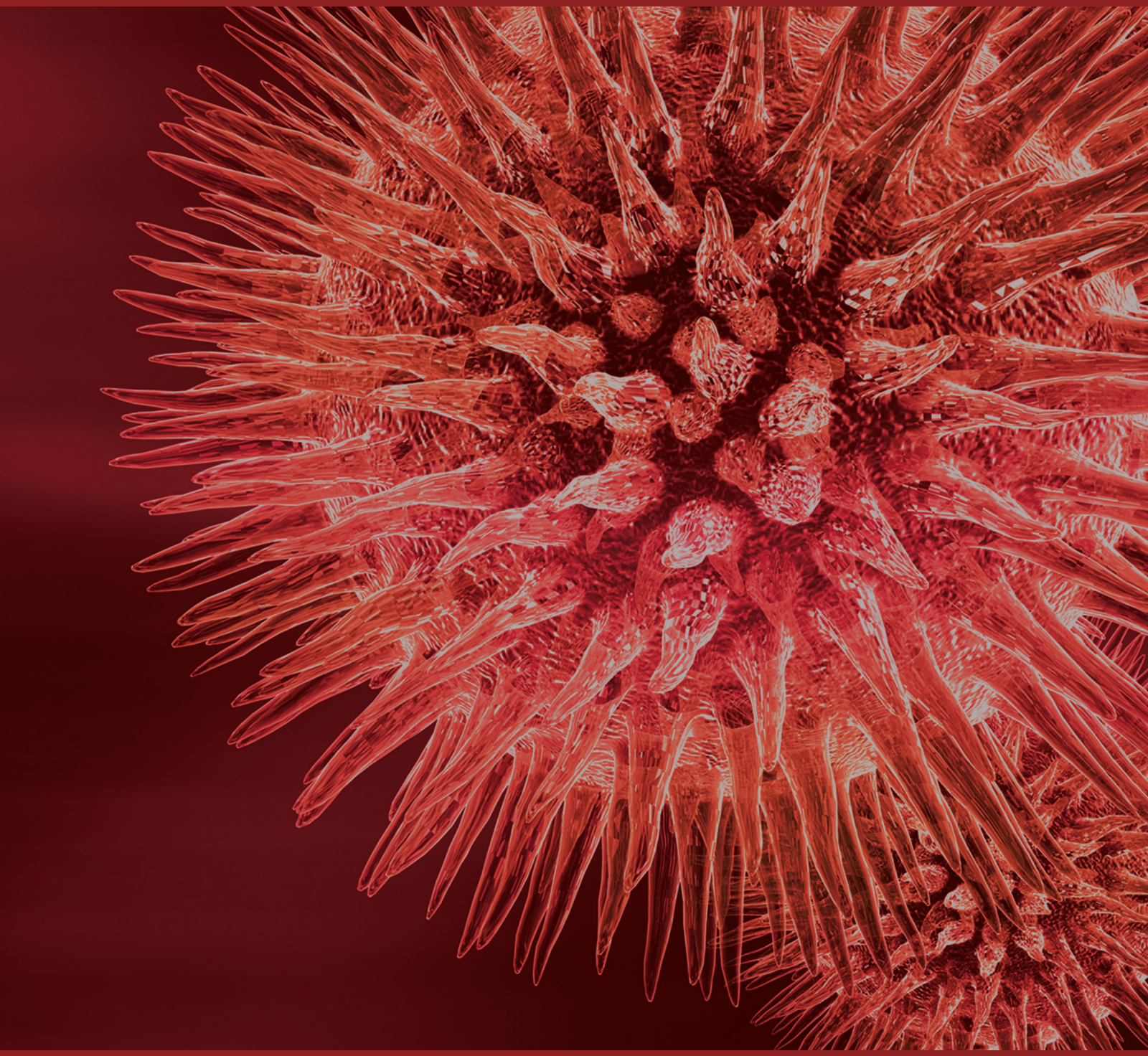
BioMed Research International

Extraskeletal Functions of Vitamin D

Guest Editors: Domenico Santoro, Katerina Sebekova, Daniel Teta, and Luca De Nicola,



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Editorial

Extraskeletal Functions of Vitamin D

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The recent identification of an expanded role for vitamin D action, beyond its traditional actions in mineral metabolism and the novel investigational approaches as well, has opened new therapeutic avenues for both the clinician at bedside and the scientist. This is the rationale of dedicating a special issue on this hot topic.

A new spectrum of vitamin D biological activities including important aspects on cellular proliferation, differentiation, and the immune system has been recently identified. Relevant is also the interaction of vitamin D with other kidney hormones such as renin and erythropoietin; this topic is specifically addressed by this paper. Indeed, the administration of vitamin D analogues has been associated with an improvement in anaemia and reduction in erythropoiesis-stimulating agents (ESA) requirements. Furthermore, vitamin D deficiency could contribute to the inappropriately activated or unsuppressed renin-angiotensin-aldosterone system (RAAS) in pathologic conditions.

Experimental data show that vitamin D may also interfere with the compensatory increase in renin synthesis occurring during chronic administration of anti-RAAS agents. In this context, use of vitamin D may be therefore suggested in patients treated with anti-RAAS but not reaching the safe threshold level of proteinuria. As further support to the hypothesis on the use of vitamin D analogues as antiproteinuric agents in proteinuric chronic kidney disease (CKD) are the data evidencing its anti-inflammatory effects. In this special issue, J. Egido et al. provided novel insights into the association between vitamin D deficiency and the activation of the RAAS, which is critical if one considers that local activation of this latter system mainly contributes to inflammation and tissue damage in renal diseases. Based on studies in human kidney proximal tubular cells, the authors propose a novel signalling pathway explaining, at least in part, the anti-inflammatory effects of paricalcitol in chronic kidney disease (CKD). Indeed, they found that the anti-inflammatory actions of paricalcitol may depend on the inhibition of TGF-α/ADAM17/EGFR pathway stimulated by aldosterone.

I. Guessous reviewed the role of vitamin D, as a marker of health status rather than a predictor of health outcomes. He analyzed the potential impact of currently ongoing randomized clinical trials (RCTs). The role of vitamin D in skeletal complications is also questioned, since, according to recent studies, vitamin D might not be as essential as previously thought for maintaining bone health.

Observational data support a link between vitamin D status and cardiovascular diseases, and vitamin D deficiency can thus be considered a cardiovascular risk marker. Regarding this issue, O. Marginean and I. Mozos discussed the role of vitamin D in blood pressure control, left ventricular hypertrophy, atrial fibrillation, the metabolic syndrome, and peripheral artery disease. Genotyping for vitamin D receptor variants could help to distinguish patients at risk of developing cardiovascular disease.

In a compelling review, P. Olivier et al. first describe the trophic effect of vitamin D in the skeletal muscle. Hypovitaminosis D is consistently associated with decrease in muscle function and performance and increase in disability. Data

from randomized controlled trials and meta-analysis showed that vitamin D supplementation improves muscle strength, gait, and the risk of falls in different settings, especially in the elderly frail patient.

G. Ferlazzo et al. review data from clinical and basic research data on the role of vitamin D in inflammatory bowel disease (IBD) with regard to immune regulation, VDR (vitamin D receptor) polymorphism, disease activity and severity, and outcomes. Despite the growing evidence suggesting a role for vitamin D deficiency in the development of IBD, the exact role of vitamin D in IBD is still unclear and merits further investigation.

The article of P. Mansueto et al. focuses on the most recent epidemiological and experimental data looking at relationships between vitamin D and HIV infection. Clinical implications and potential benefits of vitamin D supplementation, in this particular setting, are explored in this comprehensive review.

Two papers in this issue discuss vitamin D in relation with gynecological and obstetrics diseases. O. Triolo et al. reviewed in detail the current knowledge of the association between VDR-mediated signalling pathways and vitamin D levels in polycystic ovary syndrome, endometriosis, and ovarian and breast cancer as well as in pathologies related to the maternal-fetal unit, such as preeclampsia, gestational diabetes mellitus (GDM), infertility, and in vitro fertilization. Pleskacova et al. compared midgestation and early postpartum vitamin D status in women with GDM versus controls. They showed that both groups present similarly low 25(OH)D levels and overall high prevalence of vitamin D deficiency. However, women with GDM history presented significantly lower 25(OH)D levels and higher prevalence of 25(OH)D deficiency postpartum. The above manuscripts suggest that, despite convincing associations between hypovitaminosis D and gynecological/obstetric diseases, a causal relationship needs to be confirmed in future investigations aimed at clarifying the mechanisms linking vitamin D metabolism and hormonal/metabolic pathways involved in these diseases.

The study by Kalousová et al. readdressed the important issue of vitamin D status in nondialysis and dialysis CKD patient. In particular, they studied the relationship between vitamin D concentration in plasma and vitamin D binding protein (VDBP). The authors confirmed that plasma levels of vitamin D are decreased in CKD, especially in dialysis patients. More importantly, they found that VDBP does not play a role in vitamin D deficiency because of enhanced production as compared with healthy controls that compensates urinary losses of this protein.

It is known that intracellular calcium concentration significantly increases in peripheral blood mononuclear cells (PBMCs) of CKD patients, possibly impairing the regulatory mechanisms maintaining cellular calcium homeostasis. L. Sikurova et al. evaluated in early CKD patients with vitamin D deficiency if treatment with cholecalciferol for 6 months may restore these abnormalities. They showed that vitamin D3 supplementation had a beneficial effect on disturbed cell calcium homeostasis in these patients with early CKD.

The study of K. Šebeková et al. addressed the question whether an excessive accumulation of advanced glycation end products (AGEs) in the skin of diabetic patients interferes with dermal vitamin D3 formation and whether hypovitaminosis D is associated with an increased formation and toxicity of AGEs. They showed that hypovitaminosis D is not associated either with enhanced AGE accumulation or with markers of inflammation. These data suggest that hypovitaminosis D seems to be of limited importance for the development of microinflammation and accumulation of AGEs.

As summarized here, the research field on the extraskeletal effects of vitamin D is continuously growing. In this special issue, we tried to give a glimpse, although somehow critical, of the most interesting studies in this topic. Clinical studies are however still needed to correctly translate in patients the results obtained in labs.

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Research Article

Paricalcitol Inhibits Aldosterone-Induced Proinflammatory Factors by Modulating Epidermal Growth Factor Receptor Pathway in Cultured Tubular Epithelial Cells

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Chronic kidney disease is characterized by Vitamin D deficiency and activation of the renin-angiotensin-aldosterone system. Increasing data show that vitamin D receptor agonists (VDRAs) exert beneficial effects in renal disease and possess anti-inflammatory properties, but the underlying mechanism remains unknown. Emerging evidence suggests that "a disintegrin and metalloproteinase" (ADAM)/epidermal growth factor receptor (EGFR) signalling axis contributes to renal damage. Aldosterone induces EGFR transactivation regulating several processes including cell proliferation and fibrosis. However, data on tubular epithelial cells is scarce. We have found that, in cultured tubular epithelial cells, aldosterone induced EGFR transactivation via TGF-α/ADAM17. Blockade of the TGF-α/ADAM17/EGFR pathway inhibited aldosterone-induced proinflammatory gene upregulation. Moreover, among the potential downstream mechanisms, we found that TGF-α/ADAM17/EGFR inhibition blocked ERK and STAT-1 activation in response to aldosterone. Next, we investigated the involvement of TGF-α/ADAM17/EGFR axis in VDRA anti-inflammatory effects. Preincubation with the VDRA paricalcitol inhibited aldosterone-induced EGFR transactivation, TGF-α/ADAM-17 gene upregulation, and downstream mechanisms, including proinflammatory factors overexpression. In conclusion, our data suggest that the anti-inflammatory actions of paricalcitol in tubular cells could depend on the inhibition of TGF-α/ADAM17/EGFR pathway in response to aldosterone, showing an important mechanism of VDRAs action.

1. Introduction

One of the earliest pathologic features of chronic kidney disease (CKD) patients is active vitamin D deficiency [1, 2]. Increasing data show that vitamin D receptor agonists (VDRAs) therapy decreases proteinuria, may reduce renal damage progression, and improves cardiovascular outcomes in CKD patients [1–3]. These beneficial effects are independent of serum parathyroid hormone, phosphorus, and calcium levels suggesting that vitamin D presents pleotropic actions, beyond mineral metabolism regulation [1, 4]. Active vitamin D (1,25–dihydroxy vitamin D(3) or calcitriol) mediates its biological effects by binding to the vitamin D receptor (VDR), which then translocates to the nuclei of target cells [1]. In experimental renal disease vitamin D or VDRAs treatment diminished fibrosis, mesangial proliferation, podocyte loss, and inflammatory cell infiltration [5–10]. However, the molecular mechanism

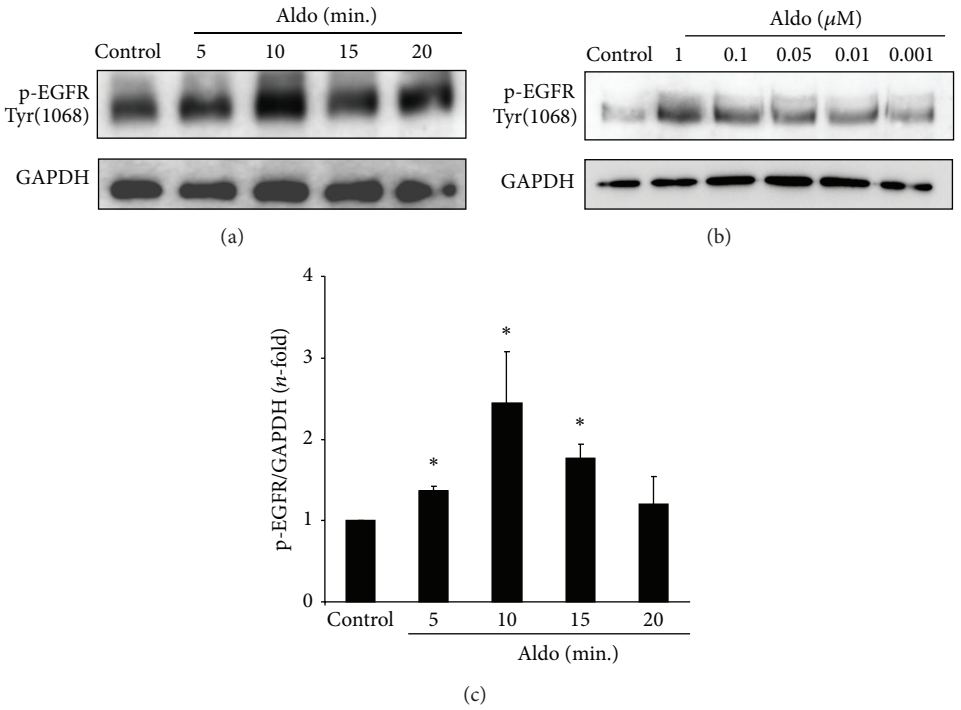

Figure 1: Aldosterone induces EGFR phosphorylation in cultured tubular epithelial cells. Human tubular epithelial cells were treated (a) with 1 μmol/L aldosterone (Aldo) for increasing times or (b) with several concentrations (range 1–0.001 μmol/L Aldo) for 15 min. (c) The figure shows a quantification of time-course EGRF phosphorylation. The data are expressed as mean ± SEM of 4 independent experiments. $^*P < 0.05$ versus control.

involved in the anti-inflammatory effects of vitamin D in the setting of CKD remains poorly characterized.

The renin-angiotensin-aldosterone system (RAAS) is a major mediator of progressive renal injury in CKD, with angiotensin II (AngII) and aldosterone (Aldo) being the most relevant RAAS components [11, 12]. Both factors promote renal inflammation, fibrosis, and podocyte injury [13–15]. There is a close relation between vitamin D and the RAAS. The hormonal form of vitamin D is a negative endocrine regulator of the RAAS by suppressing renin biosynthesis [16]. Homozygous VDR knockout mice develop high renin hypertension, cardiac hypertrophy, and increased susceptible to kidney damage following unilateral ureteral obstruction [17, 18]. Therefore, investigation of underlying mechanisms implicated in the relation between RAAS and vitamin D actions in CKD is an important field of research.

Emerging evidence suggests that blockade of epidermal growth factor receptor (EGFR) can be a therapeutic option for renal diseases. Experimental studies have shown that genetic or pharmacological EGFR blockade ameliorates renal disease progression, mainly by diminishing kidney fibrosis [19, 20]. Regarding the RAAS, both AngII and Aldo, after binding to their specific receptors, can transactivate EGFR, via "a disintegrin and metalloproteases" (ADAMs), thus regulating cellular functions, including proliferation, hypertrophy, and migration [21–23]. ADAMs are membrane-spanning metalloproteases involved in cleavage of extracellular substrates (shedding), including EGF family ligands, both constitutively and in response to regulatory stimulation [24, 25]. In the kidney, ADAM17, also known as TACE, participates in the shedding of the EGFR ligands, heparin binding EGF-like growth factor (HB-EGF), and transforming growth factor-α (TGF-α) [26–29]. Both ligands are involved in AngII-induced EGFR transactivation, although some differences have been described between cell types and tissues [30]. In mice, ADAM17-mediated TGF-α shedding contributes to AngII-induced experimental renal fibrosis [20]. Most of the studies on Aldo/EGFR pathway have been done in cultured cells, mainly in vascular smooth muscle cells [14] and in mesangial cells, the latter showing a role in cell proliferation [31]. We now demonstrated here that, in cultured tubular epithelial cells, Aldo activates the EGFR pathway via ADAM-17/TGF-α shedding, leading to upregulation of proinflammatory factors. These data are in line with our recent observation that blockade of the ADAM17/EGFR axis prevents experimental renal inflammation induced by systemic administration of the TWEAK cytokine [32], showing that this pathway, besides regulating proliferation and fibrosis, could contribute to renal inflammation. Furthermore, we now show that the beneficial anti-inflammatory effects of VDRAs such as paricalcitol in renal disease may be explained by inhibition of Aldo-mediated proinflammatory factors overexpression through modulation of ADAM17/TGF-α/EGFR signalling axis and dampening of downstream mechanisms, including ERK and STAT-1 activation. Our data add novel information about mechanisms involved in the well-known anti-inflammatory properties of

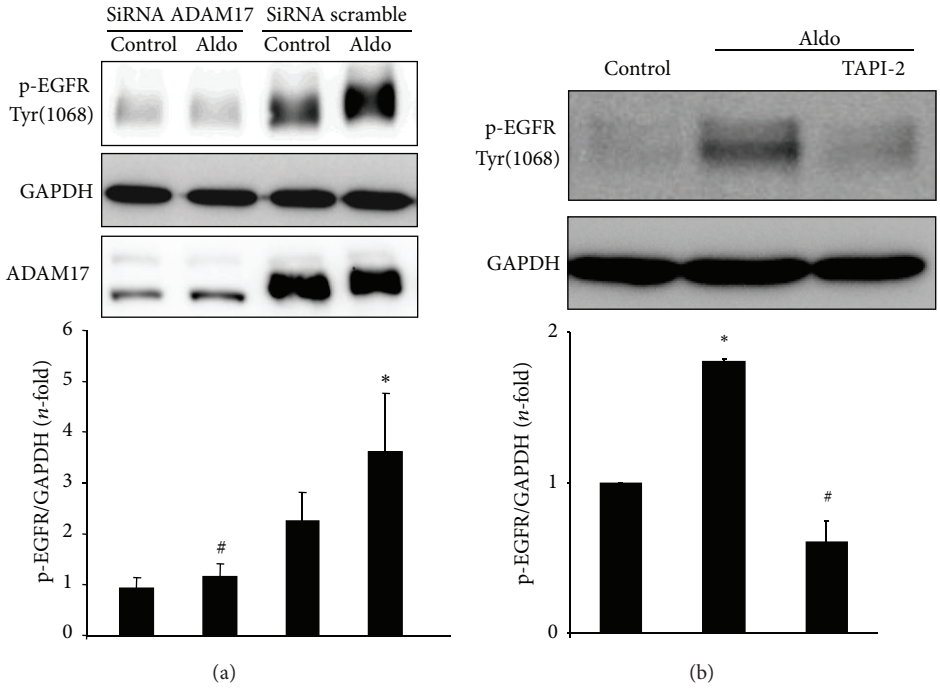

Figure 2: ADAM17 inhibition blocks aldosterone-mediated EGFR activation in tubular epithelial cells. HK2 cells were transfected with an ADAM17 siRNA or scrambled siRNA, as described in "methods," before stimulation with 1 μmol/L Aldo for 15 min. Phosphorylated-EGFR levels were evaluated by Western blot using an antibody against Y1068 phosphorylated-EGFR (p-EGFR1068). GAPDH levels were used as loading control and ADAM17 as silencing control. (a) Representative Western blot and quantification of data expressed as mean ± SEM of 4 independent experiments. $^{*}P < 0.05$ versus untreated siRNA control-transfected cells control. $^{\#}P < 0.05$ versus Aldo-treated siRNA scramble cells. (b) Cells were preincubated with the specific ADAM17 inhibitor TAPI-2 (50 μmol/L) for 1 hour and then stimulated with 1 μmol/L Aldo for 15 min. Representative Western blot and data expressed as mean ± SEM of 4 independent experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Aldo.

VDRAs and contribute to better design of future clinical trials.

2. Material and Methods

2.1. Cultured Cells. Human kidney proximal tubule epithelial cells (HK2 cell line, ATCC CRL-2190) were grown in RPMI 1640 with 10% fetal bovine serum (FBS), 1% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin-transferrin-selenite, and 36 ng/mL hydrocortisone in 5% CO_2 at 37°C. When cells reached 60 to 70% confluence, they were incubated in serum-free medium for 24 hours before the experiments.

Tubuloepithelial proximal murine cells (MCT cell line) were originally obtained from Dr. Eric Neilson (Vanderbilt University) and used for gene expression studies. These cells were grown in RPMI 1640 with 10% FBS, 1% glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 at 37°C. When reached 60 to 70% confluence, they were maintained in RPMI with 1% FBS for 24 hours.

Cells were treated with recombinant Aldo (1 μmol/L; Sigma). In some experiments cells were preincubated for 1 hour with the following inhibitors prior to stimulation: the EGFR kinase inhibitor AG1478 (100 nmol/L), the ERK inhibitor U0126 (10 μmol/L; Calbiochem), TAPI-2, a specific inhibitor of ADAM-17 (50 μmol/L, Enzo Life Sciences), and a specific inhibitor of HB-EGF, CRM197 (10 μg/mL, Sigma). In some experiments, cells were preincubated for 24 hours with a neutralizing antibody anti-TGF-α (2.5 μg/mL, Abcam) and for 48 hours with paricalcitol (12 μmol/L, Abbott). DMSO was used as a solvent in many of these reagents but had no effect on cell viability or on gene expression levels (data not shown).

2.2. Protein Studies. The EGFR phosphorylation status was analysed by Western blotting. Briefly, proteins were obtained using lysis buffer [50 mmol/L Tris-HCl and 150 mol/L NaCl; 2 mmol/L EDTA, 2 mmol/L EGTA, and 0.2% Triton X-100, 0.3% IGEPAL; 10 μL/mL protease inhibitor cocktail; 10 μL/mL PMSF and 10 μL/mL orthovanadate]. Protein content was quantified by the BCA method, using bovine serum albumin (BSA) as standard. Cell lysates (25 μg/lane) were separated on 6% to 12% SDS-polyacrylamide gels under reducing conditions. Samples were transferred to nitrocellulose membranes (Bio-Rad), blocked in 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20, and 5% milk, and incubated overnight at 4°C with the following antibodies [dilution]: anti-phosphorylated-EGFR on Tyrosine (Y) 1068 (p-$EGFR_{1068}$) [1 : 250] (Calbiochem), ADAM17 [1 : 1000] (Abcam), EGFR [1 : 250], p-ERK1/2 [1 : 200] (Santa Cruz Biotechnology), and p-STAT1 [1 : 500] (Invitrogen).

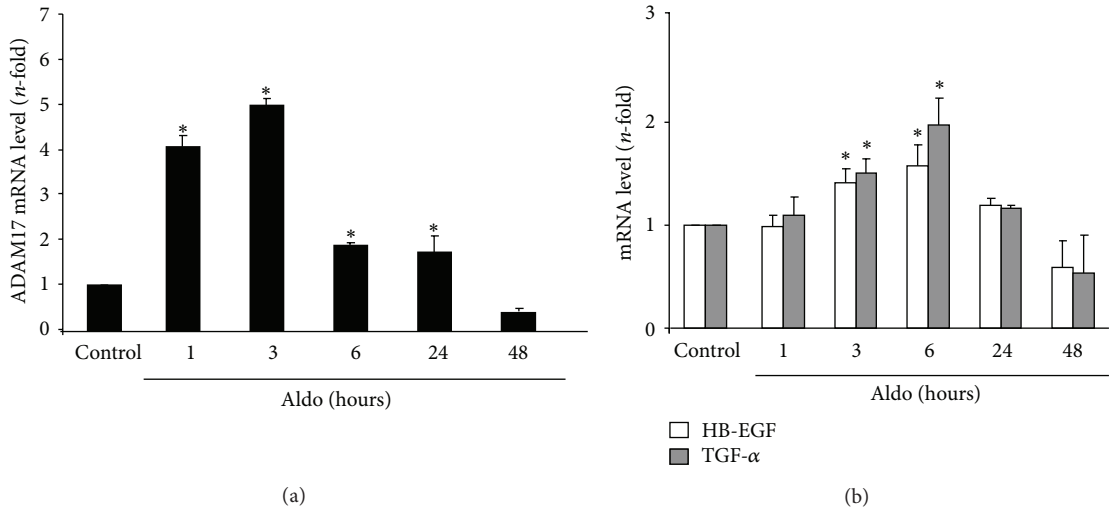

Figure 3: Aldosterone regulates genes expression of ADAM17 (a), TGF-α, and HB-EGF (b). Cells were treated with 1 μmol/L Aldo for increasing times and gene expression levels were evaluated by real-time PCR. Data are expressed as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ versus control.

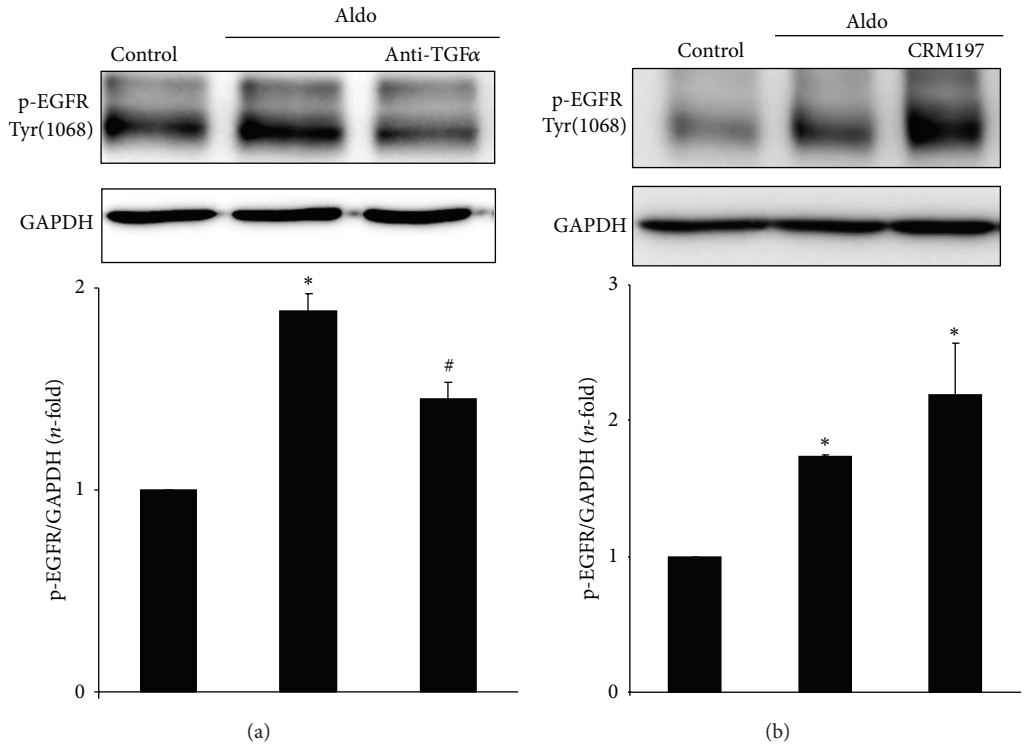

Figure 4: Aldosterone transactivates EGFR via TGFα, but not HB-EGF, shedding. Cells were pretreated for 24 h with a neutralizing antibody against TGFα (2.5 μg/mL) (a) or 1 hour with the HB-EGF pharmacological inhibitor CRM197 (10 μmol/L) (b) before stimulation with 1 μmol/L Aldo for 15 min. Western blot experiment and data are expressed as mean ± SEM of 3 or 5 independent experiments, respectively. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Aldo.

Subsequently, membranes were incubated with a peroxidase-conjugated secondary antibody and developed using the ECL chemiluminescence kit (Amersham Pharmacia Biotech). Protein quality and transfer efficiency were assessed by Ponceau red staining (not shown). To assess protein loading, membranes were incubated with anti-GAPDH [1:10000] (Chemicon International). Total proteins were used as control for phosphorylation studies. Films were scanned using the Gel Doc EZ machine imager and analysed using the Image Lab 3.0 (Bio-Rad).

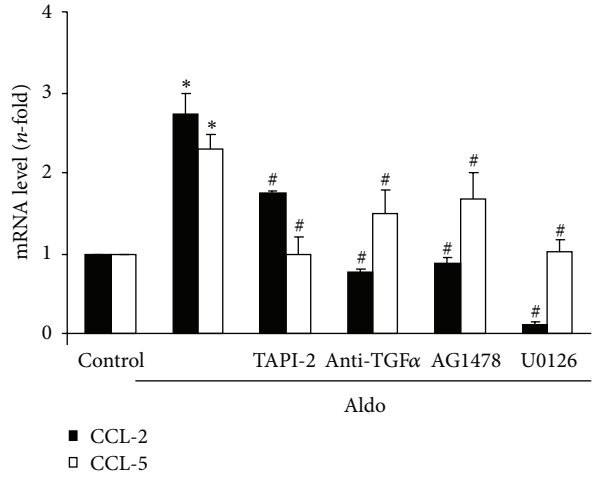

Figure 5: ADAM17/TGF-α/EGFR signaling blockade inhibits aldosterone-mediated proinflammatory factors upregulation. Cells were pretreated with the specific ADAM17 inhibitor TAPI-2 (50 μmol/L; 1 hour), a neutralizing antibody against TGFα (2.5 μg/mL, 24 hour), the EGFR kinase inhibitor AG 1478 (100 nmol/L, 1 hour), or the ERK inhibitor U0126 (10 μmol/L; 1 hour) before stimulation with 1 μmol/L Aldo for 6 hours. CCL-2 and CCL-5 gene expression levels were determined by real-time PCR. Data are expressed as mean ± SEM of 2-3 experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Aldo.

2.3. Gene Silencing. The ADAM-17 gene was silenced using an interference silencer siRNA or its corresponding scramble control (Ambion). Subconfluent cells were transfected for 24 hours with 25 nmol/L siRNA using 50 nmol/L of Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. Subsequently, cells were incubated with 10% FBS heat inactivated for 24 hours. Then, cells were incubated in serum-free medium for 24 hours before the experiment. The specificity and efficiency of silencing were checked by Western blot with an anti-ADAM-17 antibody (AbCam) [1 : 1000].

2.4. Gene Expression Studies. Total RNA was isolated in Trizol (Invitrogen, Groningen, Netherlands) from samples and mouse kidney cells. cDNA was synthesized using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using 2 μg of total RNA with random hexamer primers, following the manufacturer's instructions. We performed real-time PCR expression using probes (Taqman probes labelled with FAM fluorophore) from Applied Biosystems: VDR Mm_00437297_m1; ADAM-17 Mm_00456428_m1; CCL-2 Mm00441242_m1; CCL-5 Mm_01302428_m1; TGF-α Mm_00446232_m1. Data were normalized to 18S ribosomal RNA of eukaryotic: 4210893E (VIC). The number of copies of mRNA for each sample was calculated by the instrument software using the Ct. value (shaped point arithmetic analysis on the thermocycler). The results were expressed in relative copy number calculated relative to unstimulated cells or control mice, after normalization to 18S.

2.5. Statistical Analysis. The results shown in the text are expressed as mean ± SEM. The differences between the groups treated with agonists and controls were evaluated by the Student's t-test and Mann-Whitney test, and $P < 0.05$ was considered significant. Statistical analysis was performed using SPSS statistical software (version 11.0, Chicago, IL).

3. Results

3.1. Aldosterone Induces EGFR Transactivation via ADAM17 and Subsequent Release of TGF-α in Cultured Tubular Epithelial Cells. Aldo induced EGFR transactivation in several cells, including epithelial cells and mesangial cells [31, 33]. In cultured human tubular epithelial cells (HK2 cell line), Aldo induced a rapid activation of EGFR, as shown by increased EGFR phosphorylation on Tyr1068, in a time and dose-dependent manner, presenting a maximal effect at 1 μmol/L Aldo after 10 min (Figures 1(a), 1(b) and 1(c)). The blockade of ADAM17, by gene silencing or pharmacological inhibition using TAPI-2, markedly diminished Aldo-induced EGFR phosphorylation (Figures 2(a) and 2(b)).

ADAM17 mRNA is constitutively expressed in normal adult human kidneys and is increased in disease conditions [34]. In cultured tubular epithelial cells stimulation with Aldo rapidly increased ADAM17 gene expression, which remained elevated up to 24 hours (Figure 3(a)). Among the EGFR ligands, HB-EGF and TGF-α are released by ADAM17 and have a role in renal diseases. In tubular epithelial cells, Aldo upregulated gene expression of HB-EGF and TGF-α, observed after 3 hours (Figure 3(b)).

TGF-α blockade, using a specific neutralizing antibody, inhibited EGFR phosphorylation in response to Aldo in tubular cells (Figure 4(a)). In contrast, the pharmacological inhibition of HB-EGF by CRM197, a nontoxic mutant of diphtheria toxin that neutralizes HB-EGF binding to EGFR [29], had no effect on Aldo-mediated EGFR activation (Figure 4(b)).

3.2. Aldosterone Regulates Proinflammatory Gene Expression via the ADAM-17/TGF-α/EGFR Axis in Cultured Tubular Epithelial Cells. Aldo exerts proinflammatory actions in the

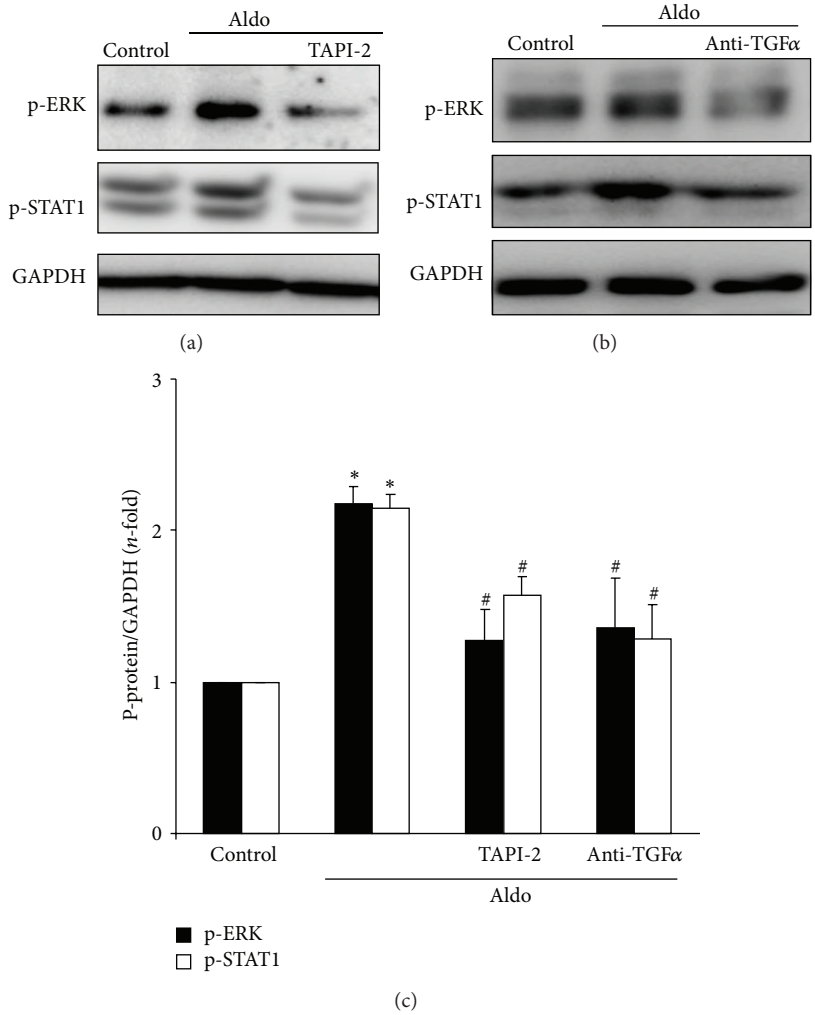

Figure 6: Aldosterone activates ERK and STAT pathways via ADAM-17/TGF-α/EGFR axis. HK2 cells were preincubated with (a) the specific ADAM17 inhibitor TAPI-2 (50 μmol/L; 1 hour) or (b) a neutralizing antibody against TGFα (2.5 μg/mL, 24 hour) and then stimulated with 1 μmol/L Aldo for 15 min. Figures (a) and (b) show representative Western blot experiment and (c) data expressed as mean ± SEM of 3 experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Aldo.

kidney, including the regulation of proinflammatory gene expression in cultured cells [35]; however, the role of the ADAM-17/EGFR pathway in these Aldo-mediated responses has not been evaluated. In cultured tubular epithelial cells, we have blocked ADAM-17/TGF-α/EGFR pathway using the following inhibitors: the ADAM17 inhibitor TAPI-2, an anti-TGF-α neutralizing antibody, and the EGFR kinase inhibitor AG1478. All of these inhibitors significantly diminished Aldo-induced gene upregulation of the proinflammatory factors CCL-2 and CCL-5 (Figure 5).

3.3. Aldosterone Activates Several Intracellular Mechanisms via the ADAM-17/TGF-α/EGFR Axis. Among the EGFR downstream signalling mechanisms, activation of the MAPK cascade has special relevance in the regulation of inflammatory events. Aldo increased EGFR phosphorylation on tyrosine 1068, which has been previously involved in ERK signalling [29, 34]. In human tubular cells, Aldo increased ERK phosphorylation and ERK blockade diminished Aldo-induced proinflammatory gene overexpression (Figure 5). Moreover, ERK activation was prevented when the ADAM-17/TGF-α/EGFR pathway was inhibited using the above-described specific blockers (Figures 6(a), 6(b) and 6(c)). Aldo also activated STAT-1 via the ADAM-17/TGF-α/EGFR pathway (Figures 6(a), 6(b) and 6(c)).

3.4. Paricalcitol Inhibits Aldosterone-Induced Proinflammatory Gene Expression in Cultured Renal Cells by Modulating the ADAM-17/TGF-α/EGFR Axis. In tubular epithelial cells, preincubation with the VDRA paricalcitol for 48 hours, inhibited proinflammatory genes induction caused by Aldo (Figure 7). These data confirm the anti-inflammatory properties of paricalcitol.

Paricalcitol blocked Aldo-induced EGFR transactivation, as shown by the downregulation of phosphorylated-EGFR levels (Figures 8(a) and 8(b)) and ADAM-17/TGF-α gene overexpression (Figure 8(c)) to control values.

During renal damage, VDR expression is downregulated [1, 3]. Interestingly, in tubular epithelial cells Aldo downregulated VDR gene and protein levels (Figures 8(a), 8(d),

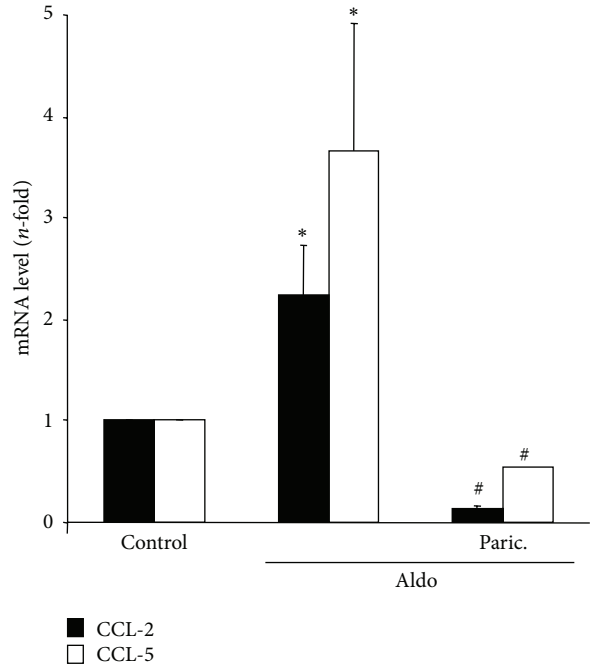

Figure 7: Paricalcitol inhibits aldosterone-induced proinflammatory genes in cultured tubular epithelial cells. Cells were pretreated with the analogue of vitamin D paricalcitol (12 μmol/L) for 48 hours before stimulation with 1 μmol/L Aldo for 6 hours. Gene expression levels were determined by real-time PCR. Data are expressed as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Aldo.

and 8(e)). Pretreatment with paricalcitol restored VDR levels, indicating that beneficial effects of paricalcitol could be due to modulation of VDR.

Finally, paricalcitol also blocked ERK and STAT-1 activation in response to Aldo stimulation (Figure 9).

4. Discussion

Chronic inflammation is a main feature of CKD. Among the factors involved in the inflammatory response in the kidney, local activation of RAAS has special relevance. AngII, the main effector peptide of this system, has been extensively demonstrated to promote renal inflammation [11]. There is previous evidence that Aldo also contributes to this process. Multiple experimental studies in models of hypertension, renal damage, and heart failure have demonstrated that selective Aldo blockade by eplerenone attenuates tissue injury in part by reducing inflammation in Aldo target organs [13, 15, 35]. Treatment with an aldosterone synthase inhibitor ameliorated experimental diabetic nephropathy by decreasing renal inflammation, matrix formation, and albuminuria [36]. Data presented here demonstrate that the ADAM-17/TGF-α/EGFR axis is an important mechanism involved in the regulation of proinflammatory factors by Aldo in cultured tubular epithelial cells (Figure 10(a)). Other data also support the involvement of the EGFR/ADAM17 axis in inflammation [37, 38], as we have recently described in TWEAK-mediated experimental renal inflammation [32]. In addition, targeting ADAM17 by pharmacologic inhibition or gene knockout attenuates the inflammatory response in animal models of vascular damage, including hypertension, atherosclerosis, and pulmonary vascular inflammation [21, 38–40].

EGFR transactivation is regulated by ligand sheddase activity [41]. Thus far, 12 EGFR ligands have been described, among them TGF-α, HB-EGF, amphiregulin, and connective tissue growth factor could be relevant in renal pathology [41–43]. Regarding the kidney, EGF modulates glomerular hemodynamics and renal metabolism, while TGF-α, HB-EGF, and amphiregulin participate in cell survival/proliferation [41, 43]. Moreover, TGF-α has been involved in genetic susceptibility to renal disease [44] and in AngII-mediated experimental renal fibrosis [20]. HB-EGF contributes to cell regeneration and repair after ischemia [41]. In HB-EGF-deficient mice with progressive glomerulonephritis, inflammatory renal infiltration and albuminuria were lower which was ascribed to EGFR pathway inhibition in podocytes [45]. In cultured tubular cells, we have found that Aldo upregulates both EGFR ligands, TGF-α and HB-EGF, but only TGF-α mediates Aldo-induced EGFR transactivation, at least at the time point evaluated. This illustrates that EGFR ligands involved in transactivation are cell and stimuli specific and indicates that future studies are needed to evaluate the EGFR ligands involved in Aldo actions in the kidney. In human renal and cardiovascular pathophysiology, ADAM17 expression may be increased [21, 34, 46]. Our findings suggest that Aldo is one of the drivers of increased ADAM17 expression in tubular cells. Moreover, ADAM17 SNPs have been associated to increases in cardiovascular mortality [47].

Activation of the RAAS has been associated to activation of EGFR signalling in renal and cardiovascular diseases. AngII via EGFR pathway regulates hypertrophy

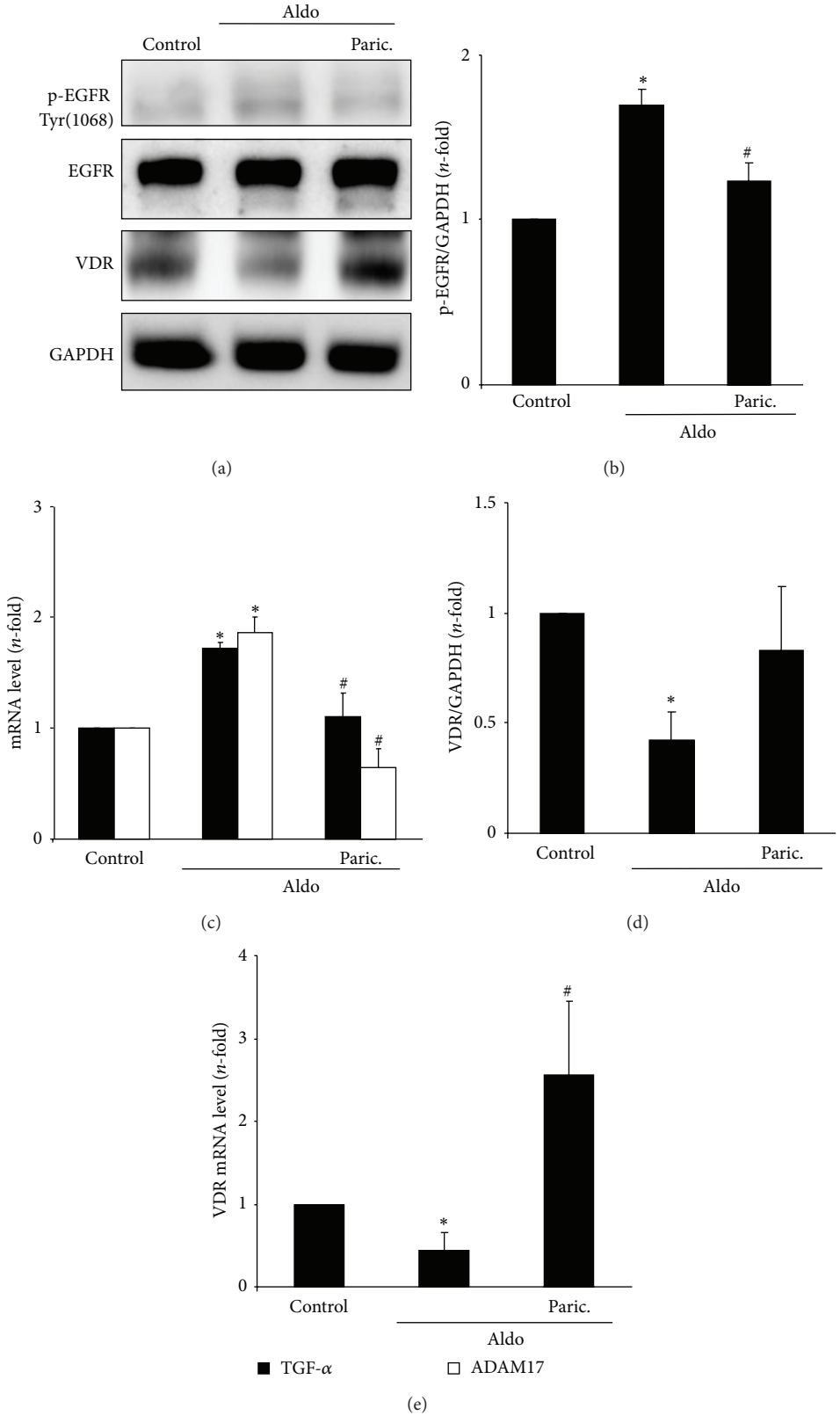

Figure 8: Paricalcitol inhibits aldosterone-induced activation of ADAM-17/TGF-α/EGFR signalling pathway. Cells were pretreated with the analogue of vitamin D Paricalcitol (12 μmol/L) for 48 hours before stimulation with 1 μmol/L Aldo for 15 min (protein) or 6 hours (gene studies). Paricalcitol inhibits Aldo-induced EGFR activation (a, b) and gene overexpression of TGFα and HB-EGF (c). Figure (a) shows a representative Western blot, in (b) quantification of p-EGFR and in (c) gene expression levels by real-time PCR. Paricalcitol restores Aldo-induced changes in VDR protein (a, d) and gene (e) expression levels. Figure (a) shows a representative Western blot, in (d) quantification of VDR protein levels and in (e) gene expression levels. Data are expressed as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Aldo.

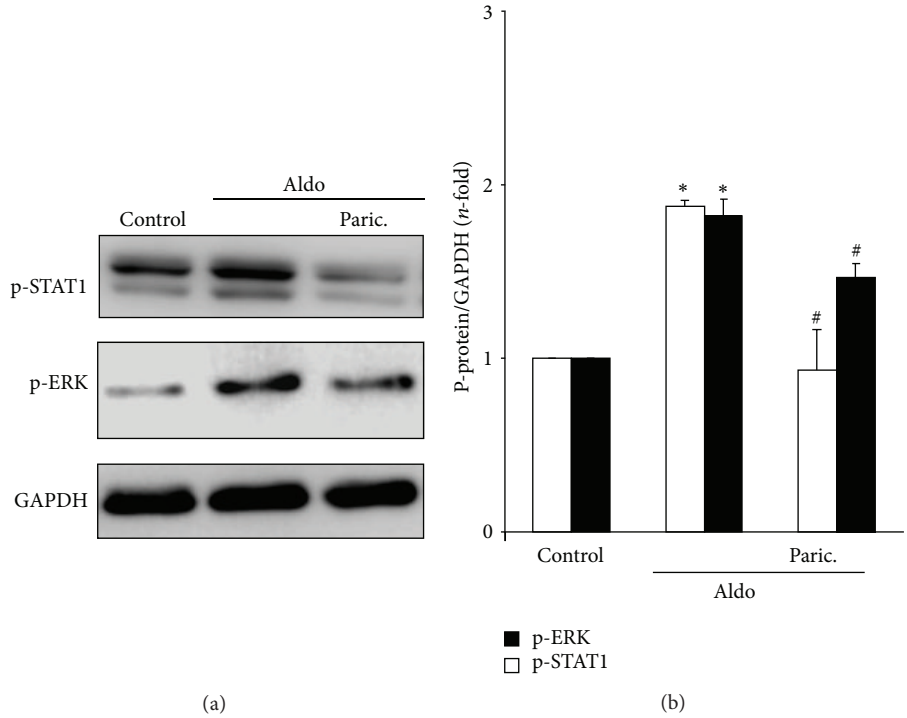

Figure 9: Paricalcitol inhibits aldosterone-mediated activation of ERK and STAT-1 pathway. HK2 cells were preincubated with paricalcitol (12 μmol/L) for 48 hours before stimulation with 1 μmol/L Aldo for 15 min. Figure (a) shows a representative experiment and in (b) quantification of p-ERK and p-STAT1 expressed as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Aldo.

and fibrosis in the kidney [20]. In porcine renal proximal tubular cells HB-EGF shedding-dependent EGFR transactivation regulates AngII-induced cell hypertrophy [48]. Interestingly, Smad activation, the main pathway controlling fibrosis, is independent of HB-EG/EGFR pathway [48]. In mesangial cells, Aldo activates EGFR linked to ROS production, ERK signalling, and modulating cell growth [31], as described in myocytes [49] and vascular cells [50]. In other cell types, Aldo/EGFR may signal through PI3-kinase/Akt/mTOR/p70S6 K1 [22]. We have described here that, in tubular cells, Aldo activates ADAM17 leading to rapid and significant activation of EGFR, which in turn activates downstream cascades, such as the MAPK ERK kinase and the STAT-1 transcription factor (Figure 10(a)).

Several lines of evidence have suggested a potential anti-inflammatory activity of vitamin D in CKD [6, 19]. In experimental glomerular diseases and obstructive nephropathy, administration of vitamin D reduces inflammatory cell infiltration [6–9, 20]. Vitamin D and VDRAs may exert their immunomodulatory actions by direct modulation of immune cells, including macrophages, dendritic cells, and T cells [51]. Vitamin D modulates dendritic cells' maturation and function, the population and function of FOXP3+ and IL-10–producing T regulatory cells [52], and regulates Th17 differentiation and decreasses IL-17A production [53]. Besides regulating immune cells, VDRAs could also exert anti-inflammatory actions by modulating responses of resident renal cells. In this sense, we have found that, in tubular epithelial cells, paricalcitol inhibits Aldo-induced proinflammatory genes upregulation. The mechanisms involved in these anti-inflammatory effects of vitamin D in epithelial cells are not well known. In monocytes/macrophages, vitamin D inhibits LPS-induced cytokine production by upregulating MAPK phosphatase-1 that inactivates p38 and JNK [54]. In tubular cells, we found that paricalcitol inhibited Aldo-induced activation of ERK and STAT-1 (Figure 10(b)), identifying a novel mechanisms of action of VDRAs.

Earlier studies showed a relation between vitamin D and EGFR signalling pathways. Those studies were focused on EGFR binding and regulation of its gene expression [55–58]. After that, many studies demonstrated that vitamin D or VDRAs increase cell proliferation via EGFR in different cell types, by a mechanism that includes changes in EGFR membrane trafficking and downregulation of EGFR growth signalling [1, 59]. However, recent studies suggest that this antiproliferative effect could be mediated by the modulation of the TGF-α/EGFR autocrine growth loop [60]. Our results show that in cultured tubular epithelial cells, the VDRA agonist paricalcitol inhibited the EGFR pathway activated by Aldo by modulating TGF-α/ADAM17/EGFR signalling pathway activation and expression, supporting this hypothesis.

5. Conclusions

Our *in vitro* data in tubular cells stimulated with Aldo suggest that the anti-inflammatory properties of the VDRA paricalcitol are, at least in part, mediated by inhibition

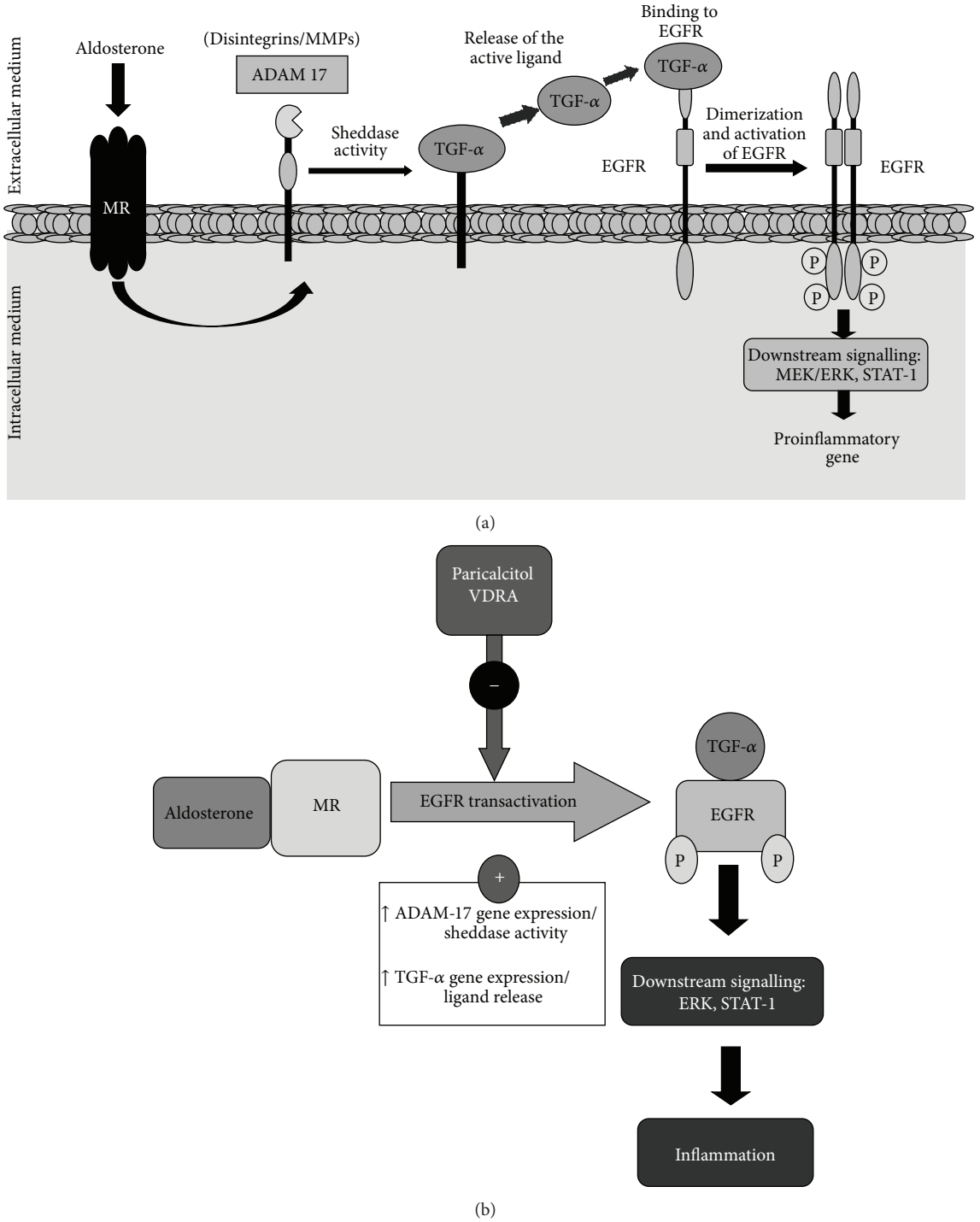

Figure 10: Aldosterone-induced inflammatory responses: role of the ADAM-17/TGF-α/EGFR axis and modulation by paricalcitol. (a) Aldosterone activates the ADAM-17/TGF-α/EGFR axis to regulate proinflammatory factors. (b) Paricalcitol inhibits aldosterone-induced activation of ADAM-17/TGF-α/EGFR signalling pathway. MR: mineralocorticoid receptor; VDRA: vitamin D receptor activators.

of the ADAM-17/TGF-α/EGFR and downstream signals, including ERK and STAT-1. In renal diseases, local activation of RAAS contributes to inflammation and tissue damage. Thus, our results show a novel signalling pathway that could be involved in the observed anti-inflammatory effects of paricalcitol in CKD and expand the current understanding of the mechanisms involved in the renoprotective effects of vitamin D and its analogs.

Conflict of Interests

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

Acknowledgments

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Review Article

Role of Vitamin D Deficiency in Extraskeletal Complications: Predictor of Health Outcome or Marker of Health Status?

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The relationship of vitamin D with extraskeletal complications, such as cardiovascular disease, cancer, and autoimmune disease, is of major interest considering its roles in key biological processes and the worldwide high prevalence of vitamin D deficiency. However, the causal relationships between vitamin D and most extraskeletal complications are weak. Currently, a heated debate over vitamin D is being conducted according to two hypotheses. In this review, we first present the different arguments that suggest a major role of vitamin D in a very broad type of extraskeletal complications (hypothesis #1). We then present results from recent meta-analyses of randomized controlled trials indicating a lack of association of vitamin D with major extraskeletal complications (hypothesis #2). We discuss different issues (e.g., causality, confounding, reverse causation, misclassification, and Mendelian randomization) that contribute to the favoring of one hypothesis over the other. While ultimately only one hypothesis is correct, we anticipate that the results from the ongoing randomized controlled trials will be unlikely to reconcile the divided experts.

1. Introduction

In humans, most circulating vitamin D is synthesized from cholesterol following exposure to ultraviolet B (UVB) in sunlight, whereas a smaller amount is derived from diet and dietary supplements. Diet contributes only between 10% and 20% to 25(OH)D levels but becomes more important when sunshine exposure is low [1]. Fish is the major dietary source of vitamin D in humans.

A summary of the synthesis and metabolism of vitamin D, which have been described in detail in multiple reviews and textbooks [1–6], is presented below. Calcitriol, or 1,25-dihydroxyvitamin D [1,25(OH)2D], is the hormonally active form of vitamin D that is derived from the following three sources: sunlight, diet, and dietary supplements (Figure 1).

There are two precursors to active vitamin D hormones, vitamins D_3 (cholecalciferol) and D_2 (ergocalciferol). Vitamin D_3 is synthesized in the skin after exposure to UVB light. Solar UVB radiation (wavelength of 290 to 315 nm) penetrates the skin and converts 7-dehydrocholesterol to previtamin D_3 by photolysis, which is rapidly converted to vitamin D_3 [4]. Vitamin D_3 may also be obtained from some dietary sources and dietary supplements. Vitamin D_2 (ergocalciferol) is derived solely from the diet (and not from UVB). Both vitamin D_3 and D_2 enter the blood circulation and are attracted to the vitamin D binding protein (VDBP).

Vitamin D in the circulation is transported to the liver, which is where it is converted by vitamin D-25-hydroxylase to 25-hydroxyvitamin D [25(OH)D]. This first hydroxylation is catalyzed by the CYP27A1 enzyme. This form of vitamin D is thought to be biologically inactive and must be converted in the kidneys by 25(OH)D 1α-hydroxylase to its biologically active form, 1,25(OH)2D [2]. This second hydroxylation is catalyzed by CYP27B1, which is located in the inner mitochondrial membrane of the proximal tubule cells of the kidneys. The 24-hydroxylation of both 25(OH)D and 1,25(OH)D to form 24,25(OH)D and 1,24,25(OH)D is the primary mechanism and the first step towards the inactivation of vitamin D metabolites.

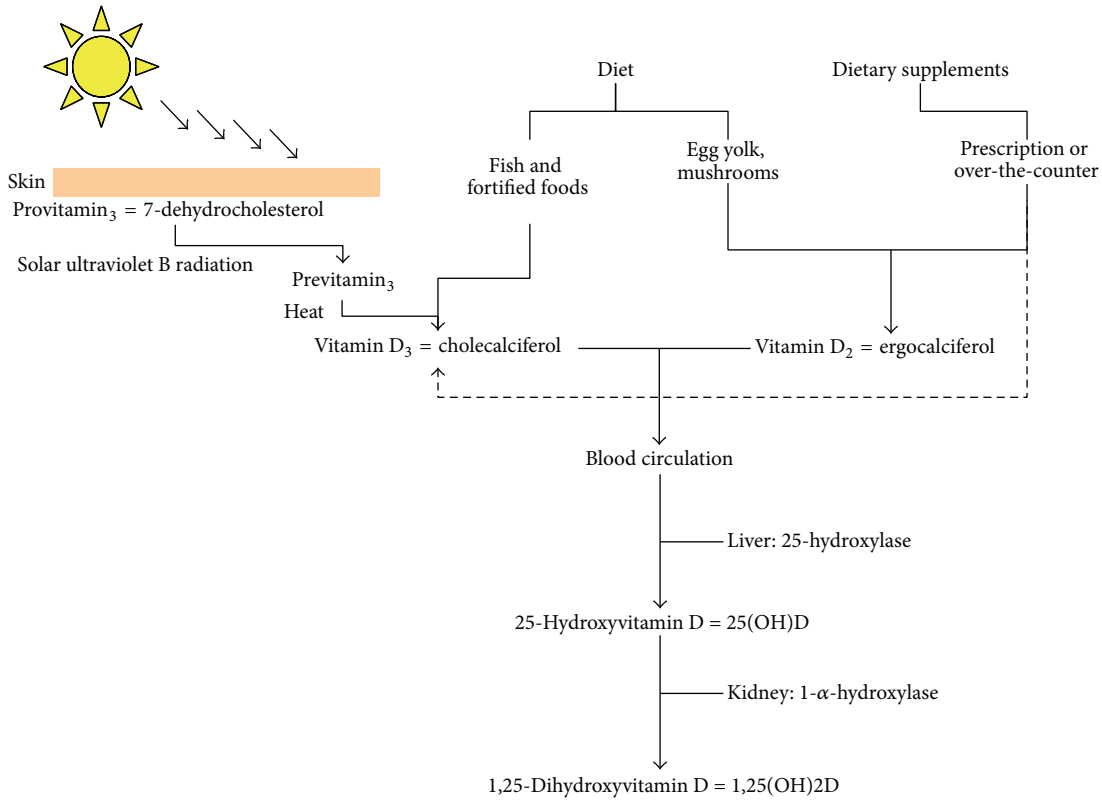

Figure 1: Sources of vitamin D and the first steps of vitamin D synthesis.

Genomic and Nongenomic Vitamin D Functions. The actions of vitamin D are largely mediated by genomic functions. Vitamin D interacts with nuclear vitamin D receptor (VDR). VDR is a ligand-induced nuclear receptor that regulates the expression of over 900 genes throughout the genome [7, 8]. It influences the transcription of genes that are responsive to the VDR-vitamin D complex. 1,25(OH)2D dissociates from serum VDBP and enters the cell. Inside the cell, it binds to and activates VDR, and the VDR-vitamin D complex translocates from the cytosol to the nucleus, where it is joined by the retinoid X receptor (RXR) partner [6]. The 1,25(OH)2D-VDR-RXR complex binds to specific sequences in the promoter regions of target genes that are called vitamin D response elements (VDREs), leading to the promotion and modulation of the expression of the targeted genes. 25(OH)D is less active than 1,25(OH)D2 because of its lower affinity for VDR.

Vitamin D has also some nongenomic rapid-response functions. In terms of its nongenomic functions, it functions as a steroid hormone by activating signal transduction pathways linked to vitamin D receptors on cell membranes.

2. Hypothesis #1: Vitamin D Is Causally Associated with Extraskeletal Complications

2.1. Vitamin D Deficiency and Associated Seasonal and Geographic Patterns

2.1.1. High Prevalence of Vitamin D Deficiency. The worldwide high prevalence of vitamin D deficiency is often presented as being clearly linked with the high burden of extraskeletal complications. In Europe, the mean serum 25(OH)D levels (conversion factor for 25(OH)D: 1 ng/mL = 2.496 nmol/L) reported in population-based studies have varied from 18 ng/mL (29 nmol/L) in Italy to 30 ng/mL (75 nmol/L) in Norway but are generally very low [9]. A recent review of vitamin D deficiency in central Europe has concluded that 25(OH)D levels are on average below 30 ng/mL [10]. Worldwide, it is estimated that one billion people have vitamin D deficiency [11], and it affects more than 40% of US and European men and women [12]. This high prevalence has also been reported in a Swiss study that has calculated a population-based estimation of vitamin D deficiency according to 25(OH)D serum levels, reporting that 39.5% of the population has 25(OH)D levels of <20 ng/mL (<50 nmol/L) (vitamin D deficiency) [13].

Of course, the prevalence of vitamin D deficiency and insufficiency depends on the definition used. Several definitions of vitamin D deficiency exist, some of which are presented in Table 1. The reasons for the differences in these definitions are partially due to the outcomes, for which the defined cutoff levels were different (e.g., bone fracture, parathyroid hormone (PTH) level, cardiovascular (CV) events, and cancer). Obviously, it is very unlikely that the risks of these outcomes all increase at the same 25(OH)D level. Given the wide variation in the definitions of vitamin D deficiency, Pilz et al. have suggested that the ideal 25(OH)D concentration for overall health-related outcomes ranges from 40 nmol/L to 120 nmol/L [14]. In fact, a more accurate

Table 1: Examples of different vitamin D status definitions.

Mayo clinic PMID*: 20675513		Institute of Medicine (IOM) PMID*: 21118827		Pilz et al. PMID*: 21682758		Kidney Disease Improving Global Outcomes (KDIGO) PMID*: 19644521	
Severe deficiency	<25	At risk of deficiency	<30	Deficiency	<50	Deficiency	<37
Moderate deficiency	25–59.9	At risk of inadequate level	30–49	Insufficiency	50–74.9	Insufficiency	37–75
Optimal	60–200	Sufficient	50–125	Optimal	75–100	Adequate	>75
Possible toxicity	>200	Possible toxicity	>125	Sufficiency	75–250		
				Intoxication	>375–500		

25(OH)D expressed in nmol/L. Conversion factor for 25(OH)D: 1 ng/mL = 2.496 nmol/L; *PMID: PubMed unique identification number.

range seems to be between 40 and 80 nmol/L according to a recent study that has suggested the presence of a J-curve between vitamin D level and overall mortality [15]. Debates on the ideal vitamin D concentration have increased after the publishing of the 2011 Institute of Medicine (IOM) report [16]. The IOM suggested that a lower 25(OH)D level should be used to define vitamin D deficiency and that a 25(OH)D serum level of 20 ng/mL (50 nmol/L) is desirable for bone and overall health [16]. While vitamin D deficiency defined as a serum 25(OH)D level of <20 ng/mL (50 nmol/L) and vitamin D insufficiency defined as a 25(OH)D level of between 20 and 29 ng/mL (50–75 nmol/L) have been extensively used in epidemiological studies, the definition of vitamin D insufficiency is more controversial. Of note, a 25(OH)D level of at least 20 ng/mL (50 nmol/L) was chosen by the IOM, with recommended dietary allowances of 600 IU/day of vitamin D for individuals aged 1–70 years and 800 IU/day for those aged 71 years and older to meet the requirements of at least 97.5% of the population. Yet other definitions of vitamin D deficiency and insufficiency are proposed in the Kidney Disease Improving Global Outcomes (KDIGO) guidelines [17]; serum levels of vitamin D are considered as adequate when the concentration of 25(OH)D is higher than 30 ng/mL, levels between 15–30 ng/mL are considered as insufficient, and values that are lower than 15 ng/mL define the diagnosis of vitamin D deficiency (Table 1).

Several factors have been proposed to explain the high prevalence of vitamin D deficiency, one of which is inadequate vitamin D intake [18]. Using data from a 10-year trend study (1999–2009, N = 9,320) performed in Geneva, Switzerland, de Abreu et al. have found that slightly more than 10% of participants complied with dietary recommendations for vitamin D intake [19]. This finding was in line with data suggesting that vitamin D is one of the critical vitamins and that its intake is below the recommended level [18, 20]. This chronic insufficient intake of micronutrients in a population without the emergence of immediate clinical signs is typical of "Hidden Hunger" [20, 21].

2.1.2. Latitude and Season. Vitamin D is often presented as the link explaining most of the observed correlations between latitude and season with extraskeletal complications. Most vitamin D is produced in the skin from 7-dehydrocholesterol by sunlight UVB exposure, which varies with latitude and season [3]. UVB exposure decreases from the equator towards the polar regions [22]. At approximately 0° latitude (i.e., the equator, e.g., the Republic of Seychelles), a high level of vitamin D-effective UV radiation is present, which varies only slightly throughout the year. On the other hand, at approximately 40° latitude (e.g., Switzerland, with a latitude of 47°), the level of vitamin D-effective UV radiation varies greatly throughout the year and decreases substantially during the winter [23].

Ecological studies have reported an inverse correlation of ischemic heart disease with sunlight and a seasonal pattern of coronary heart disease mortality [24]. We contributed to the largest (N = 230,000 from 15 countries) and most comprehensive (body mass index, waist circumference, blood pressure, total high- (HDL) and low-density lipoprotein (LDL) cholesterol, triglycerides, and glucose levels) study ever conducted to assess the seasonality of cardiovascular risk factors [25]. The results have strongly suggested that cardiovascular risk factors present a seasonal pattern, with lower levels occurring during the summer and higher levels during the winter, suggesting that at least part of the patterning might be due to changes in air/outdoor temperature, air pollution, and exposure to sunlight and thus differences in vitamin D levels [25].

Geographic and seasonal patterns of cancer incidence and mortality have also been reported in ecological studies [26, 27], suggesting a correlation between sunlight exposure (and, thus, vitamin D) and cancer.

2.2. VDR Receptor, VDR Gene, and Extrarenal 1-Alpha-Hydroxylase. The wide distribution of VDRs in humans, the influence of vitamin D on more than 3% of the human genome, and the extrarenal presence of 1-alpha-hydroxylase are often presented as factors explaining the very broad influence of vitamin D on health and disease [20].

Several biological systems have VDRs and are responsive to vitamin D. Because one of the major biological functions of this vitamin is to maintain calcium homeostasis, typical responses occur in the intestines and kidneys, which is where 1,25(OH)2D-VDR regulates genes, leading to increased calcium and phosphate absorption. Another typical action of vitamin D is the suppression of PTH synthesis by 1,25(OH)2D-VDR in the parathyroid glands. However, a recent study has reported that the 1,25(OH)2D-VDR complex controls the expression of genes and syntheses of mRNAs that are unrelated to calcium homeostasis. In fact, one of the reasons for the recent growing interest in the role of vitamin D in extraskeletal complications is emerging evidence that

VDRs are largely distributed throughout human tissues. The list of tissues in which VDRs are distributed includes the hepatogastrointestinal system (e.g., the colon), the respiratory system (e.g., the lungs), the central nervous system (e.g., neurons), the cardiovascular system (e.g., cardiomyocytes), and the kidneys [28]. Thus, tissue and cellular VDR distribution is wide. Most human tissues and cell types are responsive to 1,25(OH)2D [29, 30]. For example, the vitamin D-VDR complex controls the expression of genes involved in the inhibition of the renin-angiotensin system (RAS), which influences blood pressure, as well as genes that promote the secretion of insulin and cell proliferation and differentiation [31–34].

VDR abundance and activity seem to play important roles in individual responses to 1,25(OH)2D. Some VDR abundance and activity are determined by *VDR* polymorphisms (i.e., genetic variations that occur at a frequency of >1% [35]) [5]. The *VDR* gene lies on chromosome 12 [36]. The coding sequence of the VDR protein is comprised of eight exons, and several (>60) genetic polymorphisms have been identified [5, 37]. These polymorphisms may alter transcriptional activity and thus VDR abundance and may modulate cellular responsiveness to 1,25(OH)2D. In particular, there is a polymorphic site at the 5′ end of the *VDR* gene *Fok I* C/T. This polymorphism, in contrast with the other *VDR* variants, results in an altered amino acid sequence [5, 37]. The *Fok I* C allele generates a shorter VDR protein than the T allele, and this shorter protein is thought to be more active than the longer one [5]. Another important polymorphism is the *Cdx2 VDR* polymorphism, which is located in the promoter region of the *VDR* gene in exon 1. The *Cdx2 VDR* polymorphism has been associated with VDR transcriptional activity in the intestinal tract. The T allele has shown up to 70% greater transcriptional activity compared with the C allele [38].

It was first estimated that the number of primary 1,25(OH)2D target genes is in the order of 100–500 per tissue, but there seems to be many more genomic VDR binding sites per cell type (between 1000 and 10,000) [29]. However, as stressed by Carlberg, some of these sites may not have specific functions and may only represent noise [29].

The observations that extrarenal 1,25(OH)2D can also be produced (extrarenal 1-alpha-hydroxylase) and that 1,25(OH)2D can act locally in the tissues where it is produced (autocrine/paracrine activities) also support the potential role of vitamin D in extraskeletal complications [39].

2.3. Evidence from Molecular and Animal Studies. Molecular and animal studies have established associations of vitamin D with extraskeletal complications. Given the abundance of available literature on this topic, we will briefly present some major mechanisms involved in specific but frequent chronic extraskeletal complications, namely, CV disease (CVD) (high blood pressure, coronary heart disease, and stroke), type II diabetes, cancer, and chronic kidney disease (CKD).

2.3.1. Cardiovascular Disease: High Blood Pressure and Coronary Heart Disease. Molecular evidence has revealed effects of 1,25(OH)2D on blood pressure-related mechanisms [34]. These mechanisms include the direct inhibition by 1,25(OH)2D of the RAS. VDR is expressed in the juxtaglomerular apparatus and modulates renin synthesis. Mice lacking VDRs are hyperreninemic and present with high blood pressure and cardiac hypertrophy [40]. By contrast, the overexpression of VDR in the mouse juxtaglomerular apparatus leads to hyporeninemia [41]. In addition, vitamin D can regulate blood pressure through the prevention of secondary hyperparathyroidism [42], and it seems to have a direct effect on vascular cells and endothelial function [43].

Different mechanisms have been proposed to explain the association of vitamin D with coronary heart disease [44], some of which are indirect. Vitamin D could be related to coronary heart disease by affecting blood pressure, glycemic control, or PTH. Other proposed mechanisms are more directly related to atherosclerosis, cardiac tissues, and vasculature. Animal studies have highlighted the roles of vitamin D in cardiomyocyte remodeling in response to injury and atherosclerosis as well as in cardiac relaxation and contractility [45]. Serum levels of vitamin D seem to be inversely associated with the extent of vascular calcification in individuals at risk of ischemic heart disease [44, 46]. Vitamin D could confer protection against atherosclerosis and vascular calcification by directly affecting vascular smooth muscle cells (VSMCs). This vitamin causes an acute influx of calcium in VSMCs that might inhibit their proliferation [47]. Vitamin D could also be associated with coronary heart disease by downregulating proinflammatory cytokines (e.g., TNF-α and IL-6) and upregulating the anti-inflammatory cytokine IL-10 [44].

2.3.2. Type II Diabetes and Cancer. There is great interest in the roles of vitamin D in the susceptibilities of type II diabetes and metabolic syndrome [47]. The effects of vitamin D on type II diabetes could be mediated by its role in pancreatic β-cell function, insulin resistance, or inflammation [33, 48–50]. With respect to cancer, studies using animal models have shown that knocking out *VDR* induces many types of cancers, including mammary, prostate, and colon cancers [26, 51]. Recently, a low vitamin D level has even been proposed as a modulator of the link between diabetes and cancer [52].

2.3.3. Chronic Kidney Disease. CKD is defined as the persistence for 3 or more months of structural and/or functional abnormalities of the kidney [53]. CKD is associated with an increased risk of ischemic heart disease, stroke, peripheral vascular disease, anemia, bone disease, end-stage renal disease, and mortality. Mechanisms (beyond those related to parathormone or calcium) by which vitamin D might be associated with CKD are summarized in Figure 2. Vitamin D insufficiency correlates with mortality risk among patients with CKD and evidence has been reviewed elsewhere [54, 55]. For example, a recent meta-analysis of 20 observational studies showed vitamin D treatment to be associated with decreased risk of all-cause and cardiovascular mortality in patients with CKD not requiring dialysis and patients with end stage renal disease (ESRD) requiring dialysis [55]. This meta-analysis also highlighted the need of well-designed randomized controlled trials to formally assess the survival benefits of vitamin D.

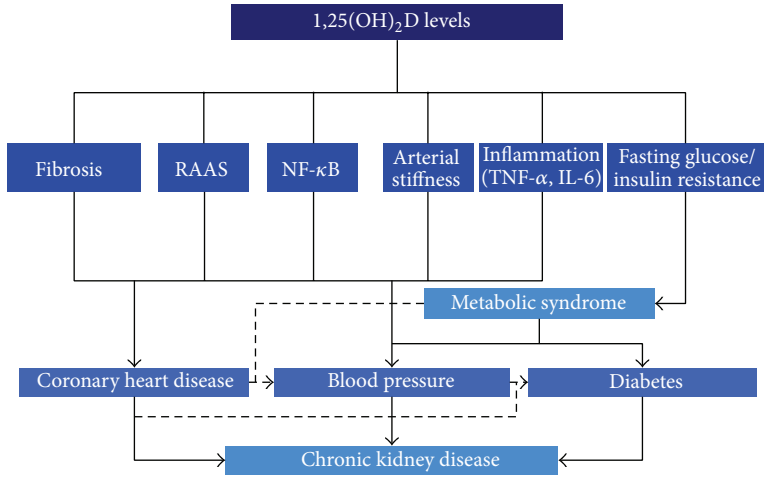

Figure 2: Main hypothetical mechanisms of action of vitamin D in chronic kidney disease. Mechanisms related to parathormone and calcium are not displayed. VSMC = vascular smooth muscle cells; RAAS = renin-angiotensin-aldosterone system; NF-κB = nuclear factor-κB; TNF-α = tumor necrosis factor-α; IL-6 = interleukin-6.

One of the mechanisms associating vitamin D with CKD involves the nuclear factor-κB (NF-κB) pathway. NF-κB is a family of transcription factors that functions as a master regulator of the immune response [56]. It regulates a wide range of genes involved in inflammation, proliferation, and fibrogenesis and is known to have a key role in kidney disease [57]. A direct inhibition by 1,25(OH)2D of the (NF-κB) pathway has been reported. Both RAS and NF-κB promote the production of profibrotic and proinflammatory factors, increase oxidative stress, and damage podocytes.

2.4. Evidence from Observational Studies. Guessous et al. have previously reviewed evidence of the roles of vitamin D in hypertension, coronary heart disease, and stroke [34]. Out of four observational prospective studies identified, two have reported an inverse association between 25(OH)D level and hypertension risk, one found no such association, and one found an association neither with hypertension nor with changes in blood pressure. Lower vitamin D levels have been found to be associated with an increased risk of myocardial infarction in a prospective study of men aged 40–75 years [58]. Higher levels of 1,25(OH)D have been associated with decreased risk of stroke among participants in a cohort study conducted in Finland [59].

2.5. Economic Impact of Correcting Vitamin D Deficiency. Finally, a potentially huge economic benefit resulting from the correction (at the population level) of vitamin D deficiency is often presented. Assuming that vitamin D deficiency is causally associated with extraskeletal complications, it has been suggested that a rise in the serum 25(OH)D levels of all Europeans to 40 nmol/L (achieved by a daily intake of 2000–3000 IU of vitamin D) would lower health-care costs by up to 17%, which represents a reduction of 187,000 million Euros/year [20, 60]. Thus, a single, simple intervention could have a major impact on the economy of health care. However, this belief is conditional on a causal relationship of vitamin D with extraskeletal complications that is far from being accepted as described below.

3. Hypothesis #2: Vitamin D Is Not Causally Associated with Extraskeletal Complications

The belief that vitamin D is not causally associated with extraskeletal complications is mainly based on the absence of robust evidence of this association from randomized controlled trials (RCTs), on the potentially unfavorable effects of vitamin D supplementation and on the precedents of other vitamin and antioxidant trials.

3.1. Randomized Controlled Trials. To date, most associations of vitamin D with extraskeletal complications reported by nonexperimental studies have not been replicated in RCTs. For example, results from observational cross-sectional and prospective studies on the inverse association of vitamin D intake or 25(OH)D level with blood pressure have not been confirmed in RCTs [34]. Only one out of three RCTs identified have found a decrease in blood pressure in the intervention arm, but daily supplementation included both vitamin D_3 and calcium (not vitamin D only) [61]. Two RCTs primarily designed to determine the effect of vitamin D supplementation on the risk of fracture have reported conflicting effects of this vitamin on coronary heart disease [62, 63], and no RCT has evaluated the effect of vitamin D supplementation on coronary heart disease as a primary outcome. Trivedi et al. have found no effect of vitamin D supplementation on stroke [62].

To evaluate the presence of biases in the associations of vitamin D with diverse skeletal and extraskeletal outcomes, Theodoratou et al. have performed a review of evidence obtained from systematic reviews and observational meta-analyses and RCTs (i.e., an umbrella review) [64]. More than 260 systematic reviews or meta-analyses that have included over 130 outcomes have been examined. This group has reported a lack of highly convincing evidence of a clear link of

vitamin D with any outcome. Even for skeletal complications, the authors have concluded that RCTs that examined vitamin D only (without calcium supplementation) have failed to demonstrate protective effects of vitamin D supplementation on fractures or falls. Moreover, a 2014 trial sequential meta-analysis (i.e., analysis that modeled the changing precision in estimates of effects as trials are reported and the likely effects of future trial results on the existing body of data) has shown that the effects of vitamin D supplementation on extraskeletal complications are below the futility boundary of 15% [65, 66]. Thus, future trials are unlikely to alter the conclusion of no causal association.

A 2009 narrative review has estimated that raising the minimum year-round serum 25(OH)D level to 100–150 nmol/L (40 to 60 ng/mL) would prevent approximately 58,000 new cases of breast cancer and 49,000 new cases of colorectal cancer each year in the US and Canada [67]. However, the most recent (2011) meta-analysis has concluded that because of the potential confounding data inherent in observational studies and the limited data obtained from RCTs, evidence is currently insufficient to draw conclusions about the efficacy of vitamin D supplementation for cancer prevention [68]. A 2014 review of vitamin D status and CVD by the UK Nutrition Society has concluded that data supporting a causal link between vitamin D status and CVD are mixed and ambiguous [69]. Similar conclusions were independently published in 2011 by the North American DRI committee [16], by the Endocrine Task Force [70], and more recently, by others [65, 71–74]. In terms of endocrine health and disease, available evidence does not show that vitamin D supplementation consistently decreases the risk of type II diabetes, Addison's disease, or autoimmune thyroid disease [75].

In a 2014 meta-analysis of observational studies ($N = 73$) and RCTs ($N = 22$) of vitamin D and mortality caused by extraskeletal complications, Chowdhury et al. have found evidence that 25(OH)D level is inversely associated with risk of death due to extraskeletal complications, but no evidence of such a relationship has been found in RCTs [76]. Stratified analysis has suggested that supplementation with vitamin D_3 and not D_2 reduces all-cause mortality [76], but most of the trials included in the stratified analysis reported a high score for the risk of bias.

Overall, the conclusions of the meta-analyses and reports are in contrast with other recent reviews, which have concluded that adequate vitamin D supplementation is an important prophylactic factor for immunity, autoimmunity, CVD, cancer, fertility, pregnancy, dementia, and mortality [77]. Therefore, most scientists have acknowledged that the prevalence of vitamin D deficiency is high (although as discussed above, the definition of vitamin D normality is not universal); however, based on the lack of robust evidence, some have questioned whether it is a health problem [78].

3.2. Unfavorable Effects of Vitamin D. Vitamin D may have unfavorable effects. For example, it could potentially contribute to arterial stiffening and hypertension. Richart et al. [79] have proposed mechanisms of the renal versus extrarenal activation of vitamin D in relation to atherosclerosis, arterial stiffening, and hypertension. Macrophages in atherosclerotic lesions can locally activate 25(OH)D to form calcitriol, which could act as a vasoactive and prooxidative substance in VSMCs. Another concern is related to severe cases of hypercalcemia observed in children with *CYP24A1* mutations [80]. Concerns extend beyond extraskeletal complications because recent evidence has also suggested that high doses of vitamin D may increase the risks of fractures and falls [81, 82].

3.3. Vitamins, Antioxidants, and Hormone Replacement Therapy Precedents. Skepticism about the curative and/or predictive potentials of vitamin D for extraskeletal complications has also been magnified by previous vitamin-related research, in which evidence reported in observational studies has not been replicated in RCTs [83]. RCTs of beta-carotene, vitamin A, and vitamin E have not replicated findings from observational studies [84]. Even worse, RCTs have reported that some antioxidants and vitamins with supposed favorable effects (based on observational studies) in fact have unfavorable effects (based on RCTs). In 2008, the SELECT trial of vitamin E and selenium in cancer prevention was stopped prematurely because the intervention arm was more (although not statistically significantly) likely to develop prostate cancer [85]. This, of course, echoes the experience and lessons learned from the Women's Health Initiative (WHI) study on hormone replacement therapy (HRT) [86]. This trial was stopped prematurely when an increase in invasive breast cancer achieved statistical significance. This trial also found increases in heart attacks and strokes in the intervention group. These findings contrasted dramatically with conclusions of previous epidemiologic studies, which indicated a lack of a convincing link between breast cancer and HRT and reported that HRT decreases cardiovascular events [87].

4. Issues Favoring One Hypothesis over the Other

4.1. Causality. A major point in the debate about the real impact of vitamin D deficiency on extraskeletal complications is whether 25(OH)D belongs to the causal pathway. In other words, does a low 25(OH)D level cause disease or is it simply a side effect of either the exposure (e.g., sedentary behavior or obesity) or disease (e.g., cancer or autoimmune disease)? While an RCT (i.e., the supreme paradigm for epidemiological research [88]) is the best study design to determine causality, risk factors, such as low 25(OH)D levels, cannot be assessed with this type of trial for obvious ethical reasons. Observational studies, which are prone to spurious results, are then conducted. This issue of study design is so central that some epidemiologists have categorized the field into *descriptive* problems (i.e., a parameter of occurrence is related to a determinant without a causal interpretation of the relationship) and *causal* problems [88]. A causal interpretation of the relationship of an outcome parameter (type II diabetes) to a determinant (25(OH)D) can be given as long as the relationship is conditional on the entire set of potential confounders (both known and unknown). This

requisite conditionality is best pursued by randomization [88] and thus the use of RCTs.

4.2. Confounding and Reverse Causation. Confounding happens when the effect of at least one extraneous factor (e.g., body mass index, BMI) is mixed with the effect of an exposure of interest (25(OH)D), thus distorting the estimate of the latter [89]. With respect to vitamin D, the list of potential known confounders is very large and includes the following major ones. (1) Age: the production of vitamin D by the skin decreases with age [2]. (2) BMI: the reasons for the inverse association between BMI and 25(OH)D level are not completely understood, but fat in the skin seems to decrease the efficacy of vitamin D synthesis, which may be due to 7-dehydrocholesterol sequestration. Of note, this inverse association could also be confounded by a decrease in sun exposure (e.g., a decrease in outdoor physical activity or comorbidities). (3) Latitude and seasons: theoretically, persons living in regions closer to the equator should present with higher levels of vitamin D synthesis than those residing in regions remote from the equator. In practice, however, because more than 90% of vitamin D arises from sunlight (in the absence of supplementation), levels of this vitamin also depend on cultural behaviors (clothing, time spent outdoors, and sunbathing habits). Overall, reports of the effect of latitude on serum 25(OH)D have been inconsistent [90–92]. A positive correlation between 25(OH)D and latitude has been found in a (25 European countries) pooled analysis, whereas the highest rate of 25(OH)D deficiency has been observed in Scottish participants (highest latitude) in a British cross-sectional study [91]. However, recent metaregression analysis did not find an influence of latitude on 25(OH)D level [92]. (4) Skin pigmentation: the packaging and sizes of melanosomes in keratocytes influence the darkness of the skin. Dark pigments in the skin reduce its ability to synthesize vitamin D from sunlight by up to 95% [22]. This fact likely explains why African-Americans have lower 25(OH)D than non-Hispanic whites in the US [22]. The darker skin pigmentation in individuals residing in southern compared to northern European countries may underlie the higher prevalence of 25(OH)D deficiency in southern Europe [93]. (5) Diet: diets typically contain only small amounts of vitamin D (vitamin D_3 or vitamin D_2). Fish is the major dietary source of vitamin D in humans. Three ounces of cooked salmon and 3.5 ounces of cooked mackerel provide 90% and 86%, respectively, of the recommended daily vitamin D intake (400–600 international units/day), whereas 3.5 ounces of cooked beef only provides 4% of the recommended intake. (6) Occasional sunscreen: sunscreen use by children and young adults is unlikely to cause vitamin D deficiency, but the chronic use of sunscreen by elderly individuals has been shown to decrease 25(OH)D and to cause vitamin D deficiency. (7) Altitude, air pollution, ozone, time of the day, and cloud cover: at higher altitudes, UVB radiation is stronger because the concentrations of aerosols and particles are lower. Air pollution decreases vitamin D-effective radiation. Ozone, time of the day, and cloud cover also influence vitamin D-effective radiation and thus vitamin D photosynthesis. Other potential confounders include genetic and epigenetic (i.e., heritable and modifiable changes in gene expression that do not affect DNA sequences) variations that have been implicated in association with vitamin D that may contribute to the interindividual variability of the impacts of its deficiency and/or supplementation [29, 94]. Disregarding the unknown potential confounders, fully accounting for all of these potential known confounders is practically impossible (residual confounding) unless randomization is used.

Temporality in causal criteria refers to the necessity that the cause precedes the effect in time (causal sequence) [89]. Thus, reverse causation refers to a situation in which an outcome precedes and causes an exposure instead of the other way around [95]. Autier et al. have performed a systematic review of vitamin D status and ill health and have concluded that the discrepancies between observational and intervention studies indicate that low 25(OH)D is a marker, not a cause, of ill health [96]. These authors have notably proposed that the wide range of disorders associated with low 25(OH)D is explained by the fact that inflammatory processes involved in disease occurrence and clinical course reduce 25(OH)D. Other groups have stressed that 25(OH)D is an unreliable biomarker of vitamin D status after an acute inflammatory insult [97].

Although subgroup analysis of a recent meta-analysis of observational studies has suggested that the inverse association of 25(OH)D level with mortality is stronger in populations with low prevalences of vitamin D supplementation or low 25(OH)D levels [76], this finding can still be attributed to reverse causation, with more severe underlying diseases being associated with lower 25(OH)D levels.

4.3. Mendelian Randomization. Randomization in observational studies is not possible, but an alternative exists, which is termed Mendelian randomization (MR). MR is being increasingly used to overcome confounding and reverse causation for exploring causal effects of an exposure on a disease in nonexperimental studies. The concept of MR refers to the random allocation of alleles at the time of gamete formation. By analogy with the fact that the random allocation of a treatment in a RCT renders confounding unlikely, a genetic variant of interest should not be associated with known and unknown confounding factors [98, 99]. MR studies have been conducted to infer causality for vitamin D and extraskeletal complications, such as high blood pressure and type II diabetes. Genetic variants that specifically alter 25(OH)D levels, which are usually identified from genome-wide association studies (GWAS), are generally used. For example, Kunutsor et al. have used 4 variants (in the *DHCR7*, *CYP2R1*, *GC*, and *CYP24A1* genes) as instrumental variables in a small sample (unknown N), failing to show a causal role of 25(OH)D in the etiology of high blood pressure [100]. Vimaleswaran et al. have used the same 4 variants but have considered allelic scores and used a large sample ($N = 146{,}581$), reporting that each 10% increase in genetically instrumented 25(OH)D concentration is associated with an 8% decreased odds of hypertension [101]. Using the same

variants as mentioned above but not considering allelic scores, Ye et al. have also estimated the unconfounded causal associations of 25(OH)D concentration with the risks of type II diabetes and other glycemic traits using an MR approach [102], reporting insignificant MR-derived estimates for type II diabetes and glycemic traits and suggesting that the association between 25(OH)D and type 2 diabetes is not causal.

Of note, there are commonly acknowledged necessary conditions for MR to provide a causal inference in observational epidemiology [98, 99]. One condition is that the genetic instruments (e.g., *DHCR7* variants) affect the outcome (e.g., extraskeletal complication) by no other means than through the exposure (25(OH)D), which is never the case with respect to common complex human diseases. Additionally, one cannot exclude that a true causal variant may be in linkage disequilibrium with the genetic instruments (genetic variants) used. Results obtained using the MR approach should therefore be interpreted cautiously.

4.4. Measurement Error and Misclassification

4.4.1. Is 25(OH)D a Good Biomarker of Vitamin D Intake and/or Function? 1,25(OH)2D is the active form of vitamin D, but because 25(OH)D has a much longer circulating half-life, circulates at higher concentrations, and is less influenced by other hormones, such as PTH, it is used to determine vitamin D status. Because of its long half-life in circulation, 25(OH)D reflects vitamin D supply and usage over a period of time. Circulating 25(OH)D is also a better marker of vitamin D exposure than indirect estimates of vitamin D exposure based solely on diet, which do not take into consideration sunlight sources [103].

However, as discussed elsewhere [104], serum 25(OH)D concentration is influenced by several factors, such as the quantity of vitamin D delivered to the liver, the amount of 25(OH)D produced by the liver, and the half-life of 25(OH)D in the serum. These factors are themselves influenced by several determinants (sunlight exposure, intestinal absorption, body fat, 25-hydroxylase activity, VDBP production in the liver, etc.) [104]. Prentice et al. have stressed that because serum 25(OH)D has little interaction with VDRs, it might be a good biomarker of intake but not of function [104]. Therefore, other biomarkers of vitamin D function have been proposed (e.g., the 1,25(OH)2D/25(OH)D ratio, 1,25(OH)2D/24,25(OH)2D ratio, and >35 additional 25(OH)D metabolites formed by the body). However, evaluating such biomarkers in large epidemiological studies is difficult. Additionally, serial measurements in the same individuals are ideal but rarely feasible. Therefore, it is not excluded that 25(OH)D, when used as a biomarker of extraskeletal complications, provides biased associations similar to many other biomarkers, as demonstrated in a recent systematic evaluation of 56 meta-analyses of emerging cardiovascular biomarkers (e.g., C-reactive protein and homocysteine) [105].

Even if we agree that 25(OH)D is sufficiently linked to active 1,25(OH)D and is the *best* marker of vitamin D status similar to the conclusion of a meta-analysis performed by Tzoulaki et al. [105], it is worth noting that results vary markedly depending on the assay used [106, 107]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) remains the gold standard technique to measure 25(OH)D [106, 108].

4.5. Deficiency versus Supplementation and Life-Course Perspective. Different factors that could explain the lack of replication in the few RCTs of the results reported by several observational studies have been discussed. For example, it has been suggested that the impact of vitamin D deficiency (as determined in observational studies) is not the same as the effect of vitamin D supplementation (as determined in RCTs), particularly in participants with sufficient vitamin D levels [109]. Other groups have suggested that the relationship of 25(OH)D with the risk of extraskeletal complications could be observable only at low levels of 25(OH)D [26, 109]. Davis has also suggested that the risk associated with low vitamin D status might be conferred earlier in life and that studies of circulating 25(OH)D in adults do not address the most relevant time period of exposure [26]. Vitamin D may play pivotal roles throughout life not only in calcium homeostasis and skeletal metabolism but also in infection and autoimmune disease development and progression [104]. Reviews have been published on vitamin D and functional outcomes, specifically in infants and young children [110], adolescents [111], and elderly individuals [112].

5. Why Results from Ongoing Randomized Controlled Trials Will Be Unlikely to Reconcile the Two Schools of Thought

Five RCTs (the CAPS, VITAL, Do-Health, FIND, and ViDa trials; see [20] for further details) are currently underway in 9 countries (Figure 3) to determine the impacts of vitamin D supplementation on extraskeletal complications. Most are well funded and sufficiently powered to detect a significant impact, if any exist. More than 42,000 participants will be included with a potential of 208,116 person-years, and $22 million has been invested just for the VITAL trial [113]. However, several experts have already pointed out fatal limitations that might invalidate the (negative or null) results of these trials [69, 114–117]. These limitations include the following: (i) the supplementation doses of vitamin D are too low, and higher doses should be tested; (ii) the contrast in 25(OH)D intake between the two arms is insufficient given that control groups are producing 25(OH)D even in the absence of the intervention; (iii) the 5-year follow up (which some experts have attributed to be based more on the funding grant cycle than on the vitamin D cycle) is too short; (iv) the optimal vitamin D concentration may not be the same for all extraskeletal complications considered; (v) participants are included regardless of their 25(OH)D level at baseline, and most participants already have an optimal 25(OH)D level prior to the start of the intervention. The effects of vitamin D likely depend on the baseline vitamin D status; and (vi) future trials are not likely to change pooled (null) estimates already reported by meta-analyses.

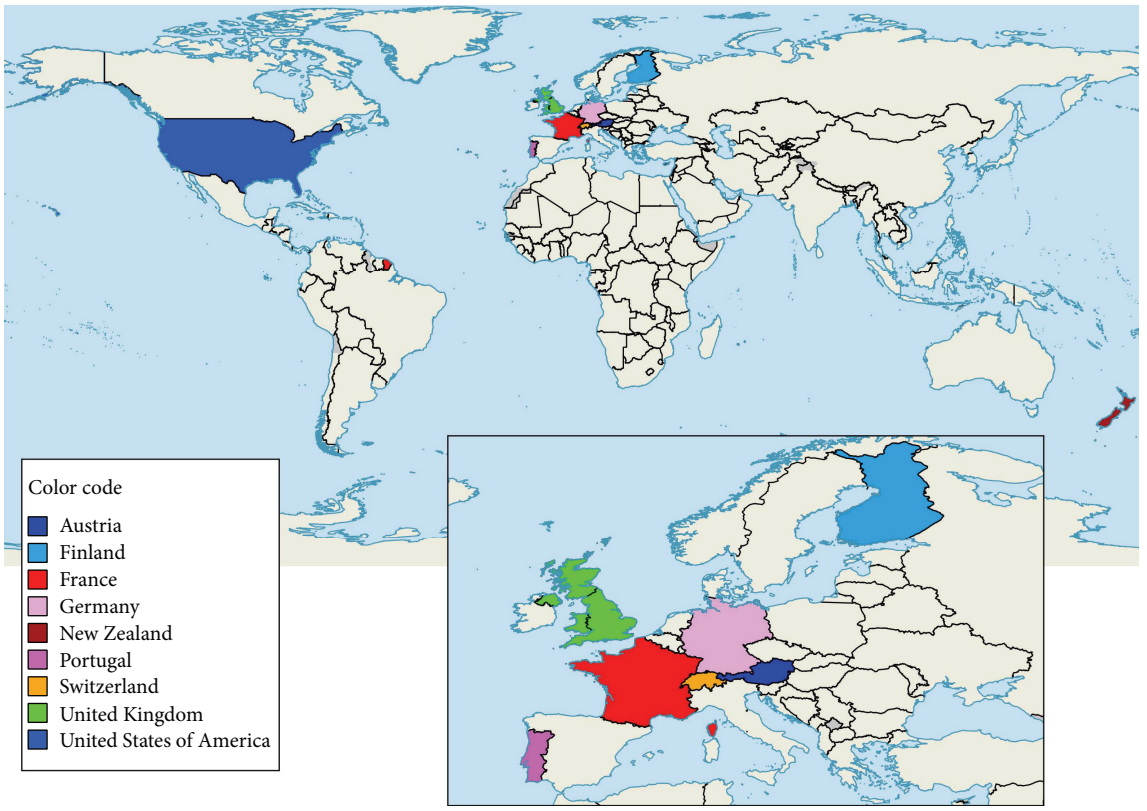

Figure 3: Countries where large randomized controlled trials are currently ongoing to determine the impact of vitamin D supplementation on extraskeletal complications. CAPS, USA; VITAL, USA; Do-Health, Austria, Germany, Finland, France, Portugal, Switzerland; FIND, Finland; ViDa; New Zealand. See [20] for further details.

We would like to add to these limitations the potential risk of "medical reversal," which is a phenomenon described by Prasad et al. involving the identification of faults with trials and the unwillingness to consider that an intervention might be ineffective [118]. We believe that medical reversal is even more likely to happen if ongoing trials report null effects (rather than negative effects, as in the WHI trial).

6. Summary and Concluding Remarks

This debate is mostly fueled by the classical divergence between observational and experimental results [119]. Observational studies are prone to reverse causation and confounding. Associations between vitamin D status and extraskeletal complications in observational studies could merely indicate that vitamin D is a "simple" indicator of health status and that compared with healthier subjects sicker subjects could have a lower vitamin D level or status. The diversity of biological systems with which vitamin D deficiency has been associated (cardiovascular, diabetes, depression, neurodegenerative diseases, cancer, etc.) could further suggest that this vitamin is a marker of health status rather than a predictor of health outcomes. However, both the wide distribution of VDRs in humans and the influence of vitamin D on more than 3% of the human genome could explain its broad effects on health.

RCTs assessing the efficacy of vitamin D supplementation in reducing extraskeletal complications are ongoing. Trials are still recruiting participants, and the first results will not be available before the year 2017. The impacts of the ongoing RCTs on clinical practice are of course difficult to predict, but at least three scenarios exist as follows: favorable and meaningful results are reported by the RCTs (scenario A); no favorable or meaningful results are reported (scenario B); or unfavorable results are reported (scenario C). All of these scenarios may or may not be followed by appropriate changes in clinical practice. Depending on the scenario, the baseline common practice, and the capacity of change in the common practice, the use of vitamin D as a biomarker could be a success or failure, as proposed by Ioannidis [120]. If it is not a success, vitamin D could eventually become a biomarker of the following: (i) "type B failure" occurs "when a biomarker shows great promise in one or more early studies, but the claims are later found to be wrong or exaggerated, and the biomarker is eventually never implemented into clinical practice" (scenario A in a practice, in which vitamin D dosing is not already common); (ii) "type D failure" occurs "when a biomarker shows no or little promise, but nevertheless is enthusiastically promoted for widespread clinical or population use" (scenario C in a practice, in which vitamin D dosing is already common); and (iii) "type A failure" occurs "when a widely used biomarker that has already been implemented in clinical practice is shown to be largely useless—or even harmful—and therefore needs to be abandoned" (scenario C

in a practice, in which vitamin D dosing is already common but the common practice can be changed).

Determining 25(OH)D dosages in the absence of robust evidence seems to already be a common practice in the US, where sales of vitamin D supplements have increased from $50 million in 2005 to $600 million in 2011 [121] and where commentators already advocate clinicians to stop costly measurements of 25(OH)D in asymptomatic patients outside skeletal-related conditions [83, 122, 123].

Finally, while our review focused on extraskeletal complications and discussed the related challenges, this debate might not remain limited to extraskeletal complications for long but may soon include skeletal complications. Indeed, recent evidence has suggested that vitamin D might not be as essential as previously thought for maintaining bone health and preventing falls [64–66, 124].

Vitamin D (the solar vitamin) is likely to remain a burning topic in coming years.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

The Impact of Vitamin D_3 Supplementation on Mechanisms of Cell Calcium Signaling in Chronic Kidney Disease

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Intracellular calcium concentration in peripheral blood mononuclear cells (PBMCs) of patients with chronic kidney disease (CKD) is significantly increased, and the regulatory mechanisms maintaining cellular calcium homeostasis are impaired. The purpose of this study was to examine the effect of vitamin D_3 on predominant regulatory mechanisms of cell calcium homeostasis. The study involved 16 CKD stages 2-3 patients with vitamin D deficiency treated with cholecalciferol 7000–14000 IU/week for 6 months. The regulatory mechanisms of calcium signaling were studied in PBMCs and red blood cells. After vitamin D_3 supplementation, serum concentration of $25(OH)D_3$ increased ($P < 0.001$) and $[Ca^{2+}]_i$ decreased ($P < 0.001$). The differences in $[Ca^{2+}]_i$ were inversely related to differences in $25(OH)D_3$ concentration ($P < 0.01$). Vitamin D_3 supplementation decreased the calcium entry through calcium release activated calcium (CRAC) channels and purinergic $P2X_7$ channels. The function of $P2X_7$ receptors was changed in comparison with their baseline status, and the expression of these receptors was reduced. There was no effect of vitamin D_3 on $P2X_7$ pores and activity of plasma membrane Ca^{2+}-ATPases. Vitamin D_3 supplementation had a beneficial effect on $[Ca^{2+}]_i$ decreasing calcium entry via CRAC and $P2X_7$ channels and reducing $P2X_7$ receptors expression.

1. Introduction

Vitamin D hormonal system has been classically implicated in the regulation of calcium homeostasis and bone metabolism. However, it has also noncalciotropic effects through the activation of tissue vitamin D receptors (VDR) [1]. Vitamin D insufficiency/deficiency is a significant risk factor for the development of various chronic diseases, and the deficiency of calcidiol ($25(OH)D_3$) as well as calcitriol ($1,25(OH)_2D_3$) is common in CKD patients [2]. Therefore, the supplementation of native vitamin D (cholecalciferol or ergocalciferol) or active vitamin D (calcitriol and VDR activators) in CKD is well established.

Free cytosolic calcium concentration ($[Ca^{2+}]_i$) is controlled by mechanisms that regulate Ca^{2+} entry from the extracellular space and Ca^{2+} release from intracellular stores and by the activity of ATP-dependent Ca^{2+} pumps and antiporters that move Ca^{2+} back into stores or out of cells [3]. Already in early stages of chronic kidney disease (CKD), $[Ca^{2+}]_i$ and calcium concentration of intracellular stores were significantly increased in comparison with healthy volunteers, and the regulatory mechanisms of calcium signaling were impaired by the disease [4–7].

Calcium enters into the cells by any of the general classes of calcium/cation channels. In nonexcitable cells like peripheral blood mononuclear cells (PBMCs), the predominant Ca^{2+} entry pathway is the store-operated one, in which the emptying of intracellular Ca^{2+} stores activates the Ca^{2+} influx. This type of the channel is known as the calcium release activated calcium (CRAC) channel in lymphocytes.

The calcium entry through CRAC channels activates certain transcription factors which regulate the gene expression for cytokines responsible for immune responses [8, 9].

Another mechanism of calcium entry into the cell is represented by purinergic P2X receptors. At the present time, purinergic signaling is accepted as a crucial component of diseases and was found to mediate a vast array of biological processes. The $P2X_7$ receptors are expressed primarily on cells of hemopoietic origin, where they participate in immune responses, cell proliferation, cell death, bone formation, and bone resorption [10]. The $P2X_7$ receptor is a bifunctional purinoreceptor that opens a nonselective cation channel and consecutively forms a large, cytolytic pore. The key factor of $P2X_7$-dependent cytotoxicity is the massive intracellular Ca^{2+} increase triggered by its activation. This can lead to membrane blebbing and cell death by apoptosis or necrosis. There is an increasing body of evidence implicating $P2X_7$ receptors in various pathological conditions [11–14].

The plasma membrane Ca^{2+}-ATPases (PMCA) is responsible for removing excessive Ca^{2+} out of the cells to extracellular environment. The decreased PMCA activity increases $[Ca^{2+}]_i$ and affects some intracellular processes.

To our knowledge, little information is available regarding the impact of vitamin D_3 supplementation on disturbed cell calcium homeostasis in CKD. Therefore, the aim of the present study was to examine the effect of vitamin D_3 supplementation on essential regulatory mechanisms of cell calcium homeostasis.

2. Materials and Methods

2.1. Patients. The study population consisted of 16 nondiabetic patients with CKD (9 patients CKD stage 2 and 7 patients CKD stage 3). All of them were screened and followed up in the outpatient department of nephrology at the Slovak Medical University. The diagnosis of CKD was based on clinical and laboratory examinations as defined by the K/DOQI criteria [15]. Causes of their renal disease were glomerulonephritis in 9 patients, tubulointerstitial nephritis in 3 cases, hypertensive nephroangiosclerosis in 2 patients, and other in 2 causes. The glomerular filtration rate was estimated by the MDRD study formula [16]. Patients with acute impairment of renal function, nephrotic proteinuria, malignancies, and derangements in mineral metabolism of nonrenal origin were excluded from the study. Concurrent treatments interfering with mineral metabolism were not allowed. Previous therapy with vitamin D_2/D_3, calcitriol, or over-the-counter vitamin D preparations had to be cancelled at least 2 months before enrollment. Hypertension was the most common comorbidity present in all patients and treated with ACE inhibitors or angiotensin II receptor blockers in 14, diuretics in 8, betablockers in 6, and calcium channel blockers in 8 cases. Dihydropyridine calcium channel blockers were allowed as they do not interfere with studied parameters and effects in PBMCs [17]. All patients had vitamin D deficiency (serum $25(OH)D_3$ concentration <30 ng/mL) and were supplemented with cholecalciferol 7000–14000 IU/week for 6 months; the dose (approximately 1000–2000 IU/day) was chosen as a common supplementary dose for the treatment of vitamin D deficiency in general population.

The study was approved by the Ethics Committee of the Slovak Medical University and all participants gave their written informed consent.

2.2. PBMCs Isolation. Human PBMCs were isolated by the Ficoll gradient centrifugation, diluted 1 : 1 with RPMI-1640 medium, layered onto an equivalent volume of medium LSM-1077, and centrifuged at 700 g for 20 min at 22°C, as previously reported [5]. The PBMC layer was washed in 40 mL RPMI, resuspended in 10 mL RPMI and 10% fetal bovine serum (FBS), and centrifuged at 300 g 10 min at 22°C and the pellet was resuspended in 2 mL aliquots of physiological salt solution. Final concentration of PBMCs was adjusted to 2.5×10^6 cells/mL. Our preparation contained lymphocytes (94–96%), monocytes (3-4%), and natural killer cells (the rest), as determined by flow cytometry (Coulter Epics XL, Ireland). The cell viability was quantified using a 0.8% solution of trypan blue and estimated to be 96–98%.

2.3. Red Blood Cell Membranes Isolation. Isolated red blood cell (RBC) membranes were used to assess the PMCA function. RBC membranes were obtained by hemolytic fragmentation in hypotonic media using standard method of Hanahan and Ekholm [18] modified in our laboratory to achieve a higher quality of the ghosts. RBCs from previous isolation were diluted 1 : 5 with physiological salt solution and centrifuged at 1270 g for 20 min at 4°C. The supernatant was removed and the procedure was repeated one more time. RBCs were then diluted 1 : 5 with tris(hydroxymethyl)aminomethane (TRIS) medium (20 mmol/L, pH = 7.4) and centrifuged at 7700 g for 35 min at 4°C. This step was repeated twice for each TRIS medium with decreasing concentrations (20, 10, and 5 mmol/L).

2.4. Intracellular Ca^{2+} Measurements. The population of 2×10^6 PMBCs/mL was loaded with fluorescence dye Fluo-3 AM at a final concentration of 2 μmol/L for 40 min at 22°C in a physiological salt solution. After incubation, the cells were centrifuged at 300 g, washed three times with a physiological salt solution, and kept at room temperature for 10 min before use. The Fluo-3 fluorescence was measured at 37°C in Fluorolog 3–11 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ, USA) with an excitation at 488 nm (bandpass 3 nm) and an emission at 526 nm (bandpass 5 nm). Each experiment was followed by $[Ca^{2+}]_i$ calibration to estimate the actual free cytoplasmic calcium concentration from the measured fluorescence signal (F) in each cell population. $[Ca^{2+}]_i$ was quantified in nmol/L according to the following equation:

$$[Ca^{2+}]_i = K_d \cdot \left[\frac{F - F_{\min}}{F_{\max} - F}\right], \quad (1)$$

where K_d = 400 nmol/L at 37°C [19]. The maximal fluorescence intensity ($F_{\max}$) was assessed by the addition of Triton X-100 (0.1%) with Ca^{2+} (5 mmol/L), and the minimum

fluorescence level (F_{min}) was determined after the addition of 25 mmol/L EGTA (pH = 9). Digitonin (20 μmol/L) was used to answer for minimal compartmentalization [20]. To assess the role of CRAC channels in Ca^{2+} entry, 2-aminoethyldiphenyl borate (2APB), a widely used inhibitor of these channels was applied [21]. Although there are already more potent and selective inhibitors of these channels [22], 2APB was used for the possibility of comparing the results with our previous studies [4, 6]. The action of 2APB (50 μmol/L) was studied in cells where Ca^{2+} entry through these channels was stimulated by thapsigargin (Tg) (1 μmol/L), a specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPase. To examine the function of $P2X_7$ receptors, AZ11645373 (50 nmol/L), a highly selective antagonist of human $P2X_7$ receptors [23], and 2′,3′-*O*-(4-benzoyl)benzoyl ATP (BzATP) (50 μmol/L), the most potent and selective agonist of these receptors, were used [24].

2.5. Cell Surface $P2X_7$ Receptors Expression. PBMCs were stained with $P2X_7$ polyclonal antibody labelled with fluorescein isothiocyanate (FITC) according to the protocol provided by the manufacturer. Briefly, 100 μL of PBMCs were incubated either with FITC-conjugated anti-$P2X_7$ (2 μg/mL) or FITC-conjugated IgG2a (2 μg/mL, control) for 20 min at 22°C in the dark. PBMCs were simultaneously stained with phycoerythrin- (PE-) conjugated CD14 antibody in order to exclude monocytes from the examined population. After the incubation, cells were washed three times, dissolved in 500 μL of PBS, and subjected to the analysis on flow cytometer (Cytomics FC 500 cytometer, Beckman Coulter, USA). Values of control samples stained with FITC-conjugated IgG2a were subtracted from the evaluated results.

2.6. $P2X_7$ Receptors Visualization by Fluorescence Microscopy. To visualize the surface $P2X_7$ receptors, PBMCs were stained with $P2X_7$ (extracellular) antibody. Cell imaging was performed by using the Axiovert 200 inverted microscope (Carl Zeiss, Germany) with mercury lamp HBO-100 and FluoArc driver. The 450–490 nm bandpass filter and FT510 dichroic mirror were used for excitation, and emission was detected with a 515 nm long-pass filter. Images were recorded by the PentaMax cooled CCD camera (Roper Scientific) with KODAK KAF-1400 chip. Images were taken with the C-Apochromat 40x/1.2 water immersion objective (Zeiss, Germany).

2.7. Ethidium Bromide Uptake by Flow Cytometry. The ethidium bromide uptake by PBMCs was measured on the Cytomics FC 500 cytometer (Beckman Coulter, USA) and results were processed with the CXP software. PBMCs at concentration of 10^6 cells/100 μL were kept at room temperature when ethidium bromide at 30 μmol/L final concentration was added to the sample. After a 5 min incubation, the mean channel ethidium fluorescence was assessed in FL3 sensor after excitation with a 488 nm laser beam. Up to 10000 events were involved in the data analysis. For the investigation of $P2X_7$ pore function, ethidium fluorescence was measured after a 10 min incubation with either BzATP (50 μmol/L) or AZ11645373 (50 nmol/L) at 37°C.

2.8. PMCA Activity Measurement. The RBCs membrane suspension was added to working medium (mmol/L): 100 TRIS, 80 KCl, 3 $MgCl_2$, 0.2 ethylenediaminetetraacetic acid (EDTA), and 1 ouabain (pH = 7.4) in the presence or absence of $CaCl_2$ (5 mmol/L). The reaction was started by the addition of 40 mmol/L of ATP, conducted for 60 min at 37°C and stopped by the addition of a 15% trichloroacetic acid (TCA). The amount of liberated inorganic phosphate was determined using a phosphate colorimetric assay kit (BioVision) on the UV–VIS spectrophotometer Shimadzu UV-1700 (Shimadzu Corp., Japan). The estimated PMCA activity was calculated as the difference between the activities of the enzyme incubated in the presence and absence of $CaCl_2$ and was expressed as nmol of Pi/mg protein/h. The protein concentration was measured by the method of Lowry [25].

2.9. Reagents. The physiological salt solution contained (mmol/L) 140 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 Na_2HPO_4, 0.5 $MgCl_2$, 5 glucose, and 5 HEPES (pH = 7.4). Thapsigargin (Tg) was procured from Calbiochem (San Diego, CA). 2-Aminoethyldiphenyl borate (2APB), ethidium bromide, AZ11645373, and 2′,3′-*O*-(4-benzoyl) benzoyl ATP (BzATP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluo-3 acetoxymethylester (Fluo-3 AM) was from Molecular Probes (Eugene, OR), lymphocyte separation medium LSM-1077 was from PAA Laboratories GmbH (Pasching, Austria), and fetal bovine serum (FBS) and RPMI-1640 medium were from GIBCO (Grand Island, NY, USA). PE-conjugated anti-CD14, FITC-conjugated IgG2a, and anti-$P2X_7$ (extracellular) FITC were from Sigma-Aldrich (St. Louis, MO, USA). The phosphate colorimetric assay kit was from BioVision (Hayward, CA, USA). All other chemicals were purchased from Sigma-Aldrich.

2.10. Analytical Procedures. Serum calcium and creatinine were measured by the Vitros 250 Analyzer, Johnson & Johnson, Rochester, NY, USA. Intact parathormone and 25-hydroxyvitamin D_3 were determined by the electrochemiluminescence immunoassays (ECLIA) (Roche-Diagnostics, Mannheim, Germany).

2.11. Statistical Analyses. All values are expressed as means ± SD. Statistical analysis was carried out by the SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to evaluate a sample normality distribution. The statistical significance of differences was tested by the independent 2-population Student's *t*-test for normally distributed data and the Wilcoxon's test for a nonparametric analysis. A P value < 0.05 was considered significant. The Spearman's correlations between variables were used as a measure of association.

3. Results

3.1. Effect of Cholecalciferol Treatment on Main Laboratory Variables. Effect of cholecalciferol treatment was evaluated

Table 1: Main laboratory variables at baseline and after the cholecalciferol treatment.

Parameters	Baseline	6 months
Total serum calcium (mmol/L)	2.28 ± 0.08	2.34 ± 0.08
$[Ca^{2+}]_i$ (nmol/L)	120 ± 6	105 ± 3***
iPTH (pg/mL)	43 ± 17	43 ± 20
25(OH)D_3 (ng/mL)	18 ± 2	36 ± 9***
Serum creatinine (μmol/L)	102 ± 21	101 ± 28
eGFR (mL/min)	64.8 ± 5.4	63 ± 4.2

Values are expressed as mean ± SD, *** $P < 0.001$, $n = 16$ for comparison with baseline.

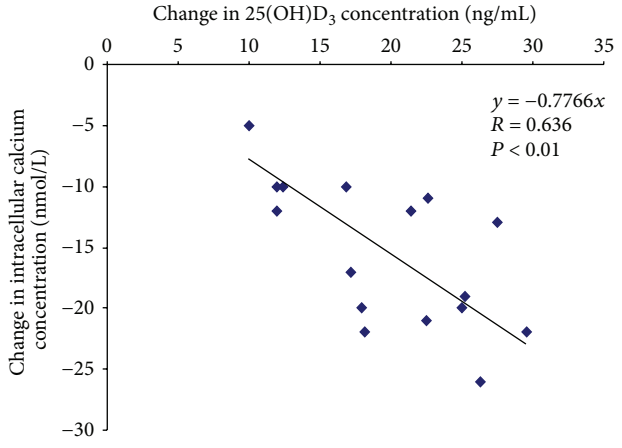

Figure 1: Relationships between absolute changes from baseline in $[Ca^{2+}]_i$ and 25(OH)D_3 concentrations after a 6-month vitamin D_3 supplementation ($P < 0.01$, $n = 16$).

in 16 CKD patients (mean age 55 ± 4, range 32–80 years, 9 males and 7 females). Table 1 shows laboratory data of CKD patients at baseline and after the vitamin D_3 supplementation. Initial 25(OH)D_3 concentrations were low (18 ± 8 ng/mL) and significantly increased after the vitamin D_3 treatment (36 ± 9 ng/mL). Eleven patients reached the recommended 25(OH)D_3 level above 30 ng/mL. The $[Ca^{2+}]_i$ in PBMCs was increased in CKD patients (120 ± 6 nmol/L). After the 6-month vitamin D_3 supplementation, $[Ca^{2+}]_i$ significantly decreased (105 ± 3 nmol/L, $P < 0.001$, $n = 16$) to values comparable with those in healthy subjects. The absolute changes from baseline in $[Ca^{2+}]_i$ were related to the absolute changes in 25(OH)D_3 concentrations ($P < 0.01$, $n = 16$) (Figure 1). The PTH concentrations were in reference range (43 ± 17 pg/mL) at basal conditions and vitamin D_3 supplementation had no effect on their values. The correlation between $[Ca^{2+}]_i$ and PTH concentrations has not been found.

3.2. CRAC Channels. CRAC channels were activated indirectly by intracellular Ca^{2+} store depletion using Tg (1 μmol/L). The 2APB (50 μmol/L), a reliable inhibitor of these channels, was applied during the sustained phase of Tg effect. It evoked a decrease in $[Ca^{2+}]_i$, which represented particularly the Ca^{2+} influx through CRAC channels. After the treatment with vitamin D_3, the enhanced Ca^{2+} entry through these types of channels (68 ± 27 nmol/L) was significantly decreased (43 ± 16 nmol/L; $P < 0.01$, $n = 16$) in CKD patients (Figures 2(a) and 7(b)).

3.3. $P2X_7$ Receptors

3.3.1. $P2X_7$ Channels. The application of $P2X_7$ receptors antagonist AZ11645373 (50 nmol/L) led to reduction in $[Ca^{2+}]_i$ in CKD patients from 120 ± 6 to 112 ± 8 nmol/L ($P < 0.001$, $n = 16$). On the other hand, after the vitamin D_3 supplementation, AZ11645373 had no effect on $[Ca^{2+}]_i$ (106 ± 4 versus 104 ± 6 nmol/L; ns, $n = 16$) (Figure 3(a)). Differences in $[Ca^{2+}]_i$ after the inhibition of $P2X_7$ receptors were significantly decreased after the vitamin D_3 supplementation ($P < 0.001$, $n = 16$) (Figure 3(b)). In BzATP stimulated cells, AZ11645373 (50 nmol/L) decreased the calcium influx before and also after the vitamin D_3 supplementation (Figures 3(c) and 3(d)), but the effect of an inhibitor was attenuated ($P < 0.01$, $n = 16$) (Figure 3(e)). The 6-month vitamin D_3 supplementation had an inhibitory effect on function of $P2X_7$ channels and thereby decreased the Ca^{2+} entry. The agonist of purinergic $P2X_7$ receptors, BzATP (50 μmol/L), caused a sustained increase in $[Ca^{2+}]_i$ in CKD patients at baseline 120± 6 to 155 ± 12 nmol/L ($P < 0.001$, $n = 16$) and also after the vitamin D_3 supplementation 106 ± 4 to 136 ± 13 nmol/L ($P < 0.001$, $n = 16$). However, the $P2X_7$ receptors activation after the vitamin D_3 supplementation did not reach the values of $[Ca^{2+}]_i$ before supplementation (Figure 4(a)). Furthermore, the effect of BzATP (50 μmol/L) on AZ11645373-inhibited calcium influx in PBMCs was evaluated. Under these conditions, a rising calcium influx through $P2X_7$ channels was found at baseline and after supplementation (Figures 4(b) and 4(c)). All these results demonstrate the inhibitory effect of vitamin D_3 supplementation on calcium entry through $P2X_7$ channels.

3.3.2. $P2X_7$ Pores. The uptake of ethidium bromide into PBMCs was measured by flow cytometry at basal conditions and with BzATP (50 μmol/L) stimulation or AZ11645373 (50 nmol/L) inhibition. The permeability of ethidium bromide through $P2X_7$ pores in PBMCs of CKD patients was significantly increased in comparison with healthy volunteers [4, 5] and remained unchanged after the vitamin D_3 supplementation. The treatment did not change the permeability of $P2X_7$ pores after the application of either BzATP (50 μmol/L) or AZ11645373 (50 nmol/L) (Figures 5(a) and 5(b)).

3.3.3. Expression of Cell Surface $P2X_7$ Receptors. The expression of cell surface $P2X_7$ receptors was 1.5-fold greater on PBMCs from CKD patients compared to healthy donors [4]. We assessed a decreased expression of these receptors after vitamin D_3 supplementation in the whole population of PBMCs ($P < 0.001$) (Figures 6(a), 6(b), and 6(c)).

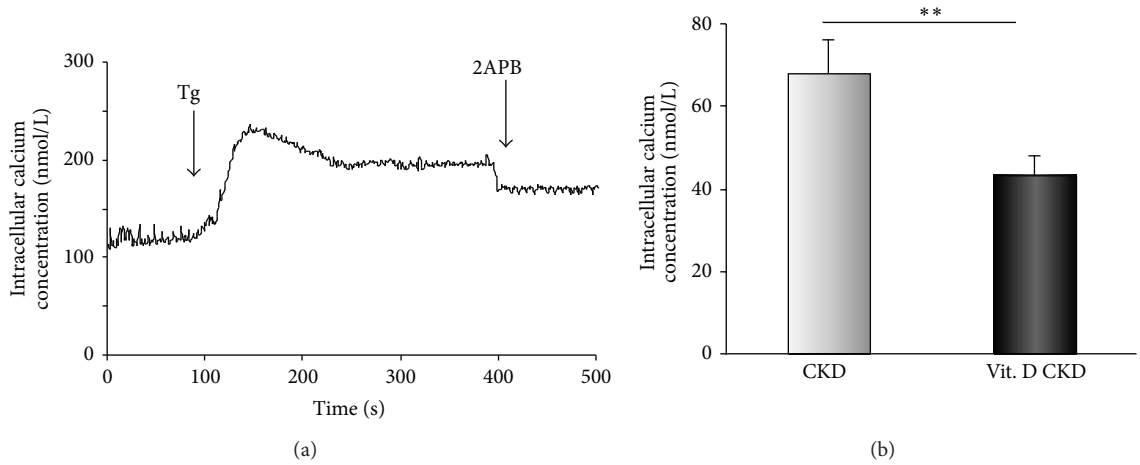

Figure 2: The calcium entry through CRAC channels. (a) A typical experiment where CRAC channels were activated by intracellular Ca^{2+} depletion by Tg (1 μmol/L) and subsequently inhibited by 2APB (50 μmol/L). The representative trace was obtained from a single experiment from PBMCs of a CKD patient. (b) The comparison of the calcium entry through CRAC channels in PBMCs of CKD patients at baseline and after a 6-month vitamin D_3 supplementation ($^{**}P < 0.01$, $n = 16$).

3.4. Plasma Membrane Ca^{2+}-ATPases. The PMCA activity of RBCs membranes is decreased by 25% in patients with early stages of CKD when compared to healthy subjects [4]. Vitamin D_3 supplementation did not increase the PMCA activity (Figures 7(a) and 7(b)). The concentrations of total plasma membrane proteins were significantly enhanced (7.7± 1.6 versus 11.8 ± 2.7 mg/mL, $P < 0.001$) which may indicate increased expression of PMCA.

4. Discussion

Disturbances in intracellular calcium homeostasis in patients with CKD represent a complex process which aggravates with CKD progression. The mechanisms of cell calcium influx and efflux are impaired in renal disease. Already in early stages of CKD, cytosolic calcium concentration ($[Ca^{2+}]_i$) and calcium concentration of intracellular stores are increased [6, 7]. The elevated calcium entry through CRAC channels and $P2X_7$ receptors, increased expression of $P2X_7$ receptors, and decreased PMCA activity contribute to this state [4]. We have previously shown that vitamin D_3 supplementation in CKD patients led to a decline in $[Ca^{2+}]_i$ to values comparable with healthy people [6]. The aim of this study was to examine the effect of vitamin D_3 supplementation on predominant regulation mechanisms of cell calcium homeostasis in nonexcitable cells from patients with early stages of CKD. The principal finding of the present study is that vitamin D_3 supplementation affected the mechanisms of intracellular calcium homeostasis as follows: the Ca^{2+} entry through CRAC and $P2X_7$ channels was decreased, while no effect was found on the permeability and functionality of $P2X_7$ pores, and the expression of $P2X_7$ receptors was reduced. Finally, the activity of PMCA was not increased after treatment.

4.1. Vitamin D_3 Supplementation. All clinical studies of vitamin D supplementation in CKD patients reported a significant improvement in $25(OH)D_3$ concentrations, although several studies did not reach a mean $25(OH)D_3$ concentration in optimal range (≥30 ng/mL). In our study, vitamin D_3 supplementation with weekly cholecalciferol dosing significantly increased $25(OH)D_3$ to the recommended levels. PTH values were in normal range and did not change after treatment, and no correlation was observed between $25(OH)D_3$ and PTH concentrations. Total serum calcium was not changed but $[Ca^{2+}]_i$ significantly decreased after supplementation. Linear regression analysis demonstrated that changes in $25(OH)D_3$ were significantly and inversely correlated with that of $[Ca^{2+}]_i$ ($R = 0.617$, $P < 0.01$). Besides our previous work [6], no studies have investigated the relationship between vitamin D status and $[Ca^{2+}]_i$ in CKD.

4.2. Ca^{2+} Entry through CRAC Channels. Dysregulation of Ca^{2+} homeostasis involving the endoplasmic reticulum (ER) and store-operated calcium channels has been manifested in patients with neurodegenerative disorders, immunodeficiency, acute pancreatitis, polycystic kidney disease, and cardiac hypertrophy [9, 26–29]. In nonexcitable cells, CRAC channels are the main pathway of Ca^{2+} entry. These channels are composed of two proteins Orai1 and STIM1 (stromal interaction molecule). Orai1 protein is located in the plasma membrane and forms the channel pore. It is activated by STIM1 located in the membrane of ER. STIM1 has a dual function of sensing the Ca^{2+} concentration in ER and activating CRAC channels. A decrease in the ER Ca^{2+} concentration induces STIM1 translocation close to the plasma membrane where it binds to and activates the Orai1 channel. Alterations in STIM1/Orai1 system may contribute to several pathophysiological conditions including cardiovascular [30, 31] and pulmonary diseases [32], hypertension [33], immunodeficiency, and autoimmune and lymphoproliferative diseases

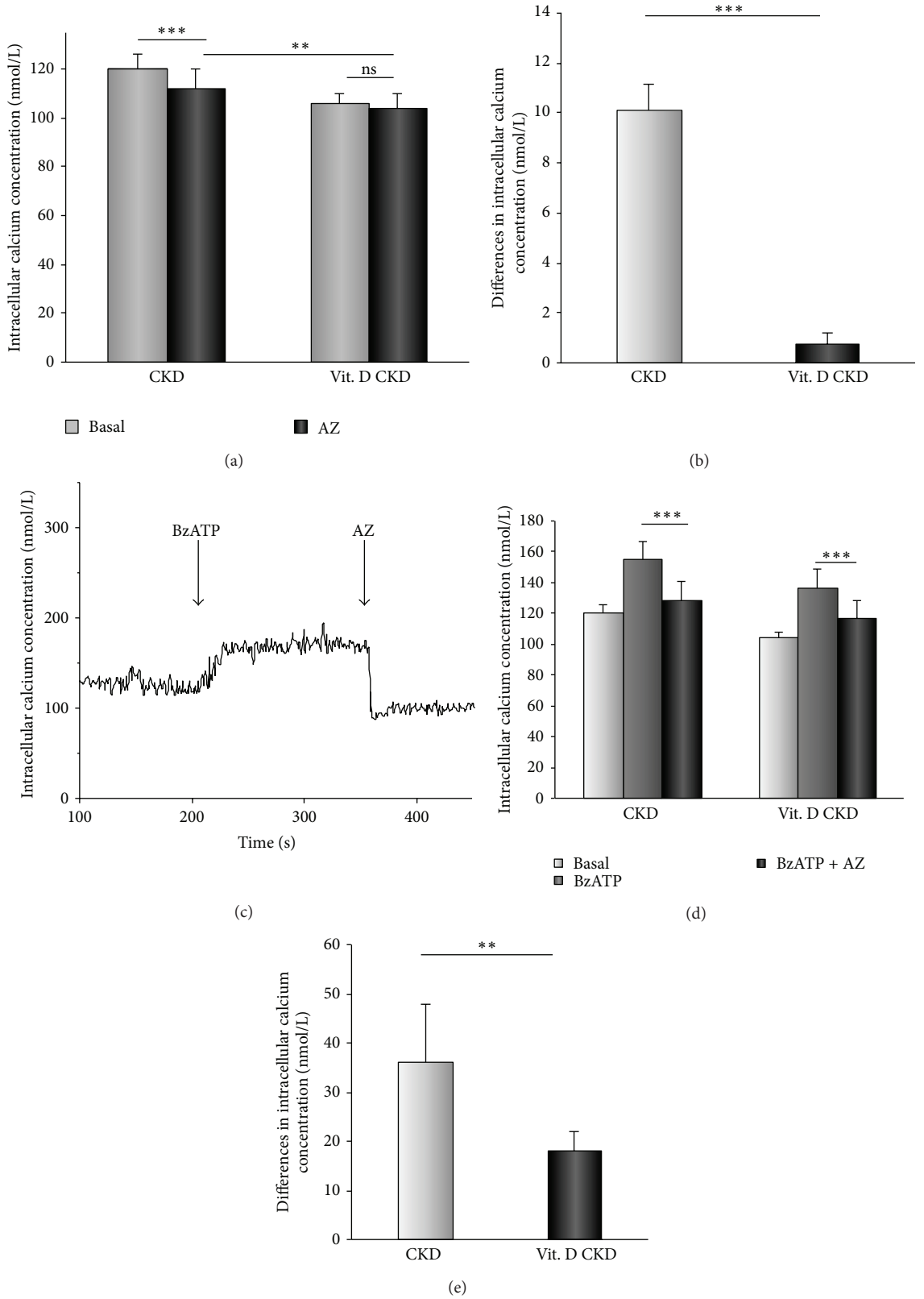

Figure 3: The effect of vitamin D_3 supplementation on an inhibitory effect of AZ11645373. (a) The comparison of the AZ11645373 (50 nmol/L) effect on $[Ca^{2+}]_i$ in CKD patients at baseline and after vitamin D_3 supplementation ($^{**}P < 0.01$, $^{***}P < 0.001$, $n = 16$). After vitamin D_3 supplementation, AZ11645373 (50 nmol/L) had no effect on $[Ca^{2+}]_i$. (b) Differences in $[Ca^{2+}]_i$ after an inhibition with AZ11645373 (50 nmol/L) at baseline and after vitamin D_3 supplementation ($^{***}P < 0.001$, $n = 16$). (c) The representative trace where AZ11645373 (50 nmol/L) inhibited the $[Ca^{2+}]_i$ rise induced by BzATP (50 μmol/L). (d) The comparison of the AZ11645373 (50 nmol/L) effect on BzATP- (50 μmol/L) activated calcium influx before and after supplementation ($^{***}P < 0.001$, $n = 16$). (e) Differences in $[Ca^{2+}]_i$ in BzATP- (50 μmol/L) activated PBMCs after the AZ11645373 (50 nmol/L) inhibition at baseline and after vitamin D_3 supplementation ($^{**}P < 0.01$, $n = 16$).

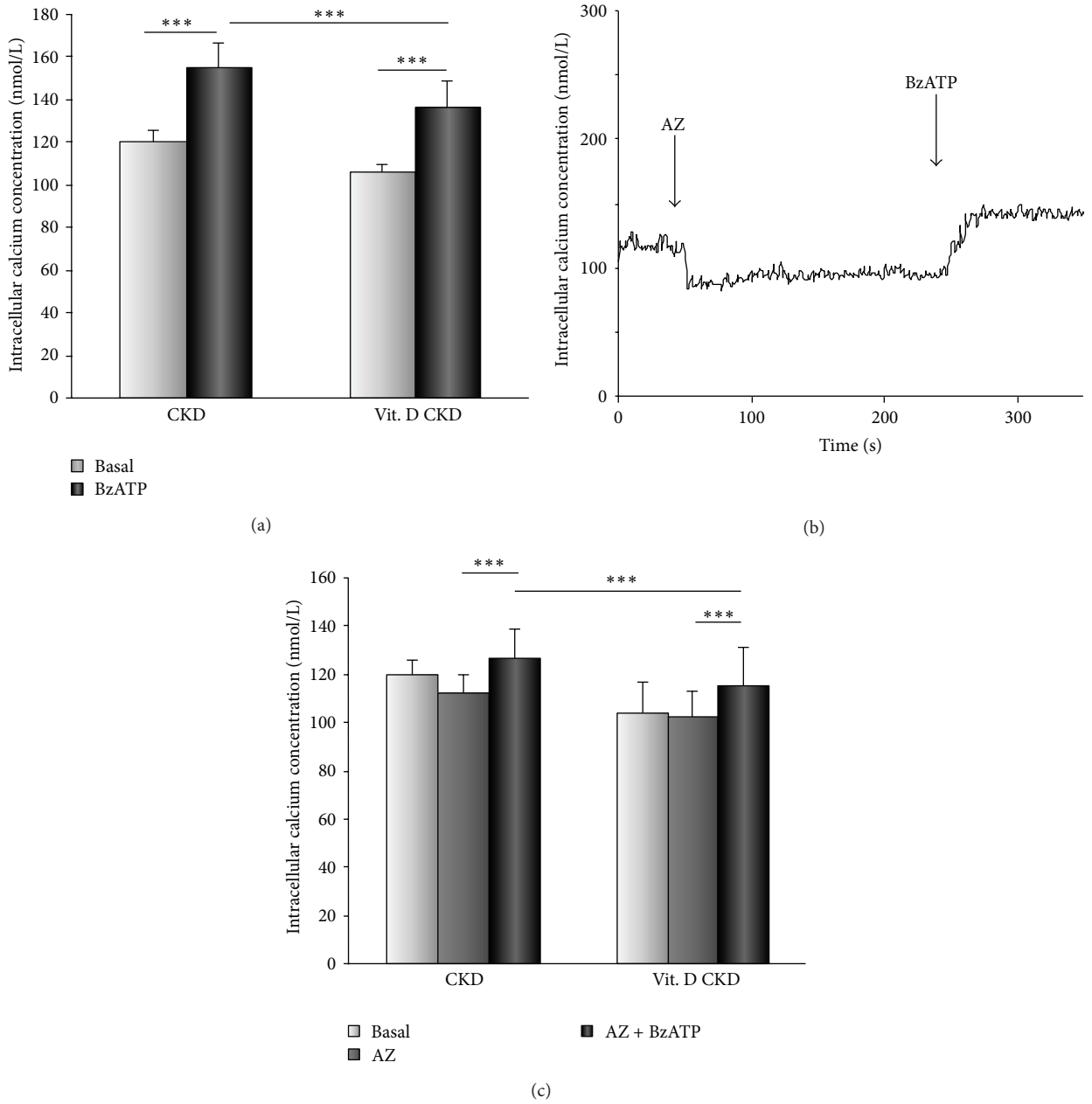

FIGURE 4: The effect of vitamin D_3 supplementation on activation by BzATP. (a) The comparison of the BzATP (50 μmol/L) effect on $[Ca^{2+}]_i$ in CKD patients at baseline and after vitamin D_3 supplementation ($^{***}P < 0.001$, $n = 16$). (b) A typical experiment where PBMCs were pretreated with AZ11645373 (50 nmol/L) prior to the application of BzATP (50 μmol/L). (c) The comparison of the BzATP (50 μmol/L) effect on AZ11645373 (50 nmol/L)-inhibited calcium influx in PBMCs before and after supplementation ($^{***}P < 0.001$, $n = 16$).

[8, 34, 35]. In our previous study, we have demonstrated that the function of CRAC channels is altered in PBMCs of patients in early stages of CKD [6]. It is not known what changes in the STIM1/Orai1 system develop over the course of CKD. In the current study, a 6-month vitamin D_3 supplementation significantly reduced the increased Ca^{2+} entry through CRAC channels which contributed to the decrease in $[Ca^{2+}]_i$. In our previous study, a 12-month vitamin D_3 supplementation decreased the Ca^{2+} entry through CRAC channels insignificantly, which could be due to less rigorous patient selection (patients with diabetes mellitus and polycystic kidney disease were included). To our best knowledge, the effects either $25(OH)D_3$ or $1,25(OH)_2D_3$ on CRAC channels were not studied in CKD.

4.3. Ca^{2+} Entry via $P2X_7$ Channels. The most recent advances provide compelling evidence for P2X receptors playing a key role in regulating physiological and pathophysiological

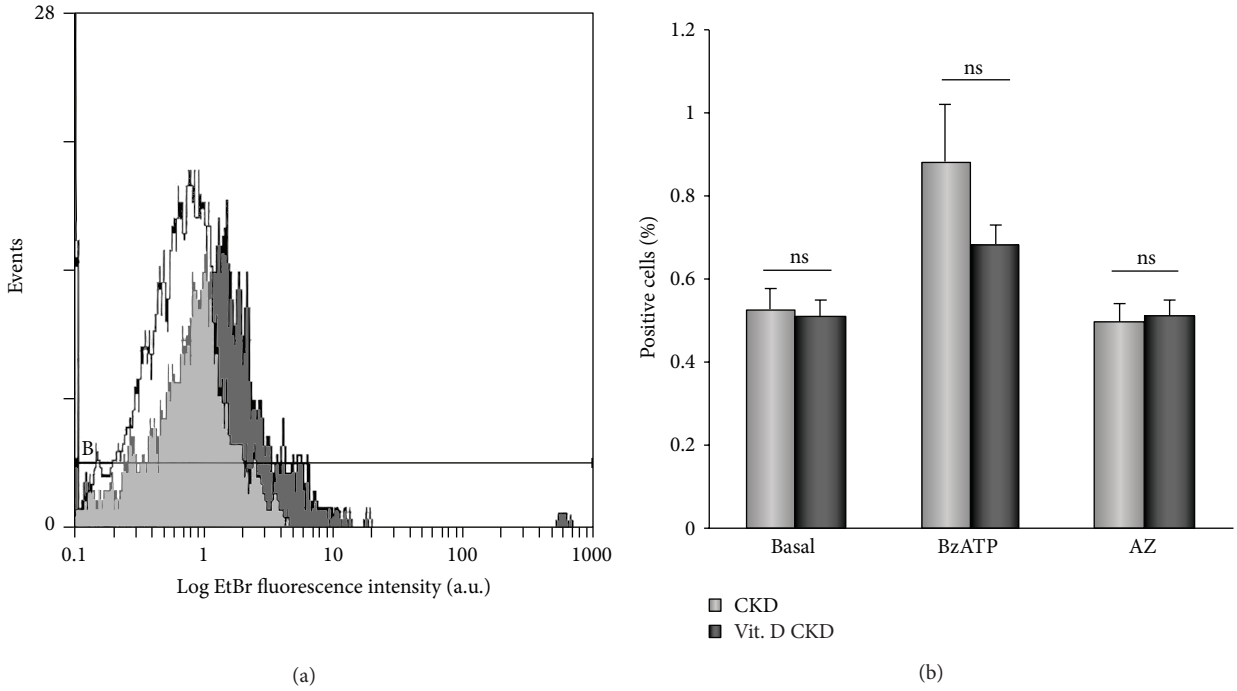

Figure 5: The effect of vitamin D_3 supplementation on permeability of $P2X_7$ pores. (a) Representative flow cytometry histograms of ethidium bromide entry into PBMCs of a CKD patient at basal conditions (white peak) and after a stimulation by BzATP (50 μmol/L) (grey peak). (b) The comparison of the ethidium bromide permeability under the basal conditions, in BzATP- (50 μmol/L) stimulated and AZ11645373- (50 nmol/L) inhibited PBMCs before and after vitamin D_3 supplementation.

processes in the kidney [36]. In our study, $P2X_7$ receptors were involved in the disrupted calcium homeostasis in PBMCs of CKD patients. We have shown that the Ca^{2+} entry via $P2X_7$ channels and pores was increased and also the permeability of $P2X_7$ pores was higher. In addition, the function of $P2X_7$ channels and pores was altered [5]. It is known that $1,25(OH)_2D_3$, an active metabolite of vitamin D, prevents Ca^{2+} increase through $P2X_7$ channels and reduces the plasma membrane permeability through $P2X_7$ pores in human PBMCs of healthy subjects [17]. $1,25(OH)_2D_3$ can also up- or downregulate the expression of several genes in many cell types. To our knowledge, the effect of $1,25(OH)_2D_3$ on $P2X_7$ receptor expression has not been studied. The data of this study disclosed that vitamin D_3 supplementation reduced Ca^{2+} influx through $P2X_7$ channels and affected their functionality. Moreover, the supplementation had no effect on permeability of $P2X_7$ pores, and the differences in responses to stimulation or inhibition were not found. Different effects of vitamin D_3 on $P2X_7$ channels and pores in CKD patients may have been made due to different sensitivity to these channels and pores. It is known that various agonists and antagonists of $P2X_7$ receptors may have a distinct effect on the function of $P2X_7$ channels and/or pores [37]. Not only the function but also the expression may be altered in some pathological conditions [38–41]. In our recent study, we found a 1.5-fold increase in the expression of surface $P2X_7$ receptors on PBMCs from CKD patients compared to healthy subjects [4]. In the current study, the flow cytometric measurement revealed that vitamin D_3 decreased the expression of $P2X_7$ receptors by 45%.

4.4. Activity of PMCA. The PMCA is critical for the maintenance of resting $[Ca^{2+}]_i$ in nonexcitable cells and may be the last gatekeeper for the control of low $[Ca^{2+}]_i$. We have observed a decreased PMCA activity in early CKD patients [4]. This finding is consistent with other studies and points out that the PMCA activity decreases with kidney disease progression [42]. The decline in PMCA activity may be caused by numerous factors such as calmodulin deficiency, an activity of other endogenous protein regulators, and inhibition of mitochondrial or glycolytic metabolism [43]. The Ca^{2+} influx through CRAC channels is also an important regulator of PMCA activity, and an altered communication between them may be another cause of PMCA malfunction [44]. $1,25(OH)_2D_3$ is known to increase PMCA expression and activity in Ca^{2+} transporting tissues such as the intestine, as well as in osteoblasts and Madin-Darby bovine kidney epithelial cells [45–47]. Several experimental studies have observed direct and/or indirect effects of $1,25(OH)_2D_3$ and/or $24,25(OH)_2D_3$ on activity and expression of PMCA in different cells, but the effects of $25(OH)D_3$ itself have not been studied. The studies targeting at the effects of native vitamin D_3 supplementation on PMCA activity in patients with early stages of CKD are also missing. In the present

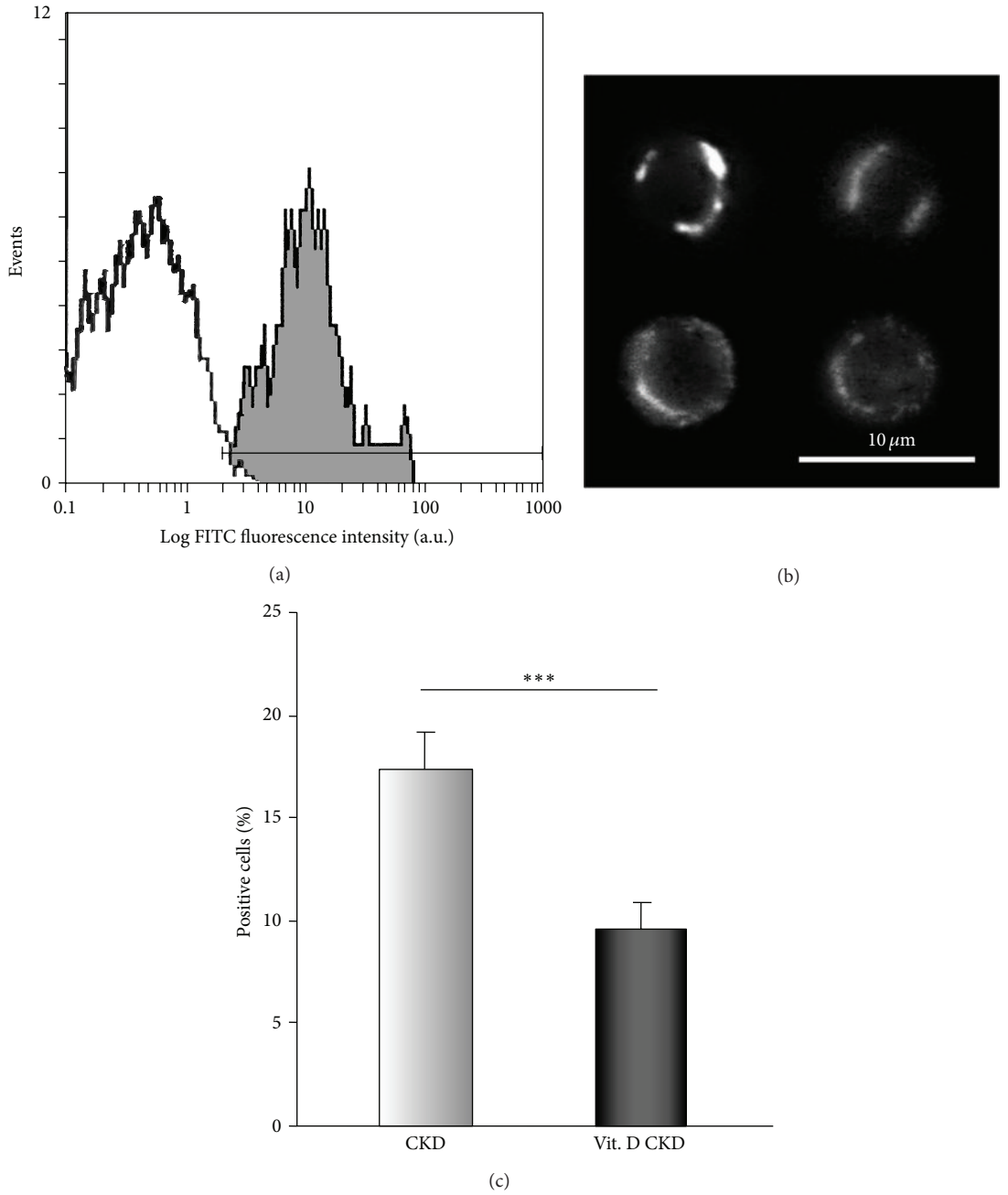

Figure 6: The expression of surface $P2X_7$ receptors. (a) Representative flow cytometry histograms of PBMCs immunostained with primary antibody for the extracellular domain of the $P2X_7$ receptor (gray peak) and an isotype-matched control (IgG2a, white peak). (b) Immunofluorescence staining of $P2X_7$ receptors in plasma membrane using anti-$P2X_7$ (extracellular) antibody of lymphocytes visualised by fluorescence microscopy. Images represent surface $P2X_7$ receptors in four different samples. (c) Decreased expression of surface $P2X_7$ receptors after vitamin D_3 supplementation in a whole population of PBMCs ($^{***}P < 0.001$, $n = 16$).

study, we did not find changes in activity of PMCA after treatment; however, the concentration of plasma membrane proteins was increased by 47%. Therefore, we cannot preclude the possibility of increased expression of PMCA.

In conclusion, we have shown that vitamin D_3 supplementation reduces elevated $[Ca^{2+}]_i$ via CRAC and $P2X_7$ channels and decreases the expression of cell surface $P2X_7$ receptors in early CKD. We did not find the effect on $P2X_7$ pores and PMCA activity. Thus, vitamin D_3 supplementation had a beneficial effect on disturbed cell calcium homeostasis in early CKD.

Conflict of Interests

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

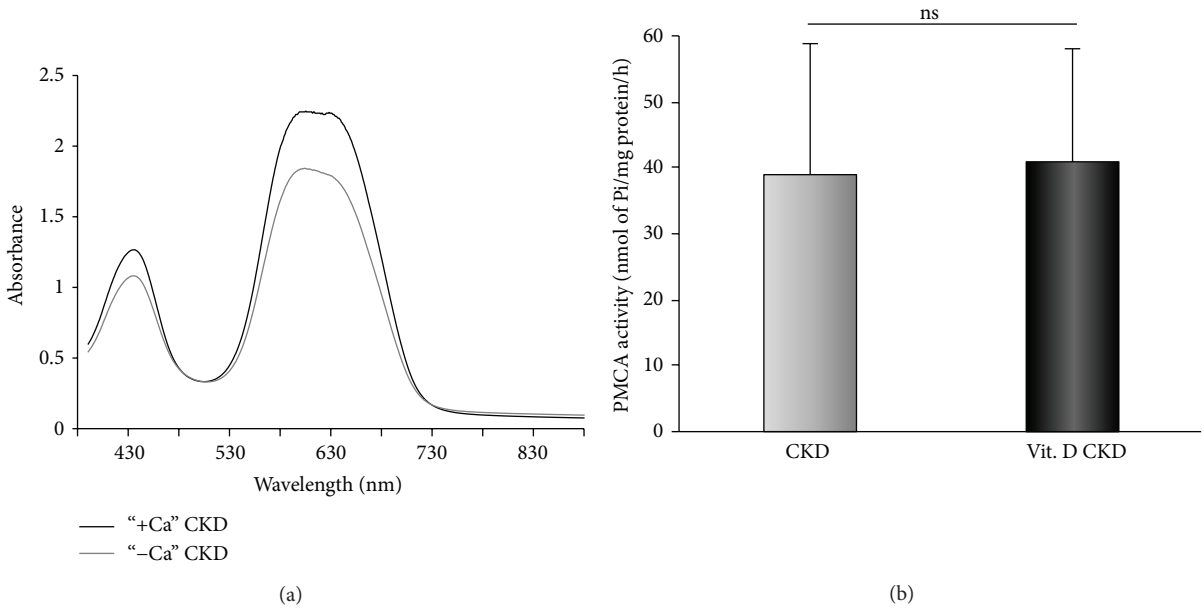

Figure 7: The assessment of PMCA activity. (a) The representative absorbance spectrum of a CKD patient PMCA in the medium with (black line) and without (grey line) Ca^{2+}. (b) The comparison of PMCA activity in CKD patients at baseline and after a 6-month vitamin D_3 treatment.

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Research Article

Vitamin D Binding Protein Is Not Involved in Vitamin D Deficiency in Patients with Chronic Kidney Disease

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Objective. This study was designed to evaluate vitamin D status with separate determination of 25-OH D_2 and 25-OH D_3 and its relationship to vitamin D binding protein (VDBP) in patients with chronic kidney disease (CKD) and long-term haemodialysis patients (HD). *Methods*. 45 CKD patients, 103 HD patients, and 25 controls (C) were included. Plasma vitamin D concentrations were determined using chromatography and VDBP in serum and urine in CKD using enzyme immunoassay. *Results*. Plasma vitamin D levels were lower in CKD (30.16 ± 16.74 ng/mL) and HD (18.85 ± 15.85 ng/mL) versus C (48.72 ± 18.35 ng/mL), $P < 0.0001$. 25-OH D_3 was the dominant form of vitamin D. Serum VDBP was higher in CKD (273.2 ± 93.8 ug/mL) versus C (222 ± 87.6 ug/mL) and HD (213.8 ± 70.9 ug/mL), $P = 0.0003$. Vitamin D/VDBP ratio was the highest in C and the lowest in HD; however, there was no correlation between vitamin D and VDBP. Urinary concentration of VDBP in CKD (0.25 ± 0.13 ug/mL) correlated with proteinuria ($r = 0.43$, $P = 0.003$). *Conclusions*. Plasma levels of vitamin D are decreased in CKD patients and especially in HD patients. 25-OH D_3 was the major form of vitamin D. Despite urinary losses of VDBP, CKD patients had higher serum VDBP concentrations, indicating compensatory enhanced production. Vitamin D binding protein is not involved in vitamin D deficiency.

1. Introduction

Vitamin D plays important physiological roles in maintaining calcium and phosphate homeostasis but also in many other biological processes. There is growing evidence that low vitamin D status is associated with several diseases including not only osteoporosis and osteomalacia but also cardiovascular disease, diabetes mellitus, multiple sclerosis, rheumatoid arthritis, and other autoimmune conditions and several cancer types [1, 2]. In general, regulating the transcription of many genes through their binding to nuclear vitamin D receptor (VDR), active vitamin D serves as antiproliferative and prodifferentiating factor [1, 2].

Vitamin D may be produced endogenously in the skin (ultraviolet irradiation converting 7-dehydrocholesterol to cholecalciferol, i.e., vitamin D_3) or obtained from food or supplements (mostly ergocalciferol, i.e., vitamin D_2, but also D_3, e.g., from fish sources). To become biologically active, both ergocalciferol and cholecalciferol must be double-hydroxylated. The product of the first hydroxylation in the liver, 25-hydroxyvitamin D (calcidiol, 25-OH D), is the major circulating vitamin D metabolite. Calcidiol is then converted by the second (1α) hydroxylation to calcitriol, that is, dihydroxylated active form of vitamin D (1, 25-OH D). For production of circulating calcitriol, renal 1α-hydroxylase is responsible, but also several nonrenal tissues and cell lines express their own 1α-hydroxylase activity [1–4]. As the liver hydroxylation is neither regulated nor rate limited, 25-OH vitamin D well represents vitamin D status in the body. Serum 25-OH D levels > 75 nmol/L (30 ng/mL) indicate sufficient vitamin D stores [1, 4].

Many CKD and mainly HD patients have low serum or plasma vitamin D concentrations [5–11]. Several explanations have been suggested: low solar radiation exposure, disturbed conversion of vitamin D precursor in the skin, low food intake of vitamin D, and loss of vitamin D binding protein due to proteinuria and accelerated vitamin D catabolism [4, 12, 13]. However, the definite role of these possible causes is not clear.

Vitamin D binding protein (VDBP) is a 58 kDa circulating alpha globulin produced primarily by the liver, binding the majority (>85%) of circulating 25-OH vitamin D [14]. It is a highly polymorphic single chain serum glycoprotein ensuring that circulating vitamin D is delivered to target tissues [15]. In principle, there are two main roles of VDBP in vitamin D physiology: enlargement of biological half-life of vitamin D (as binding protects vitamin D from biodegradation) and limiting its access to target tissues. Moreover, VDBP maintains plasma vitamin D levels through reabsorption in the kidneys [16]. The complex of VDBP with 25-OH vitamin D is filtered in the glomerulus, which is followed by receptor mediated reuptake at the brush border of tubular epithelial cells [17] involving megalin [18] and cubilin [19]. In addition to its vitamin D binding properties, there are additional actions attributed to VDBP including binding of extracellular actin and transport of fatty acids. VDBP also appears to protect the complement C5a from proteolytic degradation, enhancing its action as chemotactic protein [20]. A deglycosylated form of VDBP, VDBP-macrophage activating factor, is able to promote activation of macrophages and osteoclasts, and even native VDBP may have effect on osteoclasts [21].

There are several methodological approaches for vitamin D determination in serum or plasma. They can be grouped into immunochemical methods (based on radioactive, enzymatic, or chemiluminescence detection), chromatographic methods (HPLC: high-performance liquid chromatography), and mass spectrometry. Immunochemical methods may vary due to differential detection of D_3 and D_2 molecules (conventional analytical measurement of serum or plasma 25-OH D level reflects the sum of 25-OH D_3 plus 25-OH D_2), interference by detection using polyclonal antibodies, and nonspecific detection of other vitamin D metabolites including degradation products [22, 23]. In addition, incomplete release of vitamin D from VDBP has been identified as a potential source of variability for both manual and automated immunoassays [24]. Another analytical approach, HPLC (high-performance liquid chromatography), allows not only precise assessment of vitamin D level but also simultaneous measurement of 25-OH D_2 and 25-OH D_3 vitamins separately, informing thus about the source of vitamin D.

Therefore, in the current study, the aim was to measure vitamin D concentration in plasma using HPLC method with separate detection of vitamin D_2 and vitamin D_3 and to assess VDBP levels in serum in patients with CKD and HD patients and healthy subjects for comparison. In addition, urine levels of VDBP in CKD patients were also assessed to allow a more complex view of vitamin D status.

2. Materials and Methods

This is a cross-sectional study that includes 173 subjects with CKD, long-term HD and healthy controls. All patients were in stable clinical status at the time of the study, without signs of acute infection. Vitamin D supplementation (apart from dihydroxylated form of vitamin D3) was not prescribed in any HD or CKD patients at the time of study, and controls did not take any special alimentary supplements. The study was approved by the Ethical Committee and all patients have given written informed consent prior to entering the study.

2.1. Study Groups

2.1.1. CKD Patients. Forty-five patients (27 male and 18 female, mean age 60 ± 17 years) with CKD were included. Their median creatinine clearance was 0.39 mL/s/1.73 m^2 (IQR 0.22–0.70 mL/s/1.73 m^2). Urinary protein concentration was 0.22 g/L (median; IQR 0.09–1.10 g/L) and daily protein losses varied from 0.04 g to 13.78 g (median 0.48 g/24 hours). Duration of their follow-up for renal disease was 84 months (IQR 24–120 months). Causes of their renal disease were diabetic nephropathy in 2 patients, hypertensive nephropathy in 14 cases, chronic tubulointerstitial nephritis in 5 patients, chronic glomerulonephritis in 12 cases, polycystic kidney disease in 6 patients, and multifactorial or unknown in 6 cases. The majority of patients (42 cases) had hypertension and were treated with moderate doses of antihypertensive drugs. Cardiovascular disease was known in eight patients. Twelve patients had diabetes mellitus and were treated with insulin or peroral antidiabetics. Twenty-eight patients had dyslipidaemia and were treated with statins. Basic laboratory characteristics of CKD patients are given in Table 1.

2.1.2. HD Patients. One hundred and three patients with end-stage renal disease treated on long-term HD (63 male and 40 female, mean age 60 ± 14 years) were included in the study. Their primary renal diagnoses were as follows: diabetic nephropathy ($N = 26$), vascular nephropathy ($N = 5$), tubulointerstitial nephritis ($N = 22$), chronic glomerulonephritis ($N = 21$), polycystic kidney disease ($N = 14$), and other or unknown diagnoses ($N = 15$). Their residual diuresis ranged from anuria to 2500 mL. The majority of patients were dialyzed three times weekly for 4–4.5 hours using conventional bicarbonate-buffered dialysate. The majority of patients (97 cases in total) had hypertension and were treated with moderate doses of antihypertensive drugs. Sixty-one patients had dyslipidaemia. Cardiovascular disease was known in 34 cases. Forty-one patients had diabetes mellitus and were treated with insulin or peroral antidiabetics. Patients with secondary hyperparathyroidism were treated by synthetic calcitriol (i.e., dihydroxylated form of vitamin D_3) or paricalcitol (synthetic analogue of vitamin D_2). None of patients received any native vitamin D or its 25-hydroxylated metabolite. Basic laboratory characteristics of HD patients are provided in Table 1.

2.1.3. Control Group. The control group (C) consisted of 25 healthy adults (15 male and 10 female, mean age 49 ± 10

Table 1: Characteristics of study subjects.

	Healthy controls (C) $N = 25$	Chronic kidney disease (CKD) $N = 45$	Long-term haemodialysis (HD) $N = 103$	Significance P
Body mass index (kg/m^2)	26.13 ± 4.28	26.84 ± 5.18	27.07 ± 5.34	n.s.
S-Creatinine (umol/L)	91 ± 12	292 ± 148	777 ± 219	$P < 0.0001$ C versus CKD*** C versus HD**** CKD versus HD****
Haemoglobin (g/L)	143 ± 9	120 ± 14	112 ± 11	$P < 0.0001$ C versus CKD**** C versus HD**** CKD versus HD***
Albumin (g/L)	47.3 ± 3.4	41.4 ± 5.0	40.9 ± 3.3	$P < 0.0001$ C versus CKD**** C versus HD**** CKD versus HD n.s.
Calcium (mmol/L)	2.32 ± 0.11	2.28 ± 0.25	2.16 ± 0.18	$P < 0.0001$ C versus CKD n.s. C versus HD*** CKD versus HD**
Phosphate (mmol/L)	1.13 ± 0.20	1.19 ± 0.30	1.95 ± 0.57	$P < 0.0001$ C versus CKD n.s. C versus HD**** CKD versus HD****
Parathyroid hormone (pmol/L)	4.21 ± 2.26 3.73 (3.02–4.75)	14.03 ± 14.43 9.60 (5.15–16.73)	34.57 ± 35.81 23.97 (14.60–41.05)	$P < 0.0001$ C versus CKD n.s. C versus HD**** CKD versus. HD***
C-reactive protein (mg/L)	6.1 ± 3.0 5.6 (3.8–7.1)	7.5 ± 8.2 5.0 (2.4–7.2)	6.9 ± 6.9 5.5 (2.0–9.0)	n.s.
Proteinuria (g/L)	Not assessed (dipstick negative)	0.22 (0.09–1.10)	Not assessed	Not evaluated

Results are expressed as mean ± standard deviation and in case of high interindividual variability also as medians (interquartile ranges).
Comparison: one-way ANOVA and Tukey's multiple comparison test.
$^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$.

years). Basic laboratory characteristics of controls are shown in Table 1.

2.2. Samples. In HD patients, blood was collected from inserted dialysis needle into the arteriovenous fistula before starting HD session and prior to heparin administration. In other subjects, blood was collected after overnight fasting by puncturing the cubital vein with simultaneous blood collection for routine control examinations. Routine laboratory parameters were measured in fresh samples according to institutional standards. For vitamin D determination and VDBP assessment, blood was centrifuged for 10 minutes at 1450 g, and serum and plasma were frozen at −80°C until analysis. Additionally in CKD patients, a 24-hour urine sample was collected, frozen at −80°C, and used for analysis.

2.3. Laboratory Analyses. Vitamin D (its hydroxylated form, 25-OH D) in plasma was assessed with Chromsystems reagent kit (http://www.chromsystems.de) using high-performance liquid chromatography. This assay allows simultaneous determination of 25-hydroxycholecalciferol (25-OH D_3) and 25-hydroxyergocalciferol (25-OH D_2). Samples were treated according to the manufacturer's protocol and HPLC was performed with isocratic system with UV detection (HPLC apparatus ECOM; ECOM, http://www.ecom.cz/).

Vitamin D binding protein (VDBP) in serum and urine was assessed by standard ELISA (enzyme linked immunosorbent assay) Quantikine, RD Systems (Minneapolis, MN, USA), according to the manufacturer's protocol.

For parathyroid hormone (PTH) determination, second generation test was used (iPTH), ECLIA (Electrochemiluminescence, Modular, Roche, Germany). Other laboratory parameters were measured with standard methods.

2.4. Statistical Analysis. Statistical software GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for statistical evaluation. Results are expressed as mean ± standard deviation and in case of high interindividual variability also as medians (interquartile ranges). Vitamin D_3/VDBP and total vitamin D/VDBP ratio was calculated and evaluated. Comparison among groups was done with one-way ANOVA (analysis of variance) test followed by Tukey's multiple

Table 2: Vitamin D and vitamin D binding protein in study groups.

	Healthy controls (C) $N = 25$	Chronic kidney disease (CKD) $N = 45$	Long-term haemodialysis (HD) $N = 103$	Significance P
25-OH vitamin D_3 in plasma (ng/mL)	48.72 ± 18.35 47.10 (37.10–65.40)	30.16 ± 16.74 24.65 (19.45–38.50)	18.09 ± 15.64 13.70 (9.98–22.65)	$P < 0.0001$ C versus CKD**** C versus HD**** CKD versus HD***
25-OH vitamin D_2 in plasma (ng/mL)	Not detected	Not detected	Detected in 8 patients: 9.67 ± 7.80 8.08 (1.83–15.10)	Not evaluated
Total 25-OH vitamin D ($D_2 + D_3$) in plasma (ng/mL)	48.72 ± 18.35 47.10 (37.10–65.40)	30.16 ± 16.74 24.65 (19.45–38.50)	18.85 ± 15.85 14.45 (10.33–23.65)	$P < 0.0001$ C versus CKD**** C versus HD**** CKD versus HD***
VDBP in serum (ug/mL)	222.0 ± 87.7 209.0 (163.0–269.2)	273.2 ± 93.8 268.0 (217.4–327.4)	213.8 ± 70.9 206.6 (161.5–252.3)	$P = 0.0003$ C versus CKD* C versus HD n.s. CKD versus HD***
VDBP in urine (ug/mL)	Not assessed	0.25 ± 0.13 0.33 (0.12–0.35)	Not assessed	Not evaluated
25-OH vitamin D_3/VDBP ratio ($\times 10^{-6}$)	246.56 ± 112.44 242.54 (153.02–275.30)	129.72 ± 93.57 97.93 (72.34–191.86)	90.97 ± 74.97 70.84 (40.91–116.39)	$P < 0.0001$ C versus CKD**** C versus HD**** CKD versus HD*
Total 25-OH vitamin D ($D_2 + D_3$)/VDBP ratio ($\times 10^{-6}$)	246.56 ± 112.44 242.54 (153.02–275.30)	129.72 ± 93.57 97.93 (72.34–191.86)	95.26 ± 77.16 70.84 (42.23–120.172)	$P < 0.0001$ C versus CKD**** C versus HD**** CKD versus HD n.s.

Results are expressed as mean ± standard deviation and in case of high interindividual variability also as medians (interquartile ranges).
Comparison: one-way ANOVA and Tukey's multiple comparison test.
$^{****}P < 0.0001$, $^{***}P < 0.001$, and $^{*}P < 0.05$.
VDBP: vitamin D binding protein.

comparison test. Correlations were tested using Pearson and Spearman correlation coefficients. All tests were two-sided and results were considered statistically significant for $P < 0.05$.

3. Results

3.1. Vitamin D Status. Plasma vitamin D levels were significantly lower in CKD (30.16 ± 16.74 ng/mL) and HD (18.85 ± 15.85 ng/mL) patients versus (48.72 ± 18.35 ng/mL) in controls, $P < 0.0001$ (Table 2). In particular, low levels were in HD patients and more than 75% of HD patients were vitamin D deficient. 25-OH D_3 was the dominant form of vitamin D (Table 2) and in most subjects serum 25-OH D_2 was not detectable. This was the case for all control subjects and CKD patients. Also in HD patients, serum 25-OH D_2 was detectable only in few patients, but these concentrations were low. Overall, the total 25-OH D levels were represented mostly by 25-OH D_3. Vitamin D was not detectable in urine in CKD patients.

Vitamin D_3 significantly correlated with haemoglobin in CKD patients but not in HD patients and controls. In HD patients, but not in CKD patients and controls, plasma D_3 positively correlated with serum calcium. In CKD patients, vitamin D_3 correlated with proteinuria, protein losses, and diuresis but not with serum creatinine and creatinine clearance. No other correlations between plasma vitamin D and investigated laboratory parameters were found in HD and CKD patients, as well as in control group. Significant correlations are summarized in Table 3.

3.2. Vitamin D Binding Protein. Serum VDBP levels in CKD patients were significantly higher compared to controls as well as to HD patients (Table 2). There was no association between VDBP and vitamin D_3 in any studied group of subjects. VDBP negatively correlated with age in CKD patients which was not proven either in HD patients or in controls. VDBP did not correlate either with serum creatinine or with creatinine clearance in these patients and was not related to serum albumin, calcium, phosphate, PTH, C-reactive protein, or body mass index (BMI). Only in HD patients, VDBP correlated slightly positively with serum albumin and slightly negatively with BMI.

Vitamin D_3/VDBP ratio and total vitamin D/VDBP ratio significantly differed among studied groups with the highest levels in controls and the lowest levels in HD patients (Table 2) but did not correlate with basic laboratory parameters in any of the studied groups apart from haemoglobin in CKD patients.

Table 3: Overview of significant correlations of vitamin D and vitamin D binding protein in the study groups.

Chronic kidney disease patients			
25-OH vitamin D_3	VDBP in serum	25-OH vitamin D_3/VDBP ratio	VDBP in urine
Haemoglobin ($r = 0.40$, $P = 0.006$) Proteinuria ($r = 0.34$, $P = 0.02$) Protein losses ($r = 0.43$, $P = 0.003$) Diuresis ($r = 0.40$, $P = 0.006$)	Age ($r = -0.34$, $P = 0.02$)	Haemoglobin ($r = 0.31$, $P = 0.04$)	Proteinuria ($r = 0.43$, $P = 0.003$) Protein losses ($r = 0.39$, $P = 0.008$) Serum creatinine ($r = 0.47$, $P = 0.001$) Creatinine clearance ($r = -0.41$, $P = 0.006$)
Long-term haemodialysis patients			
25-OH vitamin D_3	VDBP in serum	25-OH vitamin D_3/VDBP ratio	
Calcium ($r = 0.22$, $P = 0.03$)	Albumin ($r = 0.22$, $P = 0.03$) BMI ($r = -0.22$, $P = 0.03$)		
Controls			
25-OH vitamin D_3	VDBP in serum	25-OH vitamin D_3/VDBP ratio	
No significant correlation.			

VDBP: vitamin D binding protein.

Urinary concentration of VDBP in CKD patients (0.25 ± 0.13 ug/mL) correlated with proteinuria. Moreover, urinary VDBP positively correlated with serum creatinine in CKD patients and similar relationship was found between creatinine clearance and VDBP in urine. No association between serum and urinary VDBP was found. Significant correlations are summarized in Table 3.

Taken together, the majority of patients with chronic kidney diseases are 25-OH vitamin D deficient. Vitamin D is represented mainly by 25-OH vitamin D_3 (25-hydroxycholecalciferol). Serum VDBP is increased in CKD patients and is measurable also in urine. Vitamin D/VDBP is the highest in healthy subjects and the lowest in HD patients.

4. Discussion

Examining a cohort of CKD and HD patients and a control group in a cross-sectional design, we assessed vitamin D status by separate determination of both 25-hydroxylated vitamin D metabolites (hydroxyergocalciferol, i.e., 25-OH D_2, and hydroxycholecalciferol, i.e., 25-OH D_3) in plasma. This allowed us to recognize the probable source of vitamin D for given subjects. Concomitant measurement of VDBP in serum and also in urine in CKD patients allowed us to evaluate the possible role of urinary VDBP losses in vitamin D status.

We found significantly lower 25-OH D plasma levels in CKD patients compared to controls. In line with previous studies, the majority of CKD patients were vitamin D deficient. In particular, low levels were found in HD patients, which is also in agreement with published data [5, 7–9]. Contrary to previous published studies, we measured separately 25-OH D_2 and 25-OH D_3 which provided us with information about the source of vitamin D in these subjects. Surprisingly, 25-OH D_2 was not detectable in plasma in the great majority of studied subjects. None of controls and CKD patients and only few HD patients exhibited detectable 25-OH D_2. Undetectable 25-OH D_2 found in plasma in all control subjects and also in the majority of renal patients points out very low intake of vitamin D from plant sources. Further studies are necessary to confirm if this observation can be generalized for our country or even for other geographical and socioeconomic areas.

Undetectable 25-OH D_2 and numerical values of 25-OH D_3 plasma concentration in our subjects indicate either solar skin irradiation and/or food containing vitamin D_3 (e.g., fish) as a vitamin D source. However, low 25-OH D_3 in CKD patients not yet on dialysis and not with limited life style indirectly shows that causes other than low sun exposure and/or disturbed conversion of skin precursor play a role in their vitamin D deficiency. Metabolic changes and their consequences that accompany CKD and end-stage renal disease may influence 25-OH D plasma levels in CKD patients and mainly in HD patients. For example, reduced hepatic synthesis of calcidiol in uraemia was described recently [25]. However, very high PTH level, which is considered as the main factor responsible for disturbed hepatic hydroxylation, was not the case in our patients.

Serum concentrations of VDBP were similar in healthy controls and HD patients. However, we observed significantly increased levels of VDBP in CKD patients compared to controls as well as to HD patients, which is in contrast to other studies [26, 27]. This increase was observed despite the urinary VDBP losses which were independent of serum

VDBP levels. Urine concentrations of VDBP were as low as several tenths of ug per mL, but total daily amount was not negligible and, however, still did not result in serum VDBP decrease. We might speculate that in vitamin D deficiency VDBP would increase. However, this would explain just the serum VDBP increase in CKD patients but not normal VDBP levels in serum in HD patients among whom many do not experience any urinary VDBP losses due to anuria. However, the interpretation of elevated VDBP should be more complex, as an association between high VDBP and risk of several cancers has been described [28]. Regardless of the mechanism leading to VDBP elevation, it is obvious that VDBP losses in urine in our patients were not associated either with vitamin D status or with serum VDBP levels similarly as in one previous study [26]. VDBP in urine was inversely related to creatinine clearance and urinary DBP correlated with proteinuria which is in line with the above-mentioned study [26], where additional urinary VDBP excretion responded to antiproteinuric treatment and was higher than that in healthy subjects. This is consistent with previously published hypothesis that urinary DBP is a marker of renal interstitial inflammation and fibrosis [29]. The occupancy of circulating VDBP by vitamin D metabolites is generally lower than 5% [14]. When calculating the 25-OH D/VDBP ratio (resp., 25-OH D_3/VDBP ratio), the main result was that this ratio is much higher in healthy controls than in CKD and HD patients. This further supports the finding that vitamin D deficiency observed in CKD and HD patients was not related to VDBP serum concentration. Also the lack of association between serum VDBP and 25-OH D levels supports the conclusion that VDBP has little effect on concentrations of vitamin D metabolites.

However, we focused only on total vitamin D serum level and we did not consider its free or so-called bioavailable fraction. According to free hormone hypothesis, only unbound fraction is biologically active [30]. With respect to vitamin D physiology, a recent study found that lower VDBP resulted in higher vitamin D bioavailability [31]. Higher bioavailability may lead to higher biological effect but also to higher biodegradation, that is, shorter half-life. Thus, the definite answer if the assessment of bioavailable fraction will bring superior information compared to total vitamin D serum/plasma level is not known at present.

Besides the slightly positive link between 25-OH D_3 and serum calcium in HD patients, vitamin D status did not correlate with any measured parameter of bone and mineral metabolism. In particular, no correlation between 25-OH D_3 and iPTH was found. Several papers report on contribution of low vitamin D status to secondary hyperparathyroidism [32]. In our patients, vitamin D status was generally low but the lack of the direct association between PTH and 25-D concentrations is not surprising as 25-OH D_3 does not represent an active vitamin D form. Active form, dihydroxylated vitamin D (calcitriol), is synthetized by kidneys but also by many extrarenal tissues and cell lines. Low serum calcitriol belongs to main stimulators for PTH production and triggers the initial as well as advanced forms of secondary hyperparathyroidism [33, 34]. We did not measure calcitriol serum concentration and thus we cannot discuss this topic in the view of our data. However, according to recent data, low calcitriol production in CKD and HD patients is related not only to kidney dysfunction but also to low substrate, that is, low 25-OH D levels [4]. Based on this, it is important to assess vitamin D status, which was the main target in our study. The positive association of plasma vitamin D_3 concentration with proteinuria was surprising and requires further investigation.

In CKD patients, but not in HD patients and controls, serum VDBP levels inversely correlated with age. The age dependency of vitamin D binding protein was described also by others [35, 36]. We did not analyse this relationship, but it is not likely to be associated with age-dependent decrease of renal function, as there was no relationship between serum creatinine and VDBP. Contrary to CKD subjects, in HD patients, serum VDBP positively correlated with serum albumin and inversely with BMI, indicating possible nutritional association.

The present study has several limitations. The age of our groups of patients was a little bit different, which theoretically might influence VDBP concentration, as we found a correlation between age and VDBP but only in CKD patients. Another issue which complicates the interpretation of our findings is well-known genetic variability in VDBP [14]. Vitamin D was assessed in plasma and VDBP in serum which was done due to the availability of material and according to manufacturers, and both methods were designed for both materials. Lastly, no vitamin D nutritional supplementation was prescribed in our patients, but the possibility of vitamin D intake cannot be rigorously excluded. Although some patients were treated with active vitamin D or paricalcitol, it was demonstrated recently that they do not affect 25-OH D levels in blood [10].

5. Conclusion

In CKD and mainly HD patients not administered vitamin D supplementation, low vitamin D status was found, with no detectable 25-OH D_2 in most cases. Thus, 25-OH D_3 was the major form of vitamin D. Despite VDBP urinary losses in nondialysis CKD patients, serum VDBP was increased in these subjects compared to healthy controls, indicating VDBP losses are not responsible for 25-OH D deficiency.

Conflict of Interests

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

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Review Article

Interplay of Vitamin D, Erythropoiesis, and the Renin-Angiotensin System

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For many years deficiency of vitamin D was merely identified and assimilated to the presence of bone rickets. It is now clear that suboptimal vitamin D status may be correlated with several disorders and that the expression of 1-α-hydroxylase in tissues other than the kidney is widespread and of clinical relevance. Recently, evidence has been collected to suggest that, beyond the traditional involvement in mineral metabolism, vitamin D may interact with other kidney hormones such as renin and erythropoietin. This interaction would be responsible for some of the systemic and renal effects evoked for the therapy with vitamin D. The administration of analogues of vitamin D has been associated with an improvement of anaemia and reduction in ESA requirements. Moreover, vitamin D deficiency could contribute to an inappropriately activated or unsuppressed RAS, as a mechanism for progression of CKD and/or cardiovascular disease. Experimental data on the anti-RAS and anti-inflammatory effects treatment with active vitamin D analogues suggest a therapeutic option particularly in proteinuric CKD patients. This option should be considered for those subjects that are intolerant to anti-RAS agents or, as add-on therapy, in those already treated with anti-RAS but not reaching the safe threshold level of proteinuria.

1. Introduction

The kidney has an important role in the regulation of several systems. In addition to excretory activity, regulation of water and electrolytes, and maintaining normal acid-base homeostasis, the kidney has also an endocrine function. It is carried out through the production of important hormones: renin, prostaglandins, erythropoietin, and calcitriol.

Renin is released from the renal juxtaglomerular apparatus (JGA). Renin production is regulated by three major mechanisms: change in renal perfusion pressure, solute delivery to the macula densa cells, and influence of renal sympathetic nerves. The negative effects of the activation of renin-angiotensin system on the progression of renal failure are well known. Indeed, blockade of the renin-angiotensin system is a widely established and utilized antiproteinuric and renoprotective modality [1, 2].

Moreover, it has been suggested that the dual-block therapy might improve outcome by preventing compensatory feedback processes that generate more angiotensin II when a single blocker is used. Indeed a number of studies on the progression of renal disease were focused on the role of blocking the activation of the renin-angiotensin system (RAS), to reduce the loss of glomerular filtration rate and delay the start of dialysis, even though the results on the safety of this intervention do suggest caution on the "aggressive" suppression of RAS [3, 4]. However, a reduced efficacy of RAS inhibitors is due to the compensatory increase of renin synthesis caused by the disruption of the feedback inhibition loop. Renin build-up in fact not only stimulates

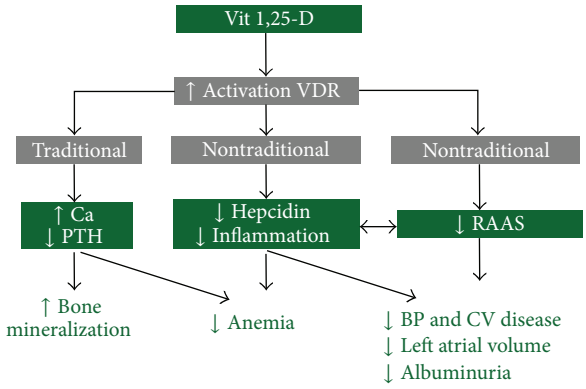

Figure 1: Traditional and nontraditional effects of active vitamin D.

the conversion of Ang I leading to Ang II accumulation but is also likely associated with detrimental effects directly induced by renin [5]. In this context it is important to value the role of vitamin D. Experimental evidence in fact has accumulated on vitamin D-related blunting of the compensatory increase of renin synthesis occurring during chronic administration of anti-RAS agents [6, 7]. In particular, in experimental diabetes, block of the compensatory increase of renin expression by vitamin D analogs dramatically increases the therapeutic efficacy of RAS inhibition (Figure 1) [7]. Beyond the known effect on blood pressure recent studies provided valuable insight into the nonhemodynamic actions of Ang II and other components of the RAS in the progression of kidney disease [8].

In patients with chronic renal disease, a slow, gradual decrease in the level of 1,25-dihydroxyvitamin D (calcitriol) and erythropoietin is observed whilst different mechanisms bring to increased activation of the renin-angiotensin system [9]. The main complications of erythropoietin and 1,25-dihydroxyvitamin D (calcitriol) deficiency are in fact anemia and secondary hyperparathyroidism. Renal anemia is due to a reduced production of erythropoietin by interstitial fibroblast in the renal cortex, between tubular epithelial cells and peritubular capillaries [10]. The origin of decreased serum levels of $1,25(OH)^2D$ is multifactorial. The leading cause is a decrease in renal mass, which causes a consequent reduction in the level of 1-α-hydroxylase available for the production of active vitamin D. In CKD, hyperparathyroidism and hyperphosphoremia can contribute to inhibit the renal bioactivation of vitamin D.

While single effects of the different renal hormones are well known, less information is available regarding the interaction between them. Recently, a number of studies suggested that vitamin D interplays with both renin-angiotensin system and erythropoietin [11, 12]. This interaction would be responsible for some of the systemic and renal effects which has recently been implicated for vitamin D. In particular, the interplay between renal hormones produces its effects on hypertension and proteinuria [13].

2. Vitamin D and Erythropoietin

Recent clinical observations suggest a possible role of vitamin D in erythropoiesis [14]. In the hemodialysis population, 1-25(OH)D repletion has been associated with dose reductions in erythrocyte-stimulating agents (ESA) and increased reticulocytosis [15, 16].

In CKD patients, the administration of either nutritional or active vitamin D has been associated with an improvement of anaemia and reduction in ESA requirements [17].

Despite these intriguing observations, there is overall paucity of clinical studies investigating whether adequacy of 1-25(OH)D affects blood hemoglobin (Hb) levels. Patel et al. show that 25D and 1,25D deficiency are independently associated with decreased hemoglobin levels and anemia in chronic kidney disease. They measured the concentrations of 25-hydroxyvitamin D (25D), 1,25-dihydroxyvitamin D (1,25D), and hemoglobin in a cross-sectional study of 1661 subjects in SEEK, a multicenter cohort study of chronic kidney disease patients in the United States, of whom 41% met the criteria for anemia. The mean hemoglobin concentrations significantly decreased with decreasing tertiles of 25D and 1,25D. These linear trends remained significant after adjustment for age, gender, ethnicity, eGFR, diabetes, and PTH [12].

To evaluate the prevalence of anemia in a population of individuals with vitamin D deficiency, Sim et al. studied for two years 554 subjects in a general population as part of normal healthcare operations. Anemia was present in 49% of 25(OH)D-deficient subjects compared with 36% with normal 25(OH)D levels ($P < 0.01$). 25(OH)D-deficient subjects had a lower mean Hb levels (11.0 versus 11.7; $P = 0.12$) and a higher prevalence of ESA use (47% versus 24%; $P < 0.05$). This study demonstrates an association between vitamin D deficiency, greater risk of anemia, lower mean hemoglobin, and higher use of ESA [18].

In end-stage heart failure subjects, vitamin D deficiency has been showed to be independently associated with low Hb values and anemia. In these subjects, the mean Hb concentrations were significantly reduced in the lower tertiles of 25(OH)D and $1,25(OH)^2D$ ($P < 0.001$). The odds ratios for anemia of the lowest tertile of 25(OH)D (<18 nmol/L) and $1,25(OH)^2D$ (<40 pmol/L) were 2.69 (1.46–5.00) and 4.08 (2.18–7.62) compared with their respective highest tertile (>32 nmol/L and >70 pmol/L). Patients with severe dual deficiency of 25(OH)D and $1,25(OH)^2D$ had an odds ratio for anemia of 9.87 (95% CI 3.59–27.1) compared with patients in the highest tertile for both vitamin D metabolites [19].

Although vitamin D appears to be associated with anemia, the mechanism is unknown.

A reverse correlation was found between PTH and Hb level [20]. Possible causes of low Hb level or anemia due to SHPT may be because of increased bone marrow fibrosis, which may lead to decreased erythropoietin and increased resistance to EPO [21]. Erythropoietin cells express calcitriol receptors, which induces proliferation and maturation of erythroid progenitor cells. Therefore, deficiency of calcitriol, a cause of hyperparathyroidism, may impair erythropoiesis (Figure 1). There are also some studies, which support

an increase in erythrocyte osmotic fragility due to high concentration of PTH in patients on dialysis, leading to low Hb level [22]. There is also indirect evidence of restoration of the hematocrit after parathyroidectomy in uremic patients due to restoration of bone marrow space after operation and rise of immunoreactive erythropoietin (EPO) serum concentrations [23]. Icardi et al. on the contrary consider that these effects are not related to parathyroid hormone (PTH) values and seem to be independent of PTH suppression [24].

The majority of studies concerning vitamin D deficiency or supplementation, and degree of renal anaemia, point out the prevalent role of inflammation in the mechanism underlying these associations. Immune cells express the vitamin D receptor (VDR) which in turn is involved in the modulation of innate and adaptive immunity. Both *in vivo* and *in vitro* studies have demonstrated that calcitriol reduces cytokines production [25]. VDR activation inhibits the expression of inflammatory cytokines in stromal and accessory cells and upregulates the lymphocytic release of interleukin-10 (IL-10) exerting both anti-inflammatory activity and proliferative effects on erythroid progenitors. In CKD patients, vitamin D deficiency may stimulate immune cells within the bone marrow microenvironment to produce cytokines, inducing impaired erythropoiesis. Immune activation involves the reticuloendothelial system, increasing hepcidin synthesis and functional iron deficiency [24]. Recently Zughaier et al. showed that 1,25-dihydroxyvitamin-D(3) ($1,25(OH)^2D3$), the hormonally active form of vitamin D, is associated with decreased hepcidin and increased ferroportin expression in lipopolysaccharide (LPS) stimulated THP-1 cells. $1,25(OH)^2D3$ also resulted in a dose-dependent decrease in prohepcidin cytokines, IL-6, and IL-1β, release *in vitro*. Further, they show that high-dose vitamin D therapy impacts systemic hepcidin levels in subjects with early stage CKD. These data suggest that improvement in vitamin D status is associated with lower systemic concentrations of hepcidin in subjects with CKD [26].

Another possible explanation may be that calcitriol directly stimulates erythroid progenitors; vitamin D has been demonstrated to affect bone marrow function [27, 28].

Furthermore, levels of 1,25 hydroxyvitamin D ($1,25(OH)^2D$), the active form of vitamin D, are several hundredfold higher in bone marrow compared with plasma [25]. Aucella et al. have shown that administration of $1,25(OH)^2D$ increased burst-forming unit erythroid proliferation in patients with ESRD. Calcitriol has a direct effect on erythroid precursors proliferation, as demonstrated both *in vitro* and *in vivo*, with a synergistic effect with epoetin alfa [29]. Vitamin D receptors have been discovered in numerous nonrenal target tissues including the bone marrow [27, 28]. Normalizing tissue 25(OH)D levels may provide an adequate substrate for local tissue production of $1,25(OH)^2D$ in hematopoietic tissues via extra-renal tissue activity of the 1-alpha-hydroxylase enzyme. Hematons, the buffy coat of bone marrow containing erythroid precursors, fibroblast, endothelial cells, lipid laden cells, and macrophages, have been demonstrated to contain significantly higher concentrations of 25(OH)D and $1,25(OH)^2D$ levels than bone marrow plasma [16]. High local concentrations of $1,25(OH)^2D$ in hematopoietic tissues may then directly activate erythroid precursor cells in a paracrine fashion.

In conclusion, only few studies with a limited number of patients explored the association between vitamin D deficiency and anemia in CKD patients. In addition, the molecular evidence about the role of calcitriol in erythropoiesis is still very limited.

3. Vitamin D and Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAS) plays a central role in the regulation of blood pressure, electrolyte, and volume homeostasis. Epidemiological and clinical studies have repeatedly evidenced the impact of vitamin D on RAS activity at the clinical, pathophysiological, and molecular level.

RAS includes a cascade that leads to the generation of angiotensin II (Ang II), the main effector of the system. The rate-limiting component of the RAS is renin, a highly specific aspartic peptidase synthesized and secreted predominantly by the juxtaglomerular (JG) cells in the nephron. The only known substrate of renin is angiotensinogen, which is enzymatically cleaved to angiotensin I by renin. Angiotensin I is further cleaved to Ang II by the angiotensin converting enzyme (ACE) [11].

Ang II exerts diverse actions in multiple organs, including the brain, heart, kidney, adrenal glands, and peripheral vasculature, to regulate the blood pressure and electrolyte and extracellular volume balance and inappropriate stimulation of the RAS has been associated with hypertension, heart attack, and stroke [30, 31].

The relationship between vitamin D and blood pressure and/or plasma renin activity has been debated in many studies. The first clinical studies suggesting an inverse relationship between calcitriol and renin levels were published by Burgess, Resnick et al. more than two decades ago [32, 33]. This correlation was recently confirmed in a large cohort study of CKD patients by Forman et al. They examined the relation between plasma 25-hydroxyvitamin D and elements of the RAS in 184 normotensive individuals in high sodium balance; these included circulating levels of plasma renin activity and Ang II, and the renal plasma flow response to infused Ang II, which is an indirect measure of the intrinsic RAS activity in the kidney. Compared to individuals with sufficient 25-hydroxyvitamin D levels ($\geq$30 ng/mL), those with insufficiency (15–29.9 ng/mL) and deficiency (<15 ng/mL) had higher circulating Ang II levels (p-trend = 0.03). Moreover, those with vitamin D deficiency had significantly blunted renal plasma flow responses to infused Ang II (mean decrease of 115 mL/min/1.73 m^2 in renal plasma flow versus 145 mL/min/1.73 m^2 among those with sufficient vitamin D levels; P value = 0.009). Although plasma renin activity was higher among individuals with insufficient levels of vitamin D, the result was not statistically significant. These data suggest that low plasma 25-hydroxyvitamin D levels

may result in upregulation of the RAS in otherwise healthy humans [34, 35].

Furthermore Park et al. studied fifteen hemodialysis patients with secondary hyperparathyroidism. They showed that, in patients receiving calcitriol, levels of plasma renin (18.5/−12.7 v 12.3/−11.0 pg/mL; $P = 0.007$) and angiotensin II (AT N; 79.7/−48.6 v 47.2/−45.7 pg/mL; $P = 0.001$) were significantly decreased [36].

Several mechanistic studies confirming negative regulation of the renin gene by calcitriol have been published by the group of Li et al., who showed that renin expression and plasma angiotensin II production were increased several-fold in vitamin D receptor-null (VDR-null) mice, leading to hypertension, cardiac hypertrophy, and increased water intake. In wild-type mice, inhibition of 1,25-dihydroxyvitamin-D(3) synthesis also led to an increase in renin expression, whereas 1,25-dihydroxyvitamin-D(3) injection led to renin suppression [37]. In another study they demonstrated that suppression of renin expression by 1,25-dihydroxyvitamin D *in vivo* is independent of parathyroid hormone (PTH) and calcium [38]. To explore the molecular mechanism, they analyzed the mouse Ren-1c gene promoter by luciferase reporter assays. The data obtained indicate that calcitriol binds to the VDR and subsequently blocks formation of the cyclic adenosine monophosphate-response element-binding protein (CRECREB- CBP) complexes in the promoter region of the renin gene, reducing its level of expression [39].

Studies on suppression of renin-angiotensin gene expression in the kidney by paricalcitol were also conducted. Freundlich et al. studied rats with the remnant kidney model of chronic renal failure (5/6 nephrectomy) to which have been given two different doses of paricalcitol thrice weekly for 8 weeks. Paricalcitol was found to decrease angiotensinogen, renin, renin receptor, and vascular endothelial growth factor mRNA levels in the remnant kidney by 30–50 percent compared to untreated animals. Similarly, the protein expressions of renin, renin receptor, the Ang type 1 receptor, and vascular endothelial growth factor were all significantly decreased. Glomerular and tubulointerstitial damage, hypertension, proteinuria, and the deterioration of renal function resulting from renal ablation were all similarly and significantly improved with both treatment doses [40].

In a recent study Fryer et al. show that, in C57/BL6 mice administered vehicle, paricalcitol produces significant, dose-dependent suppression of renin expression in the absence of hypercalcemia at doses 10-fold above those necessary for PTH suppression. Calcitriol also produced suppression of renin at doses at least 10-fold above those required for PTH suppression, but increases in iCa(2+) were observed at doses only 3-fold above those necessary to elicit renin suppression.

Interactions between vitamin D and other system RAAS components have been studied as well [41].

Aldosterone binds mineralocorticoid receptor, which belongs to the same superfamily of nuclear receptors as the VDR. Therefore, cross talk between these receptors and their agonists could potentially exist. Fischer et al. observed that plasma concentration of 1,25-dihydroxyvitamin-D(3) and aldosterone were significantly higher in mice that are genetically deficient for klotho, a membrane protein participating in the inhibitory effect of fibroblast growth factor-23 (FGF23) on the formation of 1,25-dihydroxyvitamin-D(3). High levels of calcitriol were associated with hyperaldosteronism, which is similarly reversed by a vitamin D-deficient diet [42]. Furthermore Good et al. identified a novel regulatory interaction whereby aldosterone acts via nongenomic mechanisms to enhance the genomic response to 1,25-dihydroxyvitamin-D(3). Aldosterone may influence a broad range of biological processes, including epithelial transport, by modifying the response of target tissues to 1,25-dihydroxyvitamin-D(3) stimulation [43].

It has been demonstrated that low vitamin D status adversely affects cardiac function. This effect of vitamin D seems to be mediated by the renin-angiotensin system. Indeed, VDR-knockout mice show myocardial renin overexpression and marked cardiomyocyte hypertrophy [44].

Despite many studies suggested that vitamin D may favorably influence myocardial hypertrophy, two large randomized clinical trials have shown that VDR activation did not influence or reverse left ventricular hypertrophy [45, 46]. In particular in the PRIMO trial, which included 227 patients with CKD stages 3 to 4 who were randomized to paricalcitol or placebo, the change in left ventricular mass index after 12 months did not differ between the two groups [45]. Similar results were reported in the OPERA trial, where patients with 3 to 5 CKD were randomly assigned to receive oral paricalcitol or placebo. After 52 weeks, VDR activation with paricalcitol failed to demonstrate any change in the measures of LV structure and function. However in both studies the authors found a correlation between VDR activation and hospitalization for cardiovascular events [46]. Interestingly a post hoc analysis of PRIMO trial has demonstrated that forty-eight weeks of therapy with paricalcitol significantly reduces left atrial volume and attenuates the rise of brain natriuretic peptide (Figure 1) [47].

Chronic kidney disease (CKD) is a public health priority due to the prevalence rates, around 10% in general adult population, and the ominous and costly cardiorenal outcome [48]. Nowadays, albuminuria is widely considered the main "modifiable" risk factor of global prognosis in CKD patients. Even moderate increases of albuminuria in fact remarkably enhance the risk of end-stage renal disease (ESRD) and all-cause and cardiovascular (CV) death independently of age, hypertension, and diabetes [49, 50]. More important, a recent study in a cohort of 638,150 adults from a province-wide registry in Alberta, Canada, has demonstrated that proteinuria of increasing severity is associated with a faster rate of renal decline regardless of baseline eGFR [51]. A similar main independent prognostic role of proteinuria has been confirmed in the specific setting of tertiary nephrology care [52, 53]. Indeed, the new classification of CKD recently issued by KDIGO highlights the major role of albuminuria [54].

On the other hand, a decrease of proteinuria following therapeutic interventions with anti-RAS agents heralds

a better renal prognosis over time in most patients, even in those starting with moderate proteinuria [55–57].

Therefore, albuminuria (or proteinuria) identifies patients at risk for adverse clinical outcomes and efficacious antiproteinuric (antialbuminuric) approaches improve long-term prognosis. Inhibition of the renin-angiotensin system (RAS) certainly is the cornerstone of treatment in proteinuric patients with the effect being largely independent of blood pressure control [58]. However, the high complexity of the system with multiple-level escape mechanisms prevents adequate suppression. Indeed, monotherapy with either angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) decreases proteinuria by not more than 20–30% [59]. Combined use of ACEi and ARB can be an efficacious strategy to further decrease proteinuria especially in nondiabetic CKD [60], but safety issues prevent wider implementation of dual blockade [3, 4].

Novel antiproteinuric strategies aimed at attaining remission of proteinuria (<0.5 g/24 h) are actively being sought. Under this point of view, of great interest are the experimental data linking vitamin D with albuminuria by means of its anti-RAS and anti-inflammatory effects [5, 13, 61].

Recent interventional studies have disclosed that nutritional vitamin D repletion or administration of active vitamin D, that is, a less potent and more calcemic vitamin D analog [62], reduces proteinuria in patients with milder degrees of renal disease, such as microalbuminuric diabetic nephropathy with moderate GFR impairment and IgA nephropathy with close-to-normal GFR [63, 64]. In patients with more advanced disease (CKD stages 3 to 5 and/or macroalbuminuria-proteinuria), consistent data on antiproteinuric effect (−30% on average) have been almost exclusively provided for paricalcitol, an active analogue of vitamin D with low calcemic effect when used at low dose (1 mcg/24 or 48 h), in diabetic as nondiabetic patients with residual proteinuria after anti-RAS therapy [6, 65–69]. In particular in vital study, the authors found that the use of paricalcitol was associated with a reduction of blood pressure, probably for the previously mentioned effect on renin-angiotensin system [68].

These data have been consolidated in two recent large meta-analyses also showing a substantial safety on markers of mineral bone disease (MBD) of this therapy [70, 71].

The effectiveness of paricalcitol in proteinuric CKD, as well as the awareness of albuminuria as determinant of poor renal prognosis in kidney transplant recipients (KTR) [72], has led investigators to test the antialbuminuric property of paricalcitol also in KTR patients. Two studies deserve to be mentioned. The first one was an observational work with long follow-up (up to 24 months) in 58 patients, transplanted by 6 yrs on median, with mean eGFR 35 mL/min/1.73 m^2 and proteinuria of 1.1 g/24 h [73]. In this study, paricalcitol at the dose of 1 mcg/48 h induced a 36% reduction of proteinuria that was associated with a significantly slower decline of eGFR during the 24 months of treatment as compared to the 24 months before. More recently, the group headed by Remuzzi has completed a 6-month randomized controlled trial showing that oral paricalcitol (1 to 2 mcg/day) in 43 recipients of renal transplants with secondary hyperparathyroidism induced, besides the better control of markers of MBD obtained in the absence of hypercalcemia-phosphatemia, a significant reduction of proteinuria (from 0.27 to 0.14 g/24 h on average) [74].

4. Vitamin D and Hypertension

In the last decade, observational or epidemiologic studies consistently indicated that hypovitaminosis D is associated with higher all-cause mortality rates, including those from cardiovascular diseases [75–77]. In particular, the mean serum 25-hydroxyvitamin D levels have been reported to be significantly lower in patients with stable coronary artery disease than in healthy control subjects and independently associated with extent and complexity of coronary artery disease and hypertension [78]. In this regard, previously reported evidence suggested that vitamin D inadequacy may be involved in the development of hypertension. The Third National Health and Nutrition Examination Survey showed that an inverse relationship existed between 25-hydroxyvitamin D and systolic blood pressure, and this relationship remained significant even after adjustment for age, sex, ethnicity, physical activity, and body mass index [79]. Moreover, a retrospective analysis of 2 large cohort studies showed that men whose plasma levels of 25-hydroxyvitamin D were in the lowest (<15 ng/mL) category were at the highest risk of hypertension relative to men whose levels of 25-hydroxyvitamin D were in the highest (≥30 ng/mL) category (relative risk 6.13). In the same comparison with women, the multivariate relative risk was 2.67 [80].

Among the pathophysiological mechanisms, still largely unknown, underlying the association between hypovitaminosis D and hypertension, a key role could be played by vitamin D-mediated suppression of renin biosynthesis through regulation of the renin-angiotensin system (RAS) [37]. Data from animal studies indicated that circulating active vitamin D may act as an inhibitor of renin expression in the juxtaglomerular apparatus and vascular smooth muscle cell proliferation [81]. Vitamin D receptor activation inhibits intrarenal mRNA levels and protein expression of key components of RAS (angiotensinogen, renin, renin receptors, and angiotensin II type 1 receptor) independently of calcium metabolism in mice and rats [39, 40]. Notably, these findings have not been replicated in humans since no suppressive effect on systemic RAS has been found in patients treated with vitamin D [82] and in essential hypertensives after short-term calcitriol administration and after long-term cholecalciferol therapy [83]. However, in both studies, patients were under treatment with RAS inhibitors. Recently, chronic vitamin D receptor stimulation by cholecalciferol therapy has been shown to blunt systemic RAS activity in essential hypertensive patients with hypovitaminosis D under constant salt intake and free from drugs interfering with RAS [84]. Moreover, compared with sufficient vitamin D status, vitamin D deficiency has been associated with a decreased arterial response to angiotensin II challenge (increased delta brachial pulse-wave velocity and delta aortic augmentation index)

and increased arterial stiffness in healthy humans, possibly through an angiotensin II-dependent mechanism [85].

Since suboptimal vitamin D levels are linked with development of hypertension, it could be assumed that vitamin D replacement or normalization would reduce the risk of cardiovascular disease and its effects. Results from interventional studies strongly suggested that vitamin D supplementation had a great blood pressure lowering effect and overall improved cardiovascular risk profile [75, 86–88].

The supplementation for 8 weeks with vitamin D plus calcium in 148 vitamin D-deficient elderly women significantly lowered systolic blood pressure by 9.3%, while the calcium-only supplementation lowered it by 4.1% compared to baseline [86]. These results were confirmed by a subsequent double-blind, parallel group, and placebo-controlled randomized trial in vitamin D-deficient type 2 diabetes patients, which were administered with a single dose of 100,000 international units (IU) vitamin D2 or placebo for 8 weeks. Vitamin D supplementation significantly improved flow mediated vasodilatation (FMD) of the brachial artery by 2.3% and decreased systolic blood pressure by 14 mmHg compared with placebo. The improvement in FMD remained significant after adjusting for changes in blood pressure. However, changes in FMD did not correlate with the reduction of systolic blood pressure [87]. A positive correlation between FMD and 25(OH)D was also observed in asymptomatic vitamin D-deficient subjects supplemented with 300,000 IU monthly for 3 months. FMD measurements significantly improved after replacement therapy and resulted significantly lower than controls. Additionally, posttreatment values of lipid peroxidation indexes were significantly lower than pretreatment levels, and a negative correlation between FMD and lipid peroxidation indexes was also observed [88].

Further observations demonstrated that daily supplementation of 2000 IU of vitamin D for 16 weeks optimized vitamin D levels and significantly improved carotid-femoral pulse-wave velocity, a cardiovascular surrogate marker, in 49 young black people with vitamin D insufficiency or deficiency [89].

However, despite the large number of clinical studies carried out to examine the effect of vitamin D supplementation on blood pressure, no univocal data are available on the potential antihypertensive effect of vitamin D. As discussed by a recent meta-analysis on vitamin D supplementation and cardiovascular events, this might be due to heterogeneity of patient baseline characteristics, differences in sample size and follow-up periods, and different vitamin D doses. Indeed, most of randomized controlled trials of vitamin D supplementation and blood pressure mainly have given vitamin D for short periods (<6 months) or at low doses (400 IU per day) [90]. Moreover, the use of different vitamin D formulations produced differences in blood pressure reduction, as shown by ergocalciferol or cholecalciferol with ultraviolet B demonstrating a greater decrease in systolic blood pressure (−6.2 mmHg) than calcitriol (0.7 mmHg) [75]. Notably, recent findings suggest that the association between vitamin D status and elevated blood pressure noted in observational studies may not to be causal. Indeed, vitamin D supplementation did not reduce blood pressure in individuals with pre- or stage I hypertension and vitamin D deficiency [91]. Six months of intermittent, high-dose oral vitamin D3 supplementation did not reduce blood pressure or left ventricular mass in patients with resistant hypertension [92]. Moreover, a long-term (18 months) vitamin D supplementation, increasing the mean 25-hydroxyvitamin D3 concentration >100 nmol/L, had no effect on systolic or diastolic blood pressure in healthy adults without severe vitamin D deficiency [93].

Essential hypertension is a typical example of a complex, multifactorial, and polygenic trait where different metabolic pathways are involved (inflammation, coagulation cascade, sodium reabsorption, cellular adhesion, and lipid metabolism). Some gene variants, contributing to between 30% and 50% of the variation in blood pressure among humans, have been identified so far that interact with environmental factors to produce the hypertensive phenotype. Recently, evidence for an important role of the endothelial vitamin D receptor (VDR) in regulating endothelial function and blood pressure has been provided [94]. Moreover, VDR gene polymorphisms (BsmI, ApaI, and FokI) have been shown to be associated with left ventricle hypertrophy, atherosclerosis, and essential hypertension [95–98].

A decade ago a study investigating the relationship between bone mineral density (BMD) and carotid artery intimal medial thickness (IMT), as a surrogate marker of endothelial dysfunction, among 471 Mexican women, showed that forearm BMD and IMT were significantly higher in individuals having the VDR BsmI BB genotype. Furthermore, the association of the VDR genotype with IMT was not dependent on the association between VDR and BMD [99]. In contrast, no significant difference was detected in biochemical parameters and physical examination between groups for BsmI and ApaI VDR gene polymorphisms in a subsequent study including 74 hypertensive patients (49 females/25 males) without other comorbidities, that is, diabetes mellitus, impaired glucose tolerance, and severe obesity [100]. Interestingly, a negative correlation was observed between vitamin D levels and day-time interval and early morning average blood pressure in the FokI non-FF (Ff/ff, $n = 35$) group compared with the FF one ($n = 39$). Serum cystatin-C was higher in the non-FF group, and the degree and presence of retinopathy were significantly higher in the non-FF group when compared to the FF group [100].

These results were confirmed by a large case-control study investigating the relationship between the VDR FokI polymorphism and essential hypertension in 280 patients and 200 healthy subjects. The risk for hypertension in FF homozygotes was found 2.2 times greater than in Ff heterozygotes and 2.2 times greater than in ff homozygotes, regardless of the presence of family history and smoking status However, when comparing Ff and ff genotypes, no significant difference was observed [101].

Recently, a prospective study has been carried out which aimed to evaluate the association of circulating vitamin D metabolites, VDR FokI and BsmI gene polymorphisms, and their interaction with risk of hypertension [102]. Briefly among the recruited 1,211 US men that were free of baseline hypertension 695 men developed incident hypertension

during 15.3-year follow-up. After multivariable adjustment statistical analysis showed that carriers of VDR BsmI bB or BB had a relative risk for hypertension increased by 1.25-fold compared with carriers of bb, while carriers of VDR FokI ff had a relative risk increased by 1.32-fold compared with carriers of FF and Ff combined. Moreover, evidence for an inverse association between plasma 25(OH)D and risk of hypertension was found even if the relation between plasma 25(OH)D and risk of hypertension did not differ by VDR BsmI and FokI polymorphisms [102].

Notably, it has been reported that VDR mutant mice are characterized by lower bioavailability of the vasodilator nitric oxide (NO) due to reduced expression of endothelial nitric oxide synthase, leading to endothelial dysfunction, increased arterial stiffness, increased aortic impedance, structural remodeling of the aorta, and impaired systolic and diastolic heart function at later ages, independent of changes in the renin-angiotensin system [103]. In the light of these reported observations, it may be assumed that more research is needed to further evaluate the role of vitamin D polymorphisms in hypertension development and the usefulness of vitamin D supplementation in hypertension prevention.

5. Conclusion

Beyond the traditional involvement in mineral metabolism, vitamin D may interact with other kidney hormones such as renin and erythropoietin. This interaction would be responsible for some of the systemic and renal effects associated with VDR activation. The administration of analogues of vitamin D has been associated with an improvement of anaemia and reduction in ESA requirements [15–17]. The associations found in clinical studies and the supporting mechanistic studies make it plausible that vitamin D deficiency could indeed contribute to an inappropriately elevated renin levels, as a mechanism for progression of CKD and/or cardiovascular disease [5–7]. Consequently the beneficial effects of vitamin D receptor activators in experimental chronic renal failure could be related to downregulation of the renal renin-angiotensin system and in particular to a reduced renin build-up caused by the disruption of the feedback inhibition loop [5–7].

Studies in large patients series and with adequate follow-up are definitely needed to confirm the effects of long-term paricalcitol treatment in CKD and its potential role in improving renal outcome in comparison with not only placebo but also other vitamin D metabolites and analogues [65–74]. Meanwhile, however, it is plausible to suggest that treatment with active vitamin D analogues represents a therapeutic option in proteinuric CKD that can be used in patients intolerant to anti-RAS agents or, as add-on therapy, in those already treated with anti-RAS but not reaching the safe threshold level of proteinuria (<0.5 g/24 h) [69].

Conflict of Interests

L. De Nicola has received fee as scientific consultant for ABBVIE, JANSSEN, and ASTRAZENECA. In the last 5 years, D. Teta has been consultant and/or speaker for Abbott Nutrition International, Fresenius Medical Care, Fresenius Kabi, and Shire and received an international Research Grant from Baxter USA (Baxter ExtraMural Grant Program) for research regarding peritoneal dialysis solutions; D. Santoro, S. Lucisano, D. Caccamo, K. Sebekova, and M. Buemi declare no conflict of interests.

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Review Article

The Pleiotropic Effects of Vitamin D in Gynaecological and Obstetric Diseases: An Overview on a Hot Topic

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The traditionally recognized role of vitamin D consists in the regulation of bone metabolism and calcium-phosphorus homeostasis but recently a lot of in vitro and in vivo studies recognized several "noncalcemic" effects of vitamin D metabolites. Accumulating evidence suggests that the metabolic pathways of this vitamin may play a key role in the developing of gynaecological/obstetric diseases. VDR-mediated signalling pathways and vitamin D levels seem to (deeply) affect the risk of several gynaecological diseases, such as polycystic ovary syndrome (PCOS), endometriosis, and ovarian and even breast cancer. On the other hand, since also the maternal-fetal unit is under the influence of vitamin D, a breakdown in its homeostasis may underlie infertility, preeclampsia, and gestational diabetes mellitus (GDM). According to our literature review, the relationship between vitamin D and gynaecological/obstetric diseases must be replicated in future studies which could clarify the molecular machineries behind their development. We suggest that further investigation should take into account the different serum levels of this vitamin, the several actions which arise from the binding between it and its receptor (taking into account its possible polymorphism), and finally the interplay between vitamin D metabolism and other hormonal and metabolic pathways.

1. Introduction

The traditionally recognized role of vitamin D consists in the regulation of bone metabolism and calcium-phosphorus homeostasis but recently a lot of in vitro and in vivo studies recognized several "noncalcemic" effects of vitamin D metabolites [1]. Reduced levels of vitamin D are linked with the onset and progression of various diseases such as autoimmune diseases including diabetes mellitus type 1, respiratory infections, type 2 diabetes, hypertension and cardiovascular disease [2], neuromuscular disorders, and cancer [3]. Sunlight exposure is the primary source of vitamin D. The synthesis of vitamin D starts in the bowel epithelial with the oxidation of cholesterol from food or bile to pro-vitamin D3 (7-dehydrocholesterol), which is then transported to the skin, mainly the epidermis, wherein it is isomerized to pre-vitamin D3 (cholecalciferol) by UVB radiation. It is then metabolized into two different substances within the body: 25(OH)D3 or calcidiol and 1,25(OH)2D3 or calcitriol. Vitamin D can also be taken from the diet. Decreased sun exposure limits vitamin D synthesis. There are two principal enzymes involved in the formation of circulating 1,25(OH)2D3 from dietary absorbed or skin synthesized vitamin D: the hepatic microsomal or mitochondrial vitamin D 25-hydroxylase (CYP27A1) and the renal mitochondrial enzyme 1α-hydroxylase (CYP27B1) for vitamin D and 25(OH)D3, respectively [4, 5]. These hydroxylases belong to a class of proteins known as cytochrome P450 mixed function monooxidases. Extrarenal activity of 25(OH)D3-1α-hydroxylase (CYP27B1) has been reported in various cell types including macrophages, keratinocytes, prostate, and colon cancer cells [6–8]. It was shown that 1,25(OH)2D3 is produced locally in many tissues. The potent

fat soluble seco-steroid hormone 1,25(OH)2D3 acts via binding to a corresponding nuclear receptor called the "vitamin D receptor" (VDR) [9–11]. The VDR represents the final common pathway through which vitamin D works on target tissues. The VDR is widely distributed across many tissues. This widespread distribution underlies the potential myriad of physiologic actions for vitamin D. 1,25(OH)2D are mediated by the VDR acting primarily by regulating the expression of genes whose promoters contain specific DNA sequences known as vitamin D response elements (VDRE). The VDR works in partnership with other transcriptional factors, the best-studied of which is the retinoid X receptor (RXR), and a number of coactivators and corepressors that provide context, tissue, and target gene specificity. However, some actions of 1,25(OH)2D are faster than genomic and may be mediated by a membrane-bound VDR that has been less well characterized than the nuclear VDR [12]. VDR belongs to the superfamily (>150 members) of transacting transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors [13, 14] and is encoded by a large gene (>100 kb) located on the chromosome 12q12-14 [15]. The VDR gene encompasses two promoter regions, eight protein-coding exons (namely, 2–9), and six untranslated exons (1a–1f). It has an extensive promoter region capable of generating multiple tissue-specific transcripts. It has been demonstrated that VDR requires heterodimerization with auxiliary proteins for effective DNA interaction. These auxiliary proteins have been identified as the retinoid-X receptors (RXR) α, s, and γ [13, 14, 16, 17]. Vitamin D response elements have been identified in numerous genes involved in many activities (i.e., cellular growth, differentiation, apoptosis, invasion and metastasis of tumour cells, and so on).

Considering these assumptions, in this paper we aimed to review the most updated evidence which clearly suggests a key role for vitamin D pleiotropic actions in the reproductive physiology as well as development of several gynecologic/obstetric diseases. In particular, we discuss the influence of VDR-mediated signaling pathways in polycystic ovary syndrome (PCOS), gestational diabetes mellitus (GDM), preeclampsia, infertility and in vitro fertilization (IVF), endometriosis, and breast and ovarian cancer.

2. Vitamin D, Polycystic Ovary Syndrome, and Insulin Resistance

PCOS is the most common gynaecological endocrinopathy in women of reproductive age, with a prevalence of 6–10% in the general population. It is a multigenic disorder characterized by increased ovarian and adrenal androgen secretion; hyperandrogenic symptoms such as hirsutism, acne, and/or alopecia; menstrual irregularity; and polycystic ovaries [18–21]. In addition, insulin resistance (IR) is common in PCOS women [22] who are therefore at an increased risk of type 2 diabetes [23]. There is an increasing evidence that supports the contribution of vitamin D deficiency to metabolic disturbances in women with PCOS, including insulin resistance (IR) [24–26], obesity [24, 27], hypertension [28], and menstrual dysfunction [29], findings supported by the fact that vitamin D regulates about 3% of the human genome, including genes that are crucial for glucose and lipid metabolism [30, 31]. Consistent evidence also suggests that polymorphisms in the VDR gene are associated with vitamin D deficiency in PCOS and its metabolic and endocrine disturbances [26, 32]. The VDR Cdx2 "AA" genotype is reported as an associated marker with lower fasting insulin and homeostatic model assessment-IR [26] and the ApaI "CC" genotype was associated with an increased risk for PCOS [32]. The exact mechanisms underlying the association of vitamin D and IR are not fully understood. Firstly, vitamin D may have a beneficial effect on insulin action by stimulating the expression of insulin receptor and thereby enhancing insulin responsiveness for glucose transport [33]. The vitamin D responsive element is present in the promoter of the human insulin gene [34] and the transcription of the human insulin gene is activated by 1,25(OH)D2 [35]. Secondly, vitamin D regulates extracellular and intracellular calcium that is essential for insulin-mediated intracellular processes in insulin-responsive tissues such as skeletal muscle and adipose tissue [33]. Moreover, alterations in calcium flux can have adverse effects on insulin secretion, which is a calcium dependent process [36]. Finally, as vitamin D has a modulating effect on the immune system [37], hypovitaminosis D might induce a higher inflammatory response, which is again associated with IR [38]. Literature assessing VDR polymorphisms and/or polymorphisms related to vitamin D metabolism in women suffering from PCOS in relation to vitamin D status and metabolic disturbances is scarce. Recently, Krul-Poel et al. [39] have carried out a review on this topic. They found 29 eligible trials with inconsistency in their results. The conflicting findings might be due to the small sample sizes, the lack of adjustments for confounders, the use of different definitions for PCOS, the use of different assays for serum 25(OH)D measurement, the duration of intervention, the use of different amounts of vitamin D supplementation in the intervention trials, and the lack of an optimal serum 25(OH)D level in the general population. They underline that only one well-designed randomized placebo-controlled trial demonstrating no effect of vitamin D3 supplementation on IR has been carried out until now [40]. Moreover, they found that univariate regression analyses of the weighted means revealed vitamin D to be a significant and independent predictor of IR in both PCOS and control women. The significance disappeared after adjustment for BMI in PCOS women [39]. Still, it remains unclear whether vitamin D and IR are causally interrelated or whether they constitute two independent characteristics in women with PCOS. The causal relationship between vitamin D status and metabolic disturbances in PCOS remains to be determined in well-designed placebo-controlled randomized clinical trials. Until then, screening women who are at risk of vitamin D deficiency and supplementation with vitamin D could be considered.

3. Vitamin D and Gestational Diabetes Mellitus

GDM is a condition of abnormal maternal glucose tolerance that occurs, or is detected for the first time, during pregnancy [41]. Pregnancy is a status in which the mother undergoes

physiological insulin resistance, which helps the fetus absorb more nutrients. Maternal postprandial hyperglycaemia is the reason why the fetus can take in more carbohydrates and amino acids via the placenta, thanks to a carrier passage, the functioning of which is facilitated by the different gradient (typically facilitated transport). If the mother is unable to compensate with an increase of pancreatic β-cell insulin secretion, GDM is derived from this metabolic condition [42, 43]. Women affected by GDM generally demonstrate in the puerperium, and/or later in life, a maintenance of high levels of insulin resistance, which is the effect of β-cell dysfunction, and suggests that GDM is a transient manifestation of longstanding metabolic impairment with a predisposition to reappear in the future [44]. There is a strict connection between glucose metabolism and vitamin D pathways [45]: it is widely accepted, for example, that this vitamin and PTH play a key role in the extracellular homeostasis of calcium [46], and moreover that patients affected by hyperparathyroidism develop more frequently diabetes mellitus type 2 with respect to the general population [47, 48]. Since 1,25(OH)2D is able to induce insulin secretion and to decrease insulin resistance, low levels of this vitamin are associated with the developing of GDM. A cross-sectional study conducted by Maghbooli et al. [49] on 741 pregnant women showed that prevalence of severe vitamin D deficiency (<12.5 nmol/L; <5 ng/mL) in GDM patients was higher than in normoglycaemic pregnancies and found a strong correlation between the HOMA index and serum levels of vitamin D. Confirming these results, Zhang et al. [50] found that approximately 33% of GDM cases in their study population, compared with 14% of controls ($P < 0.001$), had maternal plasma 25(OH)D concentrations consistent with a prespecified diagnosis of vitamin D deficiency (<50 nmol/L; <20 ng/mL). Moreover, each 12,5 nmol/L (5 ng/mL) decrease in 25(OH)D concentrations was related to a 1.29-fold increase in GDM risk. Finally, Zuhur et al. [51] evidenced that correlation between low levels of vitamin D and risk of GDM still remain even after adjusting for well-established risk factors (maternal age, race, family history of diabetes, and pre-pregnancy BMI) of GDM. Nevertheless, another study [52] did not find evidence of an association between first-trimester maternal levels of 25(OH)D and subsequent development of GDM. Taken together, all these results allow us to underline the strict correlation between vitamin D and glucose metabolisms, even if further studies based on larger population are needed to get crystal clear evidence about the topic.

4. Vitamin D and Preeclampsia

Impaired placentation and maternal endothelial dysfunction are principal features of the pregnancy syndrome preeclampsia that affects 3–7% of all pregnancies [53, 54]. Effective preventive or therapeutic strategies do not exist to date [53]. Vitamin D3 deficiency is associated with cardiovascular disease, hypertension, obesity, diabetes mellitus, and metabolic syndrome [54, 55]. Although the mechanisms through which low serum vitamin D levels can affect the risk of preeclampsia are still unclear, the causal relationship is biologically plausible. Vitamin D has been shown as a potent endocrine suppressor of renin biosynthesis to regulate the renin-angiotensin system [56] that plays a critical role in the regulation of blood pressure and electrolyte and plasma volume homeostasis. Therefore, normal serum vitamin D levels help prevent hypertension through suppression of the renin-angiotensin system. In addition to the effect of vitamin D on the renin-angiotensin system, vitamin D can influence blood pressure through the suppression of vascular smooth muscle cell proliferation. It can also ameliorate insulin resistance, improve endothelial cell-dependent vasodilatation, and inhibit anticoagulant activity [57]. Vitamin D may modulate macrophage activity and cytokine production. Compared with uncomplicated pregnancies, preeclampsia is characterized by marked changes in vitamin D3 and calcium metabolism [58]. A recent meta-analysis and several observational studies show a significant relationship between vitamin D deficiency and an increased risk for preeclampsia [59–61]. Studies have shown that sufficient vitamin D intake during pregnancy reduces the risk of complications, including gestational diabetes, preterm birth, and infection [62, 63]. Maternal 25(OH)D3 levels are lower in women with preeclampsia than in normotensive pregnant women [64]. Moreover, a nested case control study revealed that maternal vitamin D deficiency at less than 22 weeks of gestation is a strong, independent risk factor for preeclampsia [65]. Placenta dysfunction plays an important role in the pathogenesis of this pregnancy disorder, since preeclampsia is associated with a reduced placental and fetal vitamin D pool [66]. Normal placental development and function ensure a healthy pregnancy outcome. It is believed that, during pregnancy, 1,25(OH)2D3 may be produced not only by kidneys but also by placenta trophoblasts. Human placenta and decidua are capable of producing and secreting 1,25(OH)2D3 [67]. The existence of gene transcript of 1α-hydroxylase and the finding of VDR expression in placental trophoblasts suggest a possible autocrine loop of vitamin D signaling within trophoblasts [68, 69]. A study by Zehnder et al. [70] also found that mRNA expression of 1α-hydroxylase was higher in the first- and second-trimester than in the third-trimester placentas, whereas mRNA expressions for VDR across gestation were less pronounced compared with 1α-hydroxylase. Though preeclampsia has been linked to maternal vitamin D insufficiency/deficiency [71, 72], the information on placental vitamin D metabolic system between normal and preeclamptic pregnancies is lacking. Preeclampsia is hypothesized to be a 2-stage disorder [73]. At its first stage, placental perfusion is reduced, often secondary to abnormal implantation. The poorly perfused placenta is proposed to produce materials that, in an appropriate maternal environment, initiate the ensuing multisystem sequelae (second stage). These pathophysiological changes are proposed to be secondary to abnormal endothelial function, which is a component of a generalized increase in the inflammatory activation [74]. The active form of vitamin D, 1,25(OH)2D, has been shown to regulate the transcription and function of genes associated with placental invasion, normal implantation, and angiogenesis [75]. Therefore, insufficient serum vitamin levels can impair normal functioning of these processes. Taken

together, these findings about the association of maternal vitamin D deficiency and increased risk of preeclampsia emphasize the importance of adequate vitamin D levels and proper vitamin D metabolism during pregnancy.

5. Vitamin D, Infertility, and In Vitro Fertilization (IVF)

Accumulating evidence strongly indicates a potential role of vitamin D in human reproduction. Vitamin D receptors are present and differentially expressed in murine endometrium and ovary throughout the estrous cycle [76] whereas knockout experiments have shown that vitamin D receptor null mice experience uterine hypoplasia and impaired folliculogenesis [77]. Moreover, a study in cell cultures confirmed the expression of vitamin D receptors in human endometrial cells and demonstrated that the expression of 1-alpha-hydroxylase, an enzyme which catalyzes the hydroxylation of calcidiol to calcitriol, is upregulated in the human endometrial stromal cells of early pregnant versus cycling endometria [78]. However, in vivo data supporting a role for vitamin D in female fertility in general and embryo implantation in particular are not robust. Finally, a recent retrospective study postulated that vitamin deficiency may negatively affect pregnancy rates with an effect mediated through the endometrium, given that vitamin D deficiency was not correlated with ovarian stimulation characteristics or with markers of embryo quality [79]. Up to date, only a few cohort studies have attempted to examine the role of vitamin D levels in infertile patients [79–82]. Results from these studies are strongly contradictory, with some findings showing that maternal vitamin D deficiency is associated with lower pregnancy rates [79, 81] and others demonstrating that vitamin D deficiency does not affect the final reproductive outcome [82, 83]. In addition, the number of patients enrolled in these studies is small, whereas the major shortcoming of all of the studies is the fact that no single embryo transfer policy has been adopted, with patients receiving up to four embryos per transfer. This indeed may severely bias the results, since it might be a strong confounding factor for estimating differences in pregnancy rates between vitamin deficient and replete women. A recent innovative study of Polyzos et al. [84] has evaluated the influence of vitamin D deficiency on pregnancy rates among women undergoing IVF/ICSI and Day 5 (blastocyst stage) single embryo transfer (SET). They have found that vitamin D deficiency results in significantly lower pregnancy rates in this setting of women. Overall 368 consecutive infertile women treated within a period of 15 months were included in the study. Clinical pregnancy rates were significantly lower in women with vitamin D deficiency compared with those with higher vitamin D values. Finally, even when restricting the analysis to women undergoing elective SET, vitamin deficiency was again independently associated with pregnancy rates. Vitamin D deficiency impairs pregnancy rates in women undergoing single blastocyst transfer. Future prospective confirmatory studies are needed to validate our results and examine the exact underlying mechanism by which vitamin D levels may impair pregnancy rates in infertile women undergoing IVF/ICSI.

6. Vitamin D and Endometriosis

Endometriosis, defined as the presence of endometrial glands and stroma in ectopic locations, affects 6%–10% of reproductive-age women. It is considered a chronic, estrogen-dependent, and inflammatory disease [85] associated with dysmenorrhea, dyspareunia, chronic pelvic pain, irregular uterine bleeding, and/or infertility [86, 87]. Actually, a unifying theory regarding the origin of endometriosis has remained elusive. Accumulating evidence is suggesting that dysregulation of Wnt and/or Hox genes may affect cell migration during organogenesis and differentiation of Müllerian structures of the female reproductive tract, with possible dislocation and dissemination of primordial endometrial stem cells in ectopic regions, which have high plasticity to differentiation [88]. It is possible that, during postpubertal age, under the influence of different stimuli, these misplaced and quiescent ectopic endometrial cells could acquire new phenotype, biological functions, and immunogenicity. So, these kinds of cells may differentiate, specializing in epithelium, glands, and stroma, to form a functional ectopic endometrial tissue. This may provoke a breakdown in the peritoneal cavity homeostasis, with the consequent processes of immune alteration, documented by peripheral mononuclear cells recruitment and secretion of inflammatory cytokines in early phases and of angiogenic and fibrogenic cytokines in the late stages of the disease [89]. An association has been postulated between endometriosis and vitamin D, since endometriosis is a disease that mimics malignancy and fulfills most of the criteria of an autoimmune disease, and vitamin D is an agent with antiproliferative, anti-inflammatory, and immunomodulatory properties [90–92]. Endometriosis has many features of an autoimmune disease, and an immune-mediated defect in recognition and elimination of endometrial fragments refluxed in the peritoneal cavity has been proposed to play a crucial role in endometriosis development [93]. Activated $CD4^+CD8^+$ lymphocytes, macrophages, and dendritic cells express widely VDR and both the activating and metabolizing enzymes, 1-α hydroxylase and 24-hydroxylase [94, 95]. This suggests that 1,25(OH)2D can be produced locally in the immune system and plays an autocrine-paracrine role [96]. However the link between endometriosis as an autoimmune disease and vitamin D as an immunomodulator is more complex. In fact, endometriosis is associated with normal or high 25(OH)D reserve, rather than insufficiency/or deficiency, as would have been expected. In addition, the manifestations of endometriosis do not exhibit seasonal flares or exacerbations or seasonal changes in 25(OH)D levels as in other autoimmune diseases. It is plausible that the immunomodulatory role of vitamin D in this disease, if existent, is local, autocrine, and/or paracrine, at the level of endometriotic foci or lesions. If so, it would be missed by correlating disease manifestations with circulating serum 25(OH)D levels and could only be identified by targeted in vitro studies, which to the best of our knowledge are lacking [97]. In a recent large prospective cohort study, a greater predicted plasma 25(OH)D level was associated with a lower risk of endometriosis [98]. However, other studies have failed to demonstrate this association [90, 99]. This could be

explained by the small sample size, heterogeneity, and case-control nature of these previous studies. Finally, recently, a randomized double-blind study was conducted in order to evaluate the role of vitamin D in primary dysmenorrhea: women received vitamin D supplementation before their expected menses ($n = 20$) or placebo ($n = 20$); a significant reduction (41%) in the mean pain score was noticed in the experimental arm [100]. The greatest reduction of pain scores was observed in the subset of more symptomatic patients at baseline. The pain reduction could be attributed to the action of 1,25(OH)2D on the endometrium with a decrease in prostaglandin synthesis and an increase in prostaglandin inactivation by suppression of cyclooxygenase 2 and upregulation of 15-hydroxyprostaglandin dehydrogenase, respectively. 1,25(OH)2D may also exert anti-inflammatory effects through other pathways, such as inhibiting nuclear factor-β signaling and increasing mitogen-activated protein kinase phosphatase 5 activity, thus blocking cytokine production via p38 activation [101]. Actually, the medical treatment of endometriosis is not satisfactory, and there is a constant need to find novel drugs with better efficacy and tolerability. These findings show the role of vitamin D as a possible modifiable risk factor for endometriosis. Use of vitamin D supplementation in these patients, especially when exhibiting low plasmatic levels of 25(OH)D, may allow these women to limit the use of nonsteroidal anti-inflammatory drugs [100]. However, larger, placebo-controlled studies are needed to clarify the possible favorable effects of vitamin D supplementation in women with endometriosis.

7. Vitamin D and Breast and Ovarian Cancer

Biological and epidemiological data have revealed the protective functions of vitamin D against ovarian, breast, colorectal, gastric, liver, prostate, and nonmelanoma skin cancers [102–104] and the potential role of VDR gene polymorphisms and risk of cancer [105–107]. The most frequently studied single-nucleotide polymorphisms are the restriction fragment length polymorphisms FokI (rs2228570) and BsmI (rs1544410), as defined by the endonucleases FokI and BsmI [105]. As widely reviewed by Vuolo et al. [91], several levels of evidence support the relationship between vitamin D and cancer: firstly, low circulating levels of vitamin D are associated with increased risk of developing cancer, secondly a high intake of vitamin D is associated with a reduced risk of cancer, in addition the aggressiveness of cancer is lower in summer when the production of vitamin D is higher, and finally polymorphisms of genes encoding proteins involved in the signal pathway of vitamin D affect the risk of developing cancer. However, data about vitamin D and cancer are often conflicting, with a considerable variability [106–109]. Upregulation of VDR expression has been shown in several tumors and is thought to represent an important endogenous response to tumor progression [106, 107].

Of particular interest in this regard, it is estimated that 20% of all cancer cases are caused by obesity in women [110] and vitamin D is thought to be one of the mechanisms underlying this association. Shanmugalingam et al. [111] performed a systematic review and meta-analysis in order to assess whether vitamin D plays a role in the pathway between obesity and cancer, since no mediation analyses have been performed for this effect to date. For the topic obesity and cancer, a positive association was reported between obesity and risk of cancer, showing that the strength of this association varies between cancer sites, sex, and, in breast cancer, the menopausal status. There are several molecular mechanisms suggested to explain the increased risk of cancer in obese people: firstly, the "insulin-cancer hypothesis" [112]; secondly, in hormonally driven cancers, such as endometrial and postmenopausal breast cancer, the increase in circulating levels of sex steroid hormones. In the postmenopausal state, the majority of oestrogen is derived from adipose tissue rather than from the ovaries, potentially explaining the discrepancy between pre- and postmenopausal women. Finally, obesity is thought to result in a state of chronic inflammation. These changes lead to an increase in tumor cell motility, invasion, and metastasis. For the link between obesity and vitamin D, the meta-analysis reported a modest inverse association between obesity and low vitamin D levels. The underlying biological mechanisms are still unknown. The most likely hypothesis is that vitamin D stored in fat tissue increases local vitamin D concentrations causing activation of the VDR in adipocytes. This may lead to low energy usage and further promotion of obesity [111, 113]. Finally, authors evaluated if vitamin D is a mediator for the association between obesity and cancer. Even if the literature shows consistent evidence for an association between vitamin D and obesity, there was lack of studies showing a consistent link between vitamin D and cancer after adjustment for obesity. Authors concluded that it seems that the significance of the mediating role of vitamin D in the biological pathways linking obesity and cancer is low [111].

Breast cancer is one of the most common female cancers in the Western population and there is a growing interest in identifying the role and the relative importance of environmental risk factors, lifestyle, and diet in this type of tumor. There are laboratory data that support the hypothesis that the anticarcinogenic effects of vitamin D could be mediated via the oestrogen pathway by downregulation of the oestrogen receptor (ER) and thus attenuating oestrogenic bioresponses such as cell growth [114, 115]. Also for breast cancer, VDR has a crucial role. Both healthy and cancer breast cells express the VDR and gene ablation studies have shown a role of VDR in physiological mammary gland development. Zinser and Welsh [116] showed that, after the stimulation with the carcinogen DMBA, mice knockout for VDR gene developed a higher number of premalignant lesions compared to wild-type mice. The antiproliferative and prodifferentiating effects of vitamin D seem to regulate differentiation in the breast by a balance between the activity of the 1α-hydroxylase and 24-hydroxylase enzymes, responsible, respectively, for the synthesis and degradation of the active hormone 1,25(OH)2D. Several studies have found an increased expression of CYP24 in tumor cells compared to healthy cells, suggesting that the malignant tissue present a tendency to the degradation of active vitamin D [117]. Recently, Narvaez et al. [118] reviewed the frequency of genomic VDR changes in human breast cancers using data sets available on The Cancer

Genome Atlas. Alterations in the VDR gene were present in only 5% of human breast tumors. The most common change was a reduction in mRNA expression. Intriguingly, the Luminal B subtype had the highest frequency of VDR alterations with 10.5% of tumors displaying reduced VDR mRNA expression compared to 0–3% for Luminal A, Basal, HER2, or Claudin-Low subtypes. These results are in line with the data of Santagata et al. [119]; interestingly, this study also demonstrated that breast tumors with the highest expression of VDR, ER, and androgen receptor had the best prognosis. Several prospective epidemiological studies have investigated the relationship between vitamin D and breast cancer incidence, but with conflicting results [103, 120–122]. Recently, a meta-analysis of prospective studies reported that a 12,5 nmol/L (5 ng/mL) increase in 25(OH)D was linked to lower risk of postmenopausal (but not premenopausal) breast cancer with RR of 0.88 per 12,5 nmol/L (5 ng/mL) (95% CI, 0.79 to 0.97) [123]. However, Amir et al. [124] did not find a significant association of 25(OH)D levels with breast cancer risk. Stronger evidence is available from four randomized trials that have evaluated the effect of vitamin D supplementation on risk of bone fracture and mortality; they examined risk of cancer as a secondary outcome [125–128]. The United Kingdom trial compared vitamin D supplementation to placebo; no cancer risk reduction was observed [125]. In the Nebraska trial, patients were randomly assigned to receive calcium and vitamin D, calcium alone, or placebo; there was no direct comparison of vitamin D alone versus placebo [126, 127]. There were few cancers overall; however, those randomized to calcium/vitamin D supplementation (versus placebo) had a lower overall cancer incidence. The larger Women's Health Initiative trial did not show a reduction in risk of colorectal, breast, or any cancer in those randomized to vitamin D [127]. Finally, in the Record trial, vitamin D versus placebo administration was not associated with a reduction in cancer risk [128]. These largely negative results may reflect inadequate vitamin D dosing, small sample sizes, or the true absence of an association. The promising findings of the Nebraska study may reflect the combined use of calcium and vitamin D, rather than vitamin D alone. Regarding the cancer outcome, a recent meta-analysis of 42 RCTs reported an inverse correlation between vitamin D supplementation and all-cause mortality; cancer specific mortality was not examined [129]. For established breast cancer, a meta-analysis of eight studies has found that low vitamin D levels were linked to higher risk of recurrence and death; however, these results may be flawed by many bias and confounding factors in the included studies [130]. Three of the RCTs discussed above (United Kingdom, WHI, and Record) also evaluated cancer outcomes. No significant difference in cancer death between the vitamin D and non-vitamin D supplementation arms was identified in these three studies (HR 0.86, 95% CI, 0.61 to 1.20; HR 0.90, 95% CI, 0.77 to 1.05; and HR 0.85, 95% CI, 0.68 to 1.06, resp.) [125, 127, 128].

Ovarian cancer is one of the most lethal gynaecological malignancies, with an estimated 225,500 new cases and 140,200 deaths worldwide annually [131]. Epidemiological and laboratory studies have shown that the vitamin D endocrine system may be involved in ovarian carcinogenesis. The presence of VDR in normal ovarian epithelium, in human ovarian tumors, and in human ovarian cancer cell lines has been demonstrated [10]. For the ovarian cancer cell line, OVCAR-3, 1a,25(OH)2D reduces the proliferation induced by dihydrotestosterone through the VDR [132]. In ovarian cancer cells, 1a,25(OH)2D leads to G2/M cell cycle arrest through a p53-independent induction of GADD45, which modulates tumor formation [133]. VDR is necessary for full ovarian function through direct effects on oestrogen biosynthesis and regulation of aromatase gene expression [134]. Additionally, it may also antagonize androgen, which has been suggested to play an important role in ovarian carcinogenesis [135, 136] by inhibiting the androgen receptor expression which was found in the majority of ovarian tumors [132]. Interestingly, VDR has been found to be upregulated in ovarian tumors when compared with nonmatched normal ovarian tissue [137]. With regard to ethnical differences, substantial racial variation has been observed in the incidence of ovarian cancer, with highest rates in Caucasian women and lowest rates among Asian women [138]. This fact might be partially explained by differences in variant allele frequencies, the association of VDR polymorphisms with ovarian cancer risk being generally inconsistent among ethnic groups [134].

8. Conclusions

A large body of recent evidence suggests that abnormalities of vitamin D levels and its signaling may play a key role in the development of gynecological/obstetric pathologies in various age periods of woman's life, including selected oncological diseases. VDR-mediated signaling pathways and vitamin D levels seem to (significantly) affect the risk of several gynecological diseases, such as PCOS, endometriosis, and ovarian and even breast cancer. Moreover, since also the maternal-fetal unit is under the influence of vitamin D, a breakdown in its homeostasis may underlie infertility, preeclampsia, and GDM. According to our literature review, the relationship between vitamin D and gynaecological/obstetric diseases must be replicated in future studies which could clarify the molecular machineries behind their development. We suggest that further investigation should take into account the different serum levels of this vitamin, the several actions which arise from the binding between it and its receptor (taking into account its possible polymorphism), and finally the interplay between vitamin D metabolism and other hormonal and metabolic pathways.

Conflict of Interests

All authors have no proprietary, financial, professional, or other personal interests of any nature in any product, service, or company. The authors alone are responsible for the content and writing of the paper.

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Research Article

Vitamin D Status in Women with Gestational Diabetes Mellitus during Pregnancy and Postpartum

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Of many vitamin D extraskeletal functions, its modulatory role in insulin secretion and action is especially relevant for gestational diabetes mellitus (GDM). The aims of the present study were to determine midgestational and early postpartum vitamin D status in pregnant women with and without GDM and to describe the relationship between midgestational and postpartum vitamin D status and parallel changes of glucose tolerance. A total of 76 pregnant women (47 GDM and 29 healthy controls) were included in the study. Plasma levels of 25(OH)D were measured using an enzyme immunoassay. Vitamin D was not significantly decreased in GDM compared to controls during pregnancy; however, both groups of pregnant women exhibited high prevalence of vitamin D deficiency. Prevalence of postpartum 25(OH)D deficiency in post-GDM women remained significantly higher and their postpartum 25(OH)D levels were significantly lower compared to non-GDM counterparts. Finally, based on the oGTT repeated early postpartum persistent glucose abnormality was ascertained in 15% of post-GDM women; however, neither midgestational nor postpartum 25(OH)D levels significantly differed between subjects with GDM history and persistent postpartum glucose intolerance and those with normal glucose tolerance after delivery.

1. Introduction

Diabetes mellitus with the first onset in pregnancy—a gestational diabetes mellitus (GDM)—is a common complication of pregnancy [1]. The frequency of GDM may reach up to 18% depending on the population and diagnostic criteria used [2]. Even the normal pregnancy is characterized by a marked reduction in maternal insulin sensitivity in the second and third trimesters. However, the reduced β cells reserve or their maladaptation to higher insulin demands may lead to the development of GDM. Resulting abnormal metabolic situation during GDM pregnancy might adversely influence the foetal development (resulting most often in macrosomia with subsequent delivery complications and possibly also the postnatal health status of offspring due to the foetal programming). Moreover, GDM is a significant predictor of woman's predisposition to the development of overt diabetes mellitus type 2 later in life as documented by epidemiological studies [3, 4]. In addition, GDM strongly predicts cardiovascular disease in the future life. The risk is increased by 70% in women with a previous history of GDM compared to women without this history [5].

Vitamin D has traditionally been viewed as a key regulator of bone mineralisation [6] and calcium homeostasis [7]; however, the documented effects are far more pleiotropic. Vitamin D facilitates active calcium absorption in the small intestine by increasing calcium channel and calcium binding protein expression. Furthermore, it interacts with its receptor in osteoblasts and promotes the maturation of preosteoclasts. Besides that, growing evidence mounted that vitamin D has a number of extraskeletal functions. Vitamin D—via its binding to the vitamin D receptor (VDR)—regulates expression of hundreds of genes (directly or indirectly) including those that control key processes affecting cell fate [8]. The complexity of vitamin D action is further increased by VDR gene polymorphism. The reported associations with plethora of

phenotypes (including cancer, autoimmune, cardiovascular, metabolic, and renal and many other diseases) have been extensively meta-analysed and reviewed [9, 10]. In general, vitamin D decreases cell proliferation and stimulates cell maturation and apoptosis. Furthermore, vitamin D has a strong immunomodulatory effect; it inhibits angiogenesis [8] and is also involved in the regulation of insulin secretion and possibly insulin action [11, 12]. Interestingly vitamin D also exerts renoprotective and antiproteinuric effects with several mechanisms involved including inhibition of renin-angiotensin-aldosteron system (by decreasing renin expression), suppression of inflammation (by reducing accumulation of inflammatory cells), and restoration of glomerular filtration barrier (by attenuating podocyte damage) [13–15].

The major source of vitamin D is skin after sunlight exposure. Cutaneous vitamin D synthesis is modulated by several factors including skin pigmentation, clothing, melanin concentration, latitude, climate type, and season [16]. Vitamin D, either produced in the skin *de novo* from cholesterol (cholecalciferol) or ingested from the diet as a precursor (cholecalciferol and ergocalciferol), undergoes hydroxylation to 25-hydroxyvitamin D (25(OH)D) in the liver. Circulating plasma concentration of 25(OH)D is considered the most reliable indicator of individual's vitamin D status. 25(OH)D is further hydroxylated to the active 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) almost exclusively in the kidney upon regulation by parathormon [17]. Several studies have consistently shown that $1,25(OH)_2D$ concentration increases progressively during gestation being twice as high in late pregnancy as in postpartum or in nonpregnant controls [17, 18]. The active form $1,25(OH)_2D$ is also produced by placenta during pregnancy [19] with possible autocrine or paracrine function [20].

A number of studies focused on putative role of vitamin D deficiency in various pregnancy pathologies including GDM [21–23]. Observational studies revealed correlation between low vitamin D levels and preeclampsia or GDM [7]. Vitamin D deficiency in pregnancy was related to the incidence of GDM and serum 25(OH)D was significantly lower in women with GDM than in those with normal glucose tolerance [24–28]. Whether this association is causal remains however unclear [29]. Furthermore, several studies found inverse correlation between 25(OH)D and fasting plasma glucose (FPG), 1 hr after load plasma glucose in oral glucose tolerance test (oGTT) and glycated haemoglobin [30, 31].

Currently, little is known about postpartum vitamin D status in women with history of GDM and possible relationship between 25(OH)D plasma levels measured at the time of GDM diagnosis and the degree of glucose (in)tolerance postpartum. We hypothesise that individual's midgestational 25(OH)D plasma levels might independently reflect the risk of postpartum persistence or early reoccurrence of glucose abnormality in women with GDM history. Therefore, the aims of the present study were (1) to determine midgestational and early postpartum vitamin D status by measuring 25(OH)D plasma levels in pregnant women with and without GDM to confirm the hypothetical deficiency in GDM in central European population and (2) to describe the relationship between midgestational and postpartum vitamin D status and parallel changes of parameters characterising glucose tolerance.

2. Materials and Methods

2.1. Subjects. To avoid the confounding factor of seasonal variation of vitamin D levels, the recruitment of study subjects was confined to women whose 24–30th weeks of gestation (i.e., the first, midgestational blood sampling) spanned winter months (i.e., January, February, and March). Therefore, the inclusion criteria were (i) GDM or non-GDM diagnosed by 3-point oGTT between 24th and 30th weeks of pregnancy during January 1 to March 31 and (ii) participation in postpartum oGTT 6 weeks–12 months after delivery. A total of 76 pregnant women were included in the study (all Caucasian of Czech nationality from South Moravian Region), of those 47 had GDM (those were consenting consecutive subjects positively diagnosed with GDM and then followed from the time of GDM diagnosis till the birth at the Diabetes Centre of the University Hospital Brno) and 29 had physiological pregnancy (those were consenting women who passed midgestational GDM screening with negative result and were followed in several out-patient prenatal centres in the city of Brno until delivery). All participants completed questionnaires mapping vitamin and mineral supplementation during pregnancy. Study participants were not reporting vitamin D or multivitamin supplementation on top of routinely recommended folic acid supplementation. Exclusion criteria were diabetes mellitus type 1 or 2 before pregnancy, non-Caucasian, foreign nationality, multiple pregnancies, and severe comorbidities. Therapy for GDM consisted of diet (100%) and insulin therapy (27.7%).

GDM screening was carried out using oGTT with 75 g of glucose performed between 24th and 30th weeks of pregnancy. GDM diagnosis was established according to the WHO criteria recommended by the Czech Diabetes Society at that time of recruitment (2012): FPG $\geq$ 5.6 mmol/L, 1 hr after load glucose $\geq$ 8.9 mmol/L, and 2 hr after load glucose $\geq$ 7.7 mmol/L (any one of the three above cut-off values qualified for the GDM diagnosis). Postpartum diagnosis of diabetes/prediabetes was based on the WHO criteria for nonpregnant subjects: FPG $\geq$ 7 mmol/L alone or 2 hr after load glucose $\geq$ 11.1 mmol/L for diabetes mellitus and FPG 5.6–6.9 mmol/L or 2 hr after load glucose 7.8–11.0 mmol/L for prediabetes.

Study was approved by the Ethical Committee of Faculty of Medicine, Masaryk University, and was conducted in accordance with Helsinki declaration. Each participant provided informed consent. The paper complies with EQUATOR (Enhancing the QUAlity and Transparency Of health Research) network's guidelines.

2.2. Blood Samples and 25-Hydroxyvitamin D Measurement. Samples of peripheral EDTA-blood were taken from each participant between 24th and 30th weeks of pregnancy during their scheduled visit in prenatal centre by gynaecologist in non-GDM subjects or by diabetologist during their first visit of diabetes centre and repeatedly 6 weeks–12 months postpartum ibid. Plasma was separated by centrifugation

Table 1: Characteristics of study subjects.

Parameters	GDM ($n = 47$)	Controls ($n = 29$)	P
Pregestational parameters			
Age (years)	33 [28–35]	31 [28–33]	NS
BMI (kg/m^2)	24.45 [22.68–28.91]	21.11 [20.44–24.77]	0.014
History of previous GDM	12.8%	0%	0.0491
Family history of DM	78.7%	17.2%	0.0018
Midgestational parameters (24–30th weeks of gestation)			
FPG (mmol/L)	4.8 [4.5–5.2]	4.1 [4.0–4.4]	<0.001
1 hr after load glucose (mmol/L)	9.2 [8.3–9.6]	5.9 [5.3–6.5]	<0.001
2 hr after load glucose (mmol/L)	8.0 [7.7–8.9]	5.3 [4.8–5.8]	<0.001
BMI (kg/m^2)	27.16 [25.24–31.25]	23.53 [22.72–29.00]	0.019
25(OH)D (nmol/L)	28.5 [21.0–34.0]	31.7 [24.0–40.0]	NS
25(OH)D < 50 nmol/L	95.7%	93.1%	NS
Postpartum parameters (6 weeks–12 months after delivery)			
Weight gain during pregnancy (kg)	8.0 [5.0–10.5]	14.0 [11.0–17.0]	<0.001
Offspring birth weight (g)	3060 [2750–3480]	3400 [3100–3740]	0.016
Persisting glucose abnormality	14.9%	—	—
25(OH)D (nmol/L)	47.5 [40.0–53.0]	56.5 [48.0–69.0]	0.0041
25(OH)D < 50 nmol/L	63.8%	34.5%	0.012

Data expressed as a median [IQR] or proportions. Differences evaluated by nonparametric Mann-Whitney or Fischer's exact test, respectively.

(2 000 g, 10 min, 4°C) and stored at −70°C until analysis. 25(OH)D was measured using an *in vitro* diagnostic enzyme immunoassay kit 25-Hydroxy Vitamin D^S EIA (Immunodiagnostic Systems, Boldon, United Kingdom) according to the manufacturer's instructions and using a microtiter plate reader Spectramax 340PC (Molecular Devices, Sunnyvale, California, USA).

2.3. Statistics. Data are expressed as medians and interquartile ranges (IQR) or proportions for between-group comparisons. Nonparametric tests were used for comparison between and within the groups (Mann-Whitney and Wilcoxon tests, resp.). Fischer's exact test was used for contingency tables. Correlations were computed using Spearman's correlation coefficients. Software Statistica (StatSoft, Tulsa, Oklahoma, USA) was used for all analyses. $P < 0.05$ was considered statistically significant. Due to the specific requirements of the study—seasonally limited sampling of consecutive GDM and controls subjects—power analysis was performed *post hoc*. The power of the study to detect difference in 25(OH)D levels with given sample size was 0.91 (two means t-test).

3. Results

Characteristics of study subjects in both groups are shown in Table 1. Positive history of previous GDM was significantly more frequent in GDM group compared to controls ($P = 0.0491$, Fischer's exact test) with no previous foetal macrosomia reported and the same was true for positive family history of any form of DM ($P = 0.0018$, Fisher's exact test). Women with GDM were not significantly older but they were significantly heavier, they had smaller weight gain during pregnancy, and their offspring had significantly lower birth weight. Therefore, we first assessed correlations between 25(OH)D levels in pregnancy and pregestational BMI ($r = -0.35$, $P = 0.0019$), midgestational BMI ($r = -0.30$, $P = 0.0075$), FPG ($r = -0.36$, $P = 0.0014$), postload oGTT values (P = NS), weight gain during pregnancy ($r = 0.35$, $P = 0.0017$), and offspring birth weight (P = NS). Furthermore, we assessed correlations between postpartum 25(OH)D levels and weight gain during pregnancy (P = NS), offspring birth weight (P = NS), and parameters of glucose tolerance after delivery, where significant negative correlation with 2 hr after load glucose postpartum was ascertained ($r = -0.43$, $P = 0.0051$). All reported correlations are summarised in Table 2.

In spite of the previously assessed inverse relationship of 25(OH)D with BMI, midgestational 25(OH)D levels—both unadjusted and adjusted for midgestational BMI—did not significantly differ between pregnant women with GDM (generally heavier) and healthy controls (P = NS, Mann-Whitney). While postpartum 25(OH)D levels raised significantly in both groups ($P < 1 \times 10^{-6}$ and $P = 3 \times 10^{-6}$, resp., Wilcoxon test), postpartum 25(OH)D levels in women with GDM history remained significantly lower compared to controls ($P = 0.0041$, Mann-Whitney); see Figure 1.

Based on the results of oGTT repeated up to maximum 12 months postpartum the glucose abnormality was detected in 7 women (14.9%) with history of GDM. We compared both midgestational and postpartum 25(OH)D levels between GDM women with persistent postpartum glucose abnormality and those whose glucose tolerance returned to normal after delivery to test eventual predictive or pathogenic potential of 25(OH)D measurement. There were no statistically significant differences (both $P > 0.05$,

Table 2: Correlations between 25(OH)D levels and selected anthropometric and biochemical parameters.

Parameters	Midgestational 25(OH)D levels		Postpartum 25(OH)D levels	
	r	P	r	P
Pregestational BMI	−0.35	0.002	−0.25	0.030
Midgestational BMI	−0.30	0.008	−0.23	0.049
FPG	−0.36	0.001	−0.18	NS
1 hr after load glucose	−0.14	NS	−0.05	NS
2 hr after load glucose	−0.15	NS	−0.43	0.005
Weight gain during pregnancy	0.35	0.002	0.15	NS
Offspring birth weight	0.01	NS	−0.04	NS

Correlations were assessed using Spearman's correlation coefficient. The midgestational 25(OH)D levels were correlated with results of oGTT provided in the midtrimester, and the postpartum 25(OH)D levels were correlated with results of oGTT provided 6 weeks to 12 months postpartum.

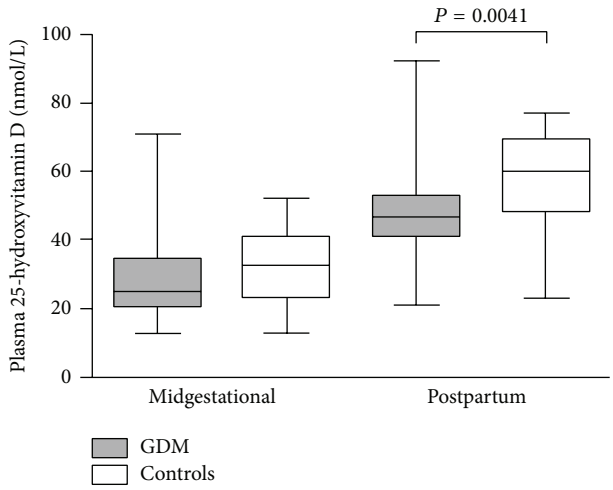

Figure 1: Plasma 25(OH)D levels. Box and Whisker plots were constructed as medians, minimum, and maximum values and IQR. Differences in 25(OH)D levels between midgestational and postpartum values in each group were significant (both $P < 5\times10^{-6}$, Wilcoxon paired test, not shown in the graph).

Mann-Whitney); however, comparison suffers from rather disparate numbers in the groups (7 versus 40).

Even though there is no consensus on physiological 25(OH)D range, most papers consider levels < 50 nmol/L as deficient [8]. Levels in the range 50–72.5 nmol/L indicate relative insufficiency and levels > 72.5 mmol/L are considered sufficient [7, 32]. In our study, we have found that midgestational vitamin D deficiency (i.e., 25(OH)D levels < 50 nmol/L) was present in majority of the study sample, that is, 45 of 47 (95.7%) women with GDM and 27 of 29 (93.1%) healthy pregnant women (P = NS, Fisher's exact test). After delivery, 30 of 47 (63.8%) women with GDM and 10 of 29 (34.5%) controls remained deficient (P = 0.012, Fischer's exact test), although majority of blood samples postpartum were taken in summer.

Finally, due to the fact that in April of 2014 Czech Diabetes Society adopted new diagnostic criteria for GDM in accordance with the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) recommendations [33] we reclassified our study sample according to the IADPSG criteria with the following thresholds: FPG ≥ 5.1 mmol/L, 1 hr after load glucose: ≥10.0 mmol/L, and 2 hr after load glucose: ≥8.5 mmol/L retrospectively. Using newly adopted criteria, 24 women would be diagnosed as having GDM and 52 as healthy subjects (note all previously classified controls remained, 23 of previously diagnosed GDM subjects became controls), and then we compared midgestational and postpartum 25(OH)D levels. Interestingly, we found statistically significant differences in both unadjusted and BMI-adjusted midgestational 25(OH)D levels between women with GDM and controls classified by IADPSG criteria (P = 0.014 and P = 0.006, resp., Mann-Whitney) and also in postpartum 25(OH)D levels between the two groups (P = 0.018, Mann-Whitney). In all comparisons 25(OH)D levels in GDM group were significantly lower (data not shown).

4. Discussion

Vitamin D seems to have several extraskeletal functions including regulation of glucose metabolism through influencing insulin sensitivity, although the mechanisms are not fully understood. The pancreatic β cells express both vitamin D receptor and enzyme 1α-hydroxylase which enables them to produce 1,25$(OH)_2$D locally [17]. The effect of vitamin D on regulation of pancreatic β cell function and insulin secretion could be mediated through intracellular changes in calcium pool. Vitamin D could also enhance insulin sensitivity by stimulating insulin receptor gene expression thereby enhancing insulin mediated glucose transport [34]. In addition, vitamin D may also be needed to ensure a normal rate of calcium flux across cell membranes and maintenance of an adequate cytosolic calcium pool, which is important for insulin-mediated intracellular signalling in insulin-responsive tissues [35]. Finally, several studies suggest that vitamin D could play a role in the pathogenesis of diabetes mellitus type 2 by affecting insulin sensitivity of β cell function [36, 37]. Vitamin D is also essential for proper foetal programming and its deficiency during pregnancy may lead to low birth weight and increased susceptibility to chronic disease later in life [38].

Although there is no general consensus on the criteria for vitamin D deficiency in pregnant women, in our study we have found high prevalence of deficiency (when using

cut-off < 50 nmol/L) in overall study sample: 95.7% of women with GDM and 93.1% of controls were vitamin D deficient during pregnancy. Reassessment up to 12 months postpartum revealed persisting 25(OH)D deficiency in 63.8% of women with GDM history and 34.5% of controls. Dovnik et al. [39] described seasonal variation of 25(OH)D levels in women of the same stage of pregnancy in Slovenia. Nearly 50% of pregnant women were vitamin D deficient in September while it was 82% in December. This fact could explain the significant difference between postpartum (blood drawn in summer) and low pregnancy 25(OH)D levels in our population which was found in both women with GDM and in controls.

Studies that measured 25(OH)D levels in different time points during pregnancy and after delivery in healthy women provided contradictory results. Holmes et al. [23] have shown that vitamin D deficiency (≤50 nmol/L) in Caucasian population (Irish women) can occur in 95% of pregnant women in 12th week of pregnancy, in 90% in 20th week, in 66% in 35th week, and in 15% 3 days postpartum. Concentrations of 25(OH)D increased in each measurement, being highest after delivery, which could be explained by season in which samples were collected (i.e., mostly during autumn). On the contrary, Haliloglu et al. [40] measured 25(OH)D levels in healthy pregnant Turkish women in each trimester and 6 weeks after delivery and reported that 25(OH)D concentration decreased significantly in each trimester being the lowest postpartum. Contradictory results could be explained by seasonal, geographical, or ethnic vitamin D variation; the paper does not unfortunately mention in what season women were included in the study.

Given the high overall prevalence of vitamin D deficiency, we did not find any statistically significant difference in 25(OH)D levels between woman with GDM diagnosed by WHO criteria and controls during pregnancy. Maghbooli et al. [25] reported higher prevalence of severe vitamin D deficiency (≤12.5 nmol/L) in GDM than in normoglycaemic pregnancies in 741 Iranian women. Nevertheless, vitamin D levels in Asian population are in general lower than in Caucasian population and criteria used in her study would classify most European women as having optimal vitamin D status. Zuhur et al. [41] described significantly lower 25(OH)D levels in 234 Turkish pregnant women with GDM compared to 162 controls. An increased risk of GDM was present only in subgroup with severe 25(OH)D deficiency (<12.5 nmol/L) after controlling for maternal age, previous history of GDM, familiar history of diabetes mellitus type 2, and pregestational BMI. Study of Burris et al. [26] found an inverse association between second trimester 25(OH)D levels < 25 nmol/L and 1 hr after load glucose (50 g) levels; however, only 5% of studied women developed GDM and as mentioned above threshold for vitamin D deficiency differed from our study. Soheilykhah et al. [28] reported that prevalence of vitamin D deficiency is higher among women with impaired glucose tolerance or GDM in 204 Iranian women but they did not find correlation with BMI or FPG. Clifton-Bligh et al. [30] found significantly lower 25(OH)D levels in women with GDM than in healthy pregnant women in the group of 307 Australian women; however, when 4 ethnic subgroups were analysed separately, no association was confirmed between 25(OH)D levels and GDM. Our results are in agreement with other published studies reporting lack of association between vitamin D levels in pregnancy and GDM. For example Makgoba et al. [42] found no statistically significant difference in maternal 25(OH)D levels between GDM and control group in first trimester in 248 British women and Farrant et al. [43] did not find association between maternal vitamin D status in 559 nondiabetic pregnant women from South India and the risk of GDM.

Furthermore, the present study replicated findings of inverse correlation between 25(OH)D and FPG during pregnancy and lack of correlation with age ascertained by others [25, 30, 31]. We also found inverse correlation between 25(OH)D and 2 hr after load glucose after delivery. Contrary to published data [28, 43], we found significant negative correlation between 25(OH)D levels and pregestational and midgestational BMI. Zhang et al. [35] found negative correlation between 25(OH)D levels and pregestational BMI earlier in pregnancy (16th week). Interestingly, we have found positive correlation between total weight increment during pregnancy and midgestational 25(OH)D levels. The findings that GDM women are generally heavier but have lower weight increment during pregnancy and lower offspring birth weight could be explained by the effect of a stricter dietary regime in GDM subjects.

Interestingly, the results appear criteria-dependent and this might be a critical issue in all available studies so far. When applying IADPSG diagnostic criteria for GDM to the same study sample there are statistically significant differences in 25(OH)D levels between women with GDM and those with normoglycaemia not only postpartum but also in midtrimester of pregnancy. Still, since the IADPSG criteria were applied *post hoc*, these results have to be considered hypothesis driving and conclusions speculative.

Several studies investigated vitamin D supplementation in women with GDM [23, 31]. Lau et al. [31] studied whether vitamin D supplementation may improve glycaemic control in women with GDM. Despite the fact that 147 Australian women with GDM were advised to take daily prenatal multivitamins containing 400 IU or 500 IU vitamin D, 41% of the participants had vitamin D deficiency. Asemi et al. [44] assessed the effect of calcium and vitamin D cosupplementation on GDM in a randomised placebo-controlled study (56 Iranian women with GDM) and they observed a significant reduction in FPG, serum insulin levels, and HOMA-IR and increase in QUICKI compared with placebo, and also an increase in glutathione and a reduction in serum LDL-cholesterol and total cholesterol and a significant elevation in HDL-cholesterol were common. Rudnicki and Mølsted-Pedersen [45] reported that supplementation with an active form of vitamin D ($1,25(OH)_2D$) was associated with significant decrease of plasma glucose level and possible effect on insulin sensitivity.

Finally, there are certainly limitations of the current study: first of all, a relatively small sample size. For the sake of homogeneity, the enrolment into the study spanned only one quarter of the whole year with the aim to eliminate a possible seasonal effect on vitamin D levels. Moreover,

due to the low compliance of GDM women in postpartum screening the sample size was reduced further. According to published data reviewed in [46, 47] only about 50% of women with GDM return after delivery to repeat recommended oGTT and this applied to our study stays in approximately same proportion. As for healthy pregnant women, their participation in postpartum oGTT was entirely voluntary and this resulted in even smaller number of control subjects.

5. Conclusions

Our study in pregnant women of central European population did not replicate sporadic previous findings of significantly decreased levels of vitamin D in GDM pregnancy; however, results seem to be criteria-sensitive (WHO versus IADPSG) and the topic warrants further study. We confirmed overall high prevalence of vitamin D deficiency in pregnant women in spite of the GDM presence. The novel and most striking observations of the current study are significantly lower absolute 25(OH)D levels together with significantly higher prevalence of early postpartum 25(OH)D deficiency in women with GDM history compared to those without. Potentially beneficial effect of vitamin D supplementation and the plausible pathogenic role of 25(OH)D deficiency in the subsequent development of diabetes mellitus type 2 in women with GDM history has to be further explored considering the role of vitamin D in modulating insulin sensitivity and glucose metabolism.

Conflict of Interests

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

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Review Article

Links between Vitamin D Deficiency and Cardiovascular Diseases

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The aim of the present paper was to review the most important mechanisms explaining the possible association of vitamin D deficiency and cardiovascular diseases, focusing on recent experimental and clinical data. Low vitamin D levels favor atherosclerosis enabling vascular inflammation, endothelial dysfunction, formation of foam cells, and proliferation of smooth muscle cells. The antihypertensive properties of vitamin D include suppression of the renin-angiotensin-aldosterone system, renoprotective effects, direct effects on endothelial cells and calcium metabolism, inhibition of growth of vascular smooth muscle cells, prevention of secondary hyperparathyroidism, and beneficial effects on cardiovascular risk factors. Vitamin D is also involved in glycemic control, lipid metabolism, insulin secretion, and sensitivity, explaining the association between vitamin D deficiency and metabolic syndrome. Vitamin D deficit was associated in some studies with the number of affected coronary arteries, postinfarction complications, inflammatory cytokines and cardiac remodeling in patients with myocardial infarction, direct electromechanical effects and inflammation in atrial fibrillation, and neuroprotective effects in stroke. In peripheral arterial disease, vitamin D status was related to the decline of the functional performance, severity, atherosclerosis and inflammatory markers, arterial stiffness, vascular calcifications, and arterial aging. Vitamin D supplementation should further consider additional factors, such as phosphates, parathormone, renin, and fibroblast growth factor 23 levels.

1. Introduction

Vitamin D exists in two forms: D2 (ergocalciferol) and D3 (cholecalciferol). Vitamin D3, "the sunshine vitamin," is synthetized in the human epidermis via ultraviolet irradiation, or it may be consumed in the form of oily fish or supplements. Vitamin D2 is found in plants, as a product of irradiation of ergosterol [1]. The vitamin is converted in the liver and kidney to calcidiol and calcitriol, respectively, and acts on specific target tissues via vitamin D receptors. Calcitriol, the active form of vitamin D, binds to vitamin D receptors in the intestines, bones, and kidneys to increase calcium absorption from the intestines, promote calcium deposition in bones, and decrease parathyroid hormone concentrations (PTH). Its extraosseous effects are less known. Vitamin D receptors were found in other tissues, as well, including the brain, cardiomyocytes, vascular smooth muscle cells, endothelial cells, pancreatic beta-cells, skeletal muscle, breast, prostate, colon, macrophages, and skin, exerting several pleiotropic effects, and their expression decreases with age. The vitamin D receptor is closely related to the thyroid, retinoid, and peroxisome proliferator-activator receptors [2]. Recent studies have found active 1 alpha hydroxylase in several extra renal tissues, such as the heart and vascular smooth muscle cells [3–5]. Activated vitamin D may influence cellular growth, proliferation and apoptosis, oxidative stress, membrane transport, matrix homeostasis, cell adhesion, and immune system functions and may regulate a large number of genes and healthy aging [6, 7].

Vitamin D insufficiency is a common public health problem, very often unrecognized and untreated, associated with rickets, dental caries, and growth retardation in children and osteomalacia, osteopenia, osteoporosis, decreased muscle strength, falls, and increased risk of fracture in adults.

Vitamin D insufficiency is associated with indoor lifestyle, sun avoidance strategies, obesity, diabetes mellitus, low HDL cholesterol, older age, distance from the equator, darker skin, winter season, air pollution, smoking, malabsorption, renal and liver disease, and medication (anticonvulsants, glucocorticoids, antirejection, and human immunodeficiency virus therapy) [1–11]. The biologically active form of vitamin D is 1,25 dihydroxyvitamin D, but the best indicator of vitamin D status in individuals free of kidney disease is 25-hydroxyvitamin D, the substrate for the renal and nonrenal production of calcitriol, with a longer biological half-life and a higher concentration than 1,25 dihydroxyvitamin D, reflecting the total endogenous and exogenous production of vitamin D [12, 13].

Recent research has linked inadequate vitamin D status to nonskeletal major chronic diseases, especially cardiovascular diseases [8]. Existing data from laboratory studies, epidemiologic and experimental research and prevention trials, suggest that vitamin D reduces the risk of cardiovascular disease, and a large, randomized, primary prevention trial, with adequate dosing, combining cholecalciferol and omega-3 fatty acids, is ongoing: the VITAL study. Poor vitamin D status was associated with cardiovascular and overall mortality, despite unconvincing results of vitamin D supplementation on mortality [13]. Food-based strategies for enhancement of vitamin D status in the population could lower cardiovascular risk if a causal link between low vitamin status and cardiovascular pathology would be demonstrated [14].

The aim of the present paper was to review the most important mechanisms explaining the possible association of vitamin D deficiency and cardiovascular diseases, focusing on recent experimental and clinical data.

2. Definition of Vitamin D Deficiency

Optimal serum concentration of 25-hydroxyvitamin D considers only bone health and was defined as the concentration that maximally suppresses serum parathyroid hormone [15]. Most experts define vitamin D deficiency as a calcidiol level of <20 ng/mL and insufficiency as 21–29 ng/mL [1, 16]. Vitamin D is sufficient if >30 ng/mL, and vitamin D intoxication is considered if >150 ng/mL [16]. There are variations among professional bodies regarding the cut-off values for insufficient or deficient vitamin D level [17].

According to a report of the Institute of Medicine (IOM), vitamin D at doses of 600 IU/day is beneficial for the bones, but it is not certain if higher doses could reduce the risk of chronic diseases, including cancer and cardiovascular pathology [17]. A threshold effect between vitamin D status and cardiovascular risk was suggested [11]. Zittermann et al. found a vitamin D level of 30–35 ng/L as the best choice for risk reduction in cardiovascular mortality [18].

3. Vitamin D and Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAA) maintains vascular resistance, due to angiotensin II synthesis, and extracellular fluid volume homeostasis, considering the release of aldosterone [19]. Studies by different groups, in both animals and humans, demonstrated that vitamin D *decreases RAA activity* [20], suppressing *renin gene expression* [12]. Vitamin D regulates the genes involved in renin production, through a *cis*-DNA element in the renin gene promoter [1, 21], downregulating the RAA system.

Vitamin D receptor-null mice had a sustained elevation of renin expression, maintaining a normal level of blood electrolytes [19]. Increased renin synthesis leads to an elevated angiotensin II production, which is a strong vasoconstrictor, enabling the development of hypertension and left ventricular hypertrophy [19]. Similarly, 1-alpha-hydroxylase deficient mice, unable to synthetize the active metabolite 1,25-dihydroxyvitamin D, develop also high blood pressure and left ventricular hypertrophy [4]. Studies using renal arteries from hypertensive patients reported that calcitriol *reduces the expression of the angiotensin-1 receptor in endothelial cells,* improving endothelial function and preventing reactive oxygen species overproduction [22]. Secondary *hyperparathyroidism* in vitamin D receptor-null mice may also contribute to renin upregulation [19], considering that intravenous infusion of PTH increases plasma renin activity and renin release [23, 24].

Vitamin D regulation of renin expression is independent of calcium metabolism, and calcitriol markedly suppresses renin transcription by a vitamin D receptor—mediated mechanism in cell cultures [19]. Ferder et al. suggested a possible feedback link between vitamin D and the renin-angiotensin system (RAS), considering that vitamin D and angiotensin II receptors are distributed in the same tissues, changes in RAA activity and activation of the vitamin D receptors seem inversely related, and vitamin D deficiency could be explained by the cellular *inflammatory response activity* induced by the RAA system [25]. Therapy should combine RAS blockade and VDR stimulation [25].

D hypervitaminosis induces vascular and soft-tissue calcifications. *Calcium deposition in the vascular smooth muscle cells* may also lead to RAA activation [26–28].

Suppression of renin production and downregulation of the RAA may explain the direct myocardial and vascular effects through *modulation of hypertrophic stimuli* [10].

4. Vitamin D and Atherosclerosis

Vitamin D suppresses inflammation via several pathways, such as inhibition of prostaglandin and cyclooxygenase pathways, upregulation of anti-inflammatory cytokines, decrease of cytokine induced expression of adhesion molecules, reduction of matrix metalloproteinase 9, and downregulation of the RAA [11, 25]. Vitamin D deficiency stimulates *systemic and vascular inflammation,* enabling atherogenesis [1]. On the other hand, as already mentioned, hypertension is also associated with lack of vitamin D, due to activation of the RAA system, enabling *endothelial dysfunction*, the first step in plaque formation. The proinflammatory nuclear factor kB mediates partly the association between endothelial dysfunction and low vitamin D status [11].

Large epidemiological studies have highlighted vitamin D deficiency as a marker of cardiovascular risk [29], promoting

accelerated atherosclerosis [12, 30] and subsequent cardiovascular events [11]. Chronic vitamin D deficiency causes *secondary hyperparathyroidism, increasing insulin resistance*, impairing beta-pancreatic cell function, and enabling the development of metabolic syndrome and diabetes mellitus [1]. Calcitriol regulates the genes involved in insulin production in the pancreas [1].

Vitamin D has also some *antiatherogenic functions*, inhibiting the formation of foam cells, cholesterol uptake by the macrophages, and enabling HDL transport [31]. Lower serum 25-hydroxyvitamin D was associated with the metabolic syndrome and its components, especially HDL cholesterol concentration [32].

Vascular smooth muscle cells and endothelial cells express receptors for vitamin D, enabling conversion of calcidiol to calcitriol [12], and vitamin D is involved in *regulation of growth and proliferation of smooth muscle cells and cardiomyocytes* [12, 33, 34]. Vitamin D inhibits proliferation of vascular smooth muscle cells by acute influx of calcium into the cell and increases calcification of smooth muscle cells [34].

The cardiovascular protective effects of vitamin D include also the anti-inflammatory effects, inhibition of vascular smooth muscle cells proliferation, suppression of proatherogenic T lymphocytes, preservation of endothelial function [22, 35–40], and protection against advanced glycation products [2].

Vitamin D deficiency was associated with *vascular stiffness*, which is a known predictor of cardiovascular morbidity and mortality [11] and a marker of subclinical atherosclerosis.

5. Vitamin D Insufficiency and Hypertension

Low vitamin D levels have been associated with increased prevalence of hypertension [41–43] or elevated diastolic blood pressure [44–46]. Clinical studies demonstrated an *inverse, dose-response relationship* between plasma 1,25(OH)2D3 concentration and blood pressure or *renin activity* in both normotensive and hypertensive patients [43, 47–49]. Wang et al. reported associations of hypertension risk and plasma 25-hydroxyvitamin D and vitamin D receptor polymorphism, respectively [8]. Rats with experimentally induced vitamin D deficiency developed hypertension and cardiomegaly [43, 50]. Mice lacking vitamin D receptor had an increased renin expression and angiotensin II production and developed also hypertension and cardiac hypertrophy [19, 43, 51]. Lower end-diastolic pressures were noted in Dahl salt-sensitive rats treated with paricalcitol, a vitamin D receptor activator, compared to untreated animals [52].

Ultraviolet light exposure enables vitamin D synthesis and has blood pressure lowering effects [53, 54]. Hypertensive patients exposed to a tanning bed significantly raised their concentration of 25-hydroxyvitamin D after 3 months and became normotensive [54]. Vitamin D3 supplementation reduces blood pressure in patients with essential hypertension [55, 56] and reduces also plasma renin activity and angiotensin II levels in hyperparathyroidism patients [57, 58]. Erythemal and preerythemal doses of UV irradiation decrease vascular resistance, with diffuse skin vasodilatation, related to nitric oxide release [2, 59].

Calcitriol exerts a protective effect on human renovascular function, restoring the impaired *endothelium-dependent relaxation* in renal arteries, accompanied by the normalization of oxidative-stress related proteins [22]. The augmented production of *reactive oxygen species* is induced by angiotensin II in human renal arteries and endothelial cells and impairs vascular function enabling the development of hypertension [22]. Vitamin D metabolites reduced endothelium-dependent vascular smooth muscle contractions and vascular tone in hypertensive rats by affecting calcium influx across endothelial cells [60]. *In vitro*, vitamin D receptor activation induces a concentration-dependent increase of nitric oxide production in endothelial cells and improves the angiogenic properties of endothelial progenitor cells [61, 62].

Hypertensive patients with vitamin D deficiency were associated with a twofold risk of cardiovascular events, including myocardial infarction, angina, prolonged chest pain with documented ECG changes, stroke, transient ischemic attack, peripheral claudication, and heart failure [12].

Not all studies demonstrated blood pressure lowering effects of vitamin D. High-dose intermittent vitamin D therapy was given every 2 months in patients with resistant hypertension, on, at least, 3 antihypertensive agents, for 6 months, with no reduction of office blood pressure, 24-hour ambulatory blood pressure, or left ventricular mass [63].

The relation of vitamin D deficiency and *preeclampsia* is controversial. Vitamin D deficiency in pregnancy has been associated with an increased risk of preeclampsia [64, 65], suggesting that vitamin D supplementation in early pregnancy could prevent preeclampsia [64] and decreased calcidiol was found at diagnosis of early onset severe preeclampsia [66]. On the other hand, several authors reported no adverse pregnancy outcomes, including preeclampsia, with low 25-hydroxyvitamin D [67, 68]. An inverse association was reported between calcium intake and maternal blood pressure, as well, as the incidence of preeclampsia syndrome, explained, probably, by the influence on parathyroid hormone release and intracellular calcium availability, but the relationship between calcium and risk of hypertension in pregnancy seems to be inconsistent and inconclusive [69]. Preeclampsia is associated with reduced placental perfusion and maternal endothelial dysfunction [62]. Vitamin D increases capillary formation in endothelial colony forming cells, probably mediated by increased expression of *vascular endothelial growth factor and promatrix metalloproteinase activity* [62].

The mode of *vitamin D prophylaxis during infancy* (continuous daily supplementation, bolus doses of vitamin D forte every three months during the first year of life, or bolus doses during winter combined with continuous daily drops during summer) did not influence the blood pressure level in early adolescence, and no adverse effects were reported despite exceeding daily doses [70].

Vitamin D levels were significantly lower in patients with *orthostatic hypotension*, but lower vitamin D status was not associated with impaired orthostatic hemodynamics [46]. Vitamin D deficiency may also be involved in orthostatic hypotension development in elderly patients [71]. Serum levels of vitamin D should be checked during the evaluation of those patients, considering that orthostatic hypotension is associated with falls, fractures, cardiovascular events, and significant mortality in the elderly [46, 71]. Vitamin D receptors are found in vascular smooth muscle, endothelial and cardiac cells, enabling involvement in the cardiac and vascular response during orthostasis [72], and vitamin D deficiency downregulates the RAA system and was also associated with endothelial dysfunction [46]. On the other hand, both orthostatic hypotension and vitamin D deficiency are more prevalent in the elderly.

Women's Health Initiative study of *postmenopausal women* found no reduction in blood pressure with supplementation of 1,000 mg/day of calcium and 400 IU/day of vitamin D3, probably because the vitamin dose was insufficient for beneficial blood pressure effects [73].

The parathormone (PTH) is a crucial regulator of calcium and phosphate balance. Higher PTH concentrations were associated with several cardiovascular risk factors, including hypertension and arterial stiffness [74]. The mechanisms linking PTH and blood pressure include upregulation of RAA due to increase of serum calcium and sympathetic activity [74]. PTH correlated with blood pressure and hypertension incidence in a cross-sectional study, including 1,205 elderly subjects; serum vitamin D was not associated with blood pressure, probably due to the relatively high levels in the study population [75].

The antihypertensive properties of vitamin D include suppression of the RAA system, renoprotective effects including antiproteinuric and anti-inflammatory effects, direct effects on endothelial cells and calcium metabolism, inhibition of growth of vascular smooth muscle cells, prevention of secondary hyperparathyroidism, and beneficial effects on common cardiovascular risk factors and are important in vitamin D deficient, hypertensive patients [42, 44, 76]. Observational studies suggest that vitamin D deficiency is associated with high blood pressure, but randomized clinical trials did not yield conclusive results [20].

6. Vitamin D and Metabolic Syndrome

Cardiovascular risk factors such as hyperlipidemia, abdominal obesity, hypertension, and diabetes often cluster in the same individual [42]. A small cross-sectional study, including middle-aged men, found that vitamin D metabolites were related to lipid and glucose metabolism and serum urate [77]. Serum calcitriol was inversely correlated to the blood pressure and triglycerides and calcidiol to fasting insulin and lipoprotein lipase activity both in adiposal tissue and in skeletal muscle [77].

Statins exert beneficial effects, not only on lowering cholesterol but also on diabetes, bone metabolism, and inflammatory states, probably related to vitamin D [78]. Atorvastatin used in patients with acute coronary syndromes decreased cholesterol levels and significantly increased vitamin D levels, due to the shared metabolic pathway of cholesterol and vitamin D [42, 79].

Obese individuals are at higher risk of hypertension, hypercholesterolemia, diabetes, cardiovascular mortality, and vitamin D deficiency. Besides vitamin D sequestration in subcutaneous fat and making stores less available to become biologically activated, obese persons may have a sedentary lifestyle and be less active outdoors, and skin production of vitamin D may be impaired due to clothing habits [42, 80].

Many cellular, experimental, and observational studies support the role of vitamin D in the pathogenesis of type 1 and type 2 diabetes [81]. Type 1 and type 2 diabetic patients have a higher incidence of hypovitaminosis D [81]. A lower incidence of type 2 diabetes was found after vitamin D supplementation in high risk individuals, supporting the hypothesis that high vitamin D status protects against type 2 diabetes [82]. Insulitis and type 1 diabetes mellitus were prevented by pharmacologic doses of vitamin D in nonobese mice, possibly by immune modulation and direct effect on beta-cell function [81]. An inverse association between circulating 25-hydroxyvitamin D and incident type 2 diabetes was demonstrated by a systematic review and meta-analysis, including only prospective studies [83]. The plausible mechanisms explaining the mentioned associations involve vitamin D receptors in pancreatic beta-cells influencing insulin secretion and vitamin D effects on insulin sensitivity, or through the effects of vitamin D on calcium metabolism, but there is no demonstrable evidence of causality yet [27, 83, 84]. Sun exposure implies greater outdoor physical activity, improving insulin sensitivity [84]. Further mechanism connecting vitamin D and diabetes mellitus involves pancreatic tissue and cells of the immune system expressing not only vitamin D receptors but also vitamin D binding protein, some allelic gene variations involving vitamin D metabolism and vitamin D receptors, associated with glucose intolerance, insulin secretion and sensitivity, and inflammation [81]. Thus, vitamin D has an important role in glycemic control, which may influence cardiovascular outcomes [27].

Vitamin D may influence several components of the metabolic syndrome, especially hypertension, hyperglycemia, insulin resistance, and hyperlipidemia.

7. Vitamin D, Coronary Heart Disease, and Heart Failure

A strong association was found between vitamin D deficiency and *slow coronary flow, endothelial dysfunction and subclinical atherosclerosis,* in patients with normal or near-normal coronary arteries at coronary angiography [85]. Decreased levels of vitamin D binding protein were found in the plasma of survivors of a myocardial infarction at young age, statistically correlated with the number of affected coronary arteries [86]. Low vitamin D levels have been linked to inflammation, *higher coronary artery calcium scores, increased mean platelet volume,* and *increased vascular stiffness* [11, 28]. Abnormally high mean platelet volume has been associated with cardiovascular diseases, considering the higher risk to block arteries, due to the ability to aggregate more rapidly with

collagen, the higher thromboxane A2 level, and expression of more glycoprotein Ib and IIb/IIIa receptors than smaller platelets [28]. The increased release of proinflammatory cytokines in patients with vitamin D deficiency increases oxidative stress and enables release of immature and activated platelets from the bone marrow, with an increased mean platelet volume [28].

Vitamin D deficiency was associated with coronary heart disease and myocardial infarction [87] and was found in a high proportion of patients with myocardial infarction [9, 88]. Vitamin D status is prognostic for major *postinfarction adverse events*, such as heart failure hospitalizations, recurrent acute myocardial infarction, death [89, 90], or restenosis after percutaneous coronary intervention [11]. A significant, moderate association was found between circulating vitamin D concentration and the risk of all-cause mortality, especially deaths due to coronary disease [6].

Vitamin D level was not associated with the severity of coronary lesions in patients with ST-segment elevation myocardial infarction [88]. On the other hand, the severity of coronary artery stenosis, assessed according to the Gensini score, a validated measure of the angiographic severity of coronary heart disease, was associated with vitamin D deficiency [91].

Arnson et al. examined the effects of short-term vitamin D supplementation on *inflammatory cytokines* after an acute myocardial infarction, reporting a reduction of vascular cell adhesion molecules, C-reactive protein, and interleukin-6, supporting the cardioprotective anti-inflammatory effects of vitamin D on the vascular system [92].

Racial differences have been reported regarding the associations between serum 25-hydroxyvitamin D and the risk of coronary heart disease in a multiethnic community-based cohort of adults without clinical cardiovascular disease. The association of low calcidiol and increased risk of coronary heart disease was demonstrated in white and Chinese study participants, but not in black or Hispanic [93].

Vitamin D exerts biological effects on cardiac myocytes, stimulating calcium-ATPase activity and calcium uptake in cardiac myocytes [11]. Lack of vitamin D could cause diastolic dysfunction, and the Hoorn study found a trend towards increased risk of diastolic dysfunction in persons with vitamin D deficiency, considering 614 persons from a population-based cohort of older men and women [94]. No significant association was found between vitamin D levels and left ventricular diastolic performance, including left atrial volume index in a retrospective observational study including 1,011 unselected patients (involving patients with hypertension and diabetes) [95]. Several explanations were found for the lack of association between vitamin D level and diastolic dysfunction, such as the cross-sectional study design and insufficient information about the duration of vitamin D deficiency [95].

The majority of congestive heart failure patients have insufficient vitamin D, due to reduced sunlight exposure, difficult mobilization and outdoor activity, nutritional factors, and malabsorption of vitamin D due to intestinal edema in severe right heart failure and comorbidities, such as obesity and renal and hepatic failure [10]. Lack of vitamin D causes hypocalcemia and *secondary hyperparathyroidism*. Indeed, serum parathormone (PTH) was elevated in patients with congestive heart failure due to ischemic or dilated cardiomyopathy and hypovitaminosis D was present [96]. Osteomalacia and osteoporosis and fracture rates should be, probably, evaluated in individuals with congestive heart failure [10]. Osteoporosis and cardiovascular pathology share common backgrounds, including osteoprotegerin and receptor activator of nuclear factor kappa-B ligand, involved in osteoclast activation and vascular calcification and atherosclerosis; bone morphogenetic protein, involved in osteoblastic differentiation and atherosclerotic lesions, and age-related estrogen deficiency [97]. An inverse association between PTH and vitamin D level persists until the vitamin D value exceeds 30 ng/mL [72]. The presence of hypocalcemia, osteopenia, or osteomalacia could justify vitamin D supplementation in heart failure patients, despite controversial causative link between vitamin D deficiency and heart failure. An *autocrine/paracrine vitamin D system* also exists, independent of PTH level, besides the endocrine vitamin D system [74].

It is important to determine the vitamin D level in patients with myocardial infarction and correct deficient levels. Vitamin D repletion to prevent cardiac remodeling after a myocardial infarction deserves future study [87]. Vitamin D signaling plays an important cardioprotective role after myocardial infarction through anti-inflammatory, *antifibrotic, and antiapoptotic* mechanisms [98]. Despite several studies revealing vitamin deficiency in congestive heart failure patients, no clear data on improvement of outcome with vitamin D supplementation exist, despite reduction of inflammatory markers and PTH level [10]. The RECORD randomized controlled trial demonstrated that vitamin D supplementation might *protect against cardiac failure* in the elderly, but not against myocardial infarction or stroke [99].

Vitamin D receptor knockout mice had upregulated matrix metalloproteinases, involved in cardiac remodeling, impaired cardiac relaxation and contractility, and developed left ventricular hypertrophy [27, 100]. It seems that both matrix metalloproteinases and inhibitors of metalloproteinases expressions are modulated by vitamin D [100].

Vitamin D decreases fibrosis in mesenchymal multipotent cells through the increased expression and nuclear translocation of vitamin D receptors, decreasing profibrotic factors (transforming growth factor B1 and plasminogen activator inhibitor) and several collagen isoforms and increasing expression of antifibrotic factors [101].

Vitamin D deficiency is associated with an increased prevalence of coronary heart disease with adverse outcomes, considering the proatherogenic and profibrotic effects, impaired coronary perfusion, and cardiac remodeling.

8. Vitamin D and Left Ventricular Hypertrophy

Murine models, lacking vitamin D receptor, exhibit increased ventricular mass, higher atrial natriuretic peptides, and impaired homeostasis of metalloproteinases and fibroblasts, leading to ventricular dilatation and impaired electromechanical coupling [2]. Considering *hypertension* associated

with low vitamin D levels, left ventricular hypertrophy could also be a consequence. O'Connell et al. demonstrated that calcitriol increases *myocyte protein levels and cell size,* suggesting that it induces cardiac myocyte hypertrophy [33]. Blocking the S phase of the cell cycle is the mechanism by which 1,25(OH)2D3 regulates *myocyte proliferation* [33].

Vitamin D reduces cardiac hypertrophy in spontaneously hypertensive rats [102] and in salt-sensitive rats via modulation of several protein kinase pathways [52, 103]. Among the proposed cardioprotective effects of vitamin D, reduced expression of mediators of myocardial hypertrophy, including atrial natriuretic peptides, and growth factors promoting cell proliferation were mentioned [10].

Intravenous calcitriol treatment, used to control secondary hyperparathyroidism in hemodialysis patients, caused regression of myocardial hypertrophy and reduction of QT interval dispersion, suggesting a cardioprotective effect of vitamin D [104]. The addition of calcitriol to cardiomyocytes inhibits cell proliferation without apoptosis, promoting cardiac differentiation [87]. A significant relationship between vitamin D level and interventricular septum and left ventricular mass index was found after adjusting for age, hypertension, and vitamin D therapy status, in a large retrospective study, suggesting the role of vitamin D in ventricular remodeling [95]. *Calcium* is also involved in cellular proliferation and activates AKT, a protein kinase involved in the development of cardiac hypertrophy [105]. Calcium increases after vitamin D supplementation and could also enable cardiac hypertrophy, and calcium overload causes also myocyte apoptosis and cardiac arrhythmias [105].

The results reporting the effect of vitamin D on left ventricular hypertrophy are not convincing, ranging from favorable influences to negative results [106].

9. Vitamin D and Atrial Fibrillation

Conflicting results were also found regarding the association of low vitamin status and atrial fibrillation. A relationship between vitamin D deficiency and nonvalvular atrial fibrillation was reported by several studies [107, 108]. Serum 25-hydroxyvitamin D level correlated with the *left atrial diameter, high-sensitive C reactive protein, and pulmonary systolic pressure* and was significantly associated with atrial fibrillation in Chinese patients with nonvalvular persistent atrial fibrillation [108]. Direct *electromechanical effects* on the left atrium were revealed by Hanafy et al. for vitamin D, enabling prevention or termination of atrial fibrillation [109].

Rienstra et al. evaluated 2,930 participants of the Framingham Heart Study during a follow-up period of 9.9 years and found no relation between vitamin D status and incident atrial fibrillation, concluding that vitamin D deficiency does not promote the development of atrial fibrillation [110].

10. Vitamin D and Stroke

Epidemiological studies have shown that vitamin D deficiency is an independent risk factor for arterial hypertension and stroke [111]. A recent "umbrella" review stated that the association between high vitamin D level and low stroke risk is possible, but not convincing [112].

Additional *neuroprotective* actions of vitamin D have also been reported [111], which may reduce cognitive impairment in poststroke patients [113], and the neuromuscular and osteoprotective effects may improve mobility. It is premature to recommend vitamin D supplementation for the prevention and treatment of stroke, considering that randomized controlled trials did not confirm that vitamin D reduces stroke incidence [111]. The high prevalence of vitamin D deficiency in patients with hypertension and stroke, associated with musculoskeletal pathology, could justify the evaluation, prevention, and treatment of vitamin deficiency in these patients [111].

11. Vitamin D and Peripheral Arterial Disease

Vitamin D receptors may be also found in the vascular wall, suggesting that vitamin D status might play a role in the pathogenesis of arterial disease [114]. Among individuals with peripheral artery disease, low vitamin D status was associated with a *faster decline of functional performance* but not with mortality [115]. Vitamin D deficiency was highly prevalent in patients with occlusive and aneurysmatic arterial disease, independent of traditional cardiovascular risk factors, and showed a strong association with the severity of the arterial disease and atherosclerotic markers: *carotid artery intima-media thickness and ankle-brachial index and high sensitive C reactive protein* [114]. It was suggested that the relationship between low vitamin D status and arterial disease is mediated by an independent arterial wall effect [114]. Severe vitamin D deficiency results in a disrupted adaptive *immune response and an inflammatory milieu,* promoting vascular dysfunction and insulin resistance [2, 116].

Only few studies examined the effects of vitamin D on vascular function and the results are contradictory [2]. Vitamin D also affects *aortic stiffness and vascular aging* [117, 118]. Activation of the RAA system and subsequent synthesis of angiotensin II increase vascular tone and arterial stiffness, preceding the development of hypertension [2]. A study, including 62 diabetic participants, identified no beneficial effects on cardiovascular risk, insulin resistance, and arterial stiffness after 24 weeks of vitamin D supplementation [119]. The lack of reduction in arterial stiffness might be due to the negative effects of vitamin D supplementation on arterial-stiffness related cardiovascular risk factors and the insufficient duration of the therapy [119].

Vascular calcifications, the result of calcium-phosphate deposition, major determinants of mortality and morbidity in affected patients, are associated with excessive vitamin D and hyperphosphatemia. Arterial calcifications occur in the vascular intima, associated with atherosclerosis and lipid accumulation, or in the media, associated with arteriosclerosis due to age, diabetes, and end-stage renal failure; both forms increase vascular stiffness [34, 120].

Physiologic vascular vitamin D actions include inhibitions of proatherogenic processes and intimal and medial artery calcification, including release of proinflammatory

cytokines and adhesion molecules, migration, and proliferation of vascular smooth muscle cells [121].

12. Renal Implications of Vitamin D Deficiency Related to Cardiovascular Pathology

The kidneys are involved in the synthesis of the metabolically active form of vitamin D, since the second hydroxylation, stimulated by the parathyroid hormone, occurs in the kidneys. Serum phosphate levels influence the renal hydroxylation of vitamin D through a negative feedback mechanism [122]. Individuals with renal disease have a deficiency of 1,25-dihydroxyvitamin D, impairing calcium and phosphate balance [122].

Cardiovascular diseases are more prevalent in patients with chronic kidney disease compared to patients with normal kidney function, and several links between vitamin D deficiency and poor cardiovascular outcomes were described in patients with renal disease [43].

Mortality, due mainly to cardiovascular causes, was associated with low vitamin D levels and high parathyroid hormone in patients with chronic renal disease [43]. The randomized Japan Dialysis Active Vitamin D Trial (J-DAVID), with the following primary outcomes: fatal or nonfatal cardiovascular events, coronary interventions, and lower limb artery intervention in hemodialysis patients, will, probably, provide valuable data regarding cardiovascular events in patients with chronic kidney disease stage 5, considering active vitamin D [43].

Vascular calcifications were found also in experimental uremic models with low levels of vitamin D [123]. They are associated with an increased cardiovascular mortality in stage 5 chronic kidney disease [120], and renal osteodystrophy and its therapy, the use of warfarin, and, probably, other elements of the uremic milieu may contribute to its etiology [34].

Vitamin D deficient patients with chronic renal failure had *enhanced atherosclerotic lesions* with arterial stiffening and reduced flow-mediated dilatation [97]. Animal studies evaluating the effects of vitamin D compounds on uremic vascular calcifications and pulse wave velocity revealed a dose-response relationship on vascular calcifications and a differential effect of different compounds, suggesting different mechanisms of action [43, 120]. Low doses of calcitriol and paricalcitol, sufficient to correct secondary hyperparathyroidism, were protective against aortic calcification in a mouse model of chronic kidney disease, but higher doses stimulate further calcification [124]. Calciphylaxis, a highly morbid and severe type of vascular calcification, was reported to be more prevalent in patients treated with calcitriol, but not with selective vitamin D analogues [43].

Patients on *chronic dialysis* are at increased risk of vitamin D deficiency, and six months of cholecalciferol therapy did not improve *blood pressure, arterial stiffness, and cardiac function* [125].

Elevated PTH contributes probably to the development of *uremic cardiomyopathy*, considering the correlations between PTH and left ventricular hypertrophy in chronic renal failure [126]. Vitamin D given in hemodialysis patients enabled regression of myocardial hypertrophy and reduction of QT interval dispersion, a marker of ventricular arrhythmia risk [104].

Vitamin D receptor polymorphisms, such as B alleles of BsmI, with altered vitamin D signaling, are genetic risk factors for the development of left ventricular hypertrophy in kidney disease [127–129]. Left ventricular hypertrophy is a strong cardiovascular risk marker in patients with end-stage renal disease [127–129]. The possible mechanisms responsible for the increased mortality associated with BsmI polymorphism in hemodialysis patients are as follows: modification of vitamin D receptor sensitivity and expression in cardiac and vascular tissues, modification of the circulating levels of vitamin D due to the influence of vitamin D receptors on the feedback mechanism for the regulation of alpha-1-hydroxylase, hyperparathyroidism with calcium-phosphate imbalance, which predisposes to cardiac and vascular calcifications, and hampered calcitriol effects [130].

13. Why Conflicting Results?

At present, the data regarding the causal link between low vitamin D status and cardiovascular disease are mixed, conflicting, and ambiguous. Several reasons for conflicting results were found, including significant heterogeneity of vitamin D doses, baseline concentration, therapy duration and compounds, differences of absorption and metabolism among individuals, genetic differences in the vitamin D receptor, private use of vitamin D, biases due to different diseases, study-design related factors, variations in definitions [43], several potentially confounding factors, including age, body mass index, medication, diet, sunlight exposure, physical activity, and concomitant intake of calcium [114, 131, 132], latency of the effect of vitamin D, inappropriate follow-up time or lack of a control group with normal vitamin D level [9], lack of standardization of 25-hydroxyvitamin D assay, different ethnic populations [83], autocrine and paracrine vitamin D systems, local tissue vitamin D intoxication, concomitant hyperphosphatemia, PTH level, and counterregulatory hormones, such as fibroblast growth factor 23 [74, 133]. Assessment of vitamin D status only from dietary questionnaires has, probably, a high degree of subjectivity. Future investigations should focus also on bioavailability rather than total 25-hydroxyvitamin D [43]. A high cardiovascular disease incidence and prevalence were found at high latitudes and geographical areas with low exposure to ultraviolet B radiation [20]. Winter and spring months would probably show higher proportions of patients with vitamin D deficiency. The risk of mortality was significantly higher in studies with lower baseline use of vitamin D [6]. Calcium intake may affect the results, because oral calcium may increase the risk of cardiovascular disease [134]. Most of the studies are observational and they should be replicated in randomized controlled trials [112]. The studies are very different, including observational studies of plasma vitamin D concentrations, cross-sectional, longitudinal, systematic literature reviews, and randomized controlled trials of vitamin D supplementation and experimental studies exploring the mechanisms of the associations. Even meta-analyses of

randomized studies may not be convincing, especially due to limited sample and low level of significance. Bias due to selection of participants, comparability of study groups, and selection of outcomes of interest [6] could also contribute to different results.

It is still not clear if vitamin D supplementation is needed only if vitamin D is deficient in order to exert its cardioprotective effects. Which type of vitamin D or vitamin D analogue is effective is another question still requiring an answer. Lower doses in vitamin D2 supplementation and shorter intervention periods were associated with a higher mortality [6].

The question about the benefit of vitamin D supplementation for cardiovascular outcomes cannot be answered certainly for the moment [76], but perhaps the outcomes of the VITAL prevention trial and J-DAVID trial will provide more answers.

Another concern is related to the vitamin D level with benefic effects for cardiovascular disease, considering that doses recommended for osteoporosis treatment are neither beneficial nor harmful in cardiovascular disease [29]. Consumption of high amounts of vitamin D may interfere with the regulation of phosphate metabolism by fibroblast growth factor 23 and the Klotho gene product [133]. It is therefore important to identify and use new markers for phosphate homeostasis, such as salivary phosphate secretion [105], during vitamin D therapy.

It still remains uncertain whether the association between low vitamin D status and cardiovascular diseases is causal or just a bystander. It is likely that unidentified factors and relationships with other endocrine networks are also involved in vitamin D biology, emphasizing the need of further research in this area [74].

14. Conclusions

Maintaining an optimal vitamin D serum level seems important not only for calcium homeostasis but also for cardiovascular risk, blood pressure control, prevalence of stroke, metabolic syndrome, and peripheral artery disease. Observational data support the link between vitamin D status and cardiovascular diseases, and vitamin D deficiency can be considered a cardiovascular risk marker. Vitamin D exerts its cardiovascular effects by reducing the activity of the renin-angiotensin-aldosterone system, lowering blood pressure values, and having an anti-inflammatory, antiproliferative, antihypertrophic, antifibrotic, antidiabetic, and antithrombotic effect and beneficial modulation of classical cardiovascular risk factors. The mentioned effects might be very important for public health, considering the high prevalence of vitamin D deficiency, the aging population, and the indoor oriented lifestyle.

Vitamin D deficiency is treatable and supplementation is inexpensive. Vitamin D could be combined with antihypertensive agents in order to control blood pressure, as a simple, inexpensive, and important prophylactic method in order to prevent cardiovascular morbidity, especially in the elderly. Even small gains in prevention are important from a public health perspective. Further proteomics and basic research studies are needed in order to identify the missing pieces in the vitamin D-cardiovascular disease puzzle. Large randomized controlled trials could confirm the promising findings of observational studies, considering endothelial function, arterial stiffness, and patients undergoing percutaneous coronary interventions. Guidelines are needed in order to establish optimal vitamin D level and intake, to maintain a healthy vitamin D status in patients with cardiovascular diseases, and to include vitamin D blood tests, genotyping for vitamin D receptor variants, and serum calcium and phosphates level and bone mineral density as mandatory in evaluating patients with cardiovascular disease. The benefits of screening and treating vitamin D deficiency would be, probably, reflected by reduced cardiovascular morbidity and mortality. Vitamin D supplementation should further consider additional factors, such as phosphates, PTH, RAA, and fibroblast growth factor 23.

Conflict of Interests

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

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Review Article

Vitamin D: A Review on Its Effects on Muscle Strength, the Risk of Fall, and Frailty

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Vitamin D is the main hormone of bone metabolism. However, the ubiquitary nature of vitamin D receptor (VDR) suggests potential for widespread effects, which has led to new research exploring the effects of vitamin D on a variety of tissues, especially in the skeletal muscle. *In vitro* studies have shown that the active form of vitamin D, calcitriol, acts in myocytes through genomic effects involving VDR activation in the cell nucleus to drive cellular differentiation and proliferation. A putative transmembrane receptor may be responsible for nongenomic effects leading to rapid influx of calcium within muscle cells. Hypovitaminosis D is consistently associated with decrease in muscle function and performance and increase in disability. On the contrary, vitamin D supplementation has been shown to improve muscle strength and gait in different settings, especially in elderly patients. Despite some controversies in the interpretation of meta-analysis, a reduced risk of falls has been attributed to vitamin D supplementation due to direct effects on muscle cells. Finally, a low vitamin D status is consistently associated with the frail phenotype. This is why many authorities recommend vitamin D supplementation in the frail patient.

1. Introduction

Vitamin D is the main hormone regulating calcium phosphate homeostasis and mineral bone metabolism. The discovery that a variety of tissues can express vitamin D receptor (VDR) has opened new ways of research related to vitamin D biological effects and molecular pathways [1–3]. There is evidence that vitamin D is implicated in the regulation of the immune system, the cardiovascular system, oncogenesis [4], and cognitive functions [5].

Loss of muscle mass and frailty are prevalent in many chronic diseases such as chronic obstructive pulmonary disease, cardiac insufficiency, cancer, and chronic kidney disease (CKD) [6].

Vitamin D deficiency is indeed extremely frequent in the above diseases. More than 3 decades ago, the clinical observation that patients with rickets and osteomalacia displayed proximal myopathy suggested a direct link between hypovitaminosis D and muscle function [7]. Recent evidence has confirmed that vitamin D may modulate muscle growth. In this review, we will specifically address the effect of vitamin D on skeletal muscles and its clinical implications, especially frailty and the risk of fall.

2. Methods

This is a review article not intended to meet the full range of criteria required for a systematic review. However, we used a rigorous methodology for the selection of the material presented here. In October 2014, we performed a comprehensive literature search in the bibliographic database "Pubmed," looking at studies discussing the following topics: "hypovitaminosis D and physical performance: observational studies," "can vitamin D supplementation improve muscle function?," "relationship between vitamin D status, muscle and falls," and "link between vitamin D and frailty." The study period considered was from January 1, 2000, to September 30, 2014. Keywords used were "vitamin D," "muscle," "strength," "fall," "frailty," "risk," "chronic kidney disease," "supplementation," "randomized controlled trial," "review," "systematic review," and "meta analysis." Only articles in English were considered. From the studies identified in this way, we selected only

observational trials, randomized control trials (RCTs), and meta-analysis. Case reports and series were excluded. In studies testing vitamin D supplementation, the dose of vitamin D should have been specified, physical performance/gait should have been reported by objective measurements, and blood level of vitamin D should have been reported. In studies regarding frailty, the terminology of frailty should have been defined by objective criteria. Finally, we did not include the totality of studies meeting the above criteria, in order to prevent redundancies. Studies confirming results from previous relevant studies and providing similar conclusions were voluntarily not cited.

3. Vitamin D Metabolism

Vitamin D metabolism is orchestrated by the skin, the liver, and the kidney. The role of sun exposure is instrumental since UVB-induced vitamin D3 production in the skin accounts for 80–90% of vitamin D formation, whereas nutritional intake (fatty fish, eggs, fortified milk, and plants) only accounts for 10–20% of vitamin D3 provision. UVB converts 7-dehydrocholesterol to previtamin D which is then converted to cholecalciferol (or vitamin D3). Cholecalciferol subsequently binds to vitamin D binding globulin and this complex is transported to the liver where it is hydroxylated in 25-hydroxyvitamin D3 (or 25(OH) D3), the major circulating form. 25-Hydroxyvitamin D3 undergoes a final hydroxylation in the kidney proximal tubule in order to produce 1,25 dihydroxyvitamin D3 or calcitriol, the biologically active form [8]. The 1-hydroxylation is stimulated by the parathyroid hormone (PTH) and inhibited by the Fibroblast Growth Factor 23 (FGF-23). Calcitriol interacts with vitamin D receptor (VDR) in the cell nucleus to mediate biological effects through activation of calcium channels. Vitamin D synthesis depends on environmental factors such as sunlight exposure and sun cream application [9] and biologic factors such as skin's pigmentation and kidney function. In elderly people, dietary vitamin D (vitamin D2 from plants and vitamin D3 from animals) may become the major source of vitamin D3 because of reduced 7-dehydrocholesterol concentration [10] and impaired hydroxylation in the liver and the kidney [11]. This is why the relevance of vitamin D status is highlighted in this population. This is particularly true for the elderly frail patient clinically characterized by a low nutritional intake and muscle loss. Whether vitamin D deficiency may aggravate muscle function and frailty is thus a very important question.

4. Vitamin D Deficiency and Insufficiency

Because of unregulated hydroxylation by the liver, 25-hydroxyvitamin D is used as the marker of vitamin D status. Low levels of serum vitamin D (25-hydroxyvitamin D) may define vitamin D deficiency versus insufficiency [12]. However, the definitions of low levels of vitamin D vary among authors and nutritional societies/authorities. For some authors, vitamin D deficiency is the level below which osteomalacia may appear [13]. In general, this occurs at a concentration below 25 nmol/L [12]. Vitamin D insufficiency may be defined as the level below which PTH begins to rise. Depending on the studies, this level may vary from 25 to 75 nmol/L. Another definition is only based on serum concentration thresholds without considering biological or clinical abnormalities [14]. According to this definition, vitamin D status may be divided into 4 group levels: severe deficiency defined by a concentration of less than 27.5 nmol/L, deficiency between 27.5 and 49.9 nmol/L, insufficiency between 50 and 75 nmol/L, and optimal above 75 nmol/L [15, 16]. Differences of definitions between authors may explain, at least in part, conflicting resulting from meta-analysis addressing vitamin D status and outcomes. Vitamin D deficiency/insufficiency is highly prevalent but the magnitude of hypovitaminosis D may vary depending on the population studied and regional and seasonal considerations. We will only comment on examples of prevalence in the elderly population. Among healthy elderly patients in Argentina, Oliveri et al. found prevalence between 52% and 87% of vitamin D insufficiency (serum level below 50 nmol/L) in the winter depending on the latitude [17]. In another study addressing institutionalized elderly patients from Buenos Aires, 41% of the residents had a vitamin D serum level of less than 25 nmol/L [10]. In a study looking at elderly Italian women, the prevalence of low vitamin D, defined as a serum level below 30 nmol/L, was 51% in the December–May period and 17% in the June–November period [18].

5. Vitamin D and the Skeletal Muscle: Molecular Pathways

Vitamin D receptors (VDR) are expressed in a large number of human cell types, including skeletal muscle cells, indicating the potential for widespread effects [2, 19, 20]. Two mechanisms by which vitamin D may act in skeletal muscle have been proposed (Figure 1). VDR acts as a nuclear receptor which mediates the so-called genomic effects; VDR also acts via nonnuclear receptor mediating nongenomic actions. Genomic effects have been well characterized in studies *in vitro* [8, 21]. VDR is a ligand-dependent transcription factor, which belongs to the steroid-thyroid hormone receptor gene superfamily. Once transported in the nucleus by an intracellular binding protein, calcitriol binds to its nuclear receptor which results in gene transcription and subsequent *de novo* protein synthesis. At the nuclear level, the activation of VDR induces heterodimerization between the active VDR and the retinoic receptor (RXR). This leads to the activation of the vitamin D response element (VDRE), a complex of genes coding for the "genomic effects" of vitamin D. Genomic effects of VDR include the increase in calcium handling by enhancing the activities of the calcium binding protein (calbindin-D9K) in cell sarcoplasm [22, 23], muscle cell differentiation and proliferation through effects on insulin growth factor expression which in turn induces skeletal muscle hypertrophy [24]. Mechanisms leading to vitamin D nongenomic effects are not definitely elucidated. 1,25 vitamin D appears to bind a membrane receptor which activates a transduction signal inducing MAP kinase (MAPK) and phospholipase C (PLC) pathways, which in turn lead to a rapid influx of calcium into the cell [8, 21]. The origin of this

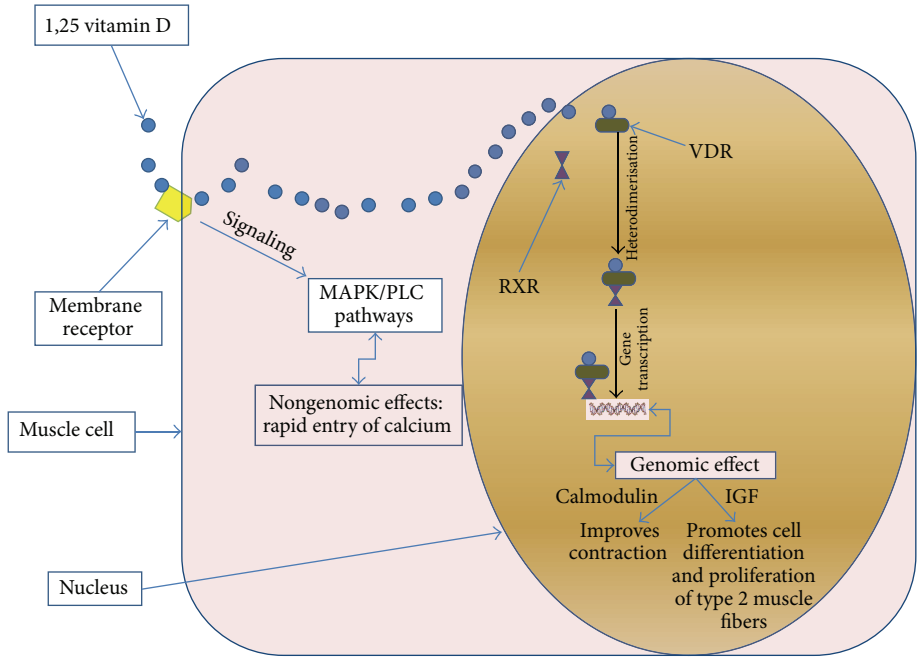

Figure 1: Effects of 1,25 vitamin D on muscle cells: molecular and nuclear pathways. 1,25 vitamin D binds the vitamin D receptor (VDR) in the nucleus, where it forms a complex with the retinoid receptor (RXR). The complex 1,25 vitamin D/VDR/XDR activates gene transcription, leading to known genomic effects. On the other hand, 1,25 vitamin D binds a putative membrane receptor which activates MAP kinase (MAPK) and phospholipase C (PLC) pathways leading to nongenomic effects.

membrane receptor is controversial. Some authors claimed that this membrane receptor is the intranuclear VDR receptor itself, which translocates from the nucleus to the plasma membrane, while others suggest that this is a distinct receptor [25, 26]. Some studies have explored vitamin D molecular pathways and VDR, through vitamin D supplementation. For instance, Ceglia et al. showed that, in elderly women, a supplementation of vitamin D (4000 IU/day) during 4 months was associated with a 30% increase in intramyonuclear VDR concentration and a 10% increase in muscle fiber cross-sectional area, especially type 2 fibers [27]. The VDR has several polymorphisms, some of which may have clinical significance. Geusens et al. showed that the presence (allele bb) or absence (allele BB) of a restriction fragment (BsmI) may determine muscle strength; that is, subjects with the bb phenotype had 23% higher muscle strength in the quadriceps than those with the BB phenotype [28].

6. Hypovitaminosis D and Physical Performance: Observational Studies

Many observational studies have investigated clinical relationships between vitamin D serum concentration and physical performance. In the Invecchiare in Chianti (InCHIANTI) study (966 individuals, 435 men and 531 women) with a mean age of 75 years, a significant association between low levels of vitamin D and poor physical performance as assessed by the handgrip strength test and a short physical performance battery test (ability to stand from a chair and ability to maintain balance in progressively more challenging positions) was found [29]. Individuals with serum vitamin D below 25 nmol/L performed lower than those with a level above 25 nmol/L. Muscle strength using a handgrip test was also significantly greater in subjects with vitamin D levels higher than 50 nmol/L than in those with levels below this threshold [29]. Mastaglia et al. reported that, in healthy women aged over 65 years ($n = 54$), vitamin D levels above 50 nmol/L were associated with a higher muscle strength from lower limbs (stronger knee extensor of 13.4 ± 2.7 versus 11.6 ± 2.5 kg, $P < 0.03$) [30]. Zamboni et al. measured serum vitamin D in elderly women ($n = 175$) and men ($n = 94$) and disability was self-reported using different questionnaires. Individuals reporting disability had a lower serum vitamin D level than those without self-reported disability [31]. This observation was confirmed in the Longitudinal Aging Study Amsterdam (LASA) prospective study which followed 1200 elderly men and women (600 men and 634 women) during 3 years. A physical assessment was performed at baseline and after 3 years. Subjects with vitamin D serum levels below 25 nmol/L had a greater chance of showing a decline in physical performance, defined by a change in the Edwards-Nunnally Index, than those with levels higher than 75 nmol/L (OR: 2.21, 95% CI 1.00–4.87) [32]. Using data from the same cohort, Visser et al. demonstrated that elderly individuals with a low vitamin D level (<25 nmol/L) had a 2.5-fold increase in the risk of developing sarcopenia, defined as a loss of handgrip strength of more than 40% or a loss of muscle mass of more than 3%, in a 3-year time period, compared with those with levels of > 50 nmol/L [33]. In a study investigating

elderly patients with falls (230 men and 370 women), a higher serum concentration of vitamin D was associated with a 3 times faster "time and get up" (TUG) test (i.e., the time required for the patient to stand up from a standard chair, walk a distance of 3 meters, turn around, walk back to the chair, and sit down again), with a five times *faster* "sit to stand" test in men, and with a 2.5 times faster TUG test in women. The data suggest possible differences in the effects of vitamin D according to the gender [34]. However, in the Progetto Veneto Anziani (Pro.V.A) study, which included 2694 community-dwelling elderly patients (1597 females and 1097 males, mean age of 74 years, 40% of women and 20% of men with a serum vitamin D below 50 nmol/L), it was shown that lower vitamin D levels were associated with a lower 6-minute walking test and weaker strength, independently of gender [35]. Positive effects of vitamin D reserves are not only observed in older persons. In a study including 1000 healthy European adolescents (470 males and 530 females), handgrip test performance was positively associated with vitamin D levels in females [3]. In another study including young women (age between 19 and 29 years), there was a correlation between vitamin D level and the handgrip test in both dominant and nondominant arms [36]. An additional study in healthy men ($n = 205$) and women ($n = 214$) (mean age of 43 years) showed a relationship between vitamin D and isometric/isokinetic arm strengths in multivariate analysis [37]. In the particular setting of CKD, 3 small studies, one study in CKD treated conservatively and 2 studies in dialysis patients, suggest a positive relationship between vitamin D status and functional ability [38–40].

A few studies did not confirm an association between vitamin D status and physical performances. Pramyothin et al. performed a study in older Hawaii women of Japanese ancestry, a population known for its very low rate of falls, a high dietary intake in vitamin D, and a large exposure to sunlight [41]. In this population, mean vitamin D level was 80 nmol/L and no subject presented vitamin D deficiency. There was no relationship between vitamin D level, physical strength test (except for the quadriceps), falls, and daily activities. The authors conclude that the absence of relationship was due to the very high level of vitamin D at baseline. In addition, another study in young men (mean age of 47 years) from Ceglia et al. including more than 1000 individuals did not demonstrate a link between vitamin D levels and physical performances [42]. However, only 20% of the subjects had a vitamin D level below 50 nmol/L [42]. These 2 negative studies, along with other observations, suggest the existence of a threshold in vitamin D level, below which hypovitaminosis D may negatively affect muscle function [29, 30]. However, in one study including 367 elderly individuals aged more than 80 years with 80% prevalence in vitamin D insufficiency, there was again no relationship between vitamin D level and physical performance, as assessed by gait speed, hand grip test, and a static balance test. In this case, the authors explain the absence of association by the decrease in VDR expression observed in very old individuals [43].

To sum up, most of the observational studies report a significant association between hypovitaminosis D and muscle dysfunction in all categories of ages, except in very old individuals. On the contrary, vitamin D levels greater than 50 nmol/L are associated with the lowest probability of muscle dysfunction. Some studies suggest that gender may influence the association between vitamin D and skeletal muscle function.

7. Can Vitamin D Supplementation Improve Muscle Function?

Several RCTs and meta-analyses have investigated the effect of vitamin D supplementation on muscle function. Two RCTs in Asians, one in healthy young volunteers [44] and another one in elderly women, compared the supplementation of daily or weekly (Table 1) vitamin D combined with calcium versus calcium alone or placebo [11]. These studies reported a benefit of vitamin D supplementation during 3 to 6 months on muscle function, that is, an improvement in quadriceps strength as measured by an isokinetic dynamometer device [11] and an improvement in 6 min walk test [44]. Another trial in 300 elderly women with a level of vitamin D <60 nmol/L demonstrated a benefit with a daily supplementation of 2000 IU of vitamin D on the TUG test. The subjects from the lowest quartile had an additional improvement in muscular strength [45]. Benefits of vitamin D supplementation were also shown in teenager females. Ward et al. randomized 69 postmenarchal females to receive either 4 doses of 150,000 IU of vitamin D2 or placebo, over one year. In the interventional group, mean vitamin D level was greater than 50 nmol/L and this was associated with improved jump velocity [46]. Another small interventional trial including young elite ballet dancers (11 males and 13 females, mean age: 28) showed an improved isometric strength and less injuries in the group receiving a daily dose of 2000 IU vitamin D [47]. In contrast, 2 trials did not show a benefit of vitamin D supplementation [48, 49]. However, one of these studies included healthy men, with very good physical performance at baseline and without vitamin D deficiency/insufficiency [48]. In the other study, vitamin D was administrated at a lower dose, that is, 8400 IU of vitamin D3 once weekly, and this failed to improve physical performance, but in the latter study, there was a positive effect on balance in a subgroup of patients with markedly low balance at baseline [49]. Two additional RCTs using intermittent large amounts of vitamin D did not show a benefit on physical performance [50–53]. One study tested the effect of 150,000 IU of cholecalciferol every 3 months in 689 elderly women (mean age above 76 years) for 9 months. Muscle strength was assessed at baseline and every 3 months with a dynamometer, and mobility was measured by a TUG test [52]. There was no difference between the treatment and the control groups. However, vitamin D was measured in only 40 subjects of the 700 subjects with indeed a high baseline level (65.8 nmol/L). In the other study, 173 young healthy females (mean age of 21 years) were given a supplementation of 60,000 IU of cholecalciferol, once a week for 8 weeks, and then 60,000 IU every 2 months for 4 months. There was no difference in muscle strength at 6 months. These findings suggest that intermittent high doses of vitamin D may not be effective at improving muscular strength. This

Table 1: Summary of RCT and meta-analysis regarding effects of vitamin D on muscle function and falls.

Type of study	Author	Number of subjects	Type of subjects	Mean age (years)	Intervention	Duration	Results
RCT	Kenny et al. (2003) [48]	65	Healthy men	76	1,000 IU/d vitamin D versus placebo	6 months	No increase in muscle strength or improvement in physical performance.
RCT	Songpatanasilp et al. (2009) [11]	72	Postmenopausal females	70	Ca 1500 mg/d + alfacalcidol 0.5 ug/d versus Ca 1500 mg/d	12 weeks	Improvement in muscular strength.
RCT	Lips et al. (2010) [49]	226	Elderly males and females with vitamin D <50 nmol/L	77	8400 IU/week vitamin D versus placebo	16 weeks	Improvement of balance in a subgroup with severe balance impairment at baseline.
RCT	Ward et al. (2010) [46]	69	Postmenarchal females (12 to 14 years old) with vitamin D <25 nmol/L	13	4 doses of 150,000 IU vitamin D every 3 months versus placebo	12 months	Increase in jump velocity in girls with low vitamin D levels. No improvement in strength in others.
RCT	Gupta et al. (2010) [44]	40	Healthy males and females	31	60,000 IU/week for 8 weeks followed by 60,000 IU/month for 4 months vitamin D + 1000 mg Ca/d versus placebo	6 months	Enhanced skeletal muscle strength and physical performance.
RCT	Zhu et al. (2010) [45]	300	Elderly females with vitamin D <60 nmol/L	77	1,000 IU/d vitamin D + Ca 1000 mg/d versus Ca 1000 mg/d + placebo	12 months	Enhanced skeletal muscle strength and physical performance in patient with the lowest vitamin D level.
RCT	Taskapan et al. (2011) [39]	25 (CKD stages 3-4) 47 (PD)	CKD and PD with vitamin D <50 nmol/L	NA	50000 IU/week vitamin D	4 to 8 weeks	Improvement in physical performance tests.
UCT	Schacht and Ringe (2012) [56]	2100	Males and postmenopausal females	75	1 mcg/d calciferol	6 months	Improved physical performance.
RCT	Glendenning et al. (2012) [52]	690	Elderly females (age >70)	77	150,000 IU/every 3 months vitamin D versus placebo	9 months	No differences in falls and physical performance between the groups.
RCT	Goswami et al. (2012) [53]	173	Healthy females	22	60,000 IU/week every 8 weeks then 60, 000 IU/fortnight + Ca 500 mg/d versus 60,000 IU/week every 8 weeks then 60, 000 IU/fortnight + placebo versus Ca 500 mg/d + placebo versus placebo	6 months	No differences in muscle strength between the groups.
RCT	Ceglia and Harris (2013) [21, 27]	21	Females with limited mobility	78	4000 IU/d vitamin D	4 months	Increase of intramyonuclear VDR concentration. Increase in muscle fibers.
RCT	Wyon et al. (2014) [47]	24	Elite ballet dancers	28	2000 IU/d vitamin D versus placebo	4 months	Increased muscle performance and less injury.

Table 1: Continued.

Type of study	Author	Number of subjects	Type of subjects	Mean age (years)	Intervention	Duration	Results
Meta	Muir and Montero-Odasso (2011) [54]	2268 (13 RCTs)	Elderly males and females (age >65)	78	Vitamin D supplementation		Beneficial effects on strength and balance.
Meta	Stockton et al. (2011) [55]	5072 (17 RCTs)	Males and females of all ages	NA	Vitamin D supplementation		Increase in muscle strength in adults with baseline vitamin D <25 nmol/L.
RCT	Bischoff et al. (2003) [63]	122	Elderly females	85	Ca 1200 mg and 800 IU/d vitamin D versus Ca 1200 mg/d	12 weeks	Reduced risk of fall.
RCT	Pfeifer et al. (2009) [62]	242	Community-dwelling elderly males and females	77	800 IU/d vitamin D + Ca 1000 mg/d versus Ca 1000 mg/d	12 months	Reduced number of falls and improvement in muscle function.
Meta	Gillespie (2003)	461 (3 RCTs)	Elderly males and females	NA	Vitamin D supplementation		No reduction in the risk of fall.
Meta	Bischoff-Ferrari et al. (2004) [66]	10001 (10 RCTs with sensitivity analysis) 1237 (5 RCTs without sensitivity analysis)	Elderly males and females, age >65	70	Vitamin D supplementation		Reduced risk of fall.
Meta	Michael et al. [64] (2010)	5809 (9 RCTs)	Elderly males and females	NA	Vitamin D supplementation		Reduced risk of fall.
Meta	Murad et al. (2011) [65]	45782 (26 RCTs)	Males and females (all ages)	NA	Vitamin D + Ca supplementation		Reduced risk of fall.

RCT: randomized control trial, UCT: uncontrolled trial,Meta: meta-analysis, NA: nonavailable, CKD: chronic kidney disease, PD: peritoneal dialysis, VDR: vitamin D receptor, and Ca: calcium.

lack of clinical effect may be explained by the inability of high intermittent doses of vitamin D to maintain high serum levels for a sustained period, as suggested by Gupta et al. [44].

The meta-analysis from Muir and Montero-Odasso, which pooled results from 13 RCTs in individuals older than 60 years old, supported a small benefit of daily vitamin D supplementation (800 IU to 1000 IU per day) for muscle strength and balance [54]. However, another meta-analysis by Stockton et al. looking at 17 RCTs in individuals of all ages including younger subjects only showed a benefit in muscle strength in subjects with vitamin D serum levels below 25 nmol/L at baseline [55].

Whether 1,25 vitamin D is also effective to improve muscle function has been insufficiently investigated. A prospective uncontrolled trial, not meeting the criteria of our search but including 2000 elderly subjects (mean age of 75 years, 80% females), showed that a daily supplementation of 1 mcg of alfacalcidol leads to significant improvements of the TUG, chair rising test, and tandem gait tests, used as surrogates of muscle performance and risk of fall [56].

Overall, data from RCT and meta-analysis support a positive effect of daily vitamin D supplementation on muscle function, especially in older individuals with vitamin D insufficiency/deficiency at baseline. A daily dose of 1000 UI appears to be sufficient to obtain significant improvements. In contrast, large intermittent doses of vitamin D do not appear to be efficient at improving muscle strength.

8. Relationship between Vitamin D Status, Muscle, and Falls

The known association of vitamin D insufficiency and increased risk of falls and fractures in the elderly [50, 51, 57–59] was thought to depend on bone remodeling via the rise of PTH [60]. However, current understanding has highlighted the importance of a direct effect of vitamin D on muscle strength and function [21] to explain this association. Because vitamin D has an effect on type 2 muscle fibers, it was tempting to speculate a protective effect of vitamin D on falls, via improvement in muscle function. A high number of RCTs have investigated whether vitamin D supplementation had an effect on muscle function and the incidence of falls. A Cochrane review published in 2003 [61] evaluated the efficacy of supplementation with vitamin D analogs, either alone or with calcium as cosupplementation at preventing falls. The overall analysis of vitamin D versus control found no significant difference in the rate of falls when applied to unselected community-dwelling and hospitalized elderly subjects (RR 0.87, 95% CI 0.70–1.08). In contrast, in more recent studies, benefits of vitamin D supplementation were significant. Pfeifer et al. demonstrated a reduction in falls of 27 and 39% at one year and 20 months, respectively, in community-dwelling seniors supplemented with 800 IU vitamin D and calcium daily versus with calcium only [62]. This reduction in falls was correlated with an improvement in quadriceps strength and an improvement in the TUG test. These results are consistent with the study of Bischoff et al. who showed a 49% reduction of falls in elderly women from a geriatric ward supplemented with 800 IU per day of vitamin D [63]. In the same period, results from 3 meta-analyses demonstrate a significant reduction in the odd ratio of falls in individuals supplemented with vitamin D [64–66].

Figure 2 shows the effects of vitamin D on muscle function, gait, and falls. Table 1 summarizes the studies which investigated the clinical effects of vitamin D on muscle strength, function, and the risk of falls. The most recent report of the Endocrine Society Clinical Practice Guidelines recommends vitamin D supplementation depending on age and clinical circumstances, in particular in order to prevent falls in populations at risk [15]. However, a recent paper from the Institute of Medicine from the United States questioned the effect of vitamin D supplementation on extraskeletal outcomes [67], in particular in the setting of falls, arguing that the meta-analysis of Bischoff-Ferrari et al. provided misleading conclusions, that is, a vitamin D-associated decrease of 22% in the risk of falls, due to heterogeneity considerations in the 5 RCTs considered [66].

9. A Link between Vitamin D and Frailty?

The term "frailty" is becoming more and more popular in geriatric medicine. However, its definition is vague. The Oxford dictionary defined it by "the condition of being weak and delicate." A more precise definition is given by Fried who defined frailty as "a biologic syndrome of decrease reserve and resistance to stressors that results from cumulative declines across multiple physiologic systems and causes vulnerability to adverse outcomes [68]." Criteria of the frail phenotype have been described in order to translate the above theoretical definition into clinical indicators [68]. These are as follows: unintentional weight loss, self-reported exhaustion, weakness (grip strength), slow walking speed, and low physical activity. According to these clinical criteria, 3 phenotypes have been identified: robust: 0 criteria; prefrail: between 1 and 2 criteria; frail: 3 or more criteria. The majority of these criteria are related to locomotion and physical strength. Thus, it looks readily conceivable that hypovitaminosis D may lead to frailty, through negative effects on muscle strength and/or function.

The association between vitamin D status and frailty has been studied in a number of observational studies. Data from an observational study from Hirani et al. which included 1659 community-dwelling men, with a 10% prevalence of frailty, showed that low vitamin D levels were independently associated with frailty [69]. A similar association was found by Tajar et al. in another cohort of elderly men. Subjects with vitamin D levels <50 nmol/L had an odd ratio of 2,37 of being classified into the "frail" versus the "robust" phenotype [70]. Using data from the third National Health and Nutrition Survey (NHANES), Wilhelm-Leen et al. found an association between frailty and a low vitamin D status in both elderly men and women, with overall 4-fold increase in the odd ratio of frailty [71]. Vitamin D not only is associated with frailty but also appears to be associated with an increased risk to develop frailty over time in women. In a prospective study including elderly women (age > 69 years), nonfrail women at baseline but displaying a vitamin D level of less than 50 nmol/L had

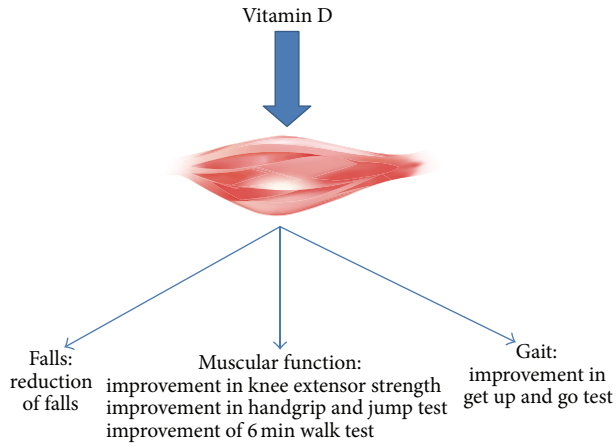

Figure 2: Clinical effects of vitamin D on muscles gait and falls.

a higher risk of becoming frail during the 4.5 years of follow-up than women with a higher level of vitamin D [72]. In a study from patients with cardiac insufficiency, Boxer et al. found an association between low vitamin D levels and the frail phenotype. In particular, vitamin D levels and the result of the 6-minute walking test were correlated [73]. In cardiac diseases, this functional test is known to predict survival [74]. Thus, low vitamin D is hypothesized to link with mortality in this setting. A prospective study including 4000 individuals (1943 men and 2788 women, mean age: 70), followed up to 12 years, indeed found a link between lower levels of vitamin D, frailty, and mortality. An assessment of vitamin D status and the physical phenotype (robust/prefrail/frail) were performed at baseline [75].

Mortality was positively associated with frailty. Frail individuals with a low vitamin D level were at increased risk (hazard ratio of 2.98) of death during the follow-up compared to robust individuals with a high level of vitamin D. Thus, overall, a clear association between vitamin D level and frailty has been demonstrated. Furthermore, interplays between vitamin D status, frailty, and mortality appear plausible. Whether vitamin D supplementation in frail subjects may reduce mortality is challenging and needs to be investigated in the future.

10. Conclusion

Consistent relationships exist between vitamin D status and muscle function, especially in the elderly frail patient. There is evidence that hypovitaminosis D is associated with a decline in muscle function. Vitamin D supplementation has beneficial effects on muscle strength, balance, and gait in diverse settings including adolescents, the elderly, and CKD patients. However, the effects of vitamin D on the prevention of falls are still a matter of debate due to conflicting interpretation of data. Differences in the dose of supplementation, type of vitamin D, and discrepancies in the threshold to define vitamin D deficiency/insufficiency may partly explain these disagreements. A low vitamin D status is consistently associated with frailty. Considering that vitamin D supplementation is safe and inexpensive, it is worthy to recommend vitamin D supplementation in patients at risk for falls, such as elderly patients, nursing home residents, frail patients with gait and balance and visual impairments, and patients with chronic diseases. These patients are most likely to have low levels of vitamin D and muscle loss/dysfunction, thus justifying supplementation independent of a putative effect on the prevention of falls.

Conflict of Interests

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

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Research Article

Is Vitamin D Deficiency Related to Accumulation of Advanced Glycation End Products, Markers of Inflammation, and Oxidative Stress in Diabetic Subjects?

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Objectives. In diabetes accumulated advanced glycation end products (AGEs) are involved in the striking cardiovascular morbidity/mortality. We asked whether a hypovitaminosis D associates with an increased formation and toxicity of AGEs in diabetes. *Methods*. In 276 diabetics (160 M/116 F, age: 65.0 ± 13.4; 43 type 1,T1DM, and 233 type 2 patients, T2DM) and 121 nondiabetic controls (60 M/61 F; age: 58.6 ± 15.5 years) routine biochemistry, levels of 25-hydroxyvitamin D_3 (25-(OH)D), skin autofluorescence (SAF), plasma AGE-associated fluorescence (AGE-FL), N^{ε}-(carboxymethyl)lysine (CML), soluble receptor for AGEs (sRAGE), soluble vascular adhesion protein-1 (sVAP-1), high sensitive C-reactive protein (hs-CRP), and renal function (eGFR) were determined. *Results*. In the diabetics SAF and AGE-Fl were higher than those of the controls and correlated with age, duration of diabetes, and degree of renal impairment. In T2DM patients but not in T1DM the age-dependent rise of SAF directly correlated with hs-CRP and sVAP-1. 25-(OH)D levels in diabetics and nondiabetics were lowered to a similar degree averaging 22.5 ng/mL. No relationship between 25-(OH)D and studied markers except for sVAP-1 was observed in the diabetics. *Conclusion*. In diabetics hypovitaminosis D does not augment accumulation of AGEs and studied markers of microinflammation and oxidative stress except for sVAP-1.

1. Introduction

Advanced glycation end products (AGEs) are a heterogeneous group of compounds implicated in the pathophysiology of aging, diabetes mellitus, and chronic kidney disease (CKD). They are formed by nonenzymatic glycation of proteins, lipids, and nucleic acids and under conditions of oxidative and carbonyl stress [1, 2]. Other factors involved in accumulation of AGEs are their impaired renal removal in kidney dysfunction [3], consumption of highly heat-treated foods with an elevated AGE content [4, 5] and inhalation of tobacco smoke [6]. AGEs exert their deleterious effects directly by modifications of long-lived intra- and extracellular proteins, which affect their structural and functional properties. Cross-linking of collagen promotes vascular stiffness [7] and also injures the skeletal muscle [8]. Indirect harmful effects arise from interactions of AGEs with their receptors (particularly RAGE) at the cell membrane. RAGE activation induces nuclear transcription factors (e.g., nuclear factor kappa-B, NF-κB), generation of oxygen radicals, synthesis of proinflammatory cytokines/chemokines, fibrogenic growth factors (transforming growth factor-β-1, TGF-beta-1), vascular adhesion molecules and cell proliferation [9], and reduction of nitric oxide (NO) formation [10]. AGEs may also interrupt key steps in reverse cholesterol transport [11]. Beside cardiovascular disturbances, AGE accumulation is linked to an enhanced cancer incidence, in part due to an AGE-induced genomic damage [12, 13].

Diabetes mellitus is associated with an excessive accumulation of AGEs [14]. Subsequently a microangiopathy

(nephropathy, neuropathy, and retinopathy) and an accelerated atherosclerotic vasculopathy (including coronary heart, cerebrovascular, and peripheral artery disease) develop [15]. AGEs may directly contribute to induction or aggravation of diabetes causing progressive insulin secretory defects and pancreatic beta cell deaths [16] and by enhancing insulin resistance via decreased biological activity of glycated insulin [17].

Elevated levels of circulating AGEs such as pentosidine, N^{ε}-carboxymethyllysine (CML), and AGE-associated fluorescence (AGE-Fl) were related to coronary and peripheral artery disease (PAD), renal damage, and total cardiovascular mortality in the general population [18–20], in particular in patients with type 2 diabetes [21] and end-stage renal disease [22]. The toxic effects of AGEs are partly neutralized by soluble RAGE (sRAGE), which represents the truncated form of the receptor acting as a decoy [23].

In the past several years, a noninvasive measurement of skin AGE-associated autofluorescence (SAF) has been developed. SAF is closely related to AGE accumulation in the tissues and reflects the "long term cumulative metabolic and oxidative stress." SAF is an independent predictor of cardiovascular complications, morbidity, and mortality [24–27].

Similar to the consequences of AGE accumulation, vitamin D deficiency may be involved in numerous biochemical and clinical disturbances, besides the musculoskeletal disorders induced by secondary hyperparathyroidism [28]. Observational and prospective studies showed associations of vitamin D_3 deficiency with cardiovascular disease [29], hypertension (via stimulation of the renin-angiotensin-aldosterone system [30–32]), vascular stiffness [33], coronary artery calcification [34, 35] heart hypertrophy, stroke [36], renal damage, autoimmune diseases (type I diabetes, multiple sclerosis, and rheumatoid arthritis) [28], infections (lowered antimicrobial peptide cathelicidin [37]), and impaired cognitive function including Alzheimer disease [38]. Vitamin D deficiency likewise seems to be involved in impaired glucose tolerance or type 2 diabetes [39]. It may predispose to an impaired insulin secretion via the vitamin D receptor (VDR) in the beta cells of the pancreas [40–42] and an impaired insulin sensitivity, resulting in insulin resistance [43–45]. In type 1 diabetes severe vitamin D deficiency predicts all-cause mortality [46]. Moreover reduced muscle function may be associated with both vitamin D deficiency [47] and AGE accumulation [8].

Data about potential relationships between vitamin D_3 deficiency and AGE accumulation are, so far, scarce. In in vitro studies it was shown that the deleterious effects of AGE-modified albumin on endothelial cells could be prevented by coincubation with calcitriol, the active form of vitamin D [48]. In diabetic rats administration of vitamin D reduced systemic oxidative stress and the deposition of AGEs (CML) in the aortic wall [49]. In the current paper we investigated the impact of vitamin D status on the AGE levels in skin and plasma and markers of microinflammation and oxidative stress as well as on muscle function in diabetic patients to elucidate the potential interactions.

2. Subjects and Methods

This cross-sectional noninterventional study was conducted according to the Declaration of Helsinki and a protocol approved by the Ethics Committee of the Medical Faculty of the University of Würzburg. Signed written informed consent was obtained from all participants.

A total of 276 consenting diabetic patients (age range: 16–94 years; 18% type 1 diabetes mellitus, (DM) duration: newly diagnosed to 56 years) visiting the ambulance of the KfH-Kidney Center Würzburg and the Practice of Internal Medicine (Dr. Werner Stürmer) in Würzburg were recruited. Inclusion criteria were type 1 or type 2 DM. Control subjects (n = 121; age range: 16–96 years) were recruited from participants of regular check-ups in the same practice during the same time period as well as the staff. Exclusion criteria for both controls and diabetics comprised any acute illness, autoimmune diseases, malignancies, dermatosis, scars and pigment disorders, pregnancy or lactation in women, current smoking (self-reported), use of glucocorticoids, vitamin D supplements (during the last 6 months), regular visits to a solarium, and use of tanning cream (during the last 14 days). Patients with hypertension and/or diabetes were treated according to the current guidelines.

Weight and height were measured and body mass index (BMI) was calculated. SAF was measured on the volar side of the forearm using the AGE-Reader (DiagnOptics BV Groningen, Netherlands) as previously described [21]. Hand-grip muscle strength was measured using the Baseline Hydraulic Hand Dynamometer (White Plains NY, USA).

Venous blood was collected in the morning hours (7.00 to 9.00 h), after overnight fasting and analyzed for serum creatinine, haemoglobin A1c (HbA1c, HPLC method, ADAM A1c HA 8180 FAST, Axonlab, Germany), high sensitive C-reactive protein (hs-CRP, nephelometrically, Siemens reagent), and 25(OH)D (electrochemiluminescence immunoassay, ECLIA, Roche, Germany) in a certified laboratory (Labor Limbach, Heidelberg, Germany). Vitamin D deficiency was defined as 25(OH)D level <20 ng/mL, vitamin D insufficiency as 25(OH)D level 20–30 ng/mL, and vitamin D sufficiency as 25(OH)D level >30 ng/mL. Abbreviated MDRD formula was used to estimate glomerular filtration rate (eGRF).

Aliquots of plasma were stored at −80°C and transferred on dry ice to a laboratory in Bratislava for determination of total proteins (Vitros 250 analyzer, USA), AGE-associated fluorescence of plasma according to Münch et al. [50], and concentrations of CML (ELISA, Microcoat, Bernried, Germany), sRAGE (ELISA, R&D Systems, Minneapolis, MN, USA), and soluble vascular receptor adhesion protein-1 (sVAP-1, ELISA, Bender MedSystem Inc., Vienna, Austria) using commercial ELISA kits according to manufacturer's instructions.

Presence of comorbidities (hypertension (HT), coronary heart disease (CHD), and peripheral artery disease (PAD)) was tracked from documentation and was not available for 12 newly diagnosed diabetics.

2.1. Statistical Analysis. Data not distributed normally were logarithmically transformed for statistical analyses.

Table 1: Cohort characteristics.

	Controls	All DM patients	P	DM1 patients	DM2 patients	P
N (%)	121	276		43 (16%)	233 (84%)	
Males/females (n; %)	60/61 (50%/50%)	160/116 (58%/42%)	0.12^{chi}	23/20 (53%/47%)	137/96 (59%/41%)	0.52^{chi}
Age (years)	*58.6 ± 15.1*	*65.0 ± 13.4*	***<0.001***	*48.6 ± 1.8*	*68.0 ± 9.6*	***<0.001***
DM duration (yrs)	—	*15.1 ± 10.7*	—	*19.5 ± 13.2*	*15.0 ± 10.1*	*0.10*
BMI (kg/m^2)	27.2 ± 3.3	30.5 ± 6.1	**<0.001**	25.4 ± 3.7	31.4 ± 6.0	**<0.001**
Total protein (g/L)	72 ± 7	72 ± 7	0.71	71 ± 5	72 ± 7	0.24
eGFR (mL/min/1.73 m^2)	84 ± 16	71 ± 25	**<0.001**	83 ± 26	69 ± 24	**<0.001**
HbA1c (%)	*5.5 ± 0.3*	*7.1 ± 1.1*	**<0.001**	*7.1 ± 1.1*	*7.1 ± 1.1*	0.98
Fl-AGEs (AU)	*281 ± 67*	*341 ± 112*	***<0.001***	*289 ± 104*	*350 ± 111*	***<0.001***
SAF (AU)	2.3 ± 0.5	2.8 ± 0.7	**<0.001**	2.5 ± 0.7	2.9 ± 0.7	**<0.001**
CML (ng/mL)	*1045 ± 368*	*1023 ± 393*	*0.71*	*1125 ± 940*	*1008 ± 385*	*0.12*
sRAGE (pg/mL)	*989 ± 376*	*936 ± 497*	*0.23*	*1133 ± 638*	*922 ± 474*	*0.30*
sVAP-1 (ng/mL)	409 ± 166	462 ± 172	***0.049***	415 ± 138	469 ± 176	0.16
hsCRP (mg/L)	*2.0 ± 2.0*	*2.7 ± 2.4*	***0.001***	*1.9 ± 1.9*	*2.9 ± 2.4*	***0.004***
Grip strength (pounds)	*84 ± 31*	*75 ± 25*	***0.026***	*85 ± 32*	*74 ± 23*	*0.09*
*Hypertension (N/Y; %)	88/33 (73%/27%)	96/167 (37%/63%)	**<0.001**^{chi}	23/13 (53%/47%)	73/154 (32%/68%)	**<0.001**^{chi}
*PAD (N/Y; %)	119/2 (98%/2%)	220/44 (83%/17%)	**<0.001**^{chi}	34/2 (94%/6%)	186/42 (82%/18%)	0.054^{chi}
*CHD (N/Y; %)	117/4 (97%/3%)	213/51 (81%/19%)	**<0.001**^{chi}	33/3 (92%/8%)	180/48 (79%/21%)	0.07^{chi}
*Total comorbidities (N/Y; %)	87/34 (72%/28%)	74/190 (28%/72%)	**<0.001**^{chi}	22/114 (61%/39%)	52/176 (23%/77%)	**<0.001**^{chi}

DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; BMI: body mass index; eGFR: estimated glomerular filtration rate; HbA1c: haemoglobin A1c; AGE-Fl: advanced glycation end products associated fluorescence of plasma; CML: N$^{\varepsilon}$-carboxymethyllysine; SAF: skin autofluorescence; sVAP-1: soluble vascular receptor adhesion protein-1; sRAGE: soluble receptor for advanced glycation end products; hsCRP: high sensitive C-reactive protein; Y: yes; N: no; PAD: peripheral artery disease; CHD: coronary heart disease; chi: chi-square; *data missing from 12 subjects.

Descriptive statistics are presented as percentages or means ± SD. Two sets of data were compared using two-sided Student's t-test, for comparison of ≥3 sets of data analysis of variance (ANOVA) with post hoc Scheffe's test was employed. Proportions were compared using chi-square test. Pearson's correlation coefficients were calculated. Multivariate analysis was performed using the General Linear Model (GLM). SPSS statistical software (v. 16.0 for Windows; SPSS, Chicago, Illinois) was used with the significance set at $P < 0.05$. The orthogonal projections to latent structures discriminant analysis (OPLS-DA, Simca v.13 software, Umetrics, Umea, Sweden) was used to identify independent variables contributing to separation between subjects with 25(OH)D deficiency and those with sufficient levels.

3. Results

3.1. Cohort Characteristics

3.1.1. Nondiabetics versus Diabetic Subjects. Cohort characteristics are given in Table 1. The proportion of females and males, CML, and sRAGE concentrations did not differ significantly between diabetic and control subjects. DM patients were significantly older ($P < 0.001$) and had as expected higher BMI ($P < 0.001$), HbA1c ($P < 0.001$), SAF ($P < 0.001$), hsCRP ($P = 0.001$), AGE-associated fluorescence ($P < 0.001$), and sVAP-1 ($P = 0.049$) levels, presented higher frequency of comorbidities, a lower eGFR ($P < 0.001$), and grip strength ($P = 0.026$) in comparison to controls. To elucidate the independent effects of age and presence of diabetes, multivariate analysis using the GLM was employed. Selected independent variables did not affect significantly CML levels (Table 2).

3.1.2. Impact of Type of Diabetes. Type 1 diabetic patients were younger ($P < 0.001$) than their type 2 DM counterparts (Table 1). Glycemia was similar in both cohorts ($P = 0.98$) and DM was diagnosed also for a comparable time period ($P = 0.10$). Type 1 and 2 diabetics did not differ significantly in CML, sRAGE, and sVAP-1 levels and produced a similar strength in the hand-grip test. In comparison to type 2 diabetic patients, type 1 diabetics presented lower BMI, AGE-specific fluorescence of plasma, SAF ($P < 0.001$), and hsCRP ($P = 0.004$) levels and higher eGFR ($P < 0.001$). The potential impact of the type of diabetes, its duration, and subjects' age on studied markers was estimated using the GLM. It did not select type of diabetes as independent significant contributor in either setting (Table 3). Duration of diabetes significantly and independently affected eGFR, AGE-Fl, SAF, sVAP-1, and grip strength, while aging was significantly associated with decline in eGFR and rise in AGE-Fl and SAF. However, in case of CML, sVAP-1, sRAGE, and hsCRP the independent variables explained only minor percentage of variability of respective dependent variables.

3.2. 25(OH)D Status. The mean concentrations of 25(OH)D were in the lower range of 25(OH)D insufficiency in

Table 2: Multiple regression, effect of ageing, and presence/absence of DM on selected independent variables.

	25(OH)D	*HbA1c*	*AGE-Fl*	*CML*	SAF	sVAP-1	*sRAGE*	*hsCRP*	*Grips*
Corr. m.	0.84	**0.001**	**0.001**	0.93	**0.001**	**0.045**	**0.019**	**0.001**	**0.001**
Intercept	0.001	**0.001**	**0.001**	0.001	**0.001**	**0.001**	**0.002**	**0.011**	**0.001**
Age	0.83	0.06	**0.001**	0.90	**0.001**	0.23	**0.006**	**0.002**	**0.001**
DM st.	0.62	**0.001**	**0.001**	0.69	**0.001**	0.10	0.20	**0.007**	0.94
R^2	−0.01	0.35	0.15	−0.01	0.31	0.01	0.03	0.05	0.17

25(OH)D: 25(OH)D_3; HbA1c: haemoglobin A1c; AGE-Fl: advanced glycation end products associated fluorescence of plasma; CML: N^{ε}-carboxymethyllysine; SAF: skin autofluorescence; sVAP-1: soluble vascular receptor adhesion protein-1; sRAGE: soluble receptor for advanced glycation end products; hsCRP: high sensitive C-reactive protein; corr. M.: corrected model; DM st.: diabetic status, classified 0/1 as absence/presence; *italics:* due to not normal distribution statistics performed on logarithmically transformed data. In case of sVAP-1, sRAGE, and hsCRP model was significant although age and presence of diabetes explained <5% in their variability (R^2).

Table 3: Multiple regression, effect of ageing, and duration of diabetes and DM type on selected independent variables.

	25(OH)D	*eGFR*	*AGE-Fl*	*CML*	SAF	sVAP-1	*sRAGE*	*hsCRP*	*Grips*
Corr. M.	0.75	**0.001**	**0.001**	**0.049**	**0.001**	**0.002**	**0.004**	**0.016**	**0.001**
Intercept	0.001	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	0.44	**0.001**
DM dur.	0.92	**0.001**	**0.001**	0.42	**0.003**	**0.027**	**0.008**	0.89	**0.001**
Age	0.28	**0.001**	**0.001**	0.09	**0.001**	0.09	0.29	0.28	0.08
DM type	0.52	0.84	0.24	0.60	0.71	0.28	0.38	0.07	0.47
R^2	−0.01	0.27	0.20	0.03	0.24	0.06	0.05	0.03	0.17

25(OH)D: 25(OH)D_3; eGFR: estimated glomerular filtration rate; AGE-Fl: advanced glycation end products associated fluorescence of plasma; CML: N^{ε}-carboxymethyllysine; SAF: skin autofluorescence; sVAP-1: soluble vascular receptor adhesion protein-1; sRAGE: soluble receptor for advanced glycation end products; hsCRP: high sensitive C-reactive protein; corr. M.: corrected model; DM type: type 1 or type 2 diabetes; *italics:* due to not normal distribution statistics performed on logarithmically transformed data. In case of CML, sRAGE and hsCRP model was significant although age and type of diabetes explained ≤5% in their variability (R^2).

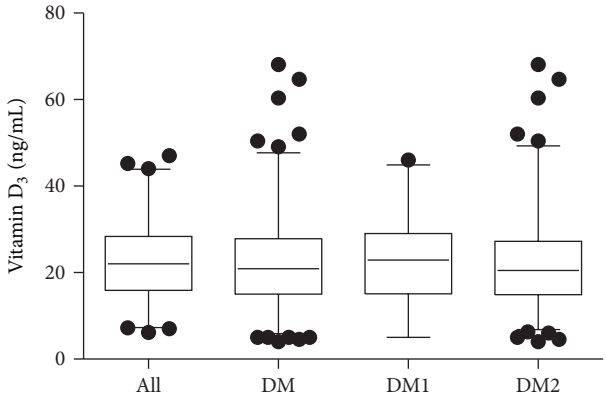

Figure 1: 25(OH)D concentration in the controls, diabetic patients (DM), type 1 (DM1) or type 2 (DM2) diabetics. Data are presented as median, interquartile range, and 95% confidence interval (CI). Dots represent outliers beyond 95% CI.

the controls and diabetics and did not differ significantly between the groups (Figure 1). Age and presence/absence of diabetes in the whole cohort, or age, duration, and type of diabetes in diabetics showed no significant impact on 25(OH)D levels (Tables 2 and 3).

In the controls no significant relationships between any other tested variable and 25(OH)D levels were revealed (Table 4). In diabetic subjects HbA1c, CML, and sVAP-1 levels correlated inversely, and grip strength directly with 25(OH)D concentrations. Similar to observations in the whole cohort of diabetics, significant associations between HbA1c, CML, sVAP-1, levels, and grip strength on the one hand and 25(OH)D concentration on the other were revealed in type 2 diabetics, while in type 1 diabetics only the relation between CML or grip strength and 25(OH)D was significant (Table 4). 25(OH)D levels showed no significant relationship with SAF either in type 1 and type 2 diabetics (Figure 2(a)) or in the controls (Figure 2(b)).

The prevalence of 25(OH)D deficiency (47% versus 44%), insufficiency (32% versus 35%), and sufficient levels (21% versus 21%) was similar among diabetics and controls (P_{chi} = 0.76).

A multivariate analysis using the OPLS-DA model was employed to elucidate which variables contribute to separation between 25(OH)D deficient subjects and those presenting sufficient levels.

In the control group a satisfactory separation between 25(OH)D deficient subjects and those with satisfactory levels was obtained (Figure 3(a)). The calculated model described 76% of variability (R^2) with an acceptable predictivity (Q^2 = 0.65). Loading scatter plot (Figure 3(b)) and VIP plot (Variables Important for Projection, Figure 3(c)) suggests that 25(OH)D deficient controls present lower total protein (VIP = 2.1) and sVAP-1 (VIP = 1.1) and higher CML (VIP = 1.0) levels. AGE-Fl, sRAGE (lower in 25(OH)D deficient subjects), and BMI (higher in 25(OH)D deficient subjects; VIP values between 0.7 and 0.5) represent variables potentially important for the separation between groups. Moreover, females tended to be more frequent in the 25(OH)D deficient group. Variables listed to the right from BMI on the VIP

Table 4: Pearson correlation coefficients independent variables to 25(OH)D_3.

	Controls		DM patients		DM1		DM2	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
ln age	0.107	0.24	−0.065	0.28	−0.097	0.54	−0.077	0.24
ln DM duration	NA	NA	NA	NA	0.201	0.20	−0.057	0.39
BMI	0.049	0.65	−0.073	0.23	0.085	0.59	−0.105	0.11
eGFR	0.082	0.50	0.051	0.42	0.028	0.87	0.066	0.34
ln HbA1c	0.060	0.62	−0.122	**0.043**	−0.025	0.087	−0.135	**0.039**
ln Fl-AGEs	0.072	0.44	0.099	0.10	0.087	0.58	0.104	0.12
ln CML	−0.185	0.20	−0.197	**0.006**	−0.444	**0.026**	−0.177	**0.020**
ln sRAGE	−0.062	0.67	−0.027	0.71	−0.319	0.12	−0.007	0.92
sVAP-1	0.040	0.79	−0.199	**0.005**	0.263	0.20	−0.184	**0.016**
ln hsCRP	0.004	0.97	−0.019	0.76	0.150	0.35	−0.059	0.39
ln Grip	0.112	0.30	0.196	**0.002**	0.506	**0.003**	0.141	**0.037**

DM: diabetes mellitus; DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; ln: logarithmically transformed data; BMI: body mass index; eGFR: estimated glomerular filtration rate; HbA1c: haemoglobin A1c; AGE-Fl: advanced glycation end products associated fluorescence of plasma; CML: N^{ε}-carboxymethyllysine; sRAGE: soluble receptor for advanced glycation end products; sVAP-1: soluble vascular receptor adhesion protein-1; hsCRP: high sensitive C-reactive protein.

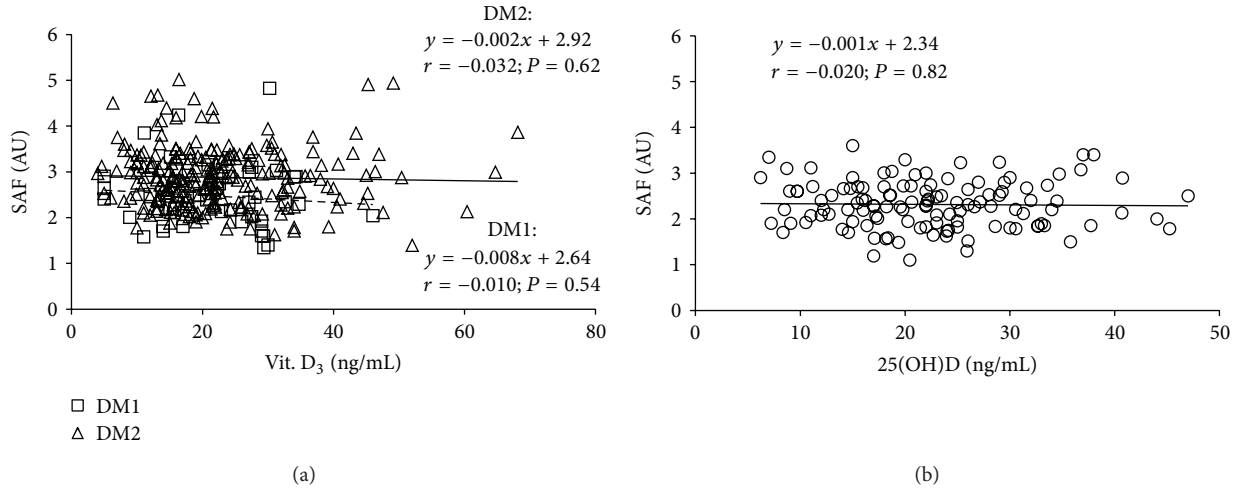

Figure 2: Relationship between skin autofluorescence (SAF) and 25(OH)D levels in type 1 and type 2 diabetics (a) and control subjects (b). DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; dotted line represents a regression in cohort of type 1 diabetics; solid line represents a regression in cohort of type 2 diabetics.

plot (VIP < 0.5; Figure 3(c)) placed in the vicinity of the intersection of *x*- and *y*-axis and zero *y*-axis on loading scatter plot (Figure 3(b)) do not have discriminatory power in this model.

However, except for 25(OH)D levels, the *t*-test did not indicate significance, only trends corresponding to results of the multivariate analysis (Table 5).

In the diabetic cohort OPLS-DA analysis revealed a satisfactory separation between the 25(OH)D deficient and sufficient subjects (Figure 4(a)), with $R^2 = 0.71$ and $Q^2 = 0.64$. Loading scatter (Figure 4(b)) and VIP (Figure 4(c)) plots suggest that 25(OH)D deficient diabetics present higher CML, total protein levels, and a weaker grip strength (VIP = 1.0, all). The levels of sVAP-1 (higher, VIP = 0.9) and AGE-Fl (lower, VIP = 0.7) represent variables potentially contributing to separation. 25(OH)D deficient diabetics comprised more females and fewer subjects suffering from CHD i compared to those presenting sufficient 25(OH)D levels. HbA1c, BMI, eGFR, sRAGE, age, and presence of hypertension or PAD did not show discriminatory power (Figures 4(b) and 4(c)).

Between-group comparison using the *t*-test confirmed the results indicated in multivariate analysis: 25(OH)D deficient diabetics presented higher CML, sVAP-1, and total proteins levels; lower AGE-Fl and grip strength and lower prevalence of CHD (Table 5). The impact of inflammation and SAF to the 25(OH)D levels was further approximated as relationship between Ln(hsCRP/SAF) and 25(OH)D. No significant association was revealed either in diabetics or in the controls (Figures 5(a) and 5(b)). However, Ln(sVAP-1/SAF) correlated inversely in all diabetic patients ($y = -0.006x - 0.006$;

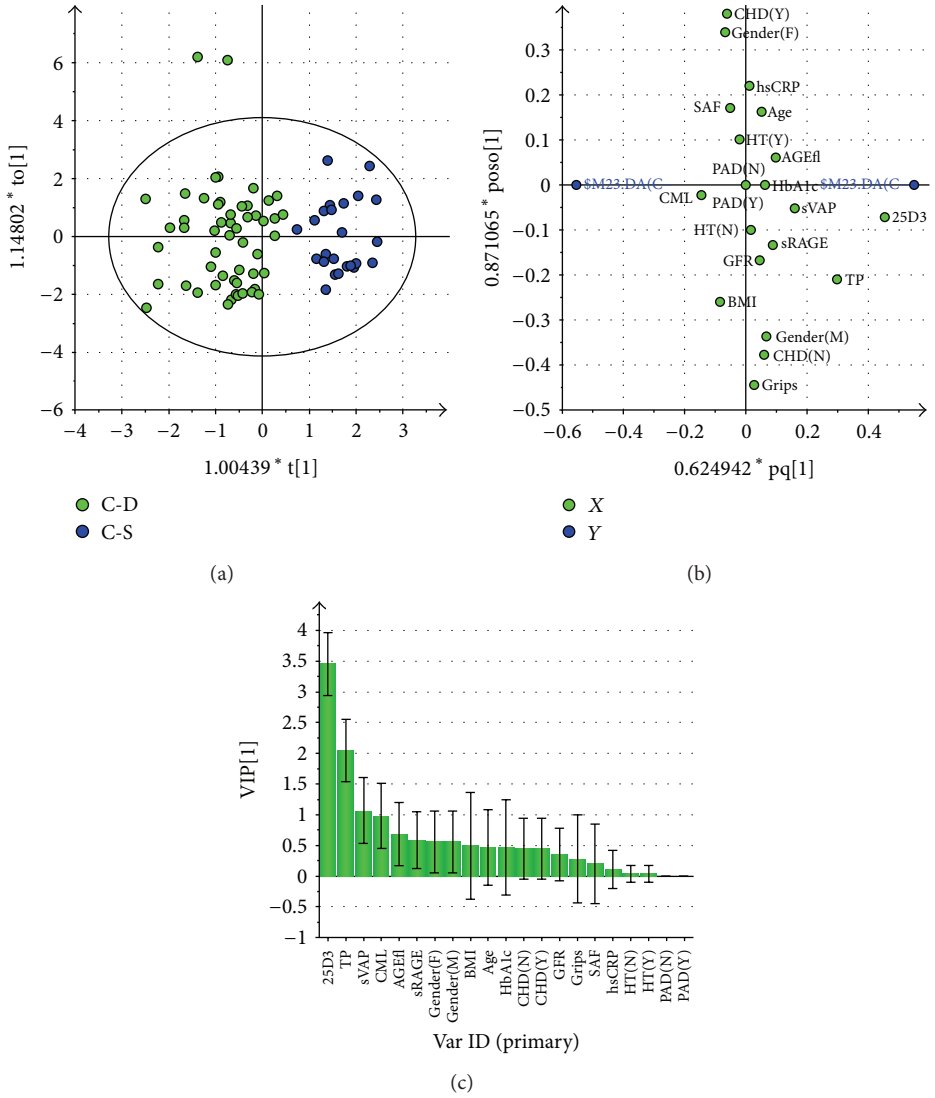

Figure 3: Multivariate analysis data from OPLS-DA model comparing 25(OH)D deficient nondiabetic subjects (25(OH)D <20 ng/mL) with those presenting sufficient levels (25(OH)D >30 ng/mL). (a) Score scatter plot of 25(OH)D deficient controls (C-D, green squares) and those presenting sufficient 25(OH)D levels (C-S, blue squares). Scores are orthogonal (=completely independent from each other), representing new variables summarizing the input of all determined variables (herein gender, presence or absence of comorbidities, age, SAF, and biochemical variables) so that one score vector corresponds to one subject, having its own score vector. Observations situated far outside Hotelling's T2 tolerance ellipse are outliers. Model reveals separation of 25(OH)D deficient and sufficient subjects (separation in direction of x-axis). Separation in direction of y-axis represents within group variability. (b) Loading scatter plot of 25(OH)D deficient controls and those presenting sufficient 25(OH)D levels. Dummy variables (blue circles) characterize the respective 2 groups categorized according to 25(OH)D levels, deficient group at left, and sufficient one at right side of the plot. Vitamin D_3 (25D3) adjacent to dummy variable representing 25(OH)D sufficient group represents the most significant component with discriminatory power determining the separation between the groups; being situated in the vicinity of vitamin D sufficient group presenting dummy variable it indicates that it is higher in this group. 25(OH)D deficient subjects also tend to present higher CML levels (positioned in vicinity of respective dummy variable), and lower total protein and sVAP-1 levels (far opposite, right to respective dummy). Variables positioned near to intersect and on y-axis are similar in 25(OH)D deficient and sufficient groups and thus do not contribute to between-group separation. (c) Plot of variables of importance contributing to between-group separation among 25(OH)D deficient controls and those presenting sufficient 25(OH)D levels. Plot of variables importance for the projection (VIP) summarizes the importance of the variables both to explain X and to correlate with dummy variables (in (a), and (b)). VIP values >1 indicate "important" X variables, <0.5 "unimportant" X variables, in the "grey interval" (0.5-to-1) the importance depends on the sample size. This plot confirms the OPLS-DA loadings scatter plot (b), showing that the variables adjacent to the origin in the former plot do not contribute to between-group separation significantly. Abbreviations used in (b) and (c): 25D3: 25(OH) vitamin D_3; TP: plasma total protein concentration; sVAP: soluble vascular receptor adhesion protein-1; CML: N^{ε}-carboxymethyllysine; AGE-fl: advanced glycation end products associated fluorescence of plasma; sRAGE: soluble receptor for advanced glycation end products; F: female; M: male; BMI: body mass index; HbA1c: glycated hemoglobin A1c; DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; eGFR: estimated glomerular filtration rate; CHD: coronary heart disease; N: no, absent; Y: yes, present; GFR: estimated glomerular filtration rate; grips: grip strength; SAF: skin autofluorescence; AOPPs: advanced oxidation protein products; hsCRP: high sensitive C-reactive protein; HT: hypertension; PAD: peripheral artery disease.

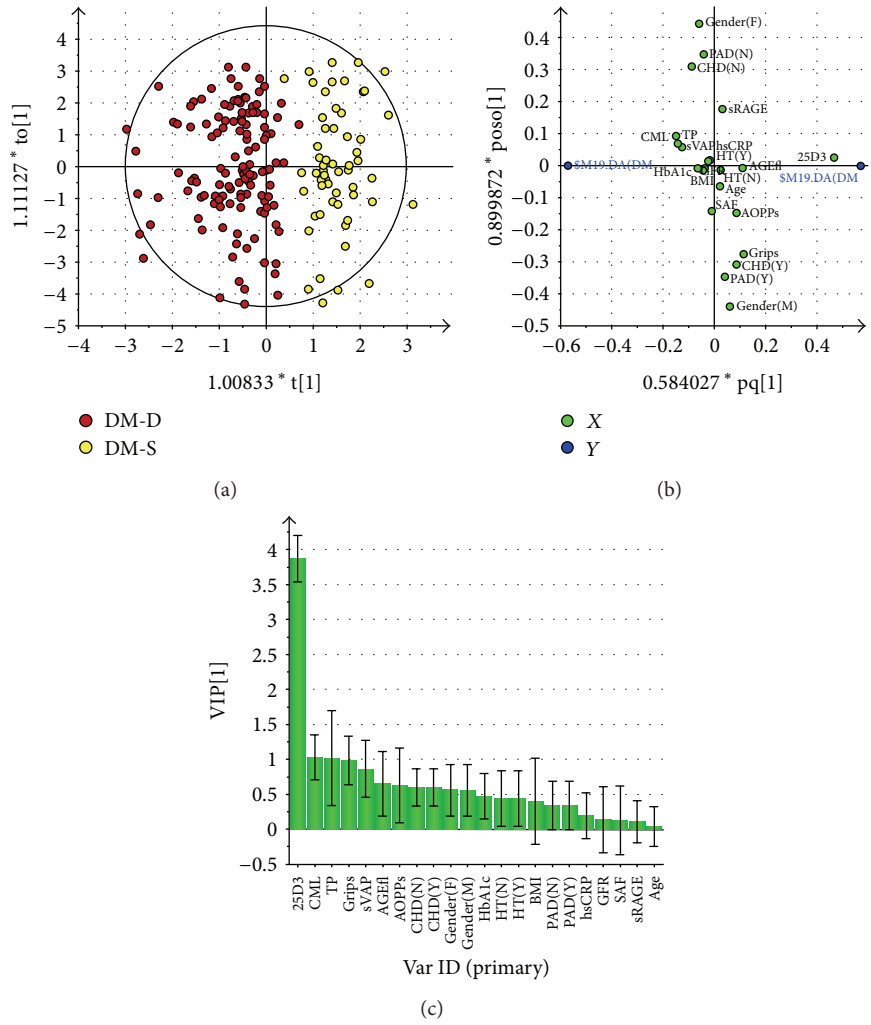

Figure 4: Multivariate analysis data from OPLS-DA model comparing 25(OH)D deficient diabetic patients (25(OH)D <20 ng/mL) with those presenting sufficient levels (25(OH)D >30 ng/mL). (a) Score scatter plot of 25(OH)D deficient diabetic patients (DM-D, red circles) and those presenting sufficient 25(OH)D levels (DM-S, yellow circles). Scores are orthogonal (=completely independent from each other), representing new variables summarizing the input of all determined variables (herein gender, presence or absence of comorbidities, age, SAF, and biochemical variables) so that one score vector corresponds to one subject, having its own score vector. Observations situated far outside Hotelling's T2 tolerance ellipse are outliers. Model reveals separation of 25(OH)D deficient and sufficient diabetic subjects (separation in direction of x-axis). Separation in direction of y-axis represents within group variability. (b) Loading scatter plot of 25(OH)D deficient diabetic subjects and those presenting sufficient 25(OH)D levels. Dummy variables (blue circles) characterize the respective 2 groups categorized according to 25(OH)D levels, deficient group at left, and sufficient one at right side of the plot. Vitamin D_3 (25D3) adjacent to dummy variable representing 25(OH)D sufficient group represents the most significant component with discriminatory power determining the separation between the groups; being situated in the vicinity of vitamin D sufficient group presenting dummy variable it indicates that it is higher in this group. 25(OH)D deficient subjects also tend to present higher CML, total protein and sVAP-1 levels (positioned in vicinity of respective dummy variable), and lower AGE-associated fluorescence of plasma, AOPPs and grip strength (far opposite, right to respective dummy). Variables positioned near to intersect and on y-axis are similar in 25(OH)D deficient and sufficient groups and thus do not contribute to between-group separation. (c) Plot of variables of importance contributing to between-group separation among 25(OH)D deficient controls and those presenting sufficient 25(OH)D levels. Plot of variables importance for the projection (VIP) summarizes the importance of the variables both to explain X and to correlate with dummy variables (in (a), and (b)). VIP values >1 indicate "important" X variables, <0.5 "unimportant" X variables, in the "grey interval" (0.5-to-1) the importance depends on the sample size. This plot confirms the OPLS-DA loadings scatter plot (b), showing that the variables adjacent to the origin in the former plot do not contribute to between-group separation significantly. Abbreviations used in Figures 3(b) and 3(c): 25D3: 25(OH) vitamin D_3; TP: plasma total protein concentration; sVAP: soluble vascular receptor adhesion protein-1; CML: N^{ε}-carboxymethyllysine; AGEfl: advanced glycation end products associated fluorescence of plasma; sRAGE: soluble receptor for advanced glycation end products; F: female; M: male; BMI: body mass index; HbA1c: glycated hemoglobin A1c; DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; eGFR: estimated glomerular filtration rate; CHD: coronary heart disease; N: no, absent; Y: yes, present; GFR: estimated glomerular filtration rate; grips: grip strength; SAF: skin autofluorescence; AOPPs: advanced oxidation protein products; hsCRP: high sensitive C-reactive protein; HT: hypertension; PAD: peripheral artery disease.

Table 5: Pertinent data of the controls and diabetic patients with 25(OH)D_3 deficiency (25(OH)D_3 <20 ng/mL) and sufficient levels (25(OH)D_3 >30 ng/mL).

	Controls			DM subjects		
	25(OH)D <20 ng/mL	25(OH)D >30 ng/mL	*P*	25(OH)D <20 ng/mL	25(OH)D >30 ng/mL	*P*
N	53	25	NA	130	58	NA
M/F (*n*; %)	23/30 (43%/57%)	15/10 (60%/40%)	0.17^{chi}	71/59 (55%/45%)	39/19 (67%/33%)	0.10^{chi}
Age (years)	*58.5 ± 17.1*	*62.6 ± 13.5*	*0.23*	*65.9 ± 13.3*	*65.5 ± 11.1*	0.94
DM duration (yrs)	NA	NA		*15.5 ± 10.7*	*14.7 ± 10.6*	0.63
BMI (kg/m^2)	*27.6 ± 3.6*	*28.6 ± 3.5*	*0.33*	*31.2 ± 7.1*	*29.7 ± 4.6*	*0.18*
Total protein (g/L)	74 ± 8	70 ± 6	0.17	74 ± 7	70 ± 7	**0.015**
eGFR (mL/min/1.73 m^2)	83 ± 12	84 ± 12	*0.71*	70 ± 26	69 ± 23	*0.70*
25(OH)D_3 (ng/mL)	*14.4 ± 3.9*	*35.5 ± 4.8*	***<0.001***	*13.9 ± 3.9*	*38.4 ± 8.7*	***<0.001***
HbA1c (%)	*5.5 ± 0.3*	*5.5 ± 0.3*	*0.53*	*7.2 ± 1.2*	*6.9 ± 1.0*	*0.17*
Fl-AGEs (AU)	*272 ± 181*	*291 ± 344*	*0.06*	*329 ± 108*	*368 ± 139*	***0.05***
SAF (AU)	2.3 ± 0.5	2.3 ± 0.5	0.85	2.9 ± 0.7	2.8 ± 0.8	0.16
CML (ng/mL)	*1098 ± 398*	*930 ± 391*	*0.27*	*1090 ± 433*	*899 ± 236*	*0.130*
sRAGE (pg/mL)	*988 ± 400*	*1070 ± 404*	*0.53*	*947 ± 550*	*949 ± 493*	*0.89*
hsCRP (mg/L)	*2.1 ± 2.4*	*1.6 ± 1.4*	*0.85*	*2.8 ± 2.6*	*2.5 ± 2.0*	*0.59*
sVAP-1 (ng/mL)	*406 ± 152*	*419 ± 177*	*0.38*	*488 ± 174*	*430 ± 181*	*0.040*
Grip strength (pounds)	*81 ± 33*	*84 ± 26*	*0.50*	*70 ± 22*	*80 ± 23*	**0.007**
*Hypertension (N/Y; %)	38/15 (72%/28%)	17/8 (68%/32%)	0.74^{chi}	39/84 (32%/68%)	23/33 (41%/59%)	0.22^{chi}
*PAD (N/Y; %)	53/0 (100%/0%)	24/1 (96%/4%)	0.70^{chi}	108/15 (88%/12%)	46/10 (82%/18%)	0.31^{chi}
*CHD (N/Y; %)	51/2 (96%/4%)	24/1 (96%/4%)	0.56^{chi}	102/21 (83%/17%)	40/16 (71%/29%)	0.08^{chi}
*Total comorb. (N/Y; %)	38/15 (72%/28%)	17/8 (68%/32%)	0.74^{chi}	34/89 (28%/72%)	18/40 (29%/71%)	0.64^{chi}

DM: diabetes mellitus; M: males; F: females; BMI: body mass index; eGFR: estimated glomerular filtration rate; HbA1c: haemoglobin A1c; AGE-Fl: advanced glycation end products associated fluorescence of plasma; AU: arbitrary units; SAF: skin autofluorescence; CML: N^{ε}-carboxymethyllysine; sRAGE: soluble receptor for advanced glycation end products; hsCRP: high sensitive C-reactive protein; sVAP-1: soluble vascular receptor adhesion protein-1; Y: yes; N: no; PAD: peripheral artery disease; CHD: coronary heart disease; comorb.: comorbidities; chi: chi-square; *data from 7 diabetics not available.

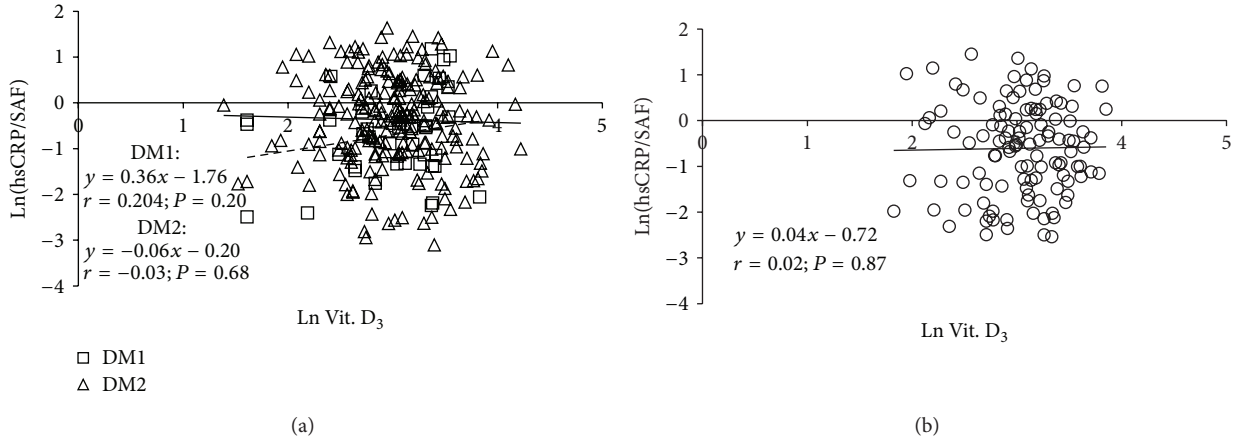

Figure 5: Regression of ln(hsCRP/SAF) over 25(OH)D concentration in type 1 and type 2 diabetics (a) and control subjects (b). DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; dotted line represents a regression in cohort of type 1 diabetics; solid line represents a regression in cohort of type 2 diabetics.

$r = 0.148$; $P = 0.040$) on account of the type 2 diabetics (Figure 4(a)). In type 1 diabetics and control subjects significance was not reached (Figures 6(a) and 6(b)).

3.3. Markers of Advanced Glycation End Products. In type 1 and 2 diabetics an age-dependent rise in AGE-associated fluorescence of plasma was described by almost parallel lines ($y = 3.6 \times \text{age} + 113$, $r = 0.636$, $P < 0.001$ and $y = 3.2 \times \text{age} + 132$, $r = 0.272$, $P < 0.001$), (Figure 7(a)) and that of SAF by fully parallel lines ($y = 0.027 \times \text{age} + 1.17$, $r = 0.671$, $P < 0.001$ and $y = 0.027 \times \text{age} + 1.03$, $r = 0.397$, $P < 0.001$, resp.) (Figure 8(a)). GLM revealed significant impact of age and diabetes duration but not of type of diabetes on SAF and AGE-Fl (Table 3).

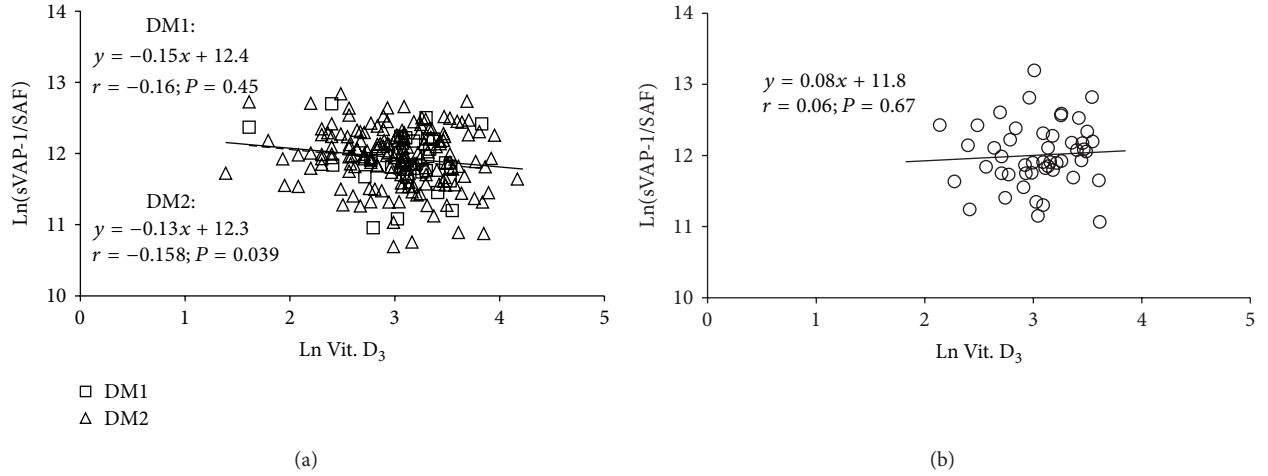

Figure 6: Regression of ln(sVAP-1/SAF) over 25(OH)D concentration in type 1 and type 2 diabetics (a) and control subjects (b). DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; dotted line represents a regression in cohort of type 1 diabetics; solid line represents a regression in cohort of type 2 diabetics.

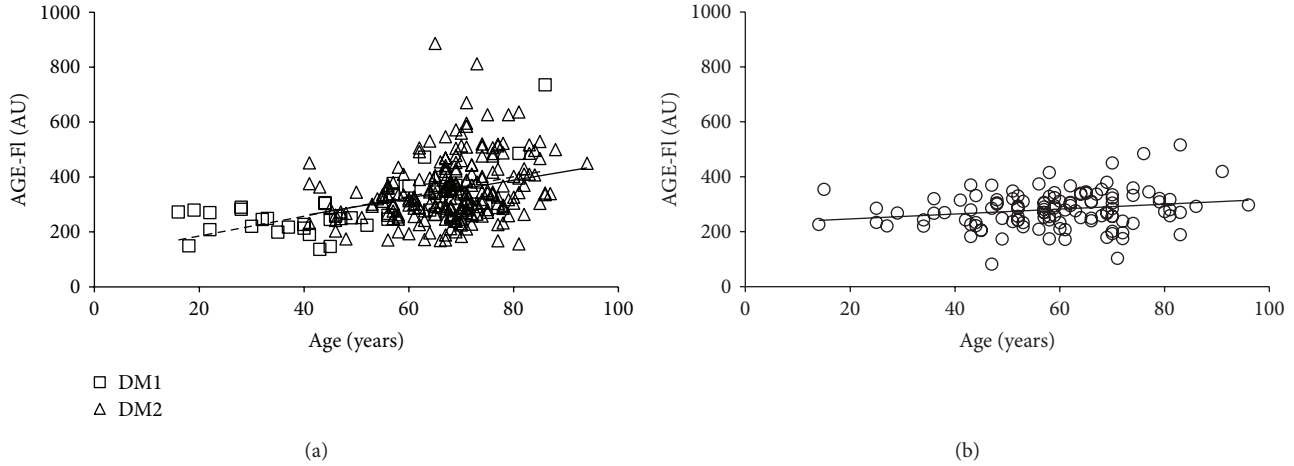

Figure 7: Age-dependent rise in AGE-specific fluorescence of plasma (AGE-Fl) in type 1 and type 2 diabetics (a) and controls (b). DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; dotted line represents a regression in cohort of type 1 diabetics; solid line represents a regression in cohort of type 2 diabetics; AU: arbitrary units.

In type 1 diabetics multivariate analysis (with age, BMI, eGFR, HbA1c, hsCRP, SAF, CML, sRAGE, sVAP-1, and duration of diabetes entered as independent variables) selected only sVAP-1 (corrected model $P = 0.005$, R^2 : 78%; $P_{\text{sVAP-1}} = 0.028$, $\beta = -0.51$, SE = 0.19) as a significant independent contributor to AGE-Fl. In type 2 diabetic subjects eGFR was selected as an independent significant contributor to AGE-Fl (corrected model $P < 0.001$, R^2: 38%; $P_{\text{eGFR}} < 0.001$, $\beta = -0.006$, SE = 0.001).

In a similar setting for SAF levels GLM selected age ($P_{\text{age}} = 0.019$, $\beta = 0.90$, SE = 0.31), sVAP-1 ($P = 0.047$, $\beta = 0.99$, SE = 0.41), and sRAGE levels ($P = 0.001$, $\beta = -1.13$, SE = 0.20; corrected model $P = 0.006$, R^2: 84%) as significant associated factors of type 1 diabetics. In type 2 diabetic subjects age ($P = 0.006$, $\beta = 1.01$, SE = 0.36), eGFR ($P = 0.022$, $\beta = -0.005$, SE = 0.002), sRAGE levels ($P = 0.016$, $\beta = -0.23$, SE = 0.09), sVAP-1 ($P = 0.014$, $\beta = 0.27$, SE = 0.11), and CML ($P = 0.050$, $\beta = -0.21$, SE = 0.11) appeared to be associated independently with SAF levels (corrected model $P < 0.001$, R^2: 37%).

In comparison with the diabetics, the age-dependent rise in SAF ($y = 0.018 \times \text{age} + 1.28$, $r = 0.520$, $P < 0.001$) and AGE-associated fluorescence of plasma ($y = 0.9 \times \text{age} + 228$, $r = 0.202$, $P = 0.028$) was much slower in the controls (Figures 7(b) and 8(b)).

GLM did not select any independent variable association independently and significantly either to AGE-Fl or to SAF in the controls.

The correction for 25(OH)D did not change the results nor was 25(OH)D selected as significant contributor if forced into either model.

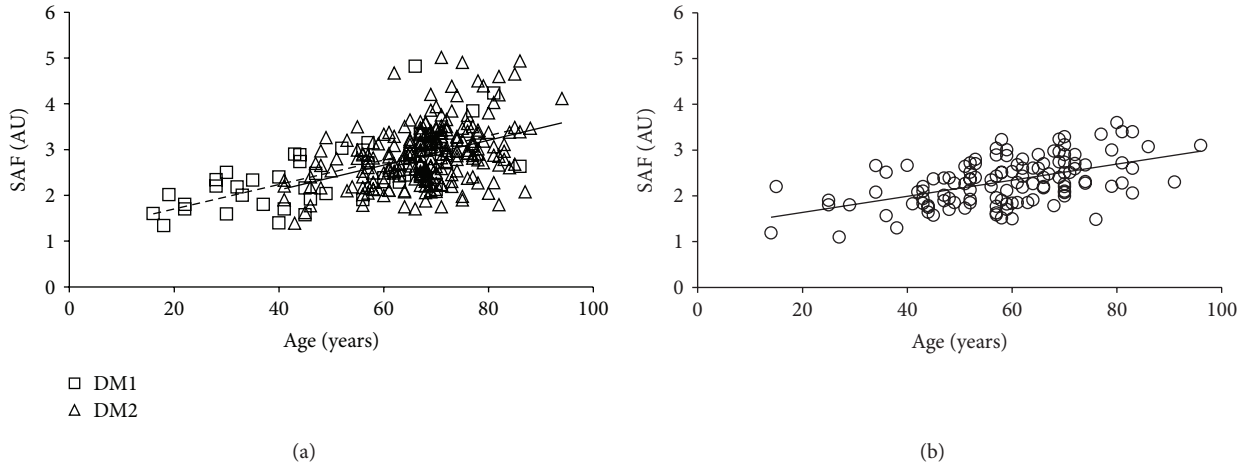

Figure 8: Age-dependent rise in skin autofluorescence (SAF) in controls (a) and type 1 and type 2 diabetics (b). DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; dotted line represents a regression in cohort of type 1 diabetics; solid line represents a regression in cohort of type 2 diabetics; AU: arbitrary units.

4. Discussion

This is one of the few studies examining in diabetic subjects the relationship between vitamin D status and AGE accumulation in plasma and skin as well as the AGE-associated biomarkers of microinflammation and oxidative stress. Surprisingly we found no association between vitamin D status and SAF or plasma AGE-Fl. Among the markers of microinflammation/oxidative stress an inverse link between vitamin D and sVAP-1 in the type 2 diabetics could be shown.

4.1. Vitamin D Status. In our study the controls presented mean vitamin D levels within the lower range of vitamin D insufficiency and averaging 22.3 ng/mL. This finding corresponds to concentrations reported for the Central European general population (17-to-33 ng/mL in mean) from cities in similar latitude as Würzburg (reviewed in [51]). They are higher than those reported for a large cohort of orthopaedic patients in Germany (18.8 ng/mL in mean in summer and 16.1 ng/mL in winter) [52].

In our diabetic subjects the prevalence of hypovitaminosis D was of the same degree as in the controls. In other studies the prevalence of low vitamin D levels was higher in diabetics and individuals with prediabetes compared to nondiabetic controls [53–56]. Consequently a low vitamin D status was assumed to be involved in the development and also progression of type 2 diabetes. This hypothesis was supported by various experimental and clinical studies which showed an enhanced insulin resistance and/or an impaired secretion of insulin in the pancreatic beta cells in the presence of hypovitaminosis D [57, 58]. In line with the Tromso study [59] and Zoppini et al. [60], we found in our diabetics an inverse relationship between vitamin D and the HbA1c concentrations. However, this is not a causal relationship since in a meta-analysis of numerous studies vitamin D supplementation was without effect on the disturbed glucose homeostasis [61]. Furthermore in a recent genetic study no proof for a relationship between vitamin D deficiency and diabetes was found [62].

4.2. Vitamin D Status and BMI. We did not reveal a significant association between vitamin D levels and BMI, in either diabetics or nondiabetic controls; however, multivariate analysis suggested that a higher BMI might be inversely associated with vitamin D status in nondiabetics. Cholecalciferol is the dominant metabolite and distributed in adipose tissue [63]. Its accumulation in fat cells probably results from its trapping. There is a significant positive association between 25(OH)D concentration in subcutaneous white adipose tissue and serum [64]. A meta-analysis of 21 studies reported that each unit of increase in BMI (kg/m^2) associates with 1.15% lower plasma concentrations of 25(OH)D [65]. Further data suggest that reduction of weight and consequently of fat in overweight and obese subjects is not associated with significant changes in white adipose tissue or circulating vitamin D_3 levels [64].

4.3. Advanced Glycation End Products. In our controls the age-dependent rise of SAF levels corresponded well with those reported for the general Dutch population [66]. As expected, in our diabetics, the age-dependent rise of SAF was much steeper than in the nondiabetic controls. These data correspond well with studies from Netherlands [67, 68] and from the Czech Republic [69]. In contrast, a large Australian study reported only a tendency towards higher SAF values [15]. The reasons are not clear, but differences in the characteristics of the controls (such as age or presence of comorbidities) and of the diabetics (duration, treatment modalities, and presence of complications and comorbidities) might be important.

Interestingly in our type 1 and type 2 diabetics the age-dependent rise of SAF was of the same magnitude and the SAF levels were directly related to duration of the disease (similar between both diabetic groups). No relationships were found to haemoglobin A1c. Also plasma Fl-AGE showed an age-dependent rise in our controls and the diabetics which is in accordance with data of Kalousová et al. [70].

4.4. Missing Interactions of Vitamin D and Advanced Glycation End Products. Since in vitro and animal studies showed that vitamin D application lowers the toxic effects of AGEs and decreases their formation [48, 49], we were particularly interested in potential relationships between both factors. We expected that low levels of vitamin D could be associated with an enhanced AGE formation, while, in the presence of sufficient vitamin D, lower concentrations of AGEs should occur. Surprisingly, we did not found any link between vitamin D and SAF.

Also plasma Fl-AGE and vitamin D levels were not interrelated. However, multivariate analysis using the OPLS-DA model suggested that vitamin D_3 deficient subjects tend to present lower AGE-associated fluorescence of plasma, regardless of presence or absence of diabetes. In a study in elderly type 2 diabetics no relationship between vitamin D_3 levels and plasma concentration of Fl-AGEs was found [55].

The missing link between SAF and vitamin D also rules out the possibility that the cutaneous AGE accumulation hinders the photoconversion of the provitamin D into vitamin D. In line with this assumption, repeated UVB radiation in hemodialysis patients was associated with a marked increase of vitamin D_3 status despite the high levels of skin AGEs in these patients [71].

4.5. Markers of Inflammation and Oxidative Stress. In the whole cohort of the diabetics the plasma levels of hs-CRP and sVAP-1 were significantly elevated. Unexpectedly, this rise was on the account of the type 2 diabetics, while in type 1 diabetics these markers of the inflammatory pathway were not enhanced. This observation is surprising with regard to the identical elevation of the HbA1c levels in both groups. This disparate pathobiochemistry may be explained by the different aetiology and pathophysiology of both diabetic states as also proposed by Kalousová et al. [70]. Type 1 diabetes mellitus is an immunological disease and characterized by dysfunction of the pancreatic β-cells. It usually develops in younger age in the absence of obesity, insulin resistance, and hypertension. In contrast, type 2 diabetes mellitus represents an insulin-resistant state and manifests in the majority of patients with a metabolic syndrome in the middle or higher age. Central obesity favors the development of dyslipidemia, microinflammation, and oxidative stress [72, 73] as well as hypertension [74]. Correspondingly, our type 2 diabetics were older, presented 1.4-fold higher incidence of hypertension and a higher BMI as compared to type 1 diabetics.

The sVAP-1 levels were elevated in hypovitaminosis D and showed an independent inverse association with the 25(OH)D concentrations in both diabetic cohorts. The augmented sVAP-1 levels might reflect involvement of oxidative stress and/or microinflammation in part due to AGE accumulation. sVAP-1, known also as semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.21), represents a molecule with a dual action: it favors lymphocyte adhesion to damaged endothelium and plays a role in the monoamine detoxification metabolizing primary amines into corresponding aldehydes, generating H_2O_2 and ammonia [75]. Increased SSAO activity in diabetes may result from enhanced SSAO substrates such as methylamine or aminoacetone.

It remains unclear whether the association of low vitamin D_3 and elevated sVAP-1 levels is a coincidence or causally related to proinflammatory consequences of hypovitaminosis D. In the latter case an augmented sVAP-1 could result from release from endothelial cells (following the shedding by metalloproteinases) or induction of the VAP-1/SSAO gene expression. In diabetes the conversion of aminoacetone by SSAO to methylglyoxal is increased and has been claimed to be related to induction of insulin resistance and development of diabetic complications [76–78]. In humans the hyperglycemia-induced rise of circulating sVAP-1 levels directly correlates with the plasma AGE concentration [79]. Moreover, sVAP-1 is associated with subclinical atherosclerotic manifestations and an increased risk of cardiovascular events and mortality rate [80].

Since both AGE accumulation [81] and vitamin D deficiency [82] may be associated with impaired muscle strength we performed the hand-grip test in the controls and diabetics. In our study we did not observe an association between elevated AGEs and impaired grip strength in control group or in diabetics. However, in contrast to the controls, in diabetic patients (type 1 and type 2) with hypovitaminosis D (insufficient and deficient vitamin D levels), we noticed impaired grip strength.

In our study, potential confounding factors to the toxic effects of AGEs and hypovitaminosis D have to be considered. First of all the impact of tight blood glucose control has to be mentioned. The mean HbA1c level of 7.1 demonstrates that in most patients glycemia was well controlled. All hypertensive patients including the nondiabetic controls were treated with either ACE-inhibitors or angiotensin II receptor blockers. These compounds exert anti-inflammatory, antioxidative, and anti-AGE forming effects as demonstrated in in vitro studies, animal models, and clinical trials [83–85]. Furthermore the frequently prescribed statins were shown to reduce microinflammation and oxidative stress [86–88] as well as the formation of AGEs [89], in part by increasing soluble RAGE [90]. Interestingly certain statins may enhance the formation of vitamin D_3 [91] which could explain the missing difference of vitamin D levels in our nondiabetic and diabetic cohorts. A very potent drug with anti-inflammatory actions is metformin which was administered to most of the type 2 diabetic patients [92, 93] except in patients with creatinine clearance less than 60 mL/min.

Taken together, in our diabetic patients, the vitamin D_3 deficiency was not unequivocally and expressively associated with markers of microinflammation and oxidative stress. This could suggest that the postulated anti-inflammatory action of vitamin D is rather limited or may even be absent under certain conditions. In fact in various inflammatory states the vitamin deficiency was not the cause, but a consequence of the disease (elective knee arthroplasty [94], critically ill patients [95]). Correspondingly in various controlled studies (in contrast to observational investigations), supplementation with vitamin D did not improve the microinflammation and oxidative stress [62]. However, in patients with chronic kidney disease, hypovitaminosis D is frequently associated with secondary hyperparathyroidism which is suppressed by vitamin D supplementation [96]. Although still needing

placebo-controlled studies, vitamin D supplementations in elderly people "seemed to decrease mortality" [97].

Summarizing, our cross-sectional study suggests that in diabetic subjects hypovitaminosis D is not associated with enhanced AGE accumulation and sufficient vitamin D levels are not linked with a lower AGE accumulation. Moreover, we conclude that an excessive rise of skin AGEs does not interfere with dermal vitamin D_3 formation. The relationships between vitamin D_3 deficiency and markers of inflammation showed a different pattern. We found higher levels of s-VAP 1 in hypovitaminosis D, but hs-CRP levels were unchanged. These data suggest that hypovitaminosis D seems to be of limited importance for development of micro-inflammation and accumulation of AGEs. With regard to equivocal results of our cross-sectional study, controlled longitudinal studies focusing on the effects of vitamin D supplementation on skin and plasma AGE and markers of microinflammation/oxidative stress are needed to elucidate a potential relationship between vitamin D status and AGE accumulation and their interaction in potentiating of toxic effects.

Abbreviations

25(OH)D: 25-Hydroxyvitamin D (calcidiol)
AGEs: Advanced glycation end products
AGE-Fl: Advanced glycation end products associated fluorescence of plasma
ANOVA: Analysis of variance
CHD: Coronary heart disease
BMI: Body mass index
CKD: Chronic kidney disease
CML: N^{ε}-Carboxymethyllysine
GLM: General Linear Model
HbA1c: Haemoglobin A1c
eGFR: Estimated glomerular filtration rate
hs-CRP: High sensitive C-reactive protein
HT: Hypertension
Ln: Logarithm
NF-κB: Nuclear factor kappa-B
NO: Nitric oxide
OPLS-DA: Orthogonal projections to latent structures discriminant analysis
PAD: Peripheral artery disease
RAGE: Receptor for advanced glycation end products
sRAGE: Soluble receptor for advanced glycation end products
SAF: Skin advanced glycation end products associated autofluorescence
sVAP-1: Soluble vascular adhesion protein-1
TGF-β-1: Transforming growth factor-β-1
VIP: Variables importance for the projection.

Conflict of Interests

Dr. F. Stäb is an employee of Beiersdorf AG, Hamburg, Germany. Other authors declare no competing interests.

Acknowledgments

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Review Article

Vitamin D Deficiency in HIV Infection: Not Only a Bone Disorder

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Hypovitaminosis D is a worldwide disorder, with a high prevalence in the general population of both Western and developing countries. In HIV patients, several studies have linked vitamin D status with bone disease, neurocognitive impairment, depression, cardiovascular disease, high blood pressure, metabolic syndrome, type 2 diabetes mellitus, infections, autoimmune diseases like type 1 diabetes mellitus, and cancer. In this review, we focus on the most recent epidemiological and experimental data dealing with the relationship between vitamin D deficiency and HIV infection. We analysed the extent of the problem, pathogenic mechanisms, clinical implications, and potential benefits of vitamin D supplementation in HIV-infected subjects.

1. Introduction

Human immunodeficiency virus type-1 (HIV) is a global health problem that has infected 60 million people and caused 25 million deaths worldwide. To date, it has been estimated that more than 33 million people, including 2 million children, live infected by HIV. However, even if the problem is far from a definitive solution, highly active antiretroviral therapy (HAART) has profoundly changed the natural history of HIV infection dramatically reducing AIDS- (acquired immune deficiency syndrome-) related morbidity and mortality [1]. Nevertheless, at least until now, HAART cannot eradicate HIV [2, 3]. Increased life expectancy exposes HIV-infected subjects both to chronic adverse drug reactions and to age-related morbidities, including neurocognitive disorders, cardiovascular and metabolic disease, renal and bone diseases (i.e., osteopenia/osteoporosis), and cancer [4–6]. Many of these appear to occur earlier in HIV patients compared to the general population. Key factors explaining premature age-associated non-AIDS-related events in patients receiving HAART are chronic inflammation and immune activation [7, 8]: plasma levels of several inflammatory and coagulopathy biomarkers, such as interleukin-6 (IL-6), highly sensitive C-reactive protein (hsCRP), and D-dimer are higher and correlate with outcome in HIV infection [9, 10].

Considering the potential role of vitamin D in many of these chronic illnesses, the scientific community focuses attention on the possible impact of its deficiency on the HIV-infected population. In this review, we first briefly describe vitamin D metabolism and its biological functions; then, we focus on the most recent epidemiological and experimental data dealing with the relationship between vitamin D deficiency and HIV infection. We analyse the extent of the problem, pathogenic mechanisms, clinical implications, and the potential benefits of vitamin D supplementation among HIV-infected subjects. We researched the PubMed database for the period from 1980 through January 31, 2015, using the keywords "HIV," "vitamin D," "neurocognitive disorders," "cardiovascular disease," "metabolic disease" (i.e., diabetes and metabolic syndrome), "renal disease," and "cancer." Articles

presenting original data as well as reviews were included in our analysis.

2. Prevalence of Hypovitaminosis D in HIV-Infected Subjects

Hypovitaminosis D is a worldwide disorder, with a high prevalence in the general population of both Western and developing countries. It has been estimated that more than 1 billion people suffer from either 25(OH)D (25-hydroxyvitamin D) deficiency or insufficiency. According to the results of the National Health and Nutrition Examination Survey (NHANES), 25(OH)D deficiency and insufficiency are at 79% among adults [11]. Thus, like the general population, it is not surprising to find high rates of hypovitaminosis D among HIV-infected subjects. The overall estimated prevalence in people living with HIV and 25(OH)D deficiency is high, ranging from 70.3 to 83.7% (Table 1).

Eckard et al. conducted an investigation on hypovitaminosis D and the possible variables associated with this pathological framework in HIV-infected pregnant women and their infants compared to healthy controls. It was found that 25(OH)D concentrations in serum cord blood were <30 ng/mL in 100% of subjects from both groups. The only variables associated with higher serum 25(OH)D concentrations were white race and non-Hispanic ethnicity [12]. These data agreed with previous observations asserting that vitamin D deficiency not only contributes to HIV disease progression and mortality in HIV-infected pregnant women, but also increases the overall risk of mother-to-child transmission by 46% [13] and the risk of death in newborns by 61% during follow-up [13]. While most infants born to HIV-infected mothers in the US will not acquire HIV infection, in utero ART (antiretroviral therapy) exposure may increase their cancer risk later in life. Thus, maternal and therefore infant 25(OH)D deficiency should not be disregarded [14].

3. Risk Factors for Vitamin D Deficiency in HIV-Infected Subjects

In the setting of HIV infection, 25(OH)D deficiency may be affected by both HIV-related and -independent risk factors; however, it is often challenging to differentiate the direct impact of HIV infection from the effect of traditional risk factors which may be normal or overexpressed in HIV-positive cohorts.

3.1. HIV-Related Risk Factor. The relationship between 25(OH)D levels, viral load, and CD4+ T-cell count is not clear cut. Some studies described a positive correlation [15, 16], some others failed to demonstrate a significant association [17, 18], and, finally, others did not find that vitamin D (in any possible formula) supplementation can increase CD4+ count [19, 20]. Different mechanisms have been hypothesized to explain the association between 25(OH)D deficiency and higher severity of HIV disease. First, 25(OH)D deficiency may be a contributory causal agent of the HIV infection itself. Second, chronic inflammation due to HIV infection and subsequent TNF-α overproduction may be responsible for renal 1α-hydroxylase impairment, reducing the PTH (parathyroid hormone) stimulatory effect on the production of the hormonally active 1,25(OH)2D (1,25-dihydroxyvitamin D). Third, infectious complications as a result of poor immunity require hospital care, which significantly reduces the duration of sun exposure for patients. Lastly, both infectious complications and hospitalization may lead to malnutrition and reduced oral intake of the few foods that contain vitamin D [21, 22].

3.2. HIV-Independent Risk Factors. Several traditional hypovitaminosis D risk factors, such as female sex, increasing age, reduced exposure to sunlight, winter season, dark skin pigmentation, non-Caucasian race (i.e., African American ethnicity), greater body mass index (BMI), low vitamin D dietary intake, gastrointestinal absorption disorders, liver and renal diseases, multiple cardiovascular disease risk factors, including diabetes mellitus, and current alcohol consumption, are similar in both HIV-positive and HIV-negative cohorts [23, 24]. An exception is represented by intravenous drug use, which has not been extensively studied in the general population [25]. Injection drug users (IDUs) often have poor nutritional status and limited/delayed access to healthcare. In addition, intravenous drug use increases the risk for a host of acute and chronic infectious and cardiopulmonary conditions. As a result, this patient population suffers a disproportionate burden of 25(OH)D deficiency, compared to other urban dwelling adults [26, 27]. In 2014, Lambert et al. evaluated the relationship between intravenous drug use, 25(OH)D deficiency, and HIV infection, analysing 950 individuals (29% of them were HIV-infected). The study found that 74% of subjects were 25(OH)D deficient (68% in HIV-infected versus 76% in HIV-uninfected, $P = 0.01$); significantly, higher odds of 25(OH)D deficiency were observed in black race, late winter/early spring season, lack of multivitamin use, and hypoalbuminemia (the latter as an expression of poor nutritional state). Notably, HIV- and HCV-infected IDUs were less likely to be 25(OH)D deficient, evoking questions regarding the role of free vitamin D measurement (not influenced by albuminemia) in these unique populations [25].

4. Vitamin D Status and HAART

Recently several *in vitro* and *in vivo* studies focused on the impact of antiretroviral drugs on vitamin D metabolism. Both protease inhibitors (PIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs) have been associated with the impairment of vitamin D metabolic pathways [28–30].

PIs, especially darunavir and ritonavir, seem to interfere with vitamin D metabolism by inhibition of vitamin D 1α- and 25α-hydroxylation both in hepatocyte and in monocyte cultures: reduction of 25(OH)D conversion to its active metabolite may potentially explain the reports of increased 25(OH)D levels in subjects with low 1,25(OH)2D. Regarding NNRTIs, there is an increasing amount of experimental data

Table 1: Prevalence of hypovitaminosis D in HIV-infected subjects reported by nation.

Authors (year), journal	Nation	Patients	Results	Comments
Dao et al. (2011) [23], Clinical Infectious Diseases	US	672 HIV-positive patients versus US general population.	70.3% patients had 25(OH)D levels below 30 ng/mL versus 79.1% of HIV-negative US adults.	Vitamin D deficiency was not different between the two groups and no relationship could be found with duration since HIV diagnosis and vitamin D deficiency.
Adeyemi, Agniel et al. (2011), Journal of Acquired Immune Deficiency Syndromes	US	1268 HIV-positive versus 510 HIV-negative women.	60% patients had 25(OH)D levels below 20 ng/mL versus 72% of controls.	Vitamin D deficiency was found in total 63% of women with the highest rates in African American women. No other predictive factors of hypovitaminosis were found in multivariate analysis.
Eckard, Judd et al. (2012), Antiviral Therapy	US	200 HIV-infected and 50 HIV-uninfected youth Americans.	77% of HIV-positive and 74% of controls had 25(OH)D <20 ng/mL.	No difference in 25(OH)D was proved between groups. However, with a 77% and 96% prevalence of vitamin D deficiency and insufficiency, nearly all HIV-infected youth suffered from these conditions.
Poowuttikul, Thomas et al. (2014), Journal of the International Association of Providers of AIDS Care	US	160 HIV-infected youth.	5% had normal 25(OH)D levels; 23.1% had 25(OH)D levels between 21 and 35 ng/mL; 71.9% had 25(OH)D level ≤20 ng/mL.	Severe vitamin D deficiency (25(OH)D ≤10 ng/mL) was related to lower CD4 counts and CD4% but not to HIV plasma RNA. CD4 counts/CD4% did not increase under vitamin D supplementation.
Crutchley, Gathe et al. (2012), AIDS Research and Human Retroviruses	US	200 HIV-infected patients.	64% had 25(OH)D <20 ng/mL and 20.5% had 25(OH)D <10 ng/mL.	Multivariate analysis showed a significant correlation between low 25(OH)D levels, African-American race, and low daily vitamin D supplemental intake.
Stein, Yin et al. (2011), Osteoporosis International	US	89 HIV-positive and 95 HIV-negative postmenopausal women (33% Afro-Americans and 67% Hispanic).	74% of HIV-positive versus 78% of HIV-negative women had 25(OH)D <30 ng/mL.	25(OH)D was significantly lower in Afro-American subjects and higher in subjects who used both calcium and multivitamins. 25(OH)D level was directly associated with current CD4 count ($P < 0.01$). No association was observed between 1,25(OH)(2)D and CD4 count or between serum 25(OH)D, 1,25(OH)(2)D, and type of cART.
Kwan, Eckhardt et al. (2012), AIDS Research and Human Retroviruses	US	463 HIV-infected patients.	24% 25(OH)D <30 ng/mL (insufficiency) 59% 25(OH)D <20 ng/mL (deficiency).	In this population, hyperparathyroidism prevalence was 30% in patients with vitamin D deficiency, 23% in those with insufficiency, and 12% in those with sufficient vitamin D levels.
French, Adeyemi et al. (2011), J Womens Health (Larchmt)	US	602 nonpregnant (480 HIV-infected and 122 uninfected) subjects.	24.4% 25(OH)D <30 ng/mL (insufficiency) 59.4% 25(OH)D <20 ng/mL (deficiency).	Only race was significantly associated with vitamin D deficiency, with no differences in HIV status.
Yin, Lu et al. (2010), Journal of Acquired Immune Deficiency Syndromes	US	100 HIV-positive and 68 HIV-negative premenopausal women.	91% of HIV-positive and 91% of HIV-negative had 25(OH)D levels <32 ng/mL; 69% of HIV-positive and 60% of HIV-negative had 25(OH)D levels <20 ng/mL; 30% of HIV-positive and 24% of HIV−negative had 25(OH)D <10 ng/mL.	In premenopausal HIV+ women, bone mineral density was lower than comparable HIV-women. Vitamin D level was not associated with differences in HIV status.

Table 1: Continued.

Authors (year), journal	Nation	Patients	Results	Comments
Rodriguez, Daniels et al. (2009), AIDS Research and Human Retroviruses	US	57 HIV-positive patients.	36.8% patients had 25(OH)D <20 ng/mL. 10.5% patients had 25(OH)D <10 ng/mL.	Lower vitamin D intake was significantly associated with severe 25(OH)D deficiency. Lactose intolerance tended to be associated with severe 25 (OH)D deficiency.
Wasserman and Rubin (2010) [17], AIDS Patient Care STDS	US	19 HIV-positive patients under NNRTI versus 37 HIV-positive patients under PI.	73.7 NNRTI recipients had 25(OH)D <50 nmol/L. 29.7 (11/37) PI recipients had 25(OH)D <50 nmol/L.	Vitamin D deficiency was not correlated to stable viral suppression. HAART receipt and tobacco use were associated with lower vitamin D levels and greater risk of deficiency and severe deficiency, respectively.
Viard et al. (2011) [21], AIDS	31 European countries, Israel, and Argentina	1985 HIV-positive among EuroSIDA study group.	23.7% had 25(OH)D <10 ng/mL. 65.3% had 25(OH)D between 10 and 30 ng/mL. 11% had 25(OH)D >30 ng/mL.	As in the general population, season (winter), age (older), and race (black) affected 25(OH)D levels (reduction). Hypovitaminosis D was independently associated with a higher risk of HIV disease progression, AIDS events, and all-cause mortality.
Allavena, Delpierre et al. (2012), Journal of Antimicrobial Chemotherapy	France	2994 HIV-positive patients.	55.6% had 25(OH)D <30 ng/mL. 31.1% had 25(OH)D <10 ng/mL.	No relationship was found in duration since HIV diagnosis and vitamin D deficiency.
Meyzer, Frange et al. (2013), Pediatr Infect Dis J	France	113 HIV-infected children versus 54 healthy controls.	70% versus 45% had 25(OH)D <30 ng/mL. 25% versus 55% had 25(OH)D <10 ng/mL.	Dark phototype was the only independent risk factor for vitamin D deficiency in HIV-infected children.
Theodorou et al. (2014) [29], Clinical Nutrition	Belgium	2044 HIV-infected subjects.	89.2% had 25(OH)D <30 ng/mL. 32.4% had 25(OH)D <10 ng/mL.	The authors also found a positive association between AIDS diagnosis and vitamin D deficiency; in particular, it was associated with cART modalities and duration.
Van Den Bout-Van Den Beukel et al. (2008) [52], AIDS Research and Human Retroviruses	Netherlands	252 HIV-positive patients.	28.96% had 25(OH)D <35 nmol/L from April to September and <25 nmol/L from October to March.	Female sex, younger age, dark skin, and NNRTI treatment were significant risk factors in univariate analysis, although in multivariate analyses skin pigmentation remained the only independent risk factor.
Bang, Shakar et al. (2010), Scand J Infect Dis	Denmark	115 HIV-positive patients.	20.0% had 25(OH)D <25 nmol/L. 4.0% had 25(OH)D <12.5 nmol/L.	Vitamin D level was not associated with age, with HIV infection, highly active antiretroviral therapy (HAART) or CD4 count.
Welz, Childs et al. (2010) AIDS	UK	1077 HIV-positive patients	91% 25(OH)D <30 ng/mL 33% 25(OH)D <10 ng/mL	Black ethnicity, sampling in winter, CD4 cell count lower than 200 cells/microl, and exposure to combination antiretroviral therapy were associated with severe vitamin D deficiency.
Gedela et al. (2014) [18], International Journal of STD & AIDS	UK	253 HAART-naive subjects.	58.5% had 25(OH)D <30 ng/mL. 12.6% had 25(OH)D <10 ng/mL.	25(OH)D deficiency was common among antiretroviral treatment-naive patients, with those of nonwhite ethnicity at highest risk; no association was found with CD4 count, HIV viral load, and HIV clinical staging.

Table 1: Continued.

Authors (year), journal	Nation	Patients	Results	Comments
Mueller, Fux et al. (2010), AIDS	Swiss	211 HAART-naive subjects.	42% had 25(OH)D <30 ng/mL in spring. 14% had 25(OH)D <30 ng/mL in fall.	Vitamin D status significantly changed in HIV-positive patients according to seasons, intravenous drugs use, and longer HIV diagnosis but remained unchanged regardless of combined cART exposure.
Haug, Aukrust et al. (1998), Journal of Clinical Endocrinology and Metabolism	Norway	54 HIV-positive patients.	54% had 1,25(OH)2D <95 pmol/L and 62% of them had undetectable levels.	HIV-patients had low 1,25(OH)2D levels, whereas they had normal serum levels of 25(OH)D and vitamin D-binding protein. Moreover, they had modestly depressed serum calcium and PTH levels. No correlations were found between these parameters and serum levels of 1,25(OH)2D. Patients with undetectable 1,25(OH)2D were characterized by advanced clinical HIV infection, low CD4+ lymphocyte counts, and high serum levels of TNF-alpha. Inadequate 1alpha-hydroxylation of 25(OH)D could be the cause of 1,25(OH)2D deficiency, possibly induced by an inhibitory effect of TNF-alpha.
Vescini, Cozzi-Lepri et al. (2011), Journal of Acquired Immune Deficiency Syndromes	Italy	810 HIV-positive patients.	47% had 25(OH)D <30 nmol/L. 3% had 25(OH)D <10 nmol/L.	Authors highlighted a correlation between 25(OH)D insufficiency and risk of cardiovascular events, diabetes mellitus, and renal disease over a median 6.5-year follow-up. 25(OH)D levels below 30 nmol/L seemed to predict faster HIV progression.
Pinzone, Di Rosa et al. (2013), Eur. Rev. Med. Pharmacol. Sci.	Italy	91 HIV-positive patients.	57% patients had 25(OH)D <30 ng/mL. 31% patients had 25(OH)D <10 ng/mL.	Vitamin D deficiency was common in HIV-infected patients. Chronic inflammation, including residual viral replication, may contribute to 25(OH)D reduction modulating vitamin D metabolism and catabolism.
Cervero, Agud et al. (2012), AIDS Research and Human Retroviruses	Spain	352 HIV-positive patients.	71.6% had 25(OH)D <30 ng/mL. 44.0% had 25(OH)D <20 ng/mL.	Higher body mass index, black race, lower seasonal sunlight exposure, men who have sex with men and heterosexual transmission categories, efavirenz exposure, and lack of HIV viral suppression were independently associated with 25(OH)D deficiency/insufficiency.
Lerma, Molas et al. (2012), ISRN AIDS	Spain	566 HIV-positive patients.	71.2% had 25(OH)D <30 ng/mL; 39.6% had 25(OH)D <20 ng/mL.	Nonwhite race and psychiatric comorbidity were predictors of vitamin D deficiency.
Teichmann et al. (2000) [56], Journal of Infection	Germany	54 HIV-positive females prior to HAART versus 50 healthy women.	1,25(OH)2D levels in HIV-positive women, 19.4 ± 7.2; 1,25(OH)2D levels in healthy women, 47.3 ± 9.1; 25(OH)D levels in HIV-positive women, 37.3 ± 7.9; 25(OH)D levels in healthy women, 61.5 ± 8.4.	Lumbar osteoporosis was found in 7 patients (14%) versus 0 controls; lumbar osteopenia was diagnosed in 31 (62%) patients and 2 (4%) controls. There was significant correlation between the CD4 counts and 1,25(OH)2D levels. Neither the CD4 counts nor the duration of disease correlated with BMD

Table 1: Continued.

Authors (year), journal	Nation	Patients	Results	Comments
Etminani-Esfahani, Khalili et al. (2012), Current HIV Research	Iran	98 HIV-positive patients.	86.7% had 25(OH)D <35 nmol/L.	Female sex, unemployment, and human hepatitis C coinfection were related to the severe serum vitamin D deficiency.
Bajaj, Misra et al. (2012), Indian Journal of Endocrinology and Metabolism	India	45 HIV-positive patients. 45 healthy controls.	93.33% patients had 25(OH)D <30 ng/mL. 73.33% patients had 25(OH)D <30 ng/mL.	51.11% patients had dyslipidemia compared to 15.55% of controls. A positive association was proved between CD4 levels and 25(OH)D. No significant difference was seen in carotid intima-media thickness in cases and controls.
Conrado, Miranda-Filho Dde et al. (2011), Journal of the International Association of Providers of AIDS Care (Chic)	Brazil	214 HIV-positive female patients on cART.	40.65% patients had 25(OH)D <30 ng/mL.	Multivariate analysis proved that hypercholesterolemia and cART ≥3 years were positively associated with 25(OH)D deficiency, whereas there was an inverse statistically significant correlation with total cholesterol.
Wiboonchutikul, Sungkanuparph et al. (2012), Journal of the International Association of Providers of AIDS Care (Chic)	Thailand	178 HIV-positive patients.	44.9% had 25(OH)D <30 ng/mL and 26.8% had 25(OH)D <20 ng/mL.	Efavirenz intake was significantly associated with low vitamin D status. The mean 25(OH)D levels in patients receiving and not receiving EFV were, respectively, 22.9 and 28.6 ng/mL.
Conesa-Botella, Goovaerts et al. (2012), International Journal of Tuberculosis and Lung Disease	Uganda	92 HIV-positive TB-positive patients (G1). 20 only HIV-positive TB-negative patients (G2). HIV-negative TB-positive. 23 HIV-negative TB-negative patients (G4).	41% patients of G1 had 25(OH)D <75 nmol/L. 35% patients of G2 had 25(OH)D <75 nmol/L. 37% patients of G3 had 25(OH)D <75 nmol/L. 65% patients of G4 had 25(OH)D <75 nmol/L.	The authors reported that the prevalence of optimal vitamin D status was relatively high in HIV-infected patients with and without TB living in Uganda near the equator.
Mastala, Nyangulu et al. (2013), PLoS One	Malawi	69 HIV-positive of 157 TB negative patients.	23.1% of HIV-positive patients had 25(OH)D <50 nmol/L.	25(OH)D deficiency seemed more common in TB patients than non-TB patients. No significant correlation was found with HIV-status.
Rwebembera, Sudfeld et al. (2013), J Trop Pediatr	Tanzania	191 HIV-exposed uninfected infants.	48.7% had 25(OH)D <30 ng/mL. 34.6% had 25(OH)D <20 ng/mL.	25(OH)D deficiency was associated with sampling during the rainy season and infant wasting, whereas infant breastfeeding, maternal CD4 T-cell count, maternal wasting status, and maternal receipt of cART were not associated.
Havers et al. (2014) [24], The Journal of Infectious Diseases	US and 8 resource-limited countries	411 patients from PEARLS trial.	49% had 25(OH)D <32 ng/mL.	25(OH)D deficiency ranged from 27% in Brazil to 78% in Thailand. It was associated with high body mass index, winter/spring season, country-race group, and lower viral load. In addition, baseline low 25(OH)D was associated with increased risk of HIV progression, death, and virologic failure after cART.

1,25(OH)(2)D: 1,25-dihydroxyvitamin D; 25(OH)D: 25-hydroxyvitamin D; AIDS: acquired immune deficiency syndrome; cART: combined antiretroviral therapy; BMD: bone mass density; HAART: highly active antiretroviral therapy; NNRTI: nonnucleoside reverse-transcriptase inhibitor; PI: protease inhibitor; PTH: parathyroid hormone; US: United States; TB: tuberculosis; TNF-alpha: tumor necrosis factor-alpha; UK: United Kingdom.

associating efavirenz (EFV). Unlike what was just reported for PIs, EFV seems to increase 25(OH)D catabolism and production of inactive metabolites, through the interaction with cytochrome P450 enzymes, some of which may affect vitamin D metabolism (i.e., induction of CYP24A1 [31, 32] and reduced transcription of CYP2R1), similar to the effects of antiepileptic drugs [33]. This hypothesis has been supported by several *in vivo* studies, which described an association between NNRTIs, especially EFV and nevirapine (NVP) use and low 25(OH)D levels (Table 2).

The weakness of most reported studies is the cross-sectional design, so that causal relationships cannot be inferred. These data suggest the need for large prospective studies, properly designed to evaluate the specific effects and clinical impact of antiretroviral drugs on vitamin D status.

5. Association between HIV, Hypovitaminosis D, and Cardiovascular Disease

Several studies have described the association between HIV and increased risk of CVD (cardiovascular disease) [34, 35]. HIV infection itself is considered an independent risk factor for atherosclerosis: the prevalence of carotid intima-media thickness (cIMT), atherosclerosis, and myocardial infarction is higher among HIV-positive subjects, occurring earlier compared to uninfected individuals [36, 37]. In these patients atherogenesis is enhanced by several factors: HIV-induced chronic inflammation and immune activation (demonstrated by increased levels of proinflammatory cytokines and endothelial activation markers), excess of traditional risk factors (e.g., 2 to 3 times higher prevalence of smoking), and antiretroviral drug-related dyslipidemia, hyperglycemia, central obesity, and lipodystrophy (especially with PIs) [38–40]. To make this framework even more complex, 1,25(OH)2D deficiency has been linked to CVD in the general population [41, 42]. Vitamin D influences cardiovascular health by suppressing the renin-angiotensin system and stimulating cellular proliferation and differentiation via 1,25(OH)2D binding to vitamin D receptors in the heart, the endothelium, and the vascular smooth muscle [43, 44].

5.1. cIMT, Brachial Artery Flow-Mediated Dilation, Coronary Artery Calcium (or Calcification), and Coronary Artery Stenosis. Considering the high prevalence of both hypovitaminosis D and CVD in patients with HIV, the evidence of a relationship between low 25(OH)D and silent and symptomatic atherosclerosis is not surprising. Even though in the general population asymptomatic CVD, as demonstrated by cIMT, brachial artery flow-mediated dilation (FMD, an early marker of endothelial dysfunction), and CAC (coronary artery calcification), has been strongly linked to the occurrence of cardiovascular events and has also been independently associated with 25(OH)D deficiency, only a few studies are available in HIV-infected populations; moreover, none of these studies shows if 25(OH)D repletion might affect cardiovascular outcomes. The clinical characteristics of the populations, the study designs, and the variables included in the analysis of results could explain the differences among the studies [45–47] (Tables 3 and 4).

5.2. Other Risk Factors for CVD in HIV-Infected Subjects. Other traditional risk factors for CVD, such as insulin resistance and diabetes mellitus, are frequently seen in HIV-positive individuals [48], and, as in the general population [49], an association between vitamin D status and type 2 diabetes, but not with insulin resistance, has been described [50, 51]. A recent Italian cross-sectional study of 1811 HIV-infected persons, enrolled in the prospective Modena (Italy) HIV Metabolic Clinic Cohort, reported lower 25(OH)D levels in subjects with Type 2 diabetes, compared to those without diabetes ($P < 0.001$), although 25(OH)D deficiency was highly prevalent in both groups. In addition, although 25(OH)D deficiency was independently associated with diabetes (OR 1.85; CI 1.03–3.32, $P = 0.038$), the association with metabolic syndrome was not significant after adjusting for vitamin D supplementation, sex, age, and BMI (adjusted OR 1.32; 95% CI 1.00–1.75; $P = 0.053$) [50]. In the setting of HIV few data are available and the effects of vitamin D(3) supplementation on insulin sensitivity need to be evaluated with large, prospective studies. However, surprising results were provided by a small prospective study conducted by van den Bout-van den Beukel et al., which showed that cholecalciferol supplementation (2,000 IU/day for 14 weeks, 1,000 IU/day until 48 weeks) led to increased HOMA measured insulin resistance, after 24 weeks, whereas no differences were seen after 48 weeks [52]. It remains to be clarified whether the results are dose- or time-dependent, but this report further suggests the importance of clinical trials extensively evaluating the pros and cons of supplementing HIV-infected individuals with cholecalciferol [52].

6. Association between HIV, Hypovitaminosis D, and HIV Disease Progression

Preclinical experiments have demonstrated that treatment of peripheral blood mononuclear cells with 1,25(OH)2D decreased the cell susceptibility to HIV infection by inhibiting viral entry, modulating expression of CD4+ cell surface antigen, damping viral p24 production, and limiting monocyte proliferation [53, 54]. Thereafter, several observational studies have shown a significant association between higher levels of 25(OH)D and rates of immune recovery [15, 55, 56]. Along these lines, some authors investigated the association between vitamin D and clinical outcomes. Baseline 25(OH)D levels lower than 32 ng/mL were independently associated with progression to more advanced HIV stage among 884 HIV-infected pregnant women in Tanzania, who were followed for a median of 70 months. The women with 25(OH)D in the highest quintile had a 42% lower risk of all-cause mortality than the women in the lowest quintile [13]. The same authors demonstrated that 25(OH)D deficiency was also associated with low BMI, oral thrush, acute upper respiratory infections, and severe anemia [57]. However, other studies failed to demonstrate an association between

Table 2: Association between HAART use and low 25(OH)D levels.

Authors (year), journal	Nation	Patients	Results	Comments
Poowuttikul, Thomas et al. (2014), Journal of the International Association of Providers of AIDS Care	US	160 HIV-infected youth (45 no ART; 67 cART with tenofovir or EFV; 48 other cART).	25(OH)D in tenofovir/EFV group: 20.3 ± 18.1 ng/mL. 25(OH)D in other cART group 21.2 ± 16.8 ng/mL. 25(OH)D in no ART group 14.6 ± 7.3 ng/mL.	Severe vitamin D deficiency (25(OH)D ≤10 ng/mL) was related to lower CD4 counts and CD4% but not to HIV plasma RNA. EFV or tenofovir therapy did not have different effects on vitamin D levels compared to other antiretroviral medications.
Viard et al. (2011) [21], AIDS	31 European countries, Israel, and Argentina	1985 HIV-positive among EuroSIDA study group (180 naive, 155 ART, and 1650 cART).	36.6% naive had 25(OH)D <12 ng/mL. 39.3% ART had 25(OH)D <12 ng/mL. 35.5% cART had 25(OH)D <12 ng/mL. 38.8% naive had 25(OH)D <20 ng/mL. 32.2% ART had 25(OH)D <20 ng/mL. 30.4% cART had 25(OH)D <20 ng/mL.	25(OH)D deficiency was frequent in HIV-infected persons (83% on combined antiretroviral therapy) and was independently associated with a higher risk of mortality and AIDS events. Patients receiving a PI-based antiretroviral regimen were at low risk of hypovitaminosis D, whereas no significant association was found with EFV or tenofovir use.
Allavena, Delpierre et al. (2012), Journal of Antimicrobial Chemotherapy	France	2994 HIV-positive patients (334 cART naive versus 2660 exposed).	79.3% had 25(OH)D <30 ng/mL among ART naive. 67.6% had 25(OH)D <30 ng/mL among cART exposed.	In multivariate analysis cART, treatment was associated with vitamin D deficiency (aOR 2.61), together with current smoking, estimated glomerular filtration rate ≥90 mL/min/1.73 m^2, vitamin D measurement not performed in summer, and CD4 <350 cells/mm^3.
Theodorou et al. (2014) [29], Clinical Nutrition	Belgium	2044 HIV-infected subjects.	1500 (73.4%) patients under HAART. 1362 (74.7%) patients under HAART had 25(OH)D <30 ng/mL.	25(OH)D levels varied according the different combinations of cART ($P < 0.0001$). Median 25(OH)D levels in patients treated with 2 NRTI + 1 NNRTI and patients 2 NRTI + 1 PI were 12.5 ng/mL versus 14.3 ng/mL, respectively, ($P = 0.0001$).
Welz, Childs et al. (2010), AIDS	UK	755/1077 HIV-positive, patients under cART.	52.1% patients under cART had 25(OH)D <10 ng/mL.	EFV treatment was significantly associated with severe 25(OH)D reduction (OR: 2.0). Tenofovir (OR: 3.5) and EFV use OR: 1.6), but not severe 25(OH)D deficiency (OR: 1.1), was associated with increased bone turnover.
Cervero, Agud et al. (2012), AIDS Research and Human Retroviruses	Spain	352HIV-positive patients (37 cART naive versus 315 cART exposed).	95.2% had 25(OH)D <30 ng/mL among cART naive. 68.4% had 25(OH)D <30 ng/mL among cART exposed.	EFV exposure was associated with 25(OH)D deficiency ($P = 0.018$). Patients receiving PIs ($P = 0.014$) or NNRTI ($P = 0.025$) had higher odds of increased PTH levels; this was significant only in 25(OH)D deficient patients ($P = 0.004$).
Van Den Bout-Van Den Beukel et al. (2008) [52], AIDS Research and Human Retroviruses	Netherlands	252 HIV-positive patients.	25(OH)D levels in white NNRTI-treated patients: 54.5 (27.9–73.8) nmol/L; 25(OH)D levels in white PI-treated patients 77.3 (46.6–100.0) nmol/L. 25(OH)D levels in black NNRTI-treated patients: 22.0 (14.7–38.4) nmol/L. 25(OH)D levels in black PI-treated patients 29.0 (20.4–5) nmol/L.	Female sex, younger age, dark skin, and NNRTI treatment were significant risk factors in univariate analysis, although in multivariate analyses skin pigmentation remained the only independent risk factor.

Table 2: Continued.

Authors (year), journal	Nation	Patients	Results	Comments
Fox, Peters et al. (2011), AIDS Research and Human Retroviruses	12 countries in Europe	256 European patients taking EFV + 2NNRTI or PI + 2NNRTI.	25(OH)D on PI + 2NNRTI 41.6 (38.6, 44.5) nmol/L. 25(OH)D on EFV + 2NNRTI 35.0 (31.0, 39.1) nmol/L.	Lower baseline vitamin D levels were associated with EFV ($P = 0.0062$) and zidovudine ($P = 0.015$) use. The increase in 25(OH)D values in about 27% of patients who discontinued EFV ($P = 0.007$) was relevant.
Brown and McComsey (2010) [32], Antivirus Therapy	US	51 HIV patients under EFV-containing treatment. 36 HIV patients under non-EFV-containing treatment.	Median 25(OH)D level before cART 52.7 nmol/L 25(OH)D reduction in EFV-treated versus non-EFV-treated patients: −12.7 ± 3.7 nmol/L.	A significant decline in 25(OH)D serum levels after the initiation of an EFV-based regimen, compared to a non-EFV-based regimen ($P < 0.001$) in HAART patients was found. In addition, subjects receiving EFV had a 1.8-fold increased probability of developing vitamin D deficiency, compared to those starting PIs.
Conesa-Botella, Florence et al. (2010), AIDS Research and Therapy	Belgium	89 HIV-positive patients before and after 12-month HAART.	43.7% had 25(OH)D <20 ng/mL before HAART and 47.1% before 12-month HAART. 70.1% had 25(OH)D <20 ng/mL before HAART and 81.6% before 12-month HAART.	A 3-fold increased risk of 25(OH)D levels below 20 ng/mL was described in subjects receiving NNRTIs ($P = 0.02$) after 12 months of HAART.
Schwartz, Moore et al. (2014), Journal of the International Association of Providers of AIDS Care	US	507 HIV-negative subjects. 358 HIV-positive patients cART naive. 893 HIV-positive patients under cART.	72% HIV-negative subjects had 25(OH)D <20 ng/mL. 18% HIV-negative subjects had 25(OH)D <30 ng/mL. 70% HIV-positive patients ART naive had 25(OH)D <20 ng/mL. 20% HIV-positive patients ART naive had 25(OH)D <30 ng/mL. 57% HIV-positive patients under cART had 25(OH)D <20 ng/mL. 24% HIV-positive patients under cART had 25(OH)D <30 ng/mL.	EFV use in cART significantly reduced the 25(OH)D levels (15 versus 19 ng/mL; $P < 0.001$). Hypertriglyceridemia was present in HIV-infected under ART (13% versus 7% of HIV-infected cART and 5% of HIV-uninfected; $P < 0.001$), with a positive relationship between 25(OH)D levels and triglycerides ($P < 0.01$). No relationships could be found between 25(OH) and cholesterol. Vitamin D deficiency was not correlated to HIV status but influenced by HIV treatment.
Fux, Baumann et al. (2011), AIDS	Switzerland	262 HIV-positive patients starting HAART (EFV versus PIs).	40.6% under EFV had 25(OH)D <30 nmol/L after 1-year therapy and 25.0% under PIs had 25(OH)D <30 nmol/L after 1 year therapy.	EFV treatment was associated with lower 25(OH)D levels compared to PIs. CYP polymorphisms and black ethnicity may define patients in whom EFV treatment will cause clinically relevant 25(OH)D deficiency.
Pasquet, Viget et al. (2011), AIDS	France	352 HIV-positive patients under cART.	41.0% patients under cART had 25(OH)D <30 nmol/L.	Authors found an association between hypovitaminosis D and exposure to NNRTIs ($P = 0.05$) but not to EFV and NVP, probably because of a lack of statistical power of their analysis. However, considering the crude and adjusted coefficients for EFV and NVP in their regression models, the authors suggested a NNRTI class effect, rather than a specific EFV or NVP impact, on vitamin D levels.

Table 2: Continued.

Authors (year), journal	Nation	Patients	Results	Comments
Ryan, Dayaram et al. (2013), Current HIV Research	US	1368 naive HIV-positive patients (686 cART with RPV; 682 cART with EFV).	In EFV arm median 25(OH)D reduction after therapy was greater in older (−3.2 ng/mL) versus younger (−1.6 ng/mL). In RPV arm median 25(OH)D remained relatively unchanged for both older (0.8 ng/mL) and younger (−0.8 ng/mL).	Progression from insufficient (50–74 nmol/L) or deficient (25–49 nmol/L) at baseline to severely deficient (<25 nmol/L) 25(OH)D at week 48 after cART was 0% in older and 2% in younger under RPV, whereas it was 13% in older and 8% in younger under EFV.
Wohl et al. (2014) [30], Antivirus Therapy	US	690 naive HIV-positive patients (345 cART with RPV; 345 cART with EFV).	In EFV arm median 25(OH)D reduction after 48-week therapy was (−2.5 ng/mL). In RPV arm median 25(OH)D reduction after 48-week therapy was (−0.2 ng/mL).	Patients with severe 25(OH)D deficiency were 5% in both groups at baseline but were significantly higher in EFV group at 48 weeks (9% versus 5%, $P = 0.032$). In addition, the patients with 25(OH)D insufficiency/deficiency at baseline, the ones who received EFV, developed more frequently severe 25(OH)D deficiency (8% versus 2%, $P = 0.0079$).
Viani, Peralta et al. (2006), The Journal of Infectious Diseases	US and Puerto Rico	303 HIV-positive patients under cART (102 received vitamin D supplementation, the others placebo).	At baseline, 54% had 25(OH)D <20 ng/mL. 45% of treatment group had 25(OH)D <20 ng/mL. 93% of treatment group had sufficient 25(OH)D levels after 12 weeks of therapy.	Oral vitamin D supplementation (50,000 IU monthly) increased 25(OH)D serum concentration from a baseline of 21.9 (13.3) to 35.9 (19.1) ng/mL after 12 weeks ($P < 0.001$) with no change for placebo. Although use of the antiretroviral efavirenz was associated with lower baseline 25-OHD concentration, efavirenz did not diminish the response to vitamin D supplementation. No toxicity was revealed.

1,25(OH)(2)D: 1,25-dihydroxyvitamin D; 25(OH)D: 25-hydroxyvitamin D; ART: antiretroviral therapy; cART: combined antiretroviral therapy; EFV: efavirenz; HAART: highly active antiretroviral therapy; NNRTI: nonnucleoside reverse-transcriptase inhibitor; NRTI: nucleoside reverse-transcriptase inhibitor; PI: protease inhibitor; RPV: rilpivirine; US: United States.

Table 3: Carotid intima-media thickness (cIMT) and hypovitaminosis D in HIV patients.

Authors (year), journal	Nation	Patients	Results	Comments
Bajaj, Misra et al. (2012) Indian Indian Journal of Endocrinology and Metabolism	India	45 HIV-positive patients and 45 controls.	93.33% patients had 25(OH)D <30 ng/mL. 73.33% controls patients had 25(OH)D <30 ng/mL. cIMT 6 mm, 51.11% patients. cIMT 7 mm, 15.55% patients. cIMT 8 mm, 13.33% patients. cIMT >8 mm, 0% patients.	No significant difference in cIMT was proved between HIV-positive patients and controls ($P = 1.00$). A positive association was seen between CD4 levels and 25(OH)D.
Ross et al. (2011) [16], Antiviral Therapy	US	149 HIV-positive patients (56 with carotid IMT), 34 controls.	5% patients had 25(OH)D <25 nmol/L. 46% patients had 25(OH)D <50 nmol/L. Mean icIMT in HIV-patients: 0.70 (0.55–0.91). Mean ccIMT in HIV-patients: 0.65 (0.55–0.75).	Authors observed a 10.62 higher probability of having cIMT above the median value in HIV-infected adults with 25(OH)D values below 30 ng/mL ($P = 0.01$). Vitamin D status was associated with CD4+ T-cell restoration after antiretroviral therapy but not with the inflammatory and endothelial activation markers, soluble TNF-α receptor 1 (sTNFR-1), and soluble intercellular adhesion molecule-1 (sICAM-1), associated with atherosclerosis and CVD development in the general population.
Choi et al. (2011) [45], Clinical Infectious Diseases	US	139 HIV-positive patients.	52% had 25(OH)D <30 ng/mL. Mean cIMT in patients with 25(OH)D >30 ng/dL: 0.87 mm. Mean cIMT in patients with 25(OH)D <30 ng/dL: 1.0 mm. Mean cIMT in patients with 25(OH)D <15 ng/dL: 1.1 mm.	An association between vitamin D insufficiency and cIMT, even after adjusting for age, sex, tobacco use, hypertension, and elevated cholesterol, was proved. The authors found that mean cIMT was 0.13 mm greater in vitamin D insufficient subjects than in normal subjects.
Eckard et al. (2013) [12], The Pediatric Infectious Disease Journal	US	30 HIV-positive patients, 31 controls.	72% patients versus 87% controls had 25(OH)D <20 ng/mL. 21% patients versus 13% controls had 25(OH)D <30 ng/mL.	After adjusting for season, sex, and race, there was no difference in serum 25(OH)D between groups ($P = 0.11$). Serum 25(OH)D was not significantly correlated with cIMT ($P = 0.34$). In HIV-infected group, 25(OH)D was negatively correlated with HOMA-IR, HIV duration, and cumulative duration of ART, NRTI, and NNRTI duration.
Portilla et al. (2014) [46], Journal of the International AIDS Society	Spain	89 HIV-positive patients (75 on ART).	80.8% had 25(OH)D <75 nmol/L. Bilateral mean cIMT in 25(OH)D deficient 0.63 ± 0.08 versus not deficient 0.56 ± 0.06 ($P = 0.09$).	High prevalence of 25(OH)D (80.9%) was found. Authors found no association between 25(OH)D insufficiency, inflammatory, or endothelial dysfunction markers and cIMT, whereas this was found between cIMT and patient age, impaired fasting glucose, and PI therapy length.

25(OH)D: 25-hydroxyvitamin D; ART: antiretroviral therapy; ccIMT: common carotid intima-media thickness; cIMT: carotid intima-media thickness; US: United States; icIMT: internal carotid intima-media thickness; CVD: cardiovascular diseases; HOMA-IR: homeostasis model assessment of insulin resistance; NNRTI: nonnucleoside reverse-transcriptase inhibitor; NRTI: nucleoside reverse-transcriptase inhibitor.

Table 4: Brachial artery flow-mediated dilation, coronary artery calcium (or calcification) and hypovitaminosis D in HIV patients.

Authors (year), journal	Nation	Patients	Results	Comments
Lai et al. (2013) [47], Vascular Health and Risk Management	US	846 HIV-infected African-American participants.	28.1% had CAC.	Logistic regression analysis revealed the factors independently associated with CAC: age, male sex, family history of CAD, years of cocaine use, total cholesterol, high-density lipoprotein cholesterol, PI treatment length, and, finally, vitamin D deficiency.
SShikuma, Seto et al. (2012), AIDS Research and Human Retroviruses	US (Hawaii)	100 patients of the HIV-Cardiovascular Cohort Study	Median 25(OH)D: 27.9 ng/mL. CAC was present in 53%.	A significant correlation was observed between 25(OH)D levels and FMD ($P = 0.01$) but not with cIMT ($P = 0.76$). Lower 25(OH)D levels were at slightly higher risk of having CAC ($P = 0.04$); these lower 25(OH)D levels were not associated with higher CAC scores ($P = 0.36$).
Gepner, Ramamurthy et al. (2012), PLoS One	US	114 healthy postmenopausal women (54 treated with vitamin D supplementation and 57 with placebo).	Median pretreatment 25(OH)D 30.3 in treatment group, 32.3 in placebo group. FMD pretreatment 0.018 in treatment group, 0.016 in placebo group. FMD posttreatment 0.001 in treatment group, 0.001 in placebo group.	Authors proved no improvement in endothelial function, arterial stiffness (as measured by brachial artery FMD, carotid-femoral pulse wave velocity, and aortic augmentation index), or inflammation markers after vitamin D supplementation in the general population.
de Boer, Kestenbaum et al. (2009), Journal of the American Society of Nephrology	US	1370 HIV-negative patients (394 with and 976 without CKD).	53% had CAC at baseline (65% with CKD and 48% without CDK). 21% of subjects who do not have CAC at baseline developed it during 3-year follow-up.	Lower 25(OH)D concentration was associated with increased risk for CAC development; each 10 ng/mL 25(OH)D reduction there was a 23% increased risk ($P = 0.049$).

25(OH)D: 25-hydroxyvitamin D; CAC: coronary artery calcification; cIMT: carotid intima-media thickness; CKD: chronic kidney disease; FMD: artery flow-mediated dilation; PI: protease inhibitor; US: United States.

25(OH)D level and clinical outcome, as in the above mentioned study by Sherwood et al. [58].

7. Association between HIV, Hypovitaminosis D, and Hepatitis C

HCV (hepatitis C virus) infection occurs at a significantly higher rate in HIV-infected persons compared to the general population, and this is especially problematic for resource-limited settings, where HCV treatment is generally not easily available [59]. HIV has a negative impact on the natural history of HCV, and, compared to HCV monoinfected patients, HIV/HCV coinfected patients have a more rapid progression from chronic active hepatitis to liver-cirrhosis, end-stage liver disease, liver cancer, and death, as well as lower response rate to traditional HCV treatment [60, 61]. Male sex, insulin resistance, acquiring HCV at an older age, heavy alcohol consumption, HCV genotype 3, and low CD4+ cell count are the factors contributing to the rapid development of liver fibrosis/cirrhosis among HIV/HCV coinfected patients [62, 63]. Other studies of HCV monoinfected patients have shown an independent association of 25(OH)D deficiency with severe liver fibrosis and treatment failure [64].

1,25(OH)2D effects on the immune system and inflammatory response have been shown to directly inhibit the proliferation and profibrotic effect of hepatic stellate cells [65]. Not surprisingly, liver fibrosis is associated with low serum levels of 25(OH)D during both HBV- and HCV-related chronic hepatitis, in both HIV-coinfected and not-coinfected patients [66]. However, low levels of 25(OH)D have been found in HBV or HCV carriers with minimal or absent liver fibrosis compared to healthy subjects [67].

On the other hand, in HIV-HCV coinfected patients, studies investigating the association between HCV sustained virologic response and vitamin D level have reported varying results, with some studies demonstrating an association [68], whereas other studies do not [69]. Mandorf et al. demonstrated that serum levels of 25(OH)D may predict the response to anti-HCV therapy. Suspicion of such a connection is strengthened by the evidence that cholecalciferol supplementation improves early and sustained virological

response (94% versus 48% in controls and 86% versus 42% in controls, resp.) in HCV genotype 1 patients treated with Peg-IFN/ribavirin [70, 71]. The exact mechanism of its antiviral (anti-HCV) effect is unknown, although it was recently shown to amplify the innate antiviral immune response upregulating IFN-β and the MxA (an IFN-induced human protein) gene expression and dampening interferon gamma-induced protein 10 (IP-10) expression [72].

8. Association between HIV, Hypovitaminosis D, and Tuberculosis

According to the World Health Organization approximately 2 billion people are exposed to *M. tuberculosis*, 8 million people per year are infected, and 2 million people die as a clinical outcome [73]. HIV is the strongest factor in the development of active TB (tuberculosis), and its spread has fuelled the resurgence of the TB epidemic. It has been proposed that in HIV infection *M. tuberculosis* escapes the local immune response within the granulomas, decreasing their containing ability and then leading to increased mycobacterial replication, dissemination, and clinical disease [74]. The rise in CD4+ count and improved immune function after HAART initiation partially restore pathogen specific immunity. In the general population, 25(OH)D levels lower than 4 ng/mL were shown to cause a 3-fold probability of having active TB [75], with higher risk of developing MDR *M. tuberculosis* infection [76]. A cross-sectional study of 174 HIV-infected and 196 HIV-uninfected individuals in Cape Town, South Africa, showed that 25(OH)D deficiency is independently associated with active TB and this association is greater in HIV-infected subjects [77]. A prospective Tanzanian cohort study enrolled 1103 HIV-infected persons initiating HAART in a randomized controlled trial (RCT) of vitamin D-free multivitamin supplementation. Baseline 25(OH)D levels lower than 20 ng/mL, but not 25(OH)D insufficiency, were associated with higher incident smear-positive TB, after a median follow-up of 20.6 months, wasting, and >10% weight loss but not with risk of malaria, pneumonia, or anaemia. Mortality hazard ratio was 2.0 for those with levels below 20 ng/mL versus those with levels above 30 ng/mL over 24 months. Reverse causality (i.e., that vitamin D deficiency occurred as a result of TB) was ruled out in this study by the exclusion of patients who developed TB within 1 month of enrolment. This finding is significant, since TB itself might contribute to vitamin D deficiency by reducing a patient's sun exposure or increasing consumption of 25(OH)D by activated macrophages [78]. Recently a systematic review was conducted to analyse studies published from 1980 to 2006 with data on serum 25(OH)D in pulmonary TB patients and controls. Five out of seven case-control studies, with a total of 531 participants, reported lower serum 25(OH)D in cases compared to controls. Several weaknesses were found: the sample sizes were small, ranging between 30 and 145 participants; some studies did not use culture for diagnosing TB; some studies included extrapulmonary TB; selection of controls was not optimal [79].

9. Association between HIV, Hypovitaminosis D, Chronic Inflammation, and Malignancy

9.1. Chronic Inflammation. HIV infection is associated with chronic inflammation (i.e., elevated TNF, IL-6, and CRP) and immune system activation (i.e., increased soluble CD14 and CXCL10), even after achieving full virologic suppression and immune recovery with the use of HAART [80, 81]. In this population, elevation of inflammation markers has been shown to be independent predictors of neurocognitive impairment, frailty, cardiovascular events, diabetes and metabolic syndrome, low BMD, malignancies, and all-cause mortality [82–85]. The same outcomes, including all-cause mortality, were also associated with chronic inflammation in the general population [86]. Thus, there seems to be a considerable overlap in the outcomes associated with 25(OH)D deficiency and chronic inflammation, in both the HIV-infected and HIV-uninfected populations.

9.2. Malignancy. Association of vitamin D deficiency with risk of cancer in the HIV-infected population remains to be determined. However, it has already been shown in the general population, including breast cancer (4-fold risk) [87], colon cancer (2-fold risk) [88], ovarian cancer (4-fold risk) [89], and prostate cancer (3-fold risk) [90]. However, to date, there is only one study that tried to correlate 25(OH)D deficiency, HIV, and cancer. Erlandson et al. enrolled 90 HIV-infected patients with AIDS-associated Kaposi's sarcoma (KS) from Zimbabwe, in a prospective pilot study investigating the effect of antiretroviral therapy on the natural history of this neoplasm. The authors demonstrated that 25(OH)D insufficiency was common and HIV-1 RNA was significantly higher in those with insufficient 25(OH)D; in contrast, tumor response, survival, and KS-associated immune reconstitution inflammatory syndrome (defined as any progression of KS occurring ≤12 weeks after initiation of HAART) were generally associated with an increased CD4+ lymphocyte count of at least 50 cells/mL above the baseline value, before or at the time of documented KS progression, and were not associated with 25(OH)D status [91].

10. Management of Hypovitaminosis D in HIV-Positive Individuals

10.1. Screening. The main arguments in favor of routine screening of vitamin D in HIV-infected patients include the potential optimization of skeletal, metabolic, and immunologic parameters with vitamin D supplementation. The arguments against routine screening include assay variability and costs, lack of a clear target range, absence of proven supplementation benefits, apart from the benefits connected with osteoporosis as in the general elderly population, limited randomized clinical trial data in HIV-infected patients, inability to distinguish the effects of vitamin D and calcium supplementation on bone, potential harm from some supplementation approaches, and increased pill regimen (possible reduction of patient compliance).

The European AIDS Clinical Society most recent guidelines suggest vitamin D status evaluation in patients with a history of low BMD or fracture, those with high risk of fracture, or those with other vitamin D deficiency associated factors (e.g., persons receiving some antiretroviral drugs, including EFV). Vitamin D replacement is recommended when 25(OH)D is lower than 10 ng/mL; for values ranging between 10 and 20 ng/mL, supplementation is recommended only for patients with osteomalacia, osteoporosis, or increased PTH [92]. McComsey et al. developed recommendations for bone disease in HIV infection, addressing vitamin D deficiency as well. They recommend 50,000 IU of cholecalciferol weekly for 8 to 12 weeks and then monthly thereafter or 2,000 IU daily for 12 weeks and then 1,000 to 2,000 IU daily thereafter. 25(OH)D levels after replacement should be measured. They recommend supplementation to achieve 25(OH)D greater than 32 ng/mL [93].

10.2. Supplementation. In the general population, recommendations regarding vitamin D supplementation are mostly derived from studies on bone health. Several large RCTs found beneficial effects of vitamin D *plus* calcium on BMD and fracture risk [94]. Meta-analyses showed that vitamin D (cholecalciferol) *plus* calcium association is superior to the use of a single drug in fracture prevention [94]. Unfortunately, the evidence for vitamin D use in clinical outcomes beyond skeletal health (i.e., on falls, CVD, diabetes, metabolic syndrome, immune response, and cancer) is inconsistent with and insufficient to base general recommendations.

10.2.1. Vitamin D Supplementation Dosage. In the general population, current recommended vitamin D oral supplementation is 800–1,000 IU cholecalciferol/day, plus calcium 1000 mg to 1200 mg daily. Serum 25(OH)D levels generally increase by approximately 1 ng/mL for every 100 IU of cholecalciferol intake. Few data from small cohorts are available on the efficacy of cholecalciferol repletion in HIV-infected subjects [95]. However, in this subgroup, it seems that a daily intake of at least 1,000–2,000 IU/day may be needed to overcome vitamin D deficiency [29].

10.2.2. Safety of Vitamin D Supplementation. Groleau et al. demonstrated that supplementation with high vitamin D(3) doses and the concomitant increased serum 25(OH)D level did not correlate with increased whole blood lead concentration in HIV-infected children and young adults. Vice versa, the more robust increase in serum 25(OH)D after 12 weeks of vitamin D(3) supplementation for participants enrolled during winter and spring was accompanied by a decrease in whole blood lead concentration [96]. Animal studies show an inverse relationship between calcium intake and lead levels. This inverse relationship was also found in pregnant women, and calcium supplementation during pregnancy was associated with reductions in blood lead. Overall, the above data provide safety information when considering higher dose vitamin D intervention [97].

10.2.3. Extraskeletal Effects of Vitamin D Supplementation. There are only a few studies investigating the effect of cholecalciferol supplementation on other cardiovascular, metabolic, and immunological outcomes in the HIV-infected population. In an RCT involving 45 subjects with 25(OH)D lower than 20 ng/mL, 12-week supplementation with daily oral cholecalciferol 4,000 IU produced an increase of approximately 5 ng/mL in 25(OH)D level compared to placebo but did not result in a statistically significant change in brachial artery FMD. Moreover, in the study group, insulin resistance increased from baseline but it was not statistically different from the placebo arm; similarly, baseline inflammatory and coagulation markers (i.e., CRP, IL-6, sTNFR-1, ICAM, vascular cell adhesion molecule (VCAM), D-dimer, and fibrinogen) did not significantly change between the groups. These results could partly be attributed to the modest increase in 25(OH)D (5 ng/mL) in subjects receiving cholecalciferol supplementation [98]. In an RCT involving 52 mostly virologically suppressed vertically infected youths aged 8 to 26 years with 25(OH)D lower than 30 ng/mL, Giacomet et al. showed that 12-month supplementation with cholecalciferol 100,000 IU every 3 months resulted in reduction of anti-inflammatory T-cell phenotype (i.e., decrease in $T_H17 : T_{reg}$ ratio) at 3 months. This effect was no longer seen at 12 months. No significant change in baseline CD4+ cell count was observed between the treatment and placebo arms [99].

Conflict of Interests

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

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Review Article

Vitamin D and Inflammatory Bowel Disease

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Vitamin D deficiency has been recognized as an environmental risk factor for Crohn's disease since the early 80s. Initially, this finding was correlated with metabolic bone disease. Low serum 25-hydroxyvitamin D levels have been repeatedly reported in inflammatory bowel diseases together with a relationship between vitamin D status and disease activity. Subsequently, low serum vitamin D levels have been reported in various immune-related diseases pointing to an immunoregulatory role. Indeed, vitamin D and its receptor (VDR) are known to interact with different players of the immune homeostasis by controlling cell proliferation, antigen receptor signalling, and intestinal barrier function. Moreover, 1,25-dihydroxyvitamin D is implicated in NOD2-mediated expression of defensin-$\beta 2$, the latter known to play a crucial role in the pathogenesis of Crohn's disease (IBD1 gene), and several genetic variants of the vitamin D receptor have been identified as Crohn's disease candidate susceptibility genes. From animal models we have learned that deletion of the VDR gene was associated with a more severe disease. There is a growing body of evidence concerning the therapeutic role of vitamin D/synthetic vitamin D receptor agonists in clinical and experimental models of inflammatory bowel disease far beyond the role of calcium homeostasis and bone metabolism.

1. Introduction

Vitamin D is a fat-soluble vitamin whose active form, calcitriol or 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), regulates bone, calcium, and phosphorus metabolism [1]. However, vitamin D also influences immune system function, and deficiency has been recognized as an environmental risk factor for autoimmune diseases like Crohn's disease (CD) [2].

In humans, vitamin D may be obtained from two sources: diet (as fat-soluble vitamin) and by ultraviolet- (UV-) mediated synthesis in the epidermal layer of the skin where UV-rays promote photolytic cleavage of 7-dihydrocholesterol (7-HDC) into vitamin D_3 [3]. The latter is the most important source of this metabolite and, at this point, vitamin D can be considered as a hormone [4]. After production, vitamin D is activated by a two-step hydroxylation, first in the carbon 5-position by 25-hydroxylase in the liver then by 1α-hydroxylase in the kidney: this active metabolite exerts its functions by interacting with the vitamin D receptor (VDR), a receptor that belongs to the superfamily of nuclear hormone receptors [1]. Binding to VDR leads to the transcription of several vitamin D-response genes, located on single loci [5]. Various tissues and, especially, immune-related cells express VDRs and are able to produce 1,25$(OH)_2D_3$. This implies that the vitamin exerts its action beyond its classic hormonal-endocrine function tending towards an autocrine role [6].

2. Vitamin D and Its Role in Immune Regulation

Vitamin D affects the immune system acting at various levels, such as antibacterial response, antigen presentation, and regulation of adaptive and innate immunity. Genome-wide analysis has revealed that a large number of genes are influenced by vitamin D levels [7]. VDRs have been discovered in almost all immune cells as activated or naïve $CD4^+$ and $CD8^+$ T cells, B cells, neutrophils, and antigen-presenting

cells (APCs) such as dendritic cells and macrophages. In particular, vitamin D_3 enhances the chemotactic and phagocytic responses of macrophages and production of antimicrobial proteins, such as cathelicidin, inhibits the surface expression of the MHC-II-complex antigen and costimulatory molecules and downregulates the production of many proinflammatory cytokines, such as interleukin- (IL-) 1, IL-6, IL-8, and TNF-α [4, 8]. An experimental study demonstrated that transferring $CD8^+$ T cells isolated from the spleen of wild type (WT) and IL-10 KO mice into immunodeficient Rag KO recipients, that is, mice with no mature B or T cells, did not induce colitis, whereas transferring $CD8^+$ T cells from VDR KO mice led to colonic inflammation, and transferring $CD8^+$ T cells from IL-10/VDR KO mice led to fulminant colitis. These data indicate that expression of VDR is required to prevent replication of quiescent $CD8^+$ T cells and that the lack of VDR induced the formation of more aggressive T cells [9]. Another study evaluated the difference of protein expression in the small intestinal mucosa between WT mice and VDR KO mice identifying a higher expression of proteins involved in cell adhesion, proliferation, and migration and stress response in VDR KO mice. The authors conclude that vitamin D and VDR play a direct, or indirect role, in balancing these functions [10].

Vitamin D/VDR status regulates development, function, and balance of T-lymphocytes dampening T-helper- (Th-) 1 cell function and cytokine patterns (IL-2 and interferon-γ (IFN-γ)) by enhancing the Th-2 cell response (IL-4, IL-5, and IL-10) [11]; moreover $1,25(OH)_2D_3$ promotes a regulatory outcome through the inhibition of Th-17 cells and their related cytokines, and the induction of regulatory T cells (Treg) that are protective against autoimmunity, stimulating the expression of the cytotoxic T-associated protein 4 (CTLA-4) and forkhead box P3 (Foxp-3), together with the induction of IL-10 [12, 13]. In addition, $1,25(OH)_2D_3$ appears to have a chemopreventive role through an antiproliferative action, for example, through VDR-mediated inhibition of the Wnt/beta-catenin pathway [8, 14, 15], inhibiting growth without inducing apoptosis and inducing differentiation in colon cancer cell lines [16, 17].

The molecular and genetic link between CD and the vitamin D/immune system axis may be in part explained by the NOD2 gene (Figure 1). The precise etiology of the inflammatory bowel disease CD is unknown. Like many chronic diseases, there are environmental factors that act on a polygenic background. Variants of the NOD2/CARD15 gene are associated with the development and phenotypic patterns of CD. This gene encodes for a protein of the family of intracellular pattern recognition receptors for bacterial components that play an important role in the innate immune system [18, 19]. Transcription of the NOD2 gene is stimulated by $1,25(OH)_2D_3$/VDR and signaling through NOD2 induces expression of DEFB2/HBD2 which stands for the antimicrobial peptide beta-defensin 2, and of CAMP which codifies for cathelicidin [20]. In a study on a VDR KO model, a downregulation of the ATG16L1 gene, together with a reduced expression of lysozyme by Paneth cells was reported [21]. These mice had an increased susceptibility to dextran sulfate sodium (DSS) colitis, whereas in human colon samples of low VDR expression correlate with ATG16L1 and a reduction of *Bacteroides* species. This finding implies that alterations of the vitamin D status might interfere with autophagy and alter the antimicrobial barrier of the intestinal mucosa and, consequently, the control of the microbiota [22].

3. VDR Polymorphisms in IBD

From the above, it appears that variants of VDR interfere with the immune system and, thus, may contribute to susceptibility to inflammatory bowel disease (IBD) [23, 24]. In fact, VDR polymorphisms have been identified in various diseases, such as cancer [25] or cancer risk [26], asthma [27], and kidney diseases [28]. The best-studied polymorphisms include BsmI (rs1544410), FokI (rs2228570), TaqI (rs731236), and ApaI (rs7975232). However, the results of these still few studies in IBD patients are contradictory (Table 1): for example, no statistical significance compared to controls was found in two studies on IBD patients for BsmI, FokI, TaqI, and ApaI [29, 30] with a borderline significance for heterozygous carriage of the FokI allele [29]. In three Chinese studies on ulcerative colitis (UC) patients [31, 32] and on CD patients, no difference [32] or an association of the Bb genotype of the BsmI variant with UC [31] was reported; whereas no association was found for ApaI, TaqI, and BsmI with CD [33].

In another study on European Caucasian patients, a significantly higher frequency of the TaqI polymorphism (genotype "tt") was reported in CD compared to UC or HC [23]. This finding was replicated in German IBD patients where the "tt" genotype was significantly more frequent in fistulizing and stenosing CD [24]. Subsequently, always in Caucasians, the finding of a lack of association of ApaI but a more frequent presence of TaqI in male IBD patients was reported [34] and confirmed 3 years later [35].

Concerning BsmI polymorphisms, the BB genotype was more frequent in Ashkenazi UC patients compared to Ashkenazi controls [36]. Finally, in a mixed IBD population investigating all 4 VDR variants, only the Fok I variant ("ff" genotype) was significantly more frequent in IBD patients [37].

Two recent meta-analyses including the same 9 studies with slightly different patient numbers (Table 1) yielded different results [38, 39]; Xue et al. [38] found that the "ff" genotype of FokI was associated with a significant risk for UC in Asians, whereas the "tt" genotype of TaqI was associated with an increased risk for CD in Europeans, but with an increased risk for both diseases, CD and UC, in Asian males. Carriage of the "a" allele (ApaI) resulted protective from CD. In contrast, Wang et al. [39] concluded that there was no association between ApaI, BsmI, and FokI and IBD, whereas subgroup analysis evidenced an increased risk for CD for ApaI and limited to East Asians, for BsmI. Conversely, TaqI variants reduced the risk for UC in Caucasians.

One study examined the influence of VDR polymorphisms on serum vitamin D levels [40] (not included in Table 1) showing a significant association of variants of the TaqI and the signal peptide, CUB domain, and EGF-like 3 (SCUBE3, rs732594) genes, the latter encodes for a protein involved in the VDR pathway, in CD patients, whereas ApaI and SCUBE3 and two variants of PHD finger protein-11

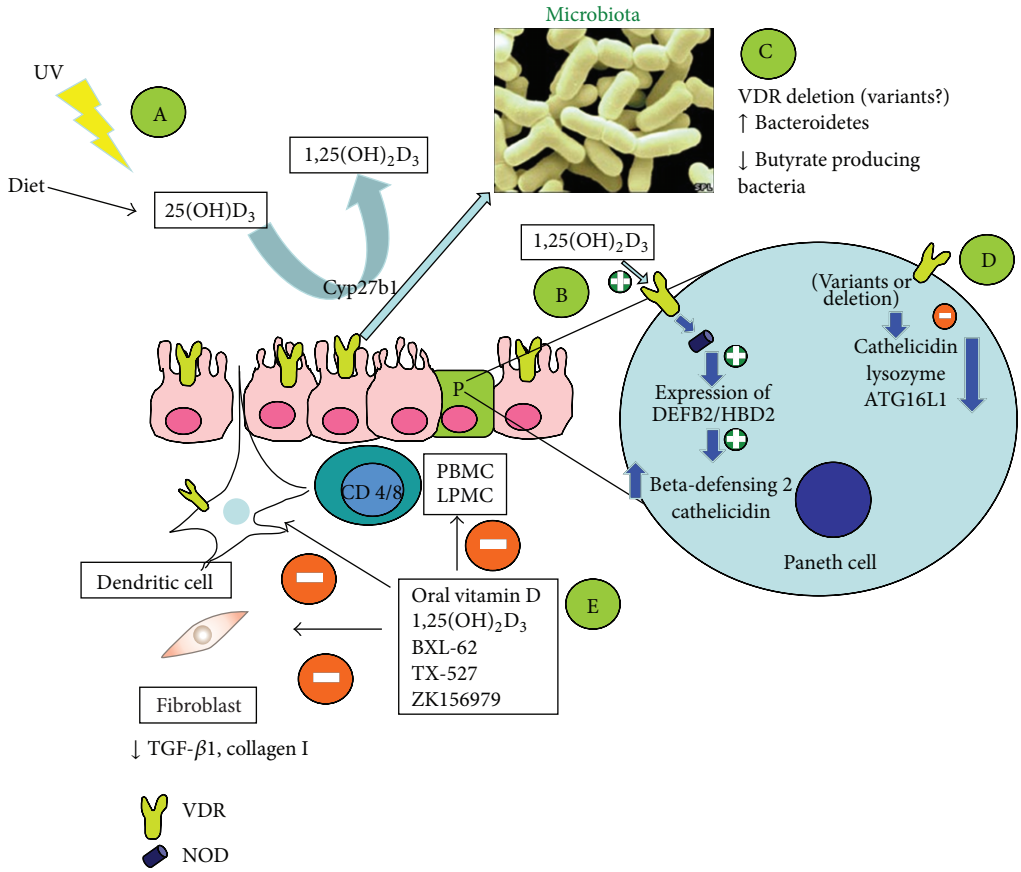

Figure 1: Potential involvement of vitamin D in the pathogenesis of inflammatory bowel disease and immunologic effects of vitamin-D-related therapeutic approaches. Scenario A: reduced UV exposure as risk factor for CD and for hospitalizations and surgery [86]; Scenario B: NOD2 gene transcription is stimulated by $1,25(OH)_2D_3$/VDR and signaling through NOD2 induces expression of DEFB2/HBD2 which stands for beta-defensin 2 and cathelicidin [20]; Scenarios C and D: variants or loss of function of VDR may lead to changes of the microbiota and reduce host defense by reducing production of cathelicidin, lysozyme, and ATG16L1 protein (autophagy) [21, 22]; Scenario E: experimental studies with vitamin D or its analogues showing inhibitory effects on PBMC, LPMC, dendritic cells, and fibroblasts in terms of cytokine production and differentiation (Table 3). VDR: vitamin D receptor; NOD: nucleotide-binding oligomerization domain.

(PHF-11) gene, namely, rs2980 and rs2981, showed a significant association with serum vitamin D levels in CD patients. PHF-11 variants have been shown to be involved with vitamin D levels in other pathologies, such as asthma [41].

Besides investigations on VDR variants, 2 SNPs of the vitamin D-binding protein (DBP), that is, the 416 variant Glu (rs7041) and the 420 variant Lys (rs4588), were analysed. A significantly reduced frequency of the 420 variant Lys was found in IBD patients compared to controls [42].

In conclusion, the influence of VDR variants on IBD risk is still poorly defined. Interesting approaches are represented by investigations on the association between polymorphisms and vitamin D levels and those examining proteins involved in vitamin D-related pathways, but all need further studies and confirmation.

4. Vitamin D Status and Related Risk Factors in IBD

Starting in the late seventies, investigations on the vitamin D status of IBD patients have been carried out with different methodological approaches and results. By comparing IBD patients (CD alone or mixed populations) versus healthy controls (HC), no differences were found for circulating $25(OH)D_3$ concentrations in 6 studies on adult IBD populations [43–48] and in 1 study on a pediatric cohort [49], whereas lower plasma levels were reported in undernourished CD patients [50], in CD patients after intestinal resections [51], in 2 studies on adult, and in 1 study on pediatric CD patients [52–54] and in 3 mixed IBD populations [55–57].

Comparing $25(OH)D_3$ levels between CD and UC patients, no differences were found in 8 studies on adult or pediatric patients in basal conditions [47, 54, 55, 57–61] and in 1 pediatric study on partially vitamin D supplemented patients [62]. Lower levels in CD compared with UC were found in 5 studies [46, 63–66].

Finally, investigations concerning the active form of vitamin D, $1,25(OH)_2D_3$, reported normal levels after bowel resections in CD [67] but no differences between well- and undernourished CD patients compared to HC or in well-nourished UC patients [50]. Similar findings were reported in a pediatric study including CD, UC and HC [61]. Lower

Table 1: Genetic polymorphisms and IBD (chronological order).

Author	Year	Population	Investigated gene polymorphisms	Main findings
Single- or multicenter studies				
Simmons et al. [23]	2000	England 158 UC, 245 CD, 164 CRADC	VDR: TaqI, ApaI, FokI	TaqI polymorphism ("tt" genotype) more frequent in CD compared to UC or controls
Martin et al. [24]	2002	Germany, 95 CD, 93 UC, 119 HC	VDR: TaqI	TaqI ("tt" genotype) significantly more frequent in fistulizing and stenosing CD
Dresner-Pollak et al. [36]	2004	Israel, 228 CD (129 Ashkenazi and 99 non-Ashkenazi), 151 UC (72 Ashkenazi, 79 non-Ashkenazi), 495 HC (352 non-Ashkenazi and 143 Ashkenazi)	VDR: BsmI	BB genotype more frequent in Ashkenazi UC compared to Ashkenazi HC
Noble et al. [34]	2008	United Kingdom, 286 CD, 154 UC, 240 HC	VDR: TaqI, ApaI	Overall no differences between CD, UC, and HC for TaqI and ApaI. TaqI variants more frequent in male IBD patients compared to (male) HC
Naderi et al. [37]	2008	Iran, 150 UC, 80 CD, 150 HC	VDR: ApaI, TaqI, BsmI, FokI	FokI polymorphism significantly higher in UC and CD. Frequency of polymorphic "f" allele and f/f genotype higher in UC and CD comparing with HC
Pluskiewicz et al. [30]	2009	Poland, 47 UC, 47 HC	VDR: TaqI, BsmI, ApaI	No differences between UC and HC
Hughes et al. [29]	2011	Ireland, 660 IBD, 699 HC	VDR: ApaI, TaqI, BsmI, FokI	Borderline significance for heterozygous carriage of the FokI allele
Pei et al. [31]	2011	China, 218 UC, 251 HC	VDR: ApaI, TaqI, BsmI, FokI	Only Bb genotype of the BsmI variant associated with UC; frequency of the BsmI polymorphic allele (B) increased in UC
Eloranta et al. [42]	2011	Switzerland, 404 CD, 232 UC, 248 HC	DBP: rs 7041, rs 4588	Significantly reduced frequency of the 420 variant Lys in IBD compared to controls
Bentley et al. [35]	2011	New Zealand, 449 CD, 448 UC, 482 HC	VDR: FokI, TaqI	No overall differences, only a higher minor allele frequency for TaqI, in male CD and UC compared to HC
Luo et al. [33]*	2013	China, 19 CD, 122 HC	VDR: ApaI, TaqI, BsmI	No significant differences in the frequencies of TaqI, BsmI, and ApaI polymorphisms
Xia et al. [32]	2014	China 382 UC, 489 HC	VDR: ApaI, TaqI, BsmI, FokI	No difference between UC and HC. The mutant allele C and genotype TC + CC of FokI were significantly increased in patients with mild and moderate UC compared to severe UC. The frequency of AAC haplotype was statistically lower in UC than HC (AAC haplotype formed by the VDR BsmI, ApaI, and TaqI gene might engender a reduced risk of UC attack)
Meta-analyses				
Xue et al. [38]	2013	ApaI: 1024 CD, 974 UC, 1551 HC FokI: 1187 CD, 1221 UC, 1746 HC BsmI: 721 CD, 813 UC, 1642 HC TaqI: 1568 CD, 1515 UC, 2152 HC	VDR: ApaI, TaqI, BsmI, FokI	FokI "ff" genotype associated with a significant risk for UC in Asians; TaqI "tt" genotype associated with an increased risk for CD in Europeans and with an increased risk for CD and UC in Asian males. ApaI "a" allele confers protection from CD

Table 1: Continued.

Author	Year	Population	Investigated gene polymorphisms	Main findings
Wang et al. [39]	2014	ApaI: 940 CD, 962 UC, 1468 HC FokI: 1098 CD, 1217 UC, 1676 HC BsmI: 713 CD, 799 UC, 1616 HC TaqI: 1553 CD, 1500 UC, 2145 HC	VDR: ApaI, TaqI, BsmI, FokI	ApaI, BsmI, and FokI are not significantly associated with IBD. Significant association between TaqI polymorphism and IBD risk. In subgroups, ApaI increases the overall CD risk and BsmI increases this CD risk only in East Asians, whereas TaqI reduces the risk for UC especially in Caucasians

CD: Crohn's disease; UC: ulcerative colitis; CRADC: cadaveric renal allograft donor controls; PCR: polymerase chain reaction; IBD: inflammatory bowel disease; HC: healthy controls; DBP: vitamin-D-binding protein; VDR: vitamin D receptor.
* Article in Chinese.

$1,25(OH)_2D_3$ concentrations compared to controls were found in 2 studies including CD and UC patients [45, 68] Conversely, elevated levels of $1,25(OH)_2D_3$ were reported after ileal resections in CD [51]. In this latter study, a positive correlation with $25(OH)D_3$ levels and PTH was reported.

Changing methodology and introducing vitamin D reference values as parameter, the importance of vitamin D in IBD has become more convincing. Defining vitamin sufficiency as serum values above 30 ng/mL, vitamin D insufficiency as values between 10/20 and 30 ng/mL, and vitamin deficiency as concentrations below 10 to 15 ng/mL, data from 27 studies from all over the world were available [44, 46, 52, 53, 56–58, 60, 61, 63, 64, 69–77], 6 of them on cohorts over 100 participants [59, 62, 66, 78–80], and one with more than 1,000 patients [81]. In synthesis, vitamin deficiency was found in 8-100% of patients with CD and in 15-60% of patients with UC, vitamin insufficiency in 12-72.3% in CD or in mixed IBD populations and in 7-64% of UC patients. Five papers [53, 61, 74, 76, 82] differentiated vitamin D levels according to seasonal variations in CD patients reporting vitamin deficiency in 50–76% in winter and in 10–19% in summer months; vitamin insufficiency, where reported [76, 82], was indicated in 73–100% in winter and 55–59% in summer months.

Studies evaluating vitamin D levels in IBD patients were all conducted after disease onset and established diagnosis, but it is not clear if vitamin D deficiency is the cause or a consequence. Pathogenesis of vitamin D hypovitaminosis in patients with IBD may depend on various mechanisms such as decreased exposure to sunlight or oral vitamin D intake, ileal resections leading to malabsorption or a disturbed enterohepatic circulation, and/or increased losses through the gastrointestinal system by protein-losing enteropathy [59].

To identify the reasons for the differences of the vitamin D status, the ability to absorb vitamin D_2 was evaluated in a study by Farraye et al. [77] comparing CD patients and HC. In this study, 42% of CD patients were vitamin D deficient $25(OH)D_3$ (≤20 ng/mL), while 29% were insufficient ($25(OH)D_3$: 21–29 ng/mL); 12 h after ingesting 50,000 IU of vitamin D_2, circulating levels of this metabolite were significantly lower in CD compared with HC indicating a significant 30% reduction of the ability to absorb vitamin D_2. In another study, on 31 CD patients and 15 HC, the capacity of absorbing orally administered vitamin D (5 μg of $25(OH)D_3$/kg body weight) was evaluated; 10% of CD patients showed decreased absorption of $25(OH)D_3$ after 4 and 8 hours [71]. Finally, a wide variability of absorption of vitamin D_2 was reported in vitamin deficient and insufficient CD patients, but vitamin D_2 absorption was significantly reduced compared with HC [77].

Several studies evaluated factors influencing vitamin D status hypothesizing reduced sun exposure as cause for hypovitaminosis, since a geographical north-south gradient was noted also for other autoimmune T helper- (Th-) 1-mediated diseases, like multiple sclerosis. The link between this gradient and the pathophysiological mechanisms that involve vitamin D status depends not only on dietary intake but also from UV exposure [83]. Indeed, a negative association between sun exposure and lower levels of 25(OH)D in CD was reported in Indian patients [52] and, most recently, also in Dutch CD [84] where reduced exposure to sunlight (defined as no sunny holidays, no solarium use, and more sun protection) was associated with low 25(OH)D serum levels.

The relationship between sun exposure and the risk of developing CD or UC has been investigated by Nerich et al. [85]. High residential sunlight exposure was associated with a significant decreased risk of CD, but not UC. Four years later, the same group published similar results, that is, an increased incidence of CD with reduced sunlight exposure, in a cohort of women living in France, whereas vitamin D intake was not associated with a risk reduction in CD or UC [86].

Reduced UV exposure seems therefore not only to increase risk for CD, but it also seems associated with a worse outcome of disease. In a recent nationwide North-American study, the influence of UV exposure on hospitalization rates, length of hospital stay, and surgeries was investigated in an impressive number of IBD patients (649,932 CD, 384,267 UC, and 288,894,297 non-IBD controls). Reduced UV exposure led to significantly longer hospitalizations in all groups and to more frequent intestinal surgeries and deaths in CD [87]. Data on $25(OH)D_3$ were not available in this study. The finding that more UV exposure is associated with a minor number of surgical procedures in CD was confirmed in

a subsequent study on 481,712 CD-related hospitalizations reporting 67,751 major surgical procedures [88].

Finally, a prospective cohort study of 72,179 women enrolled in the Nurses' Health Study addressed the question if vitamin D hypovitaminosis may, per se, represent a risk factor for the development of IBD. Incident cases of CD and UC were recorded over a follow-up period of 22 years. A $25(OH)D_3$ prediction score based on diet and lifestyle was developed and validated against effectively measured levels of $25(OH)D_3$. The authors showed that higher predicted plasma levels of $25(OH)D_3$ were associated with a significant risk reduction for CD but not for UC, suggesting that vitamin D status may contribute to the pathogenesis of CD [89].

After a series of contradictory and mostly negative studies on vitamin D levels in IBD patients compared with HC, more conclusive data have been produced introducing reference values. However, most of these studies have been aimed to investigate bone and calcium metabolism. Recent large cohort studies investigating UV exposure or vitamin D status estimating the risk to develop IBD have pushed forward our understanding on the potential role of vitamin D in the context of IBD.

5. Vitamin D Status and Clinical Outcome in IBD Patients

Several studies concerning the relationship between vitamin D status and clinical outcome in IBD patients have been published (Table 2). Almost 30 years ago, $25(OH)D_3$ levels in active CD were found to be lower than in quiescent CD [50]. Twenty years later, another study showed that low serum $25(OH)D_3$ levels were predicted by disease duration and activity scores in both, CD and UC [46]. This inverse association between disease activity and serum $25(OH)D_3$ levels was confirmed in a small prospective study in CD [52] and in a retrospective study on a much larger, mixed IBD population [59]. In this latter study, low serum $25(OH)D_3$ levels were associated with higher clinical activity scores in CD and in UC, but not with the risk for medical or surgical hospitalizations. Moreover, regression analysis found that low vitamin D levels were independently associated with quality of life (QoL) in CD patients but not in UC patients. A reduced QoL was reproduced by another study where vitamin insufficient patients had significantly lower QoL scores than those who were sufficient [82]. Finally, in a mixed IBD population, an inverse correlation between serum $25(OH)D_3$ concentrations and fecal calprotectin, a marker for gut inflammation, was found whereas serum CRP as a marker of systemic inflammation did not correlate with $25(OH)D_3$ levels [90].

Conversely, other studies on CD and UC patients failed to show a correlation between serum $25(OH)D_3$ levels and disease activity [60]. The same findings, that is, no association between $25(OH)D_3$ concentrations and disease activity, were published on a pediatric IBD population [54].

Going beyond disease activity, in a prospective study on the largest multicenter cohort involving 3,217 patients, low plasma $25(OH)D_3$ levels (<20 ng/mL) were associated with an increased risk of hospitalizations and surgery for CD as well as for UC patients [81]. In a subset of CD patients, but not UC patients, who normalized vitamin D status, a reduction of CRP levels and the need for hospitalizations was observed.

The likelihood for developing *Clostridium difficile* (Cl) colitis related to vitamin D status was investigated retrospectively. There was an increased risk for developing Cl colitis in patients with low plasma $25(OH)D_3$ levels (<20 ng/mL), and an increase by 1 ng/mL of $25(OH)D_3$ was accompanied by a 4% risk reduction of developing Cl colitis. Lastly, death from Cl colitis occurred in those with lower $25(OH)D_3$ levels compared with survivors [91]. A recent study investigated the relationship between $25(OH)D_3$ concentrations and duration of anti-TNF therapy in IBD patients. Interestingly, low vitamin D levels were associated with loss of response during maintenance therapy in CD patients [92], whereas serum $25(OH)D_3$ levels increased with anti-TNF therapy [93].

The only study that investigated plasma $1,25(OH)_2D_3$ levels found no association between $1,25(OH)_2D_3$ levels and CDAI or CAI in Japanese patients [68].

From the above, it appears that low vitamin D is inversely correlated to disease activity documented by clinical scores and surrogate markers of inflammation such as CRP and fecal calprotectin; moreover, low levels were also associated with clinical outcomes, that is, surgery, response to anti-TNF therapy, Cl superinfection, and, finally, death. Inflammation per se has been shown to upregulate conversion from $25(OH)D_3$ to $1,25(OH)_2D_3$ which may lead to a reduction of available $25(OH)D_3$. In this discussion, an observation of two recent papers may be relevant, coming from orthopaedic surgery, showing an acute reduction of $25(OH)D_3$ levels following a systemic inflammatory response induced by surgery, considering serum $25(OH)D_3$ as a negative acute phase reactant [94, 95].

6. Therapeutic Studies *In Vitro* and in Experimental Animals

As a result of this evidence, vitamin D should be proposed as a therapy for IBD. Several experimental studies, both on animals and IBD patients, have been carried out (Table 3). Starting with the former, in a model of spontaneous colitis, interleukin- (IL-) 10 knock-out (KO) mice on a vitamin D deficient diet showed growth retardation and weight loss, together with a high mortality rate (58% at week 9) compared to mice on a vitamin D sufficient diet; $1,25(OH)_2D_3$ (0.005 μg/day) supplementation starting from week 2 reduced weight loss and ameliorated histology scores, but vitamin D supplementation after symptom onset at week 7 ($1,25(OH)_2D_3$, 0.2 μg/day) did not induce significant differences compared with untreated animals, except for bowel weight indicating a reduction of inflammation in supplemented animals [96]. In another study, the efficacy of a low calcemic vitamin D analogue (22-ene-25-oxa-vitamin D (ZK156979)) was investigated in 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis [97]. Treatment was performed with $1,25(OH)_2D_3$ (0.2 μg/kg) versus ZK156979 (0.1–2.0 μg/kg), both administered intraperitoneally (i.p.) before or after colitis induction. Assessment of inflammation and colitis severity

Table 2: Vitamin D versus disease activity and outcome in IBD (chronological order).

Author	Year	Population	Methodology	Main findings
Harries et al. [50]	1985	U.S.A 40 CD 20 UC 9 HC	Single-center cohort; CD divided into 2 groups (undernourished and well nourished); 2 control groups: 20 well-nourished UC and 9 HC	$25(OH)D_3$ significantly lower in CD with active disease versus inactive disease ($P < 0.05$)
Tajika et al. [46]	2004	Japan 33 CD, 11 UC, 15 HC	Single-center cohort; $25(OH)D_3$ and disease activity assessed by CDAI and IOIBD score	Serum $25(OH)D_3$ significantly related to disease duration ($r = 0.46$, $P = 0.003$), CDAI ($r = 0.44$, $P = 0.005$), IOIBD score ($r = 0.30$, $P < 0.05$), serum ferritin ($r = 0.34$, $P = 0.03$), CRP ($r = 0.34$, $P = 0.03$)
Joseph et al. [52]	2009	India 34 CD, 34 HC	Single-center cohort; disease activity evaluated by HBI in CD	Serum $25(OH)D_3$ in CD significantly lower versus controls ($P < 0.05$). Disease activity correlated negatively with $25(OH)D_3$ level ($P < 0.004$). $25(OH)D_3$ levels were comparable to controls in mild CD but were significantly lower in moderate and severe CD
Nakajima et al. [68]	2011	Japan 47 CD, 40 UC, 41 HC	Single-center cohort; disease activity measured using CAI/CDAI scores	No decrease $1,25(OH)_2D_3$ in CD with high CDAI No significant correlation between serum $1,25(OH)_2D_3$ levels and CAI or CDAI in UC or CD
Ulitsky et al. [59]	2011	U.S.A. 504 IBD (403 CD, 101 UC)	Single-center cohort; retrospective observational study HRQOL measured with SIBDQ, disease activity measured using HBI/UCDI scores	$25(OH)D_3$ deficiency significantly associated with lower SIBDQ ($P = 0.002$) and higher mean HBI/UCDI ($P = 0.002$) in IBD versus vit D sufficient patients. Analyzed separately, vit D deficiency associated with lower HRQOL scores only in CD ($P = 0.04$), not in UC
El-Matary et al. [54]	2011	Canada 60 IBD (39 CD, 21 UC)	Cross-sectional pediatric study. Disease activity measured by PCDAI e PUCAI	No correlation between PCDAI and serum $25(OH)D_3$. Marginal evidence against the null hypothesis ($P = 0.05$) between serum $25(OH)D_3$ and PUCAI, but without statistical significance
Hassan et al. [60]	2013	Iran 60 IBD (34 UC, 26 CD)	Cross-sectional study. Disease activity measured by CDAI and Truelove index	Serum vit D lower in active versus inactive disease (non significantly). VitD deficiency was not associated with IBD activity (also considering CD and UC separately), however was associated with a history of IBD related intestinal surgery
Ananthakrishnan et al. [81]	2013	U.S.A. 3,217 IBD (55% CD, 45% UC)	Multicenter cohort; $25(OH)D_3$: Normal (>30 ng/mL), Insufficient (20–29.9 ng/mL) or Deficient (<20 ng/mL)	IBD-related surgery: CD: 10% patients never vitamin D deficient versus 13% vitamin D insufficient versus 17% vitamin D deficient. UC: vitamin D deficiency associated with elevated risk of surgery and hospitalization with effect similar to CD; no statistical significance in patients vitamin D insufficient. Normalization of $25(OH)D_3$ associated with reduction in the risk of related surgery but not in UC
Zator et al. [92]	2014	U.S.A. 101 IBD (74 CD, 27 UC)	Retrospective single-center cohort; patients on anti-TNF therapy evaluated for loss of response; $25(OH)D_3$ insufficiency: <30 ng/mL	Patients with insufficient vitamin D demonstrated earlier cessation of anti-TNF-α therapy ($P = 0.04$). This effect was significant in patients who stopped treatment for loss of response, stronger for CD than UC (P = NS)

Table 2: Continued.

Author	Year	Population	Methodology	Main findings
Ananthakrishnan et al. [91]	2014	U.S.A. 3188 IBD patients (45% UC, 55% CD)	Retrospective multi-center analysis of $25(OH)D_3$ in 35 patients who developed CDI	$25(OH)D_3$ level was significantly lower in IBD who developed CDI compared to non-CDI-IBD ($P = 0.002$). Levels below 20 ng/mL were associated with a two-fold increase in risk of CDI. $25(OH)D_3$ level was an independent predictor of CDI
Ham et al. [93]	2014	U.S.A. 37 CD	Prospectively collected samples for $25(OH)D_3$ analysis; assessment of HBI and CRP PBMC tested for VDR, Cyp	$25(OH)D_3$ levels lower in patients with active disease versus inactive disease, $25(OH)D_3$ correlated with HBI (not with CRP) PBMC: mean gene expression of VDR and CypB1 higher in active disease
Garg et al. [90]	2013	Australia 40 CD 31 UC 23 HC	Assessment of $25(OH)D_3$, fecal calprotectin and CRP	Inverse correlation between serum $25(OH)D_3$ and fecal calprotectin in CD and UC patients, but not with CRP
Hlavaty et al. [82]	2014	Slovakia 141 CD 49 UC	SIBDQ assessment in vitamin D sufficient or -deficient patients and in vitamin supplement (800 IU/day for 3 months) patients	SIBDQ was significantly better in vitamin D-sufficient patients; vitamin D supplements did not influence vitamin D status or sIBDQ
Govani et al. [88]	2015	U.S.A. 67,751 CD	Retrospective, national, analysis of UV exposure and inpatient surgery risk	UV exposure protective for inpatients surgery

Abbreviations: CD: Crohn's disease; UC: ulcerative colitis; HC: healthy controls; IBS: irritable bowel syndrome; IBD: inflammatory bowel disease; CDAI: Crohn's Disease Activity Index; IOIBD: international organization for the study of inflammatory bowel disease score; CAI: Lichtiger's clinical activity index; $25(OH)D_3$: 25-Hydroxycholecalciferol; $1,25(OH)_2D_3$: 1,25dihydroxycholecalciferol; SIBDQ: Short IBD Questionnaire; HBI: Harvey-Bradshaw index; UCDI: Ulcerative colitis disease activity index; HRQOL: health-related quality of life; PCDAI: pediatric Crohn's disease activity index; PUCAI: pediatric ulcerative colitis activity index; CDI: Clostridium difficile infection; CRP: C-reactive protein; UV: ultraviolet; TNF: tumor necrosis factor; PBMC: peripheral blood mononuclear cells; Cyp: Cyp27b1 gene; VDR: vitamin D receptor.

was established by scoring colitis, macroscopic and histological analysis, and measurement of myeloperoxidase activity (MPO) and cytokine levels. The authors found that ZK156978 reduced the severity of TNBS-induced colitis with a potency comparable with that of $1,25(OH)_2D_3$, downregulating MPO activity, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) tissue levels, and T-box transcription factor (T-bet) expression, together with an increase of interleukin IL-10 and IL-4 tissue concentrations, without calcemic effects.

Laverny et al. [98] studied the effect of an intrarectally administered vitamin D receptor agonist (1α,$25(OH)_2$-16-ene-20-cyclopropyl-vitamin D_3; BXL-62) in C57Bl/6 mice with dextran-sodium sulfate- (DSS-) induced (3%) colitis. BXL-62 treatment (1 μg/kg) compared to $1,25(OH)_2D_3$ (0.3 μg/kg) was superior in preventing weight loss and visible fecal blood, together with better stool consistency and histology scores without inducing hypercalcemia. Another synthetic vitamin D agonist, 1α,25(OH)2-19-nor-14,20-bisepi-23-yne-vitamin D_3 (TX527), has been shown to attenuate inflammation in the DSS model of colitis by downregulating IL-1, IL-6, IFN-γ, and TNF-α as well as the gastrointestinal glutathione peroxidase 2 [99].

There are three very interesting studies which associate vitamin D or its receptor with intestinal microbiota. First, in Cyp27b1-KO mice, that is, mice unable to produce $1,25(OH)_2D_3$, an increased susceptibility to DSS colitis was observed [100]. Oral vitamin supplementation reduced weight loss, whereas treatment with antibiotics greatly attenuated colitis. In these mice, a reduced expression of E-cadherin on epithelial and immune cells was observed pointing towards a more "leaky" gut. Moreover, a reduced number of tolerogenic dendritic cells were observed in the gut of Cyp27b1-KO mice. In these mice, as well as in VDR-KO mice, dysbiosis of the microbiota was observed with an increase of the Helicobacteraceae family and a reduction of the Firmicutes and Deferribacteres phyla. The authors concluded that vitamin D (production or its receptor) is involved in the regulation of the gut microbiota. Second, DSS-induced colitis was reduced together with a lower penetration of adherent-invasive *E. coli* (AIEC) in mice on a vitamin-sufficient diet compared to those fed a vitamin D deficient diet. Moreover, vitamin D hypovitaminosis and DSS colitis led to an increase of Bacteroidetes. In the same paper in Caco cells incubated with or without vitamin D and then challenged with AIEC, vitamin D maintained transepithelial resistance and prevented tight junctional protein redistribution [101]. The third paper, that reported changes of the microbiota related to interference in the vitamin D system, assessed susceptibility to DSS colitis in conditional VDR KO mice (deletion restricted to the intestinal epithelial cells), along with Paneth cell quantity and quality by means of quantification of lysozyme and ATG16L1 protein expression.

Table 3: Therapeutic studies in experimental and human IBD (chronological order).

Author	Year	Species/cells	Investigational agent	Methodology	Main findings
Animal and *in vitro* studies					
Cantorna et al. [96]	2000	IL-10 KO mice	$1,25(OH)_2D_3$ p.o.	Exp. 1. Vit. D-deficient IL-10 KO mice versus vit. D-sufficient mice (treated with cholecalciferol); Exp. 2. Vit. D-deficient IL10 KO mice versus $1,25(OH)_2D_3$-treated; Exp. 3. Vit. D treatment after onset of GI symptoms	Vitamin D sufficiency prevents enterocolitis in IL-10 KO mice up to 13 weeks; $1,25(OH)_2D_3$ treatment ameliorates inflammation
Daniel et al. [97]	2006	BALB/c mice	TNBS colitis; 22-ene-25-oxa-vitamin D (ZK156979) i.p. (vitamin D analogue)	Treatment with ZK156979 versus $1,25(OH)_2D_3$ before or after induction of colitis with TNBS; investigation of tissue MPO, TNF-α, IFN-γ, T-bet, IL-10, and IL-4	ZK156979 versus $1,25(OH)_2D_3$ prevents or ameliorates TNBS colitis decreasing pro-inflammatory and increasing anti-inflammatory cytokines
Laverny et al. [98]	2010	C57BL/6 mice	DSS-colitis, $1\alpha,25(OH)_2$-16-ene-20-cyclopropyl-vitamin D3 (BXL-62) (=VDR agonist) intrarectally	Daily administration of BXL-62 versus $1,25(OH)_2D_3$; Macro- and microscopic scoring; mucosal concentrations of TNF-α, IL-12/23p40, IL-6, and IFN-γ and assessment of mRNA	Higher potency of BXL-62 versus $1,25(OH)_2D_3$ in reducing tissue inflammation
Verlinden et al. [99]	2013	C57BL/6 mice	DSS- colitis $1\alpha,25(OH)_2$-19-nor-14,20-bisepi-23-yne-vitamin D3 (TX527)	Histological examination; measurement of transcript levels of cytokines (IL-1, IL-6, IFN-γ, and TNF-α)	TX527 reduced "clinical" disease scores and attenuated histological scores, downregulation of transcript levels of inflammatory cytokines
Ooi et al. [100]	2013	C57BL/6 mice Cyp KO VDR KO	$1,25(OH)_2D_3$ p.o.	DSS colitis; characterization of gut microbiota, and gut macrophages; E-cadherin expression	Lower expression of E cadherin and tolerogenic macrophages Less beneficial microbiota in KO mice Vitamin D treatment ameliorates colitis and reduces Helicobacteraceae
Wu et al. [21]	2014	Conditional VDR KO and IL-10 KO mice DSS-colitis cells: MEF, SKCO15, HCT116 human tissue	DSS colitis BUT feeding in IL-10 KO	VDR KO: colitis evaluation, pyrosequencing for microbiota, Paneth cells, lysozyme production, autophagy MEF ($VDR^{-/-}$ $VDR^{+/-}$ $VDR^{+/+}$) and VDR knockdown in SKCO15 with evaluation of ATG16L1 and LC3B proteins IL-10 KO: VDR and ATG16L1 expression with or w/o BUT feeding Human tissue (UC, inflamed versus normal) VDR, ATG16L1, Bacteroides concentration (FISH) HCT116 and HIEC: VDR expression with and w/o incubation with BUT	Conditional VDR KO mice: worse colitis, increased *E. coli* and Bacteroides (*B. fragilis*), and decreased BUT-producing bacteria; less and abnormal Paneth cells and reduced lysozyme and ATG16L1 protein; in SKCO15 and MEF reduced expression of ATG16L1 and LC3B proteins In UC: reduced expression of VDR and ATG16L1, increase of Bacteroides; BUT increases VDR expression in HIEC and HCT116
Tao et al. [102]	2014	C57BL/6 mice	TNBS-colitis Vitamin D sufficient or deficient diet	At week 14, assessment of ECM and total collagen production, together with determination in isolated colonic SEMF, of expression of VDR, α-SMA, and Collagen I in normal SEMF	Histological scoring, ECM, and collagen production in the colon reduced in vitamin D supplemented mice; in SEMF decreased levels of TGF-β1, Smad-3, p-Smad3, and Collagen I and induced VDR expression and decreased TGF-β1-induced α-SMA and Collagen I expression

Table 3: Continued.

Author	Year	Species/cells	Investigational agent	Methodology	Main findings
Assa et al. [101]	2015	Caco cells C57BL/6 mice	DSS- colitis Vitamin D sufficient or deficient diet $1,25(OH)_2D_3$ for Caco	Caco cells incubated with or w/o $1,25(OH)_2D_3$ challenged with AIEC C57BL/6 mice on normal or low $1,25(OH)_2D_3$ diet infected with AIEC	$1,25(OH)_2D_3$ protects Caco cells against AIEC induced loss of TER and TJ protein redistribution $1,25(OH)_2D_3$ reduces DSS colitis and AIEC invasion low vitamin D diet and DSS colitis increased Bacteroides
In vivo and *ex vivo* studies in IBD patients					
Stio et al. [105]	2007	4 CD and 4 HC	TX 527 [19-nor-14,20-bisepi-23-yne-$1,25(OH)_2D_3$], Vitamin D analogue	Single-center, *ex vivo* study; experimental study on PBMC of CD patients	TX 527 inhibits TNF-α mediated effects on PBMC and the activation of NF-κB; its action is mediated by VDR
Miheller et al. [107]	2009	37 CD	Group A treated with aVD versus group B treated with pVD	Single-center study; evaluation of bone parameters and CDAI, CRP, and SIBDQ after 6, 12, 52 weeks	In aVD, after 6 weeks (but not at 52 weeks) a significant reduction of CDAI, IBDQ, and CRP together with a significant change of bone parameters
Ardizzone et al. [103]	2009	9 UC, 8 CD	$1,25(OH)_2D_3$	Single-center *ex vivo* study; PBMC with or without calcitriol; determination of TNF-α, IFN-γ, IL-2, and IL-10	In UC PBMC $1,25(OH)_2D_3$ reduced IFN-γ and enhanced IL-10 production In CD PBMC $1,25(OH)_2D_3$ reduced TNF-α production
Jørgensen et al. [108]	2010	94 CD	Vitamin D3 versus placebo	Multi-center randomized double-blind placebo-controlled study; 1200 IU vit D3/day or placebo; estimation of clinical relapse rate	Vit. D3 significantly increased serum vit. D levels, but the decrease of relapse was not significant (13% versus 29%, $P = 0.06$)
Bendix-Struve et al. [104]	2010	108 CD	Vitamin D3 versus placebo	Randomized, placebo-controlled, clinical trial After 0, 36, and 52 weeks, PBMC tested in 10 patients treated with Vitamin D3 (1200 IU/day) and in 10 patients treated with placebo for cytokine production and proliferation	Vit. D3 treatment of CD patients increased the IL-6 levels and enhance the $CD4^+$ T-cell proliferation
Laverny et al. [98]	2010	22 CD, 21 UC	$1\alpha,25(OH)_2$-16-ene-20-cyclopropyl-vitamin D3 (BXL-62)	*Ex vivo* preparations of PBMC (+LPS) and (CD2/CD28 activated)-LPMCs incubated with or without BXL-62. Determination of mRNA and protein concentrations of TNF-α, IL-12/23p40, IL-6, and IFN-γ	Higher anti-inflammatory potency compared to $1,25(OH)_2D_3$ demonstrated by the significantly more potent inhibition in PBMC and in LPMCs of the proinflammatory cytokines TNF-α, IL-12/23p40, IL-6, and IFN-γ
Yang et al. [109]	2013	18 CD	Vitamin D3	Open-label prospective clinical trial over 24 weeks, multi-center study; vitamin D3 at 1000 IU/day; dose increase every two week of 1000 IU/day up to 5000 IU/day to achieve serum $25(OH)D_3$ >40 ng/mL	Vit. D3 supplementation significantly raised serum $25(OH)D_3$, reduced CDAI scores, and improved IBDQ scores
Bartels et al. [106]	2014	10 CD	Vitamin D3	Single-center study, oral vitamin D supplementation (or placebo) and assessment of maturation marker expression and cytokine production of monocyte-derived dendritic cells	Dendritic cells from vitamin supplemented CD patients exhibited reduced expression of CD80 and reduced production of the cytokines IL-10, IL-1β, and IL-6

Table 3: Continued.

Author	Year	Species/cells	Investigational agent	Methodology	Main findings
Ham et al. [93]	2014	PBMC	Incubation of $CD4^+$ with vit D 50 nM	Determination of $CD25^+$ and $CD39^+$ cells	3-fold increase of $CD25^+$ cells, $CD39^+$ unchanged

CD: Crohn's disease; UC: ulcerative colitis; HC: healthy controls; vit: vitamin; p.o.: per os; GI: gastrointestinal; KO: knock-out; TNBS: 2,4,6-trinitrobenzene sulfonic acid; i.p.: intraperitoneal; DSS: dextran sodium sulfate; 25(OH)D: 25-hydroxycholecalciferol; $1,25(OH)_2D_3$: 1,25-dihydroxycholecalciferol; vitamin D3 (vit D3): cholecalciferol; VDR: vitamin D receptor; MEF: mouse embryonic fibroblasts; AIEC: adherent-invasive *Escherichia coli*; TER: transepithelial electrical resistance; TJ: tight-junction; aVD: active vitamin D ($1,25(OH)_2D_3$); pVD: plain vitamin D (25(OH)vitamin D); CDAI: Crohn's disease activity index; CRP: C-reactive protein; SIBDQ: Short IBD questionnaire; PBMC: peripheral blood mononuclear cells; LPS, lipopolysaccharide; LPMCs: lamina propria mononuclear cells; IBDQ: IBD questionnaire; IL: interleukin; Cyp: Cyp27b1 gene; IFN: interferon; TNF: tumor necrosis factor; BUT butyrate; SEMF: subepithelial myofibroblasts; ECM: extracellular matrix; α-SMA: alpha smooth muscle actin; FISH: fluorescent in situ hybridization; HIEC: human intestinal epithelial cells; ATG16L1: autophagy related 16-like 1 (*S. cerevisiae*); LC3B: autophagy-related protein LC3B; SKCO15: human colorectal adenocarcinoma cells; HCT116: human colon cancer cell.

The latter is a protein involved in autophagy, and its genetic variants are well known as risk factors for CD. In this model, an increase of *E. coli* and *Bacteroides,* together with a decrease of butyrate producing bacteria was reported. Supplementing butyrate to IL-10 KO mice reverses reduced VDR and ATG16L1 expression. Similar results, that is, an increased expression of VDR and ATG16L1, were obtained incubating several cell lines with butyrate [21].

Finally, a reduction of intestinal fibrosis, assessed by production of extracellular matrix and total collagen, was seen in mice with TNBS colitis on a vitamin supplemented diet compared to mice fed a vitamin D deficient diet [102]. Moreover, in isolated subepithelial myofibroblasts from the colon, a vitamin D sufficient diet reduced concentrations of TGF-β1, Smad 3, p-Smad 3, and collagen I. It was concluded that preventive vitamin D administration reduces fibrosis inhibiting the VDR-mediated TGF-β1/Smad 3 pathway.

In the above studies, in various types of spontaneous or chemically induced colitis and in several cell lines, vitamin D and synthetic agonists have been shown to reduce colitis severity and intestinal fibrosis. Vitamin D hypovitaminosis or knocking down Cyp27b1 or VDR had the opposite results. Interestingly, these latter conditions were all associated with changes of the intestinal microbiota.

7. Therapeutic Studies in Human *Ex Vivo* Preparations

In an *ex vivo* study on PBMC obtained from IBD patients and incubated in the presence of $1,25(OH)_2D_3$, a reduction of interferon- (IFN-) γ and an increase of IL-10 production were observed in PBMC from UC patients whereas in CD the production of TNF-α were reduced [103]. The effect of orally administered vitamin D3 on monocyte-depleted PBMC from vitamin D3-treated (1200 IU vitamin D daily over 1 year) versus placebo-treated patients was investigated [104]. $CD4^+$ T-cell proliferation and T-cell cytokine production were assessed. IL-6 production in vitamin D_3-treated patients increased, whereas TNF-α, IFN-γ, and IL-4 did not. No change was observed for IL-10 and the percentage of the $CD4^+$, $CD25^+$, and $Foxp3^+$ regulatory T cells compared to placebo. The amount of proliferating $CD4^+$ T cells was significantly increased (from 41% to 56%) in the vitamin-D-treated group.

Another *ex vivo* study employed the vitamin D analogue (19-nor-14,20-bisepi-23-yne-$1,25(OH)_2D_3$; TX 527). This analogue significantly inhibited PBMC proliferation and TNF-α release in CD and HC [105]. The increase of VDR protein levels after incubation with TX 527 was higher in CD compared with HC. Moreover, in PBMC of both, HC and CD, stimulated with TNF-α, a decrease in nuclear NF-κB protein levels together with an increase in cytoplasmic IKB-α levels were observed pointing to an inhibition of TNF-α induced effects on PBMC exerted by the vitamin D analogue.

The effect of the vitamin receptor agonist BXL-62 on PBMC from CD and UC patients and lamina propria mononuclear cells (LPMC) obtained from biopsies of two CD (ileum) and two UC (colon) patients was investigated [98]. After incubation, in LPS-stimulated PBMC and in activated LPMC from IBD patients, BXL-62 significantly inhibited, with a significantly higher potency compared with $1,25(OH)_2D_3$, TNF-α, IL-6, and IL-12/23p40 transcription and cytokine concentrations measured in culture supernatants without differences between CD and UC.

In PBMC of CD patients, expression of the CYP27B1 gene, that is, the gene that encodes the enzyme that converts $25(OH)D_3$ to $1,25(OH)_2D_3$, and that of the VDR gene was investigated, showing a higher expression in active compared to inactive disease [93]. Moreover, $CD4^+$ T cells incubated in the presence of vitamin D showed a threefold increase of $CD25^+$ cells.

Finally, the effect of oral vitamin D supplementation on the maturation and cytokine production of monocyte-derived dendritic cells of CD patients was studied [106]. Compared to placebo-treated CD patients, vitamin D supplementation led to reduced CD80 expression in LPS-stimulated dendritic cells together with reduced production of IL-10, IL-1β, and IL-6.

8. Therapeutic Studies in Human IBD

There are only few studies with vitamin D addressing the clinical course of IBD (Table 3). In one of these studies, the effect of supplementation of the active form of vitamin D $1,25(OH)_2D_3$ (aVD, 1000 IU $1.25(OH)_2D_3$ daily) versus the plain vitamin D 25(OH)D (pVD; 2×0.25 μg alphacalcidiol daily) was investigated in CD patients in clinical remission (CDAI < 150) [107]. Both groups received oral calcium

supplementation (1000 mg/day). At 6 weeks, the mean CDAI and IBDQ scores, as well as the CRP concentrations, decreased in the aVD-treated group, but not in the pVD-treated group. These differences between the groups however disappeared by week 52. Serum calcium concentrations did not change at any time point. Jørgensen et al. [108] performed a randomized double-blind placebo-controlled multicenter study to assess the benefit of vitamin D3 treatment in CD. They included 94 CD patients in clinical (CDAI < 150) and biochemical remission, randomized to receive 1200 IU of vitamin D3 + 1200 mg of calcium or 1200 mg of calcium alone. During 1-year follow-up, serum $25(OH)D_3$ levels increased significantly in vitamin D-supplemented patients, on average from 27 to 38 ng/mL, but free serum calcium did not change. The relapse rate (defined as increase of CDAI >70 over baseline and CDAI ≥150) was not significantly lowered. Adjustment for the use of azathioprine and smoking resulted in minor changes of the risk estimate. However, the authors concluded that vitamin D might be effective in CD but claimed the need for larger studies.

In an uncontrolled study, 18 active CD patients were initially treated with 1000 IU vitamin D daily over 2 weeks. Thereafter, the dose was escalated (to a maximum of 5000 IU) until a serum concentration of 40 ng/mL of $25(OH)D_3$ was reached [109]. After 24 weeks, a significant reduction of the CDAI and an improvement of the IBDQ score were observed. No differences were observed for CRP, erythrocyte sedimentation rate (ESR), TNF-α, IL-17, IL-10, and vascular endothelial growth factor (VEGF). Data on serum calcium levels were not reported.

In this last paragraph, the therapeutic effects of vitamin D supplementation on disease activity mainly given to patients in remission yielded modest results; the daily administered dose ranged in these studies between 1000 and 5000 IU, with an increase of serum vitamin D levels but apparently without hypercalcemia.

9. Conclusions

Literature data highlighting the importance of vitamin D in different aspects of immune regulation, for example, in chronic immune-mediated diseases and cancer, suggest considering this metabolite not simply as a vitamin involved in bone and calcium homeostasis but as an autocrine mediator with an active role in numerous physiological processes, particularly in the innate immune system. Since most studies concerning the calcium status in IBD yielded contradictory data, in the most recent literature, the discussion has focused on the possible role of vitamin D as a risk factor for the onset and evolution of gut inflammation. The potential role of $25(OH)_2D$ as negative acute phase reactant has yet to be proven in IBD but may explain its frequently reduced levels in active disease. Besides lower vitamin D levels due to reduced UV exposure, genetic induced loss of function of VDR may contribute to defects involving vitamin D pathways. It has been shown in VDR KO animals that this deletion profoundly alters innate immune response and the gut microbiota. Further studies in this field are needed to provide more insight in the link between vitamin D/VDR and bowel inflammation.

Simple vitamin D supplementation does not seem to lead to significant improvement of the clinical course of IBD but may be indicated for a subset of patients. Vitamin D synthetic analogues of vitamin D seem to be more promising, at least in animal studies and in *ex vivo* experiments.

Conflict of Interests

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

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