS production and generate oxidative stress, leading to phenotypic features of extrinsic aging [69]. It was found that chronic exposure to PM leads to pigment spots and deep nasolabial folds [110,111]. Moreover, ultrafine particles (<0.1 μm) can penetrate tissues and localize in the mitochondria, causing an aberrant mitochondrial function because of the oxidative processes [112]. Additionally, the photo-pollution exposure may aggravate UVR-mediated skin aging [113].

UVR, predominantly UVA, by the excessive amount of ROS activates the mitogen-activated protein kinases (MAPKs) and transcription factors such as nuclear factor erythroid 2-like (Nrf2), c-Jun-N-terminal kinase (JNK), and nuclear factor-κβ (NF-κB), and increases the transcription of MMPs [114]. Activated MMPs, together with the decreased expression of MMP inhibitors (TIMP), cause a dysregulation of the ECM homeostasis and a progressive damage of collagen and elastin [115]. Additionally, UVR impairs the endogenous antioxidant enzymes, leading to an increased oxidative damage of collagen. The destruction of ECM integrity is visualized as wrinkle appearance in photo-damaged skin [93]. The activation of redox-sensitive transcription factors, the activator protein-1 (AP-1) and NF-κβ, involved in wrinkle formation and inflammation, plays crucial roles in skin aging [88]. Both factors, NF-κβ and AP-1, are elevated within hours after cutaneous exposure to low-dose UVB. The upregulation of AP-1 suppresses the transforming growth factor β (TGF-β) receptors, which further blocks the synthesis of procollagen [116,117]. Additionally, activated AP-1 stimulates the degradation of collagen by MMPs and triggers the main activator of the inflammatory response NF-κβ [118]. NF-κβ signaling is a

well-known regulator of tissue homeostasis, and its central role in skin aging was recently underlined [119]. The ROS-induced activation of NF-κβ drives an increase of proinflammatory cytokines and MMPs and decreases TGF-β and type I collagen synthesis [119]. The proinflammatory cytokines (interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α) stimulate inflammatory responses and enhance the activation of NF-κβ [93]. It was found that NF-κβ expression could increase in mitochondrial DNA (mtDNA)-depleter mice and after restoring the mitochondrial function, the NF-κβ expression could be reduced. These data confirm that NF-κβ signaling is a key mechanism contributing to the skin and hair follicle pathologies [120]. Due to the longer wavelength, UVA reaches the dermal fibroblasts in vivo with the activation of the Nrf2-mediated expression of antioxidant genes. Unlike UVA, UVB does not activate Nrf2 in skin cells or even appears to have an inhibitory effect [121]. However, vitamin $D_3$ derivatives, which are products of UVB action, do activate Nrf2 signaling [122]. The endogenous Nrf2 is essential for the protection of skin cells against oxidative insults and for regulating the redox balance during skin aging [123,124]. Many in vitro and in vivo studies confirmed the importance of the transcription factor Nrf2 and its downstream signaling in UV protection [125,126].

Indeed, the human skin aging is mainly driven by oxidative events. An extensive ROS production and insufficient scavenging activity or a mitochondrial dysfunction are crucial events in oxidative stress-induced skin aging. The high levels of ROS lead to oxidative damage of lipids, proteins, genomic, and mtDNA, and also can deplete and damage the antioxidant defense systems of the skin (both non-enzymatic and enzymatic one) [85,127].

Accumulating evidence support a strong link between the mitochondrial dysfunction and the aging process [126]. Many studies demonstrate a decrease in mtDNA content and mitochondrial number during aging. It is thought that mitochondrial dysfunction plays a role in accelerated cellular senescence, seen in advancing age [128–130]. Furthermore, mitochondria are believed to contribute to 90% of generated ROS in the cells [95]. mtDNA, as an important target for ROS, is highly vulnerable to the oxidative damage and possesses inefficient DNA repair mechanisms [96,131]. The functional decline of the mitochondria leads to vicious cycle effect contributing to further enhancement of ROS production [127,132].

## 3. Effects of Vitamin $D_3$ on the Skin

### *3.1. Impact Paths on the Skin*

Excessive exposure to solar UVR accelerates skin aging and could trigger cutaneous cancerogenesis [133]. However, UVR plays a beneficial role in the regulation of many skin functions [56,77,134]. The same UVB, responsible for the increase of non-melanoma skin cancer, is required for vitamin $D_3$ production in the skin that supplies more than 90% of the vitamin $D_3$ body's requirement [44,55,135]. In the skin, vitamin $D_3$ is essential for the formation of the epidermal barrier and hair follicles, and its deficiency has been linked to many proliferative and inflammatory cutaneous disorders [20,29,44,136].

Upon the absorption of UVB, 7-DHC is transformed to vitamin $D_3$ in the skin, a process accelerated by thermal energy. Prolonged UVB exposure can also generate tachysterol ($T_3$) and lumisterol ($L_3$) [24,97]. These reactions are non-enzymatic and dependent on the UVB dose and the temperature. Vitamin $D_3$ can be activated through canonical and non-canonical pathways with similar activation of $L_3$ to biologically active metabolites (Figure 1). In the classical pathway, vitamin $D_3$ is hydroxylated to 25-hydroxyvitamin $D_3$ ($25(OH)D_3$) by CYP2R1 and/or CYP27A1 in the liver with further hydroxylation by CYP27B1 in the kidney, skin, and other tissues to its biologically active metabolite $1,25(OH)_2D_3$ [20,21,137].

In the alternative (non-canonical) pathway, vitamin $D_3$ can be activated by CYP11A1 with further modification by other cytochrome enzymes leading to production of large number of metabolites in humans [21,36,138–142] (Figure 1), some of which are non- or low-calcemic at high, therapeutic, doses [143–146]. The major CYP11A1-derived vitamin $D_3$ products are $20(OH)D_3$ and $20,23(OH)_2D_3$ [23,139,147,148]. In addition, $20(OH)D_3$ can be defined also as a natural product because its presence in the honey [149]. The $L_3$ can

also be metabolized to biologically active derivatives [150–152], which are not recognized by the 7-DHC reductase [153].

The main genomic effects and biological responses of vitamin D metabolites in the skin are mediated through their binding to the nuclear VDR [32,61,154–156]. Notably, VDR has been reported to regulate about 3% of mammalian genome due to its broad expression in all tissues [4,34,157,158]. The skin also expresses the VDR and serves not only as a source but also as a site for the action of vitamin $D_3$ [28,39]. Additionally, the VDR activated by classical $1,25(OH)_2D_3$ can induce rapid response signaling through a non-genomic, membrane-associated mechanism based on an alternative ligand-binding site [159] or through action on $1,25D_3$-MARRS receptor [156,160,161]. Similar non-genomic activities for CYP11A1-derived hydroxyderivatives are still not established. SNPs can affect VDR activity favoring a development of melanoma and non-melanoma skin tumors [162,163]. VDR functions as a tumor suppressor [164] and a decrease in its expression is associated with progression of cutaneous melanoma [165,166]. On the opposite, the nuclear VDR expression has been found significantly elevated (moderate to strong) in squamous cell carcinomas (SCCs) and basal cell carcinomas (BCCs) compared to in normal skin [167,168]. Thus, targeting VDR with vitamin D secosteroids (especially low calcemic ones) would be rational in skin cancer prevention, attenuation, or therapy [62,64,169].

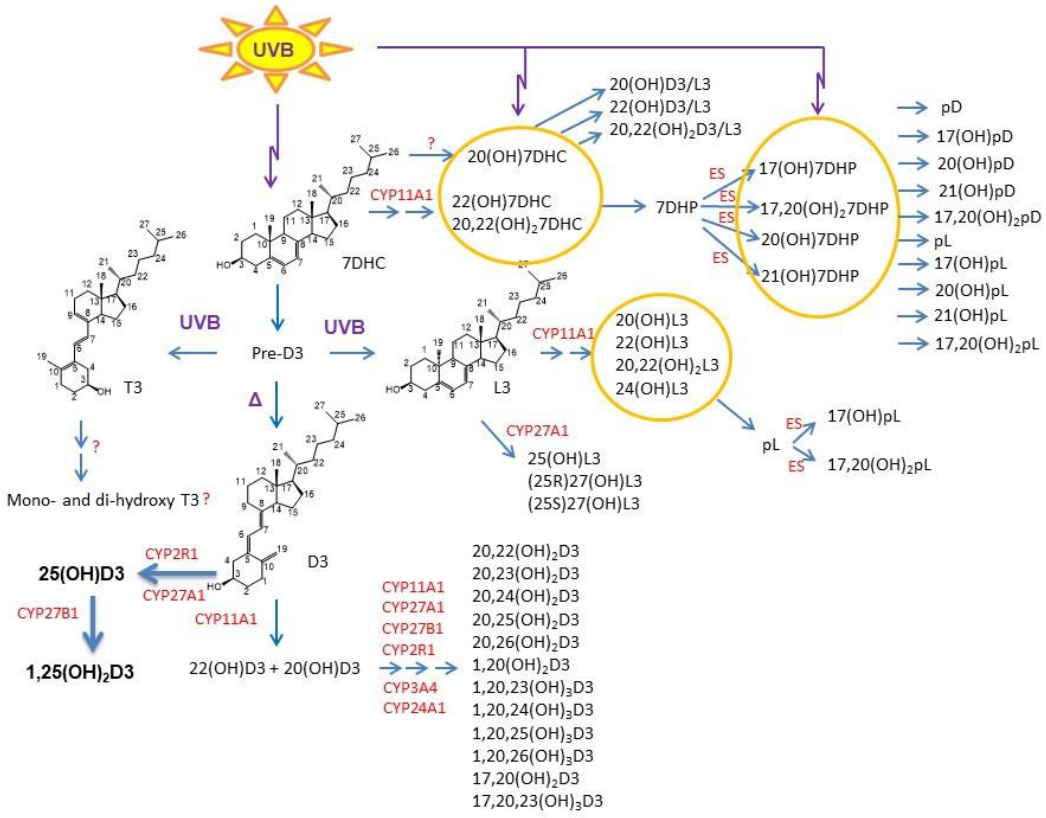


**Figure 1.** Noncanonical pathways of vitamin $D_3$ and lumisterol ($L_3$) activation (reprinted from [61] with the permission from Springer). $D_3$, $L_3$, and 7-DHC are substrates for CYP11A1 that produces the corresponding hydroxyderivatives. In the case of $L_3$ and 7-DHC, the side chain can be cleaved by CYP11A1 to produce 7DHP or pL that can be further metabolized by steroidogenic enzymes (ES). In the skin, UVB acting on 5,7-dienes can lead to the production of $D_3$, $L_3$, and $T_3$ derivatives with a full-length side chain and pD, pL, and pT derivatives with a shortened side chain. While the cut-off for UVC/UVB is 280 nm, we show the range of 290–315 nm because wavelengths below 290 nm are filtered by the ozone layer and no additional pre-$D_3$ is produced above 315 nm [170]. 7DHC, 7-dehydrocholesterol; 7DHP, 7-dehydropregnenolone; pD, preganacalciferol; pL, preganalumisterol; $D_3$, vitamin $D_3$; $L_3$, lumisterol; $T_3$, tachysterol; OH, hydroxyl group; number before OH, carbon number with OH; number in subscripts after (OH), number of hydroxyl groups.

CYP11A1-derived hydroxyderivatives can regulate some skin functions through other nuclear receptor such as retinoic acid-related orphan receptors (ROR) α and γ, which expressed in the skin [171]. The endogenously produced nonclassical vitamin $D_3$ hydroxyderivatives, $20(OH)D_3$ and $20,23(OH)_2D_3$ can act as weak RORα and RORγ inverse agonists [155,171,172]. Moreover, these hydroxyderivatives could exert anti-inflammatory effect and could inhibit tumor progression in the skin via RORγ-mediated mechanism [173].

Alternative, the classical $1,25(OH)_2D_3$ and CYP11A1-initiated vitamin $D_3$ derivatives can act as agonists on aryl hydrocarbon receptor (AhR) [174] and liver X receptors (LXR) [175]. Moreover, the activation of AhR is the top canonical pathway for $20,23(OH)_2D_3$ [174]. This receptor regulates cellular proliferation, inflammation, and melanogenesis in the skin [176]. Although many different ligands can target AhR, some functional studies and molecular modeling can predict that secosteroidal signal transduction further leads to the downregulation of proinflammatory responses [177], detoxification, and antioxidative action [61,174].

Summarizing, the biologically active classical and novel vitamin $D_3$ metabolites exert different affinities to multiple receptors in the skin and through their modulation they can influence different cutaneous pathologies. In addition to act on the VDR, the active forms of vitamin D can act on alternative nuclear receptors including RORs, AhR, LXR, and $1,25D_3$-MARRS receptor. The active forms of vitamin $D_3$ have various functions, which partially overlap in their anti-inflammatory, antimicrobial, antiproliferative, prodifferentiation, antifibrotic, and antioxidative effects on the skin [20,38,63,141,145,178]. Along with the best characterized $1,25(OH)_2D_3$, CYP11A1-derived products of vitamin $D_3$ and $L_3$ exhibit photoprotective properties against UVR-induced skin damage (Figure 2) [37,61,179–183].

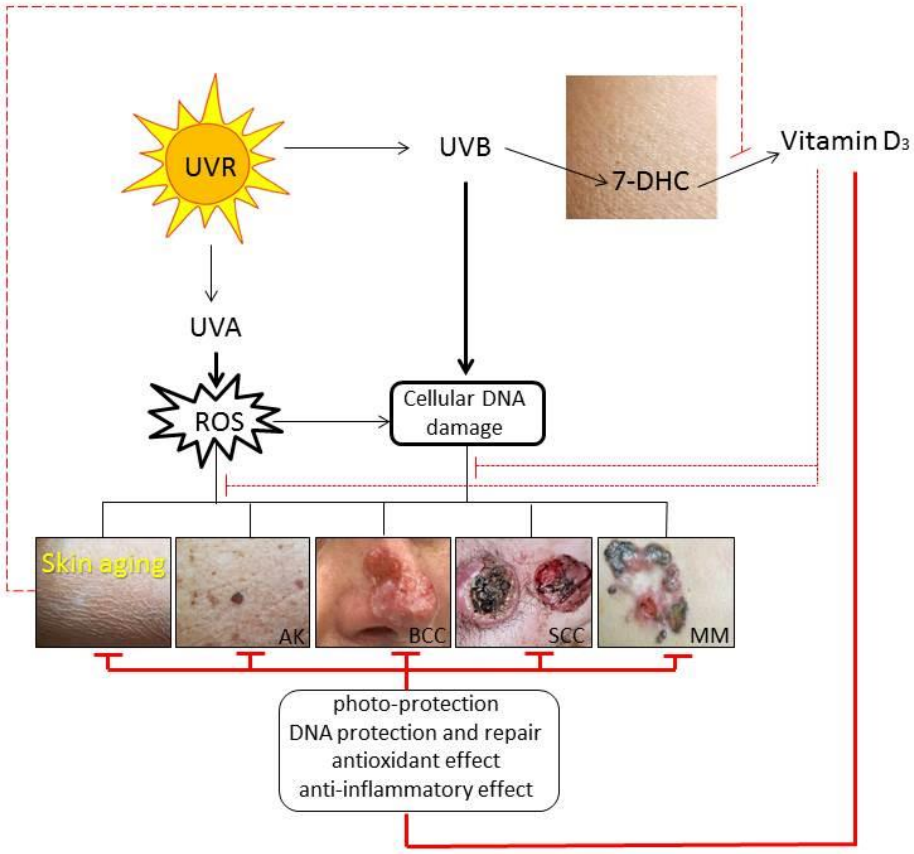


**Figure 2.** Photoprotective effects of vitamin $D_3$ in premature skin aging and cutaneous cancerogenesis. Abbreviations: 7-DHC, 7-dehydrocholesterol; AK, actinic keratosis; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; MM, malignant melanoma.

### 3.2. Effects on the Cutaneous Immune Function

Vitamin $D_3$ and its analogues and precursors play an important complex role in the regulation of both the innate and adaptive immune systems, including in the skin [8,184,185]. There is a clear connection between vitamin D deficiency and incidences of infections and immune-mediated skin diseases [31,186–188]. The expression of the VDR has been found in almost all immune cells including T- and B-lymphocytes (Lym), macrophages, mast cells, natural killer (NK) cells, and regulatory T cells (Tregs), but it is differently regulated [189]. Monocytes, for example, lose VDR expression levels during differentiation towards macrophages and dendritic cells (DCs) [190,191]. Immunomodulatory actions of active vitamin $D_3$ metabolites include the induction of Tregs [192] and Thelper-2 (Th2)-Lym, together with the downregulation of proinflammatory Th1/Th17/Th9-Lym [193]. $1,25(OH)_2D_3$ can have both direct and indirect effects on T-Lym [194]. The indirect effect is based on DC-derived cytokines, which modulate Th-Lym response [30]. Directly, $1,25(OH)_2D_3$ can suppress the immune cell production of inflammatory cytokines such as interferon gamma (IFN-γ), TNF-α, and IL-2 (Th1 cytokines), IL-17/21 (Th17 cytokines), and Th9 cytokines [193,195,196], while it enhances either the levels of anti-inflammatory IL-10 from Tregs [197] or Th2-derived IL-4 [198]. As a result, vitamin D shifts Th1 inflammatory response towards more tolerogenic Th2 response with an increase of CD4 + CD25 + Tregs reflected to a change in cytokine profile in the skin [199]. Additionally, $1,25(OH)_2D_3$ influences the activated B-Lym by inducing apoptosis [200], suppressing immunoglobulin E (IgE)-dependent mast cell activation [201,202], and upregulating IL-10 production [203]. Enhanced IL-10 synthesis contributes to a suppressed mast cell-mediated inflammation and IgE-related allergic reactions [201]. $1,25(OH)_2D_3$ and its analogues directly regulate antimicrobial peptide (AMP) gene expression in innate immune cells [204,205]. Moreover, it has been found that vitamin D is able to induce essential for antimicrobial defense, production of cathelicidin (LL37) [204] and to modulate the phagocytic activity of macrophages and NK cells [193]. Additionally, vitamin D exerts immunosuppressive effects through the modulation of epidermal Langerhans cells [206] and the proliferation of the Tregs number [184,185,197].

CYP11A1 is expressed also in immune cells [207], where vitamin D can be further metabolized to biologically active hydroxyderivatives [31,39]. Via the activation of VDR or through the inhibition of RORγ-mediated activation, $20(OH)D_3$ and $20,23(OH)_2D_3$, like $1,25(OH)_2D_3$, can attenuate Th17 differentiation, as well as the formation and activity of inflammatory cytokine IL-17 by immune cells [155,208,209]. Thus, Th17-related cutaneous inflammation could be successfully modulated via RORγ inverse agonists such as CYP11A1-derived $D_3$-hydroxyderivatives, causing the regulation of the immune system and a resistance against autoimmunity [210,211]. The most recently inhibition of collagen-induced autoimmune arthritis by CYP11A1-derived $20(OH)D_3$ was reported [212].

### 3.3. Impact on Skin Aging

The normal vitamin $D_3$ status is important for a general prevention of premature aging maintaining a healthful skin aging [213,214]. Vitamin $D_3$ metabolites including its classical ($1,25(OH)_2D_3$) and novel (CYP11A1-intitated) $D_3$ hydroxyderivatives exert many beneficial protective effects on the skin, which could influence the process of premature aging via many different mechanisms, leading to a delay or attenuation of both chronological skin aging and photoaging. Skin-resident cells (keratinocytes, fibroblasts, and sebocytes) are capable of locally activating vitamin $D_3$ [23,36,215] and exhibiting a diverse biological effect such as photoprotection and immunosuppression, similar to the UVR-induced one [179,216].

The process of chronological aging is associated with immunological alteration and the imbalance between inflammatory and anti-inflammatory mechanisms, leading to a chronic low-grade inflammation, known as “inflammaging” state [217,218]. The “inflammaging” phenotype of the skin and hair follicles is a result of both chronic antigen stimulation and continued exposure to oxidative stress caused by ROS and RNS [219,220]. With advancing

age, skin is affected by the profound remodeling of the immune system, leading to a decline in its adaptive capacity [221,222]. Th1- and Th17-related markers, together with the number of epidermal DCs are increased as a function of age [223–225]. DCs during aging appear to be functionally impaired, which contributes to an initiation of inflammatory and autoimmune skin disorders and a loss of their protective role against cutaneous infections. The active forms of vitamin $D_3$ are able to decrease the proliferation and cytotoxicity of T-Lym, as well as to suppress the differentiation of B-Lym and the maturation of DCs [193]. Therefore, vitamin $D_3$ hydroxyderivatives exert potent anti-inflammatory activities including the inhibition of TNF-$\alpha$, INF-$\gamma$, and IL-1/6/9/17 production [4,38,185], suggesting their implication in the modulation of skin inflammation. Moreover, the noncalcemic and nontoxic 20(OH)$D_3$ has shown a similar anti-inflammatory property in vivo to 1,25$(OH)_2D_3$ (hypercalcemic in high doses) through the suppression of the immune responses by T- and B-lym [155,212].

Active vitamin $D_3$ metabolites can protect skin against the hazardous effects of skin aging-triggering agents, including UVR, pollution, and microbial infections [179,226–230]. It has been shown that the oral administration of high-dose vitamin $D_3$ shortly after UVB exposure could reverse rapidly the photo-induced cutaneous damage by decreasing the inflammation and induction of the repair mechanisms of the epidermal barrier [38]. There is strong experimental evidence that active vitamin $D_3$ and $L_3$ hydroxyderivatives can induce, in a dose-dependent manner, antioxidative responses and reverse the UVB-mediated ROS production in keratinocytes by the activation of Nrf2 that works for cytoprotection and detoxification, thus attenuating photoaging [122]. Therefore, they serve as protective agents against UVB-induced oxidative stress in cells, pre-treated with each of these active metabolites for 24 h prior to UVB irradiation (50 mJ/$cm^2$) [122]. These hydroxyderivatives stimulate the expression of antioxidant-response genes downstream of Nrf2 (*GR*, *HO-1*, *CAT*, *SOD-1*, and *SOD-2*) as well as the expression of HO-1, CAT, and MnSOD at the protein level [122]. The transcription factor Nrf2 plays an important role in the detection of excessive ROS and RNS and in the induction of mechanisms counteracting the oxidative damage and skin pigmentations produced by UVA [121,125,231].

Chronic UVR irradiation, mainly UVB [232] and UVA [233], induces DNA damage and the formation of CPDs that potentially lead to premature skin aging and carcinogenesis. CYP11A1-derived $D_3$ and $L_3$ hydroxyderivatives, along with 1,25$(OH)_2D_3$, demonstrate photoprotective and reparative properties by increasing the expression and phosphorylation of p53 with its translocation to the nucleus [61,229,234,235]. The P53 gene family, in particular its isoform p63, might be an important molecular target for vitamin D action in premature aging and cancer [236], which are promoted by similar mechanisms [237].

Moreover, 1,25$(OH)_2D_3$ and 1,25$(OH)_2L_3$ inhibit DNA damage and facilitate DNA repair by the reduction of CPDs [182,235,238,239] and RNS [178,234]. The photoprotection by 20(OH)$D_3$ and 20,23$(OH)_2D_3$ is comparable to 1,25$(OH)_2D_3$ reduction of UVB-induced CPDs and DNA fragmentation in vivo [181,182] and in vitro [178]. In addition, both 20(OH)$D_3$ and 20,23$(OH)_2D_3$ stimulate differentiation, inhibit proliferation and downregulate proinflammatory responses in keratinocytes via the decrease of NF$\kappa\beta$ activity [240,241]. It was shown recently that not only the pretreatment, but also the post-treatment of keratinocytes with CYP11A1-derived $D_3$ and $L_3$ derivatives can reverse their UVB-induced damage [37,230].

Additionally, 1,25$(OH)_2D_3$ can induce rapid and dose-dependent reduction in skin cell apoptosis, and it can increase CPDs repair and decrease the oxidative DNA damage through non-genomic energy-conserving autophagy and mitophagy [227], thus contributing to the intrinsic skin photoprotection mechanism [242].

## 4. Conclusions and Future Perspectives

Vitamin $D_3$ and its active metabolites exert a variety of antiaging and (photo) protective effects on the skin. These are achieved through immunomodulation that include anti-inflammatory actions and regulation of keratinocytes proliferation and differentiation

program to build the epidermal barrier necessary to maintain skin homeostasis. In addition, they induce antioxidative responses, inhibit DNA damage and induce DNA repair mechanisms to attenuate premature skin aging and cancerogenesis. Similar actions can be assigned to lumisterol metabolites. Therefore, active forms of vitamin $D_3$ including its canonical ($1,25(OH)_2D_3$) and noncanonical (CYP11A1-intitated) $D_3$-hydroxyderivatives as well as $L_3$-derivatives are promising agents for the prevention, attenuation, or treatment of premature skin aging, when applied topically. It is expected that they will attenuate photoaging and perhaps repair the existing damage induced by external stressors. The mechanism of action would involve interaction with nuclear receptors including VDR, AhR, LXR, reverse agonism on RORα and RORγ, and nongenomic actions through $1,25D_3$-MARRS receptor and interaction with the nongenomic binding site of the VDR. The regulatory mechanism affected by $D_3$ and $L_3$ derivatives would include the activation of Nrf2 and p53 and the downregulation of NFκβ signaling pathways or the regulation of mitochondrial functions. To prevent skin aging, vitamin $D_3$ and lumisterol or their derivatives could be administrated orally and/or topically. Other forms of parenteral application of the vitamin $D_3$ precursor should be considered to avoid channeling its metabolism to $25(OH)D_3$, which is not recognized by CYP11A1 enzyme [243]. The efficacy of topically applied vitamin $D_3$ and $L_3$ derivatives needs further clinical evaluation in future trials.

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*Review Article*

# The Synergistic Interplay between Vitamins D and K for Bone and Cardiovascular Health: A Narrative Review

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Vitamins D and K are both fat-soluble vitamins and play a central role in calcium metabolism. Vitamin D promotes the production of vitamin K-dependent proteins, which require vitamin K for carboxylation in order to function properly. The purpose of this review is to summarize available evidence of the synergistic interplay between vitamins D and K on bone and cardiovascular health. Animal and human studies suggest that optimal concentrations of both vitamin D and vitamin K are beneficial for bone and cardiovascular health as supported by genetic, molecular, cellular, and human studies. Most clinical trials studied vitamin D and K supplementation with bone health in postmenopausal women. Few intervention trials studied vitamin D and K supplementation with cardiovascular-related outcomes. These limited studies indicate that joint supplementation might be beneficial for cardiovascular health. Current evidence supports the notion that joint supplementation of vitamins D and K might be more effective than the consumption of either alone for bone and cardiovascular health. As more is discovered about the powerful combination of vitamins D and K, it gives a renewed reason to eat a healthy diet including a variety of foods such as vegetables and fermented dairy for bone and cardiovascular health.

## 1. Introduction

Worldwide, a large group of people is prescribed to a supplemental regime of both vitamin D and calcium. In Europe, depending on a country and sex, between 1 and 66% of the adult population use vitamin D supplements [1, 2]. Over the last decade, large vitamin D supplementation is promoted to restore 25-hydroxyvitamin D (25(OH)D) concentrations and is considered to be safe with doses up to 4000 international units (IU) per day [3]. However, little is known about potential long-term high-dose vitamin D supplementation [2, 4].

Vitamin D is a fat-soluble vitamin that can be ingested by foods such as fatty fish, dairy products, and eggs, but is mainly synthesized by the human skin when exposed to sunlight. In the liver, vitamin D is hydroxylated to 25(OH)D, the main circulating vitamin D metabolite that is measured to assess and classify vitamin D status. Circulating 25(OH)D is further metabolized by the kidney for full biological activity into its most active form 1,25-dihydroxyvitamin D (1,25(OH)D) also known as calcitriol. Calcitriol is also produced endogenously by extrarenal production through peripheral 1-$\alpha$-hydroxylase and has positive effects on immune function and anticancer activity [5–7]. Vitamin D

plays a main role in regulating calcium metabolism by increasing intestinal calcium absorption [8]. Ample evidence recommends vitamin D supplementation for the prevention of falls and fractures [9, 10]; however, evidence suggests calcium precipitation in the vasculature and other potential side effects [4, 11–14].

Vitamin K is another fat-soluble vitamin that exists in two forms of vitamin K: vitamin $K_1$ (phylloquinone, mainly found in green leafy vegetables) and vitamin $K_2$ (menaquinone, mainly found in fermented dairy and produced by lactic acid bacteria in the intestine) [15]. Vitamin K stores are limited, but they can be recycled to a certain extent [16]. Vitamin $K_1$ is principally transported to the liver, regulating the production of coagulation factors, while vitamin $K_2$ is transported to extrahepatic tissues, such as bone and the vascular wall, regulating the activity of matrix Gla protein (MGP) and osteocalcin (bone Gla protein)—the main vitamin K-dependent proteins. They require vitamin K for carboxylation in order to function properly. When circulating concentrations of vitamin K are insufficient, a greater proportion of MGP and osteocalcin remain uncarboxylated, which is associated with unfavorable outcomes such as cardiovascular disease, lower BMD, and osteoporosis [17]. The current recommendation for vitamin $K_1$ intake is 70 $\mu$g/day for all adults defined by an adequate intake [18]. This amount is solely based on maintaining coagulation function and might not be enough for optimal carboxylation of other vitamin K-dependent proteins, which require higher amounts of vitamin K [19].

The role of vitamin K in cardiovascular health has mainly been studied in isolation [20]; however, a growing body of evidence suggests a synergistic effect of vitamin K combined with vitamin D [21–26]. Vitamin D promotes the production of vitamin K-dependent proteins, as shown in rats by Karl et al. already in 1985 [27]. These findings cannot be explained by our current understanding of the biochemical role of vitamin K, but suggest that vitamin D may influence vitamin K-dependent proteins [28].

The purpose of this narrative review is to summarize available evidence in the field of the synergistic interplay between vitamins D and K on bone and cardiovascular health. The primary focus is on the general population and includes observational studies that investigated both vitamin D and vitamin K status with outcome measures and supplementation studies that administered both vitamins D and K.

## 2. Interaction of Vitamins D and K for Bone Health

*2.1. Experimental Studies.* In experimental models, the exploration of the interaction between vitamins D and K on bone health is ongoing for decades and a fair amount of literature is available. Recent understanding suggests that vitamin D enhances vitamin K-dependent bone protein concentrations and induces bone formation in vitro [29–31] with stimulation of osteoblast-specific gene expression [32]. Osteoblast-specific expression of osteocalcin is controlled at the transcriptional level by 1,25(OH)D through the 1,25(OH)D-responsive element within the promoter of the osteocalcin gene [32]. The underlying mechanism of mineralization induced by vitamin K in the presence of 1,25(OH)D was different from vitamin K alone [33]. In rats, 1,25(OH)D receptor binding can undergo gamma-carboxylation in the presence of vitamin K. This means that 1,25(OH)D receptor carboxylation can potentially modify the intrinsic biochemical properties of the nuclear receptors and modulates its binding to DNA [34].

The effect of 1,25(OH)D and warfarin—a vitamin K antagonist—on the vitamin K cycle was studied in cultured osteoblasts [26]. Epoxide reductase, one of the key enzymes in the vitamin K cycle, was strongly inhibited by warfarin, whereas it was not affected by 1,25(OH)D, meaning that the vitamin K metabolic cycle functions normally in human osteoblasts.

Human osteoblast cell cultures indicate that glycoxidation interferes with the maturation of osteoblasts; however, this process may be counterbalanced by adding vitamins D and K, which reverses the detrimental glycoxidation on several bone markers [35]. Therefore, the addition of vitamins D and K may induce important biochemical changes in bone, which may exert therapeutic effects on bone metabolic diseases such as osteoporosis [36].

*2.2. Animal Models.* A growing body of evidence is also documenting the interaction between vitamins D and K in animal models. The effect of vitamin K of bone mineralization is enhanced by plasma 25(OH)D concentration. Vitamin K was administered to prevent osteoporosis in ovariectomized rats, but bone loss was only prevented in rats fed with a diet containing vitamin D or vitamin D supplementation [37, 38]. These findings suggest that combined treatment with vitamins D and K is more effective than vitamin K alone particularly in the early phase of estrogen deficiency after menopause.

Vitamin K and vitamin D supplementation on calcium balance was investigated in young rats fed with a normal or low calcium diet, plus vitamin K and/or vitamin D [39]. Vitamin K supplementation promoted the reduction in urinary calcium excretion and stimulated intestinal calcium absorption in rats on a normal calcium diet. Vitamin D supplementation stimulated intestinal calcium absorption with prevention of the abnormal elevation of serum PTH concentrations, prevented hypocalcemia in rats fed with a low calcium diet, and stimulated intestinal calcium absorption in rats fed with a normal calcium diet. The stimulation of intestinal calcium absorption was associated with increased 1,25(OH)D concentrations. An additive effect of vitamin K and vitamin D on intestinal calcium absorption was only found in rats fed with a normal calcium diet. This study shows the differential effects of vitamin K and vitamin D supplementation on calcium balance in young rats fed with a normal or low calcium diet.

*2.3. Observational Evidence.* Human evidence for the role of 1,25(OH)D in stimulating vitamin K-dependent proteins is scarce. In hemodialysis patients, vitamin D analog users had much higher concentrations of bone Gla protein (BGP) than nonusers indicating that vitamin D administration

may play a role in stimulating vitamin K-dependent protein activity [40]. More research on the stimulating role of vitamin D on vitamin K-dependent proteins is urgently needed to study the underlying mechanisms.

Some observational studies support the hypothesis that optimal concentrations of both vitamins D and K support bone mineralization and lower fracture risk. In a cross-sectional study among Japanese older men, lower 25(OH)D and vitamin $K_1$ concentrations were concomitantly associated with BMD, indicating a nonestrogen-dependent pathway in men [41]. In a case-control study of 184 Norwegian older adults, the combination of low vitamin $K_1$ and low 25(OH)D was synergistically associated with hip fractures: odds ratio 7.6 (95% CI 2.3, 26.7) [42]. In the NOREPOS study, another Norwegian population study, similar results were observed among 1318 older adults [43]. During 8.2-year follow-up, the combination of both low vitamin D and $K_1$ concentrations was associated with a greater hip fracture risk, hazard ratio 1.41 (95% CI 1.09, 1.82), compared to the high vitamin D and vitamin K category. No increased risk was observed in the groups low in 1 vitamin only. These results indicate that the combination of low concentrations of vitamin $K_1$ and 25(OH)D is associated with increased risk of hip fractures.

*2.4. Human Intervention Studies.* A small study among 15 healthy women indicated that 3 weeks of supplementation of 20 ml extra virgin olive oil enriched with vitamins D, K, and $B_6$ resulted in lower concentrations of uncarboxylated osteocalcin [44]. This means that a vitaminized oil can influence vitamin K-dependent proteins within multiple weeks.

An increasing amount of randomized controlled trials have demonstrated the combined effects of vitamins D and K on postmenopausal osteoporosis mostly pursued in Japan with a study duration between 8 weeks and 3 years (Table 1). A randomized trial with 4 arms (diet, menaquinone-4, cholecalciferol, and menaquinone-4 + cholecalciferol) showed that only the vitamin K plus vitamin D arm increased BMD [45]. Similar results were found in another trial with postmenopausal women with osteoporosis ≥ 5 years after menopause [46]. After 2 years of follow-up, the longitudinal changes in BMD were significant compared with those in the calcium lactate-, vitamin D-, and vitamin K-only groups ($P < 0.001$). A modest synergistic effect of vitamins D and K was found after 2 years in healthy older women from nutritionally relevant intakes of vitamin $K_1$ together with supplements of calcium plus vitamin $D_3$ on bone mineral concentration compared to either vitamin D or K alone or placebo [47]. The complementary effect of vitamin $K_1$ (1 mg/day) and a mineral + vitamin D supplement (8 $\mu$g/day) was most effective in reducing bone loss at the femoral neck after 3 years among postmenopausal women versus vitamin D alone or placebo [48]. The addition of vitamin K to vitamin D and calcium supplements compared to vitamin D and calcium alone in postmenopausal Korean women increased BMD and reduced uncarboxylated osteocalcin concentrations after 6 months compared to vitamin D and calcium alone [49]. In postmenopausal women, 1 year of oral supplementation with extra virgin olive oil enriched with vitamins $D_3$, $K_1$, and $B_6$ or extra virgin olive oil reduced uncarboxylated osteocalcin concentrations and increased the T-score of BMD [50]. These findings indicate that combined administration of vitamin D and vitamin K appears to be useful in increasing BMD in postmenopausal women. It should be noted that these studies found beneficial effects at some but not all BMD sites measured. Furthermore, treatment with vitamins D and K with calcium increased BMD in older female patients with Alzheimer's disease and led to the prevention of nonvertebral fracture odds ratio: 7.5 (95% CI 5.6, 10.1); however, no placebo capsules were administered, hampering the interpretation of the results [51].

Not all studies observed synergistic effects of vitamin D and K supplementation. A small study among adults with Crohn's disease in Ireland showed generally no effect of combined vitamin D and K supplementation versus placebo on bone mass after 1 year, except a modest increase in bone mass of the total radius [52]. Among healthy women, 1 year of vitamin D and calcium + vitamin K supplementation either by phylloquinone or menaquinone-4 supplementation had no effect on BMD compared to calcium and vitamin D alone [53]. This study does not support a combined role for vitamin D + K supplementation in osteoporosis prevention; however, the relatively short study duration and the inclusion of healthy women could explain the null finding. It is however questionable if BMD can be improved in 12 months since changes in BMD usually require at least 1 year of follow-up time.

Among healthy older men and women, no difference was observed between multivitamin and calcium and vitamin D compared with the addition of vitamin K on BMD after 3 years [19]. An additive effect was noticeable for decreased percentage of uncarboxylated osteocalcin, which indicates an improved vitamin K status in the treatment group.

The ECKO trial among postmenopausal women with osteopenia showed no beneficial effect of vitamin D and calcium + vitamin K supplementation versus vitamin D and calcium alone after 2 years of follow-up in vitamin D-sufficient women [54]. However, the risk of fractures—a clinically more meaningful endpoint—was lower in the vitamin D and calcium + vitamin K groups: hazard ratio 0.41 (95 CI 0.1, 1.18) at 2 years and 0.45 (95% CI 0.20, 0.98) after 4 years of follow-up. This result on fracture risk indicates that bone quality rather than quantity is more important as not all trials showed synergistic effects of vitamin D and K supplementation on bone mineral density.

The protective effect of vitamin D with K on prednisolone-induced loss of BMD in patients with chronic glomerulonephritis after 8 weeks of treatment was similar in the vitamin D-only group [55], meaning that the addition of vitamin K had no synergistic effect. The elevation in serum calcium concentrations in the vitamin D group was, however, attenuated in the vitamin D + K group.

Taken together the evidence for combined vitamin D and K supplementation, the majority of the studies found beneficial effects for BMD among postmenopausal women.

Table 1: Summary of clinical trials of combined vitamin D and K supplementation on bone health.

| Author, year | Country | Participants | Treatment | Study duration | Outcome | Results for the highest versus the lowest quartiles |
|---|---|---|---|---|---|---|
| Iwamoto et al., 2000 [46] | Japan | $N = 92$ osteoporotic women ≥ 5 years after menopause, mean age 64 years | (i) Calcium (calcium lactate, 2 g/day)<br>(ii) Vitamin $D_3$ 0.75 $\mu$g/day<br>(iii) Vitamin $K_2$ 45 mg/day<br>(iv) Vitamin $D_3$ plus vitamin $K_2$ | 2 years | Bone mineral density % change | Combined vitamins D and K increased BMD |
| Ushiroyama et al., 2002 [45] | Japan | $N = 126$ postmenopausal women with osteopenia and osteoporosis, mean age 53 years | (i) Diet<br>(ii) Vitamin $K_2$ 45 mg/day MK-4<br>(iii) 1-$\alpha$ hydroxylcholecalciferol 1 $\mu$g/day<br>(iv) Vitamin $K_2$ + 1-$\alpha$ hydroxylcholecalciferol | 2 years | Bone mineral density % change | K + D group increased BMD % change at 2 years $P < 0.001$ |
| Braam et al., 2003 [48] | Netherlands | $N = 155$ postmenopausal women between 50 and 60 years | (i) Placebo<br>(ii) Mineral + vitamin D (8 $\mu$g/day)<br>(iii) Mineral + vitamin D + vitamin $K_1$ 1 mg | 3 years | Bone loss | Mineral + vitamin D + vitamin K showed reduced bone loss of the femoral neck |
| Yonemura et al., 2004 [55] | Japan | $N = 60$ patients with chronic glomerulonephritis, mean age 32 years, 53% female | (i) Control<br>(ii) Vitamin D (alfacalcidol 0.5 mg)<br>(iii) Vitamin $K_2$ MK-4 45 mg/d<br>(iv) Vitamins D plus K | 8 weeks | Bone mineral density | The preventive effect in groups K and D + K was similar to D |
| Sato et al., 2005 [51] | Japan | $N = 200$ older women with Alzheimer's disease, mean age 78 years | (i) Placebo<br>(ii) 45 mg menatetrenone, 1000 IU ergocalciferol, and 600 mg calcium | 2 years | Bone mineral density and fractures | BMD increased in vitamin D + K group<br>Odds ratio nonvertebral fractures 7.5 (95% CI 5.6, 10.1) |
| Bolton-Smith et al., 2007 [47] | UK | $N = 244$ healthy women, mean age 68 years | (i) Placebo<br>(ii) 200 mg/d vitamin $K_1$<br>(iii) 400 IU vitamin $D_3$ + 1000 mg calcium<br>(iv) Vitamins $K_1$ and $D_3$ plus calcium | 2 years | Bone mineral content | Combined vitamin K with vitamin D plus calcium associated with an increase in bone mineral content at the ultradistal radius |
| Booth et al. 2008 [19] | US | $N = 401$ healthy men and women, mean age 69, 59% female | (i) Multivitamin + 10 $\mu$g vitamin D and 600 mg calcium<br>(ii) Multivitamin + vitamin D + calcium + 500 $\mu$g vitamin $K_1$ | 3 years | Bone mineral density | No differences in change in BMD<br>Vitamin D + K group lower uncarboxylated osteocalcin concentrations |
| Cheung et al., 2008 [54] | Canada | $N = 440$ postmenopausal women with osteopenia, mean age 59 years | (i) 1500 mg calcium + 800 IU vitamin D<br>(ii) 5 mg of vitamin $K_1$ + calcium and vitamin D | 2–4 years | Bone mineral density | No effect on BMD |
| Binkley et al., 2009 [53] | US | $N = 381$ postmenopausal women, mean age 62 years | (i) Calcium 315 mg + vitamin $D_3$ 200 IU<br>(ii) Phylloquinone 1 mg + calcium and vitamin $D_3$<br>(iii) MK-4 (45 mg day) + calcium and vitamin $D_3$ | 1 year | Bone mineral density | No effect on BMD |
| Je et al., 2011 [49] | South Korea | $N = 78$ Korean postmenopausal women, mean age 68 years | (i) Vitamin D 400 IU + calcium (630 mg)<br>(ii) Vitamin D + calcium +45 mg of vitamin $K_2$ | 6 months | Bone mineral density | BMD increased significantly in the vitamin D + K group |

Table 1: Continued.

| Author, year | Country | Participants | Treatment | Study duration | Outcome | Results for the highest versus the lowest quartiles |
|---|---|---|---|---|---|---|
| O'Connor et al., 2014 [52] | Ireland | $N = 46$ adults with Crohn's disease, mean age 45 years | (i) Placebo<br>(ii) Phylloquinone 1 mg, vitamin D 10 $\mu$g, and calcium 500 mg/d | 1 year | Bone mineral density | Small effect on BMD of the total radius for vitamin D + K group |
| Mazzanti et al., 2015 [50] | Italy | 60 healthy postmenopausal women, mean age 55 years | (i) Extra virgin olive oil<br>(ii) Extra virgin olive oil enriched with vitamins $D_3$, $K_1$, and $B_6$ | 1 year | Bone mineral density | Vitaminized oil D, K, and $B_6$ increased the T-score of BMD |

BMD: bone mineral density; MK-4: menaquinone-4.

## 3. Interaction between Vitamins D and K for Cardiovascular Health

Besides bone health, also, the interaction between vitamins D and K with regard to cardiovascular health receives growing research interest. MGP—the vascular marker of vitamin K status—needs $\gamma$-glutamate carboxylation to inhibit vascular calcification [56]. In an experimental rat model, warfarin was administered to induce vitamin K deficiency and caused arterial calcification [57], which was accelerated when given toxic doses of vitamin D and resulted in premature death.

The Czech MONICA study cross-sectionally observed that subjects in the highest quartile of dephosphorylated-uncarboxylated MGP (dp-ucMGP) plus the lowest quartile of 25(OH)D concentrations had the highest pulse wave velocity in middle-aged healthy adults [58]. Further, potential interaction between vitamin K status and polymorphisms of the vitamin D receptors was investigated. Pulse wave velocity was higher with the number of G-allele polymorphisms and highest in the top quartile of dp-ucMGP for the GG vitamin D receptor genotype.

A Dutch prospective cohort indicates that the combination of low vitamin D < 50 mmol/L and low K status ≥ 323 mmol/L dp-ucMGP was associated with increased systolic and diastolic blood pressures and incident hypertension after 6 years of follow-up [59]. Up to now, no study investigated the combination of optimal vitamin D and K status in relation to coronary artery calcification and cardiovascular events after long-term follow-up. This would give valuable insight if vitamins D and K are involved in developing cardiovascular disease.

So far, two human intervention studies in healthy populations have investigated the combined effect of vitamins D and K on vascular function and calcification (Table 2) [60, 61]. In postmenopausal women, after 3 years of supplementation (1000 $\mu$g/d vitamin $K_1$ + 320 IU vitamin D), the vitamin D + K group maintained vessel wall characteristics of the carotid artery, whereas the control group and the vitamin D-only group significantly worsened over 3 years of follow-up [60]. However, vitamin K status was not measured as a marker of compliance to investigate what would have occurred following supplementation. Further, in a 3-year, double-blind, randomized controlled trial in older men and women free of clinical CVD, daily supplemental vitamin K in amounts achievable by high dietary intake of green, leafy vegetables (500 $\mu$g/day) combined with 600 mg calcium carbonate and 10 $\mu$g (400 IU) vitamin D did not result in lower coronary artery calcium progression as assessed by computerized tomography compared to the calcium + vitamin D group. In a subgroup analysis of participants who were ≥85% adherent to supplementation, there was less coronary artery calcium progression in the vitamin K + calcium and vitamin D groups than in the calcium and vitamin D group alone [61]; however, MGP carboxylation status was not determined. These data are hypothesis generating, and further studies are warranted to clarify the mechanism.

Among overweight type 2 diabetic patients with coronary heart disease, cosupplementation for 12 weeks of vitamins D (10 $\mu$g) and K (180 $\mu$g) and calcium (1000 mg) had beneficial effects on maximum levels of left carotid intima-media thickness and insulin metabolism markers [62]; however, no effect on right intima-media thickness was found and the results could be a chance finding. Unfortunately, circulating markers of vitamin K concentrations and vitamin K-dependent proteins were not taken into account to get a better mechanistic understanding.

Two trials studied the effect of vitamin D versus vitamin D + K in nondialyzed CKD patients on vascular calcification and cardiovascular risk factors for 9 months [63, 64]. In 42 CKD patients, the increase in carotid intima-media thickness (IMT) was significantly lower in the K (90 $\mu$g menaquinone-7) + D (10 $\mu$g vitamin D) group compared with the D-only group after 9 months [63]. Another small trial ($n = 38$) from the same research group did not show differences between the D versus D + K groups on cardiovascular risk markers [64]. These few studies show some potential for the combined effect of vitamins D + K versus D alone on subclinical CVD risk markers. It should be noted that very few clinical studies have been conducted in this field and that vitamin D + K supplements have been often combined with different micronutrients making it difficult to solely pinpoint the effect of vitamin D + K. These limited studies indicate that joint supplementation might benefit cardiovascular health.

## 4. Vitamins D and K with Glucose Metabolism and Inflammation

Another pathway that might affect CVD risk is via disturbances in glucose metabolism. Among Iranian vitamin D-deficient women with polycystic ovary syndrome—a dysmetabolic disorder—cosupplementation of calcium (1000 mg) and vitamins D (400 IU) and K (180 $\mu$g) for 8 weeks improved markers of insulin metabolism and lipid concentrations compared to placebo [65]. The joint supplementation of vitamins D and K might improve insulin metabolism through an effect on upregulation of the insulin receptor genes, the regulation of insulin secretion from the pancreatic beta-cell, the enhancement of $\beta$-cell proliferation, and suppression of parathyroid hormone [66–69].

Further, another feature in which both vitamins D and K overlap is on inflammation, which is strongly related to the development of CVD and osteoporosis [70]. In the same Iranian clinical trial among vitamin D-deficient women with polycystic ovary syndrome, the joint supplementation of calcium with vitamins D and K had beneficial effects on endocrine and oxidative stress markers, however no effect on inflammatory markers [71].

## 5. Effects of Long-Term Vitamin D Supplementation

A large group of people uses both vitamin D and calcium for the prevention of falls and fractures. Given the fact that 25(OH)D is converted to 1,25(OH)D, vitamin D supplementation stimulates the production of 1,25(OH)D [72]. This means that long-term vitamin D supplementation could promote the production of large amounts of vitamin K-

Table 2: Summary of clinical trials of combined vitamin D and K supplementation on cardiovascular health and disease.

| Author, year | Country | Participants | Treatment | Study duration | Outcome | Results for the highest versus the lowest quartiles |
|---|---|---|---|---|---|---|
| Braam et al., 2004 [60] | Netherlands | $N = 181$ postmenopausal women, means age 55, 100% female | (i) Placebo<br>(ii) Minerals + 8 $\mu$g vitamin D<br>(iii) Minerals + 8 $\mu$g vitamin D + 1 mg vitamin $K_1$ | 3 years | Vessel wall characteristics | MDK group unchanged, placebo and minerals + vitamin D decreased elastic properties |
| Shea et al., 2009 [61] | US | $N = 388$ healthy men and postmenopausal women, mean age 66 y, 60% female | (i) Multivitamin + 10 $\mu$g vitamin D and 600 mg calcium<br>(ii) Multivitamin + vitamin D + calcium + 500 $\mu$g vitamin $K_1$ | 3 years | Coronary artery calcification | No difference between vitamin $K_1$ group and control group |
| Asemi et al., 2016 [62] | Iran | $N = 66$ overweight diabetic patients with coronary heart disease, mean age 65 y, 47% female | (i) Placebo<br>(ii) Vitamin D (10 $\mu$g), K (180 $\mu$g), and calcium (1000 mg) | 12 weeks | Carotid IMT | Lower left carotid intima-media thickness and improved insulin metabolism markers |
| | | | *Chronic kidney disease patients* | | | |
| Kurnatowska et al., 2015 [63], 2016 [64] | Poland | $N = 42$ nondialyzed CKD patient stages, mean age 60 y, 3–5, 45% female | (i) 10 $\mu$g cholecalciferol<br>(ii) 10 $\mu$g cholecalciferol + 90 $\mu$g MK-7 | 270 days | Carotid IMT | Reduced progression IMT, reduced dp-ucMGP and osteocalcin |

IMT: intima-media thickness; dp-ucMGP: dephosphorylated-uncarboxylated matrix Gla protein; MK-7: menaquinone-7.

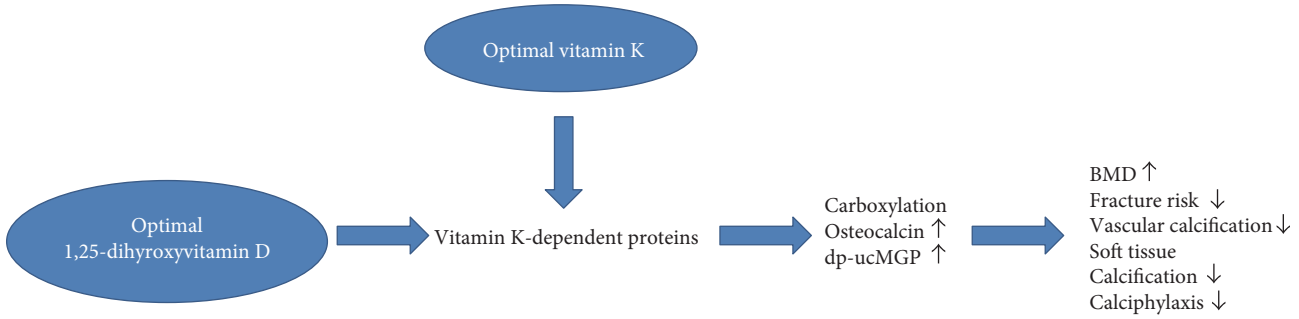


Figure 1: Simplified overview of potential synergy between vitamins D and K and bone and cardiovascular health. dp-ucMGP: dephosphorylated-uncarboxylated matrix Gla protein: BMD: bone mineral density. Genetic, molecular, cellular, and human evidence support that optimal concentrations of both vitamin D and vitamin K are beneficial for bone and cardiovascular health. Vitamin K is needed for the carboxylation of vitamin K-dependent proteins such as osteocalcin and matrix Gla protein, while vitamin D promotes the production of vitamin K-dependent protein concentrations. These vitamin K-dependent proteins are needed for extrahepatic organs such as the bone and the vascular system. This will result in bone mineralization and will inhibit soft tissue calcification, which will ultimately lead to lower risks of fractures and coronary heart disease.

dependent proteins, which remain inactive because there is not enough vitamin K to carboxylate (Figure 1). We propose a new hypothesis that if vitamin D concentrations are constantly high, there might not be enough vitamin K for activation of vitamin K-dependent proteins. Consequently, excess vitamin D diminishes the ability of vitamin K-dependent proteins to function properly, to stimulate bone mineralization, and to inhibit soft tissue calcification.

Further, increasing vitamin D intake through dietary or supplemental source increases intestinal calcium absorption, particularly when combined with calcium supplementation, and promotes hypercalcemia [73]. In this context, a human trial was performed in older women who received either 1200 mg calcium or 1200 mg calcium and 800 IU vitamin D per day over a 12-week period [74]. At the end of the 12 weeks, neither group observed a change in calcium concentrations, meaning that calcium was either excreted or stored somewhere. Increased calcium intake by itself may not be problematic as long as there is a steady state between optimal vitamin D and vitamin K concentrations. The disbalance between vitamin D and vitamin K promotes an environment in which excess calcium will be deposited into our vascular tissue instead of bone. The migration of calcification into the vascular tissue is described by the double burden of atherosclerosis and osteoporosis [75–77]. Additionally, as vitamin D increases calcium absorption, it might also promote hypercalcemia as seen in the Women's Health Initiative, which found a 24% higher risk of myocardial infarction in individuals taking calcium and vitamin D supplements and a greater risk for urinary tract stone occurrence: hazard ratio 1.17 (95% CI 1.02, 1.34) [11, 13, 14]. One prospective study found that higher 1,25(OH)D concentrations were strongly associated with the incidence of hypertension, while 25(OH)D was inversely associated with hypertension risk [78]. Higher 1,25(OH)D was associated with lower urinary calcium excretion, which could mean that the calcium meant for bone is stored somewhere else. Unfortunately, vitamin K status was not measured which would have given valuable insight into the association between vitamins D and K with calcium excretion.

## 6. Calciphylaxis and Vitamin K Antagonist Use

Calciphylaxis is a syndrome of calcification of the blood vessels, coagulopathy, and skin necrosis. It is seen mostly in patients with end-stage kidney disease, but can occur in the absence of kidney failure. Vitamin K antagonist use may contribute to its development [79]. The syndrome may cause a substantial morbidity and mortality. However, it should be acknowledged that the term calciphylaxis refers to a heterogeneous disorder that is characterized by soft tissue and vascular necrosis and has a clinical presentation from mild to severe. The underlying causes of calciphylaxis are not well understood; however, reported risk factors include female sex, obesity, elevated calcium*phosphate product, warfarin use, and vitamin D derivatives, for example, calcitriol, calcium-based binders, or systemic steroids, low blood albumin concentrations, and type 2 diabetes [80]. A recent study among patients with hemodialysis with calciphylaxis versus hemodialysis showed that cases had higher plasma uncarboxylated MGP concentrations than controls, which suggest a role of MGP in the pathophysiology of calciphylaxis. The fraction of total MGP that was carboxylated was also lower in cases than in controls. Vitamin K deficiency-mediated reduction in relative carboxylated MGP concentration may play a role in the pathogenesis of calciphylaxis [81]. This could be further mediated by the combined use of vitamin D derivatives and warfarin. Further, another study indicated that vitamin K antagonist use predisposes to the development of calciphylaxis in end-stage renal disease [82]. More evidence on the combined role of vitamin K antagonist use and vitamin D on bone and cardiovascular health is urgently needed.

## 7. Vitamin D and K Supplementation

Based on the current body of evidence, there is not enough evidence to recommend combined vitamin D and K supplementation for the prevention and treatment of osteoporosis. Most trials studied low-dose vitamin D in isolation (400–800 IU daily), which demonstrated only

modest or null effects on BMD and fracture prevention in mostly ≥65 years postmenopausal women [6–8]. Large clinical trials of moderate–high dose (≥800 IU daily) vitamin D supplementation (cholecalciferol) are currently in progress.

The most widely used vitamin K form for supplementation is vitamin $K_2$ and more specifically menaquinone-4 and menaquinone-7. Menaquinone-4 is more used in trials with bone outcomes, while menaquinone-7 is more in trials with cardiovascular outcomes with dosages between 90–360 $\mu$g. Menaquinone-7 has a higher bioavailability and may be of particular importance for extrahepatic tissue [83]. No cut-off value for vitamin K status nor vitamin K supplementation is available yet. Future studies are needed to determine whether vitamin D combined with vitamin K rich foods or vitamin K supplementation could improve bone and cardiovascular health.

## 8. Recommendations for Future Research

The recommendations for future research are as follows:

(i) Evaluate the role of vitamin D administration in vitamin K-dependent proteins in human populations

(ii) Question the possible long-term consequences of high-dose vitamin D supplementation

(iii) Assess the combined role of vitamin K antagonist use and vitamin D in bone and cardiovascular health

(iv) Investigate the joint supplementation of vitamins D and K on hard clinical endpoints

## 9. Conclusion

Taken together, animal and human studies suggest that optimal concentrations of both vitamin D and vitamin K are beneficial for bone and cardiovascular health as supported by genetic, molecular, cellular, and some human studies. However, vitamin D and calcium supplementation along with vitamin K deficiency might also induce long-term soft tissue calcification and CVD, particularly in vitamin K antagonist users and other high-risk populations. At this moment, we should be careful about supplementing high-dose vitamin D, unless indicated differently. More clinical data about the potential interplay between vitamin D and vitamin K metabolism is urgently needed before broader treatment recommendations can be given.

The consumption of a well-balanced diet is key for population-based primary prevention of chronic diseases. As more is discovered about the powerful combination of vitamins D and K, it gives a renewed reason to eat a healthy diet including a variety of foods such as vegetables and fermented dairy for bone and cardiovascular health.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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