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Special Issue Reprint

Vitamin D in Health and Disease

Edited by
Giuseppe Murdaca and Sebastiano Gangemi

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Editorial

Vitamin D in Health and Disease

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Vitamin D (VD) is a fat-soluble hormone that plays a fundamental role not only in calcium homeostasis and bone metabolism, but also has anti-inflammatory and antioxidant properties, acting on both innate and adaptive immunity. Therefore, it is now scientifically considered an immunomodulating agent. It has been shown that VD and microbiomes act in concert and direct the etiopathogenesis and evolution of many allergic diseases. Among the many activities that are in current times attributed to VD, it is necessary to remember the antiproliferative, antiangiogenic and prodifferentiation activity that, undoubtedly, attributes to VD itself, playing a role in cancer carcinogenesis that has not yet been completely gutted. However, it would appear that some of its carcinogenic effects may be attributed to binding to its receptor (VDR) and the subsequent modulation of tumor miRNA expression. In this Editorial, I would like to briefly summarize the main concepts described in the eight papers published in the Special Issue "Vitamin D in Health and Disease". The first impression that came from reading the titles of the articles is that VD is implicated in the pathogenesis of both chronic respiratory and cardiovascular diseases. It goes without saying that it is likely that VD acts in the development and clinical evolution of many chronic immune-mediated diseases, including allergic ones. The authors of the various papers underlined the association between low serum levels of VD that favor release, and Th2 cells of proinflammatory cytokines, including IL-4, IL-5 and IL-13, which might guide the inflammatory state at the base of many chronic diseases. The role of these cytokines in chronic rhinosinusitis (CRS), which is characterized by a diffuse inflammation of the mucosa, whose etiopathogenesis is not yet fully understood, has now been widely demonstrated [1]. It should be remembered that CRS is classified into two subtypes, depending on the presence or absence of nasal polyposis (CRSwNP and CRSsNP, respectively). Notably, IL-4, IL-5 and IL-13 seem to direct the development of CRSwNP, while, on the contrary, IFN-γ released by Th1 cells, would favor the development of CRSsNP [2,3]. Several studies confirmed that low VD levels could promote an increased cytokine release from inflammatory cells and fibroblasts [3], perpetuating chronic inflammatory sinus diseases and the degree of severity of NP. In particular, I would like to underline the work of Christensen et al. [4], who confirmed how maintaining VD serum levels allows to control the secretion of IL-4, IL-5 and IL-13, and, at the same time, induce the production of IL-10 at the level of sinuses, with the consequent inhibition of inflammation. Furthermore, Camargo et al. [5] confirmed the importance of VD supplementation to prevent the winter exacerbation of atopic dermatitis. Confirming the importance of bringing VD levels back to adequate values, Afzal et al. [6] discuss the therapeutic potential of VD in inflammatory lung diseases (ILDs), including asthma, lung cancer, bronchiectasis, pulmonary fibrosis, acute respiratory distress syndrome and *chronic obstructive pulmonary disease* (COPD), in which inflammation represents a significant component of the disease [7]. On the other hand, it is well established that the insufficiency of calcitriol supports the inflammatory process in COPD. Therefore, maintaining physiological levels of VD allows to exploit the anti-inflammatory action of VD itself. Notably, VD carries out its anti-inflammatory activity by acting on cellular elements

that play a role in inflammatory processes. Macrophages, an important cell type in the innate immune system, express the VD receptor (VDR). There are bacterial strains capable of regulating the expression of VDR and, consequently, the signals that derive from the VD/VDR interaction. In particular, *M. tuberculosis* activates the expression and activity of VDR and CYP27B1 by inducing the intracellular signal capable of stimulating the synthesis of cathelicidin, which favors the death of *M. tuberculosis*. It should be remembered that VD inhibits endoplasmic reticulum stress by counterbalancing the stimulating activity on macrophages by IFN-γ, as well as inhibiting the proliferation and activity of human dendritic cells (DCs). In summary, RV can modulate innate immune responses [6]. There are now scientific data confirming the importance of VD in improving the clinical picture in asthmatic patients. On the other hand, the anti-inflammatory action of RV is also expressed through its ability to reduce the serum levels of IL-17A and increase the levels of IL-10, as demonstrated in patients with asthma. RV has been shown to inhibit the profibrotic action of TGF-b1 in patients with pulmonary fibrosis [6,8]. Oriano et al. [9] confirmed that VD-binding protein (DBP) polymorphisms and the resulting isoform are involved in the pathogenesis and severity of bronchiectasis, where DBP is coded by the GC gene. Notably, the GC1f isoform (rs7041/rs4588 A/G) correlated with a more severe disease, a higher incidence of chronic infections and a lower bronchiectasis etiology and comorbidity index (BACI) score. On the other hand, the GC1s isoform had a milder phenotype with increased VD levels and a lower comorbidities score.

Endothelial cells line the inner lumen of all vessels while preserving the integrity of the vascular system. Endothelial dysfunction participates in the phenotypic expression of accelerated atherosclerosis and, therefore, in the increased risk of cardiovascular diseases. VD deficiency is associated with endothelial dysfunction, partially because of the downregulation of the VDR [10]. A significant number of studies have correlated vitamin D deficiency with an increased risk of developing heart arrhythmias, arterial hypertension, diabetes mellitus and sudden cardiac death [11,12]. Scrimieri et al. [13] investigated the potential protective role of VD on human umbilical vein endothelial cells (HUVECs). The increase in lipogenesis with a greater quantity of available triglycerides and the reduced oxidation of fatty acids favor the negative evolution of endothelial dysfunction. Furthermore, the glucose-induced thioredoxin-interacting protein (TXNIP) upregulation induces the accumulation of reactive oxygen species and, as a consequence, of lipid droplets. Low VD levels favor the development of coronary artery calcifications, the onset of atrial fibrillation (AF) and heart failure, while the downregulation of CYP27B1 (1-hydroxylase) has been associated with the severity of cardiovascular diseases [14]. Notably, if the serum levels of RV are not sufficient, they can affect cardiac electrical activity with consequent negative impacts on ventricular repolarization with the risk of the onset of severe arrhythmias [15]. Therefore, Barsan et al. [16] focused on the need to periodically monitor VD levels to choose the appropriate time for VD supplementation and the achievement of protective physiological levels. In support of this goal, Scrimieri et al. [13] demonstrated that adequate serum levels of VD downregulated TXNIP by preventing oxidative stress and by correcting the lipid metabolism and storage in lipid droplets, thus, restoring endothelial function. Marino et al. [17] demonstrated that the antiatherogenic protective effect of VD could be ascribable to the regulation of proteins involved in lipid transport and clearance. Indeed, VD decreases fatty acid accumulation in THP-1-derived macrophages exposed to an excess of free fatty acid (FFA). In particular, the addition of VD increases peroxisome proliferator-activated receptor gamma (PPAR)-γ1 levels, CPT-1A and ABCA1 proteins, while, by contrast, it blocks the increase in both CD-36 and C/EB proteins induced through FFA administration. Abulmeaty et al. [18] reported that VD deficiency is frequently present in heart transplant (HT) patients, but a 2-year supplementation of VD did not seem to have a positive impact on the bone mineral density (BMD). Furthermore, obesity represents an independent risk factor for the onset of cardiovascular diseases, for which vitamin D levels have not appeared to have any positive impact. Confirming this, VD supplementation failed to reduce the incidence rate of cardiovascular diseases and mortality. The link between VD and

the composition of the gut microbiome is well established. Singh et al. [19] demonstrated that low VD levels induced changes in the composition of the gut microbiome in children, dominated by *Prevotella* as opposed to *Bacteroides*. The authors suggest to consider that host genetics and baseline gut microbiota compensate in interpreting the VD status and designing better personalized strategies for therapeutic interventions. Eight papers developing the role of VD in cardiovascular and airway diseases were published in this Special Issue. The general conclusion would confirm that low levels of VD and alterations in the VD/VDR signal and in the microbiome are, undoubtedly, able to influence the immune system, supporting the pathogenesis of cardiovascular diseases and chronic inflammatory processes affecting the airways, including NP, COVID-19, asthma, lung cancer, COPD, bronchiectasis, pulmonary and cystic fibrosis, pneumonia and tuberculosis. It should be emphasized that the microbiome and RV deeply influence each other. In particular, RV levels may represent a risk factor for the increased incidence of respiratory tract infections, as demonstrated in childhood and adolescence. Finally, VD represents a valid aid to control the redox balance and reduce the onset of endothelial dysfunction and, thus, of accelerated atherosclerosis. Therefore, the maintenance of optimal VD serum levels could represent a relevant strategy for reducing the risk of cardiovascular and airway diseases.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Role of Vitamin D in the Clinical Course of Nasal Polyposis

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Abstract: Vitamin D is a lipo-soluble hormone well known for its effects on calcium homeostasis and bone metabolism. Recently, there has been growing interest in the extraskeletal effects of vitamin D. In particular, recent studies have highlighted how vitamin D plays a fundamental role in immunomodulation processes in the context of both innate and adaptive immunity, with consequent anti-inflammatory and anti-oxidant effect in different immune-mediated pathologies, such as systemic sclerosis, psoriasis, atopic dermatitis and rheumatoid arthritis; as well as in various pro-inflammatory processes affecting the airways, including chronic rhinosinusitis with (CRSwNP) or without (CRSsNP) nasal polyposis. We analyze the role of vitamin D in the genesis and progression of CRSwNP/sNP and its supplementation as a safe and valid therapeutic strategy capable of improving the clinical outcome of standard therapies.

Keywords: vitamin D; chronic rhinosinusitis; nasal polyposis; biologics

1. Introduction

Over the years, several studies have confirmed that vitamin D is strongly involved in immunomodulation processes, with a consequent anti-inflammatory and anti-oxidant effect in different immune-mediated pathologies. In this context, it was highlighted that some diseases of the upper respiratory tract, such as chronic rhinosinusitis with (CRSwNP) or without (CRSsNP) nasal polyposis, recognize an immune-mediated pathogenetic mechanism, in which vitamin D seems to play a fundamental role in improving the clinical and therapeutic outcome. The aim of our review is to summarize and analyze the influence of vitamin D on the genesis and clinical progression of CRSwNP/sNP and the therapeutic potential of this hormone, in addition to current treatments in the management of this disease—for which we have collected a total of 26 articles from literature, matching the search criteria with the keywords vitamin D, chronic rhinosinusitis, nasal polyposis, and biologics.

2. Chronic Rhinosinusitis and Nasal Polyposis

Chronic rhinosinusitis (CRS) is a disease of the upper respiratory tract characterized by diffuse inflammation of the mucosa [1] with unknown etiologic and pathophysiologic aspects. Anatomic factors, fungal allergies, infectious causes, and immunological disorders have been identified as favoring factors [2]. CRS may be divided into two subtypes, with (CRSwNP) and without (CRSsNP) nasal polyposis [3]. The first is characterized as an end product of Th2 cell skewing, mediated by IL-4, IL-5, and IL-13. On the other hand, CRSsNP is typically considered a result of a Th1 inflammation via, with dominant production of IFN-γ [4]. More recently, it has been suggested that classification of CRS by endotype is defined by the predominate type of inflammatory infiltrate as either eosinophilic (eCRS) or non-eosinophilic (non-eCRS) [5].

3. Vitamin D3

Vitamin D3 (VD3) is a steroid hormone that enters the circulation through epidermal transfer or intestinal absorption. Once circulating, it is hydroxylated in the liver to form 25-hydroxyvitamin D3 (25-VD3), the largely inactive form of the vitamin. To be converted into its active form, 1,25-hydroxy VD3 (1,25-VD3), it needs a second hydroxylation step in the kidney [6]. The hydroxylation process of vitamin D3 in the liver occurs by the cytochrome P450 2R1 (CYP2R1) and cytochrome P450 27 (CYP27A1) enzymes. The active metabolite 1,25 (OH) 2D3 is hydroxylated in the kidney by the enzyme CYP27B1.

CYP27B1 is also expressed by other cell types, including immune cells, which are therefore capable of synthesizing 1,25 (OH) 2D3, which plays an important role in immunomodulation processes [7,8]. Due to its steroid nature, 1,25-VD3 is able to pass through the cell membrane by binding to its cytoplasmic receptor (VDR), expressed by several human cells, including lymphocytes and dendritic cells, suggesting that vitamin D may have pleiotropic effects [9]. However, 1,25 (OH) 2D3 acts primarily through vitamin D receptors (VDRs) [10]. VDR acts as a transcription factor in different varieties of tissues, including the intestine, liver, and adipose tissue [11,12]. VDR plays a key role in modulating the immune response as it is expressed in different types of immune cells, including CD4 + and CD8 + T cells, B cells, neutrophils, and antigen presenting cells (APC) [12–15]. Approximately one billion people worldwide suffer from vitamin D deficiency [16], as determined by serum 25 (OH) D concentrations below 30 ng/mL [17]. Dietary Reference Intakes (DRIs) for vitamin D are age-dependent: 400 IU of vitamin D/day for children < 1 year, 600 IU of vitamin D/day for people aged 1 to 70, and 800 IU of vitamin D/day for people > 70 years [18,19]. Several studies performed on VD3 have highlighted the fundamental role that it plays not only as a proskeletal agent, but also as an immunomodulator [20]. In recent years, moreover, there has been a focus on the role that vitamin D plays in the pathophysiology of chronic inflammatory respiratory disorders such as allergic rhinitis, chronic rhinosinusitis, and asthma [21,22]. In particular, allergic rhinitis has shown an imbalance in the Th1/Th2 ratio favoring Th2 [23,24]. Regarding this, it has been shown that vitamin D acts by suppressing the production of IL-12 and thus reducing the differentiation of type 1 (Th1) helper T cells in favor of greater proliferation of associated type 2 (Th2) T helper cell allergy. The proliferation of Th2 cells leads to an increase in interleukin 31 (IL-31) synthesis, an effector cytokine that plays an important role in the pathogenesis of atopic and allergic diseases [25,26]. Given these correlations, a significant correlation between vitamin D deficiency and inflammation in patients with chronic rhinosinusitis with and without nasal polyps is shown [27–30].

4. Immunological Correlation between VD3 and CRSwNP/CRSsNP

Christensen et al. [5] also reported how vitamin D is able to reduce CD4+ T-cell production of signature Th2 cytokines, such as IL-4, IL-5, and IL-13, and promotes release of IL-10, and may also modulate IL-8 expression, as 1α-hydroxylase has been shown to reduce gene expression of IL-8 in fibroblasts and keratinocyte in sinonasal tissue. According to recent studies, increased levels of IL-6 and IL-8 may participate in the pathology of primary changes as well as recurrences of chronic sinusitis and NP [31]. Furthermore, Tomaszewska et al. [32] demonstrated the presence of VDR protein expression in the sinonasal mucosa, and a statistically significant decrease in VDR nuclear staining in CRSsNP and CRSwNP patients versus controls. VDR-expressing cells believed to play a role in the pathogenesis of CRSwNP include human synonasal fibroblasts (HSNFs). These are involved in the recruitment of inflammatory cells, tissue edema, and the production and resultant of extracellular matrix (ECM) tissue remodeling [33,34]. Furthermore, vitamin D derivatives could significantly inhibit TNF-α-induced matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) secretion in fibroblasts involved in nasal polyp genesis [34]. Given the role that vitamin D plays in the pathogenesis of CRS and nasal polyps, low vitamin D levels could promote increased cytokine release from inflammatory cells and fibroblasts. This could be the reason for the perpetuation of chronic

inflammatory sinus diseases and the degree of severity of nasal polyposis [35,36]. In fact, several studies reported a significant correlation between the serum vitamin D levels and severity of disease in patients with CRSwNP [37–39]. As a result, VD3 supplementation that has antiproliferative and antiinflammatory properties is suggested to be used as an adjunct therapy to decrease the incidence of inflammation and polyposis and also in reducing the recurrence of this last following endoscopic sinus surgery in patients with CRSwNP [40]. Figure 1 reports the effects of the VD on the immune pathogenesis of CRS and nasal polyposis.

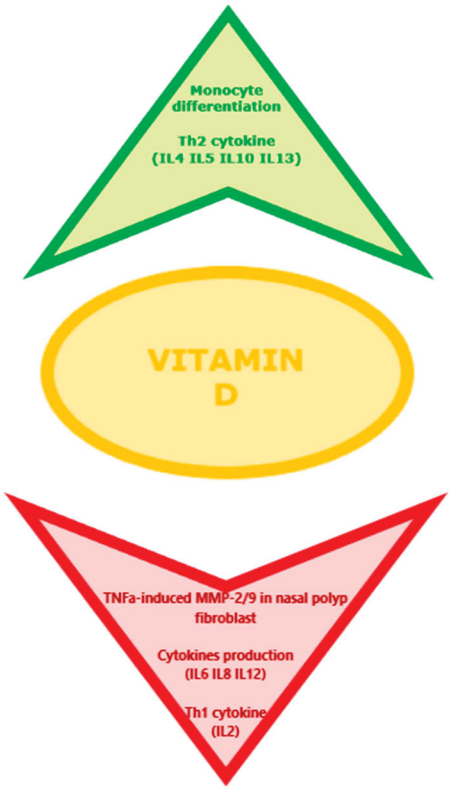

Figure 1. Effects of the VD on the immune pathogenesis of CRS and nasal polyposis.

Based on what has been discussed, vitamin D is known to act on both innate (through inhibitory effects on Toll-like receptors) and adaptive immunity (through inhibitory effects on cytokines secretion resulting in inhibition of T-cell proliferation). In addition to its cellular effects, vitamin D is capable of modulating a great variety of pro-inflammatory cytokines, thus playing a key role in the pathogenesis of many allergic disorders [41–43] such as asthma, atopic dermatitis, and food allergies [43–46]. Particularly in allergic diseases, vitamin D acts on the human immune system through inhibitory functions on the growth cycle of human dendritic cells and the functions of T cells, stimulating the secretion of specific cytokines such as IL-10 [47,48]. It has been widely discussed how patients diagnosed with allergic disease are characterized by below normal levels of vitamin D and how this hormonal deficiency leads to a greater severity of symptoms [49–51]. In fact, the metabolite of vitamin D 1,25 (OH) 2VD3 can act by inhibiting T-helper 1 (TH1) and stimulating the responses of TH2 cells. It also stimulates the differentiation of TH17 cells, resulting in upregulation of regulatory T cells (TReg) and type 1 regulatory T cells (TR1). However, 1,25 (OH) 2VD3 also inhibits the proliferation of B lymphocytes and their differentiation into antibody-secreting cells [52]. Considering these anti-inflammatory and immunomodulatory effects of vitamin D, several studies have focused on the correlation between the deficiency of this hormone and the higher prevalence of allergic diseases [53]. Some randomized studies have evaluated the role of vitamin D supplementation also in the prevention of the winter exacerbation of atopic dermatitis, demonstrating how winter

supplementation of vitamin D can be useful for patients with atopic dermatitis, in terms of clinic exacerbation [54,55].

5. Results

In consideration of what has been said, we have collected a total of 26 articles corresponding to the search characteristics, and, for of each of them, we have analyzed and reported the outcomes. Of these, seven articles report how low serum vitamin D levels are common in CRS patients, particularly in the CRSwNP form, compared to control subjects. Furthermore, six of the collected works highlight how vitamin D is involved in the pathogenesis of CRS disease, as it is able to stimulate the inflammatory process mediated by T cells and the production of mediators stimulating the growth and proliferation of fibroblasts of the nasal mucosa. In six other papers, low vitamin D levels are reported to be correlated with a more severe form of CRSwNP. Finally, seven of these highlight how vitamin D supplementation could represent a valid and safe therapy able to support standard treatments, reducing the severity and relapse of the disease. In Table 1, the 26 articles collected are reported.

Table 1. Articles collected and analyzed.

Author	Year	Type of Study	N Patients	Comorbidities	Objective	Outcome
Ali Faghih Habibi, Hooshang Gerami, et al.	2019	Case-Control Study	117		compare serum level of 25-OH-VitD in CRSw/sNP patients and control groups	serum 25-OH-VitD was significantly lower in CRS patients
Ankur Kumar Chandrakar, Arun Alexander et al.	2014	cross-sectional study	80 patients with nasal polyposis and 80 healthy subjects	Atopy	Assessment of the levels of 25-hydroxy vitamin D and high sensitivity C-reactive protein (hs-CRP) in patients with nasal polyposis and control subjects, and identified their association with disease severity in nasal polyposis.	The severity of polyposis correlated negatively with serum levels of 25-hydroxy vitamin D and positively with hs-CRP
Anna Bonanno, Sebastiano Gangemi et al.	2014	Case-control study	28 controls (HC), 11 allergic rhinitis (AR) patients, and 35 allergic asthma with rhinitis (AAR) patients	Allergy and asthma	whether low vitamin D is linked with circulating IL-31 and IL-33 in children with allergic disease of the airways	low levels of 25(OH) Vit D might represent a risk factor for the development of concomitant asthma and rhinitis in children with allergic disease of the airways independently of IL-31/IL-33 Th2 activity
Arash Shahangian, Rodney J. Schlosser	2016	Review	0		Explore some of the contributions of VD3 to chronic rhinosinusitis with nasal polyposis and its role as a disease-modifying agent	There is likely a role for VD3 use as a disease-modifying agent in treatment of patients with recalcitrant CRSwNP
B Rostkowska-Nadolska, E Sliupkas-Dyrda et al.	2010				investigate the influence of calcitriol and tacalcitol on the secretion of IL-6 and IL-8 by fibroblasts derived from NP	Calcitriol and tacalcitol are capable of affecting pro-inflammatory cytokine (IL-6 and IL-8) levels in NP cultures
Badr El-Din Mostafa, Mohammed Shehata Taha et al.	2016	Case-control study	74		Measure VD3 levels in patients with AFRS and chronic rhinosinusitis (CRS)	Serum level of VD3 in patients with CRSwNP and AFRS is significantly lower than that of patients with CRSsNP and control subjects
Binayak Baruah, Ajay Gupta et al.	2020	retrospective 1-year study	200		Comparison of incidence of vitamin D deficiency in CRS patients to normal population and evaluation of the beneficial role of its supplementation in treatment	Higher prevalence of vitamin D deficiency in CRS patients and that vitamin D supplementation went a long way in alleviating their symptoms

Table 1. *Cont.*

Author	Year	Type of Study	N Patients	Comorbidities	Objective	Outcome
Bo Li, Miaowei Wang et al.	2021	meta-analysis	337 chronic rhinosinusitis patients and 179 healthy controls	asthma and/or atopic status	Compare the serum vitamin D levels between patients with chronic rhinosinusitis and healthy controls and evaluate the associations of vitamin D level with its occurrence	Detection of a significant association between lower serum vitamin D status and chronic rhinosinusitis, especially in chronic rhinosinusitis with nasal polyps patients
E Ritter Sansoni, Nathan B Sautter et al.	2015	Case-control study	57 (CRSsNP (n = 31), CRSwNP (n = 14), and controls (n = 12))		Correlation between 25-VD3 levels and sinonasal mucus monocyte chemoattractant protein-1 (MCP-1), regulated upon activation normal T cell expressed and secreted (RANTES), and basic fibroblast growth factor (bFGF) levels in patients with CRS	25-VD3 may play a role in regulation of RANTES and bFGF expression in CRSwNP. This may occur through regulation of NP fibroblasts or other immune cells
F Bavi, R Movahed, M Salehi, et al.	2019	cross-sectional study	166 cases with CRSwNP and 172 healthy subjects		Serum vitamin D3 levels in patients with CRSwNP and its association with disease severity	Disease severity, based on imaging, endoscopic and clinical criteria, was inversely associated with serum vitamin D levels
Farnaz Hashemian, Sonya Sadegh, et al.	2020	triple-blind placebocontrolled clinical trial			Investigate the effects of oral VD3 on the recurrence of polyposis after FESS	Efficacy and safety of vitamin D supplementation in the reduction of polyposis recurrence after FESS in patients with CRSwNP
Feng Wang, Yang Yang, Haihong Chen	2019	Retrospective analysis of collected data		Atopic status and asthma	Serum vitamin D level in patients with chronic rhinosinusitis with nasal polyps and its correlation with the disease severity	Serum 25-hydroxyvitamin D3 levels are lower in Chinese CRSwNP patients. These 25-hydroxyvitamin D3 levels are associated with SNOT-22 score. Preoperative 25-hydroxyvitamin D3 level may impact the symptom improvement after surgery
Iordanis Konstantinidis, Maria Fotoulaki et al.	2017	Case-control study	152 adult participants were included in five phenotypic groups: CF with NP (CFwNP) (n = 27), CF without NP (CFsNP) (n = 31), CRS with NP (CRSwNP) (n = 32), CRS without NP (CRSsNP) (n = 30), and controls (n = 32)		Investigate if deficiency of VD3 is associated with the presence of NP in patients with cystic fibrosis (CF) and patients with chronic rhinosinusitis (CRS)	VD3 deficiency seemed to be associated with the presence of nasal polyps in the patients with CRS and in the patients with CF in a similar manner

Table 1. *Cont.*

Author	Year	Type of Study	N Patients	Comorbidities	Objective	Outcome
Jenna M Christensen, Jasmine Cheng et al.	2017	cross-sectional study	31 patients (8 CRSsNP, 10 CRSwNP, and 13 controls)		Determine expression of genes encoding the vitamin D receptor (VDR), 25-hydroxylase (CYP2R1), 1α-hydroxylase (CYP27B1), and 24-hydroxylase (CYP24A1)	Vitamin D may be dysregulated at multiple levels, with decreased transcription of the metabolic gene CYP27B1 and increased transcription of the catabolic gene CYP24A1 observed
Jennifer K Mulligan, Whitney N Pasquini et al.	2017	Observational studies			Impact of VD3 deficiency on inflammation and VD3 metabolism in an Aspergillus fumigatus (Af) mouse model of chronic rhinosinusitis (Af-CRS)	VD3 deficiency causes changes in sinonasal immunity. Both VD3 deficiency and Af-CRS were associated with reductions in local levels of the active VD3 metabolite even with adequate circulating levels
Ling-Feng Wang, Chih-Feng Tai et al.	2015	Observational study			Understand the role of vitamin D in chronic rhinosinusitis with nasal polyps (CRSwNP) by investigating its effect on the secretion of matrix metalloproteinase-2 (MMP-2) and MMP-9	Vitamin D derivatives could significantly inhibit TNF-α-induced MMP-2 and MMP-9 secretion in nasal polyp-derived fibroblasts
Ling-Feng Wang, Chih-Hung Lee et al.	2013	Case-control study			Determine if serum Vitamin D level is lower in chronic rhinosinusitis with nasal polyposis (CRSwNP) patients and if low serum Vitamin D level is correlated with the severity of CRSwNP	A significantly lower vitamin D level was found in a group of Taiwanese CRSwNP patients, which revealed an association with greater nasal polyp size
Malgorzata Tomaszewska, Elzbieta Sarnowska et al.	2019	Case-control study	52 patients with CRS without nasal polyps (sNP), 55 with CRS with nasal polyps (wNP), and 59 in the control group	Atopy	Relationships between the total concentration of vitamin D, vitamin D receptor (VDR) expression, 1α-hydroxylase expression, and clinical data, including age, gender, Sino-Nasal Outcome Test (SNOT-22), computerized tomography (CT) scan, allergy status, and vitamin D supplementation in CRS patients with (CRSwNP) and without nasal polyps (CRSsNP), and in a control group	vitamin D and its receptor and enzymes may play a role in CRS

Table 1. *Cont.*

Author	Year	Type of Study	N Patients	Comorbidities	Objective	Outcome
Murdaca, G., Tonacci, A. et al.	2019	update			Find a correlation between vitamin D levels and its effect upon several autoimmune diseases	Inverse association between vitamin D and the development of several autoimmune diseases
Omer Erdag, Mahfuz Turan, et al.	2016	case-control study	46 subjects with NP (NP group) and 40 volunteers (control group)		Assess the relation between levels of vitamin D receptor (VDR) gene expression and serum vitamin D with NP	VDR gene expression may be associated with the pathogenesis or progression of NP
Patrick J Stokes, Joanne Rimmer	2016	systematic review	539	asthma and/or atopic status	Relationship among serum VD3 levels, CRS phenotype, and disease severity by using outcome assessments	Significantly lower VD3 levels in the polypoid phenotypes of CRS compared with controls. Low VD3 levels were often associated with an increased degree of inflammation
Pooja Thakur, Praneeth Potluri	2020	prospective casecontrol study			Evaluate the association of serum vitamin D levels with chronic rhinosinusitis (CRS) in population residing at high altitudes and to assess its correlation with severity of CRS	Lower vitamin D level is associated with CRS, irrespective of presence or absence of nasal polyposis in adults residing at high altitudes. Vitamin D is an independent predictive factor for CRS. There is an inverse moderate correlation of severity of CRS with vitamin D
Rodney J. Schlosser, Zachary M. Soler, et al.	2014	Retrospective review			Determine if CRSwNP populations are at risk for vitamin D3 (VD3) deficiency and if VD3 levels correlate with radiographic measures of disease severity or eosinophilia	VD3 insufficiency/deficiency is common in CRSwNP patients, especially those of African American race. Lower levels of VD3 are associated with worse LMS on CT
Sule Ozkara, Erol Keles, Nevin Ilhan et al.	2012	case-control study	60 adult patients and 40 healthy volunteers	Allergic rhinitis	Study Th1/Th2 cell balance by measuring the levels of cytokines IL-4, IL-10, and IFN-γ and determine the correlation between Th1/Th2 cell balance and 1α,25-dihydroxyvitamin D(3)	vitamin D is effective on Th1/Th2 balance in patients with allergic rhinitis and that there is a significant relation between vitamin D deficiency and allergy

Table 1. *Cont.*

Author	Year	Type of Study	N Patients	Comorbidities	Objective	Outcome
Vahid Zand, Mohammadhossein Baradaranfar et al.	2020	cross-sectional study	93 patients suffering from chronic rhino sinusitis with nasal polyposis (CRS w NP)		Investigate the association between the serum vitamin D levels and severity of disease in chronic rhino sinusitis (CRS) patients	There was a significant relationship between the serum vitamin D levels and severity of disease in patients with CRS w NP
William W Carroll, Rodney J Schlosser et al.	2016	Case-control study	15 patients with CRSwNP and 12 control subjects		Investigate VD3 deficiency and HSNF proliferation in CRSwNP	VD3 deficiency is associated with increased HSNF proliferation in CRSwNP

6. Discussion

On the basis of what has been reported, we have shown how vitamin D plays an important role as an immunomodulator in various pro-inflammatory processes affecting the airways and influences at different levels the different pathogenetic mechanisms involved in the genesis of CRS; in particular, lower levels of VD3 are closely associated with the form CRSwNP. In addition to the known immunomodulatory effects of vitamin D, several studies have reported that it is also endowed with important antiproliferative, anti-angiogenic, and pro-differentiative effects, mainly in some cancers such as ovarian, cervical, prostate, bladder, colorectal, gastric, leukemia, melanoma, and lung. These effects are mediated through the perturbation of several important signaling pathways mediated through genomic and non-genomic mechanisms. Specifically, vitamin D seems to be able to modulate the expression of tumor miRNAs through its action at the VDR level. Recently, an overexpression of catabolic vitamin D enzymes has been found in cancer, thus suggesting that low vitamin D levels are associated with greater tumor severity and therefore a poor prognosis [56–58].

Although CRS is a common disease, its treatment remains difficult in many cases, owing to varied mechanisms involved in its etiopathogenesis [59]. According to the 2016 International Consensus Statement on Allergy and Rhinology, the management of both CRS phenotypes is currently based on pharmaceutical treatment, consisting mainly of anti-inflammatory drugs like local intranasal glucocorticoids with natural high-volume saline irrigations (>200 mL) [60].

In the literature, it is reported that approximately 25–30% of patients with CRS develop nasal polyps. Histologically, nasal polyps are characterized by an infiltrate consisting predominantly of eosinophils, known as "eosinophilic CRSwNP". This form of CRSwNP has proved to be more common in the West than in the East.

Eosinophilic infiltrate of nasal polyps has been shown to correlate with greater clinical severity of the disease and less response to conventional corticosteroid treatments. There are also studies confirming the relationship between mucosal eosinophilia and postoperative nasal recurrence.

In addition to serving as a biomarker for disease severity, it is also possible that eosinophils contribute directly to the pathogenesis of CRSwNP, resulting in a type 2 inflammation shift [61–64].

However, this current type of CRS treatment has an estimated success rate of around 50%. For some phenotypes, including nasal polyposis, comorbid asthma, aspirin-exacerbated respiratory disease (AERD), and allergic fungal rhinosinusitis (AFRS), failure of medical and surgical management is more common [65]. Low levels of VD3 are also correlated with a greater severity of CRSwNP and a worse clinical outcome of this. In consideration of the new knowledge on the pathogenetic mechanisms of this disease particularly given the involvement of Th2 and pro-inflammatory cytokines produced by them, and the high failure rate of current therapeutic protocols, new biological drugs have recently been introduced, capable of acting at the level of specific molecular targets [66,67]. In this context, vitamin D supplementation, already when levels are equal to the upper ones of the range are reached, could represent an effective and safe additional therapeutic strategy in order to slow the progression of the disease to more severe forms of CRSwNP. Should this treatment fail, the therapeutic indication remains the use of biological drugs and/or surgical treatment. Not only that, the dietary supplementation of vitamin D, even in the presence of a mild state of deficiency, would seem able to improve the clinical outcome of some allergic diseases, in particular food allergies, asthma, and atopic dermatitis. In this review, we have discussed an abundance of evidence regarding the relationship between VD3 and the different types of CRS, especially with CRSwNP. Furthermore, we have highlighted how low levels of vitamin D are correlated with a greater severity of the disease. Its integration could therefore represent a valid therapeutic strategy capable of assisting surgical and biological treatment, thus improving the clinical outcome of patients.

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Impact of Vitamin D Supplementation on Bone Mineral Density and All-Cause Mortality in Heart Transplant Patients

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Abstract: Vitamin D (VD) deficiency is frequently reported in heart transplant (HT) recipients and routinely supplemented. However, the efficacy of VD supplementation on bone mineral density (BMD) and its association with all-cause mortality is underinvestigated. The VD levels and BMD were studied for two years, and the association of VD and BMD with all-cause mortality risk was investigated. Ninety-six HT patients (38.18 ± 12.10 years old; 74% men) were followed up during VD, Ca, and Mg supplementation. Anthropometric measurements, BMD by Dual-energy X-ray absorptiometry (DEXA) scan, VD concentrations, and related biochemical parameters were analyzed before, 1 year, and 2 years after HT. Despite significant improvement of VD_3 and 25-hydroxy VD (25OHVD) levels especially in the men, BMD parameters were insignificantly changed. After 2 years, the all-cause mortality rate was 15.6%. High pretransplant levels of 25OHVD failed to improve the survival probability. Cox's regression showed a 32.7% increased hazard ratio for each unit increase in body mass index (95% CI: 1.015–1.733, $p = 0.038$), in the VD-deficient group rather than in the VD-sufficient one. In conclusion, VD supplementation improves the biochemical status, especially in VD-deficient HT. However, its impact on the BMD and mortality was not as usually expected. Further investigation of the disturbed VD metabolism in HT is warranted.

Keywords: vitamin D; bone mineral density; supplementation; all-cause mortality; heart transplant

1. Introduction

Vitamin D deficiency (VD-D) is highly prevalent among patients with end-stage organ failure especially heart failure. Further, VD-D is frequently reported in heart transplant (HT) patients [1,2]. Patients eligible for HT are hardly exposed to sunlight due to frequent hospitalization and defective hepatic metabolism for VD due to heart failure-associated hepatic congestion. The VD status is frequently represented by the level of 25 hydroxyvitamin D (25OHVD). The International Osteoporosis Foundation (IOF) reported the definition of the VD-D by having a serum level of 25OHVD less than 25 nmol/L [3]. However, many cutoff points for defining VD-D were also reported, e.g., serum level of 25OHVD < 30 nmol/L [4], and 25OHVD < 50 nmol/L [5]. The 25 nmo/L was suggested as an arbitrary cutoff value especially with a lack of sufficient evidence for the optimal value for non-skeletal effects of VD [6,7], especially in populations with

endemic VD-D such as Saudi Arabia. In Saudi Arabia, VD-D (with 25 nmol/L cutoff point) was reported as 44.5% in adults and 49.5% in school children [8]. Heart transplant is still growing in Saudi Arabia, with only 2 cardiac centers available to do HT with a rate of about 30 HT/year [9]. In a recent study, VD-D (reported by 25OHVD < 25 nmol/L) was reported in 10% of the heart transplant patients and 55% of those with orthotopic heart transplants [10]. However, the data about VD status, bone mineral density (BMD), and the impact of VD on survival are deficient.

Osteopenia or osteoporosis may develop in HT patients especially in the first year and with improper preventive management [11]. Glucocorticoids bind osteoprotegerin (OPG); this molecule is necessary for limiting bone resorption. Thus, the decreased amount of OPG reduces BMD in transplantation patients [12]. Notably, it was reported that patients referred to cardiac transplantation generally have low BMD and about 14% of them suffer from osteoporotic vertebral compression fractures [13]. In another report, the prevalence of vertebral fractures was reported as 35% after HT heart transplantation due to increased bone loss [12]. Previous reports from our center showed osteopenia (35%) and osteoporosis (8%) at the lumbar spine of pre-transplant patients, besides significant reduction in pre-transplant BMD compared with that at 1 year after heart transplantation [14]. Nutritional supplementation with VD, Ca, Mg, Zn, and vitamin C are frequently recommended to support BMD in HT patients [2,15]. The International Society for Heart and Lung Transplantation recommended a daily calcium dose of about 1000–1500 mg, and VD 400–1000 IU for HT patients to maintain serum 25OHVD level > 75 nmol/L but with a low level of evidence [16].

The impact of VD and low BMD on all-cause mortality in HT patients is underinvestigated. Some reports showed that a reduced level of $1,25(OH)_2$VD, measured on the 21st day after HT, was associated with 1-year mortality in HT recipients [17]. This relationship between $1,25(OH)_2$VD (calcitriol) and mortality, is unclear. Low $1,25(OH)_2$VD may be because of immunosuppressive medications such as calcineurin inhibitors on the renal enzyme system, or due to bad general health as a consequence following organ transplantation. In another study that used the most common indicator of VD status monitoring (25OHVD), VD supplementation in a dose of 4000 IU did not affect the mortality in patients with end-stage heart failure compared to placebo [18]. Another European randomized controlled trial (NTC01212406) used VD in a high dose of 100,000 IU/month in lung transplantation patients and reported low survival for patients who received VD [19]. Data from another area on the map such as Saudi Arabia are very lacking. Thus, this work aimed to study the changes in VD levels and BMD in VD-supplemented HT patients for two years and to analyze the association of VD levels and BMD with the survival of Saudi HT recipients.

2. Materials and Methods

2.1. Study Design and Setting

A total of ninety-six heart transplant patients at King Faisal Specialist Hospital & Research Centre (KFSH&RC) in Riyadh, Saudi Arabia were investigated and followed up for two years after the procedure had been done. Participants were divided into two groups based on the baseline level of 25OHVD: Group I in which 25OHVD < 25 nmol/L, and Group II with 25OHVD $\geq$ 25 nmol/L. The Research Ethics Committee (REC) in the College of Applied Medical Sciences, King Saud University reviewed and approved this study protocol under reference number CAMS 93-36/37; date: 01/03/2016. In addition, the REC at KFSH&RC approved it under reference No. 2161051.

2.2. Given Supplementations and Medications

Study participants were on medications as seen in Figure 1. According to the KFSH&RC's local protocol, a routine dose of vitamin D_3 was 10,000 IU (250 μg/day) for patients with VD insufficiency (25OHVD < 50 nmol/L), while those with proven VD-D (by 25OHVD < 25 nmol/L) were given 50,000 IU as a weekly oral dose for 3 months followed by a 10,000 IU mainte-

nance dose. Calcium (Ca) in an oral dose of 1200 mg/day, and magnesium (Mg) 200 mg/day per oral were also routinely given to all patients. Commitment percentages of study participants on other supplements and medications were shown in Figure 1.

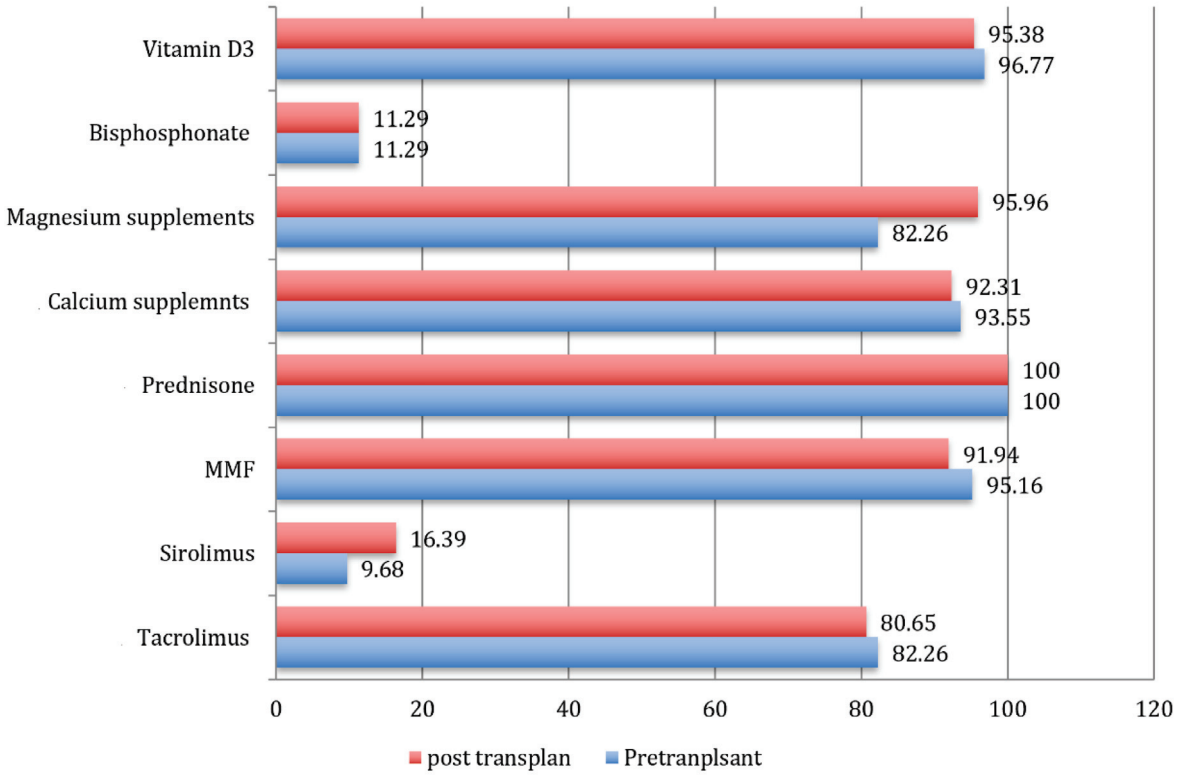

Figure 1. Medications intake by study population in the pre-transplant and post-transplant phases (numbers refer to frequencies of medication use, *MMF* = mycophenolic acid).

2.3. Anthropometric Parameters

Bodyweight (kg) and height (cm) were used for calculating body mass index (BMI) using the formula; BMI = weight (kg)/height $(m)^2$. Weight was measured to the nearest 0.1 kg by (Scale-Tronix scale, Chicago, IL, USA) while a stadiometer (Seca Co, Hamburg, Germany) was used for height measurement.

2.4. Measurement of VD and Biochemical Parameters

Serum cholecalciferol (VD_3), 25OH vitamin D (25OHVD), and intact parathyroid hormone (iPTH) levels (pmol/L) were measured on pretransplant assessment workup appointment, one year, and two years after the transplant had done by using electrochemiluminescence immunoassay, Cobas e411 autoanalyzer (Roche Ltd., Basel, Switzerland). Despite previous reports, the definition of VD-D was suggested in this study to be below 25 nmol/L of the 25OHVD level [20–22]. Moreover, levels of Alkaline phosphatase (ALP) (U/L), calcium (mmol/L), phosphorus (mmol/L), magnesium (mmol/L), creatinine (umol/L), urea (mmol/L), potassium (mmol/L), and chloride (mmol/L) were measured by Roche/Hitachi modular Cobas c 701/702 tests.

2.5. Measurement of BMD

The BMD was measured at scheduled appointments for follow-up at pre-transplant, 1 year, and 2 years post-transplant phases and the reports were used for analysis. BMD was assessed by the DEXA scan using a GE medical system Lunar iDEXA (GE Healthcare, Madison, WI, USA). Two sites were selected: lumbar spine (LS) and femoral neck (FN). Due to the relatively young age of our samples, Z-scores (rather than T-scores) were

calculated as standard deviations from the mean of the gender- and age-matched controls. Tertials of the BMD results were created as follows: (a) normal BMD: Z-scores above −1; (b) osteopenia: Z-scores between −1 and −2.5 gm/cm^2; and (c) osteoporosis: Z-score below −2.5 gm/cm^2 [23].

2.6. Sample Size and Satistical Power

The sample size and statistical power were calculated by G*Power software 3.1.9.4 (University of Kiel, Kiel, Germany), considering medium effect size (f = 0.25), alpha error probability at 0.05, power (1-β error probability) = 0.95, number of repeated measurements = 3, and the number of groups = 2. The estimated total sample size was 44 participants (22/group) and the actual power was 0.9557.

2.7. Statistical Analysis

The SPSS tool version 25 (SPSS, IBM, Chicago, IL, USA) was used for processing and analyzing these data. Continuous data were expressed as means ± SD, while dichotomous variables were expressed as percentages and categories. The normal distribution of continuous variables was tested by the Shapiro–Wilk test. For comparison of the three related samples, the Friedman ANOVA test was used with pairwise comparisons. Gender differences and comparisons between groups I and II were done via the Mann–Whitney U test. For survival analysis, Cox's proportional hazard regression analysis was used with 95% CIs. Results were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Basal Characteristics of Study Participants

Figure 2 shows participants' flow throughout the study. As shown in Table 1, pre-transplant data of our sample showed younger age, significantly lower BMI, and BMD in the women's group. Levels of VD_3, 25OHVD, PTH, Ca, ALP, Urea, Na, and K were insignificantly different. However, Mg and Cl levels were significantly higher, while creatinine was lower in the women group. Figure 3 shows the cardiovascular events which were diagnosed in our participants. The majority had dilated cardiomyopathy (52.22%), followed by ischemic cardiomyopathy (32.22%), and chemo-induced cardiomyopathy and post-partum cardiomyopathy (1.11%).

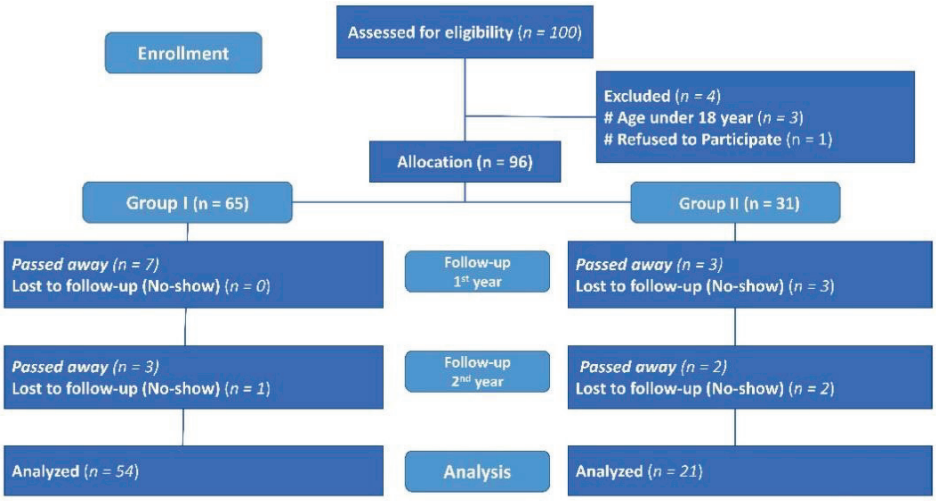

Figure 2. CONSORT diagram showing participants' flow throughout the study.

Table 1. Pretransplant characteristics of the study sample.

Variables	Total (n = 96)	Men (n = 71)	Women (n = 25)	p-Value
Age (Years)	36.17 ± 13.53	39.84 ± 12.22	32.35 ± 9.31	0.031
Height (cm)	165.33 ± 9.02	168.56 ± 7.57	156.16 ± 6.02	<0.001
Weight (kg)	65.05 ± 17.29	69.68 ± 15.87	51.90 ± 14.31	<0.001
BMI (kg/m^2)	23.62 ± 5.37	24.50 ± 5.21	21.12 ± 5.11	0.006
BMD lumbar spine (gm/cm^2)	1.05 ± 0.16	1.07 ± 0.16	0.95 ± 0.11	0.041
Lumbar spine Z-score	−0.31 ± 1.10	−0.25 ± 1.15	−0.48 ± 0.96	0.380
BMD femoral neck (gm/ cm^2)	0.59 ± 0.49	0.67 ± 0.48	0.38 ± 0.45	0.012
Femoral neck Z-score	−0.10 ± 0.91	−0.04 ± 0.99	−0.26 ± 0.60	0.293
25OHVD (nmol/L)	27.80 ± 23.78	27.72 ± 24.63	28.04 ± 21.63	0.954
Vitamin D_3 (nmol/L)	14.82 ± 14.73	15.23 ± 13.63	13.68 ± 17.76	0.654
Intact parathyroid h. (mmol/L)	78.15 ± 65.13	80.17 ± 61.88	72.40 ± 74.67	0.611
ALP (U/L)	96.07 ± 78.76	99.97 ± 80.56	84.98 ± 73	0.416
Calcium (mmol/L)	2.22 ± 0.18	2.20 ± 0.18	2.28 ± 0.19	0.054
Phosphate (mmol/L)	1.14 ± 0.34	1.15 ± 0.34	1.11 ± 0.35	0.600
Mg (mmol/L)	0.95 ± 0.55	0.86 ± 0.17	1.19 ± 1.02	0.011
Creatinine (umol/L)	91.14 ± 36.80	96.01 ± 36.14	77.32 ± 35.83	0.028
Urea (mmol/L)	10.14 ± 6.59	10.71 ± 5.75	8.50 ± 8.45	0.149
Sodium (mmol/L)	136.67 ± 6.43	136.04 ± 5.87	138.44 ± 7.63	0.110
Potassium (mmol/L)	4.05 ± 0.57	4.03 ± 0.56	4.10 ± 0.60	0.571
Chloride (mmol/L)	97.62 ± 6.89	96.52 ± 6.24	100.64 ± 7.80	0.010

BMI is body mass index; BMD is bone mineral density; 25OHVD is 25 hydroxyvitamin D; ALP is alkaline phosphatase; Mg is magnesium.

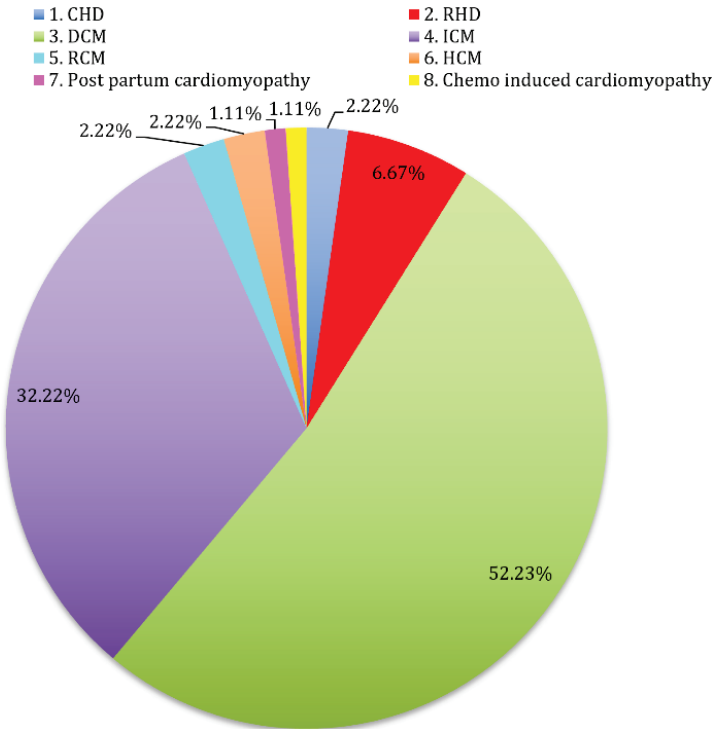

Figure 3. Percentages of main pre-transplant diagnoses among study recipients. (CHD: congenital heart disease, RHD: rheumatoid heart disease, DCM: dilated cardiomyopathy, ICM: ischemic cardiomyopathy, RCM: restrictive cardiomyopathy, HCM: hypertrophic cardiomyopathy).

3.2. Changes in VD and BMD throughout the Study Period

After 2 years, 15 participants passed away (33% women), and 6 dropped out (83% women). The data of remaining participants (n = 75; 20% women) are presented in Table 2 and analyzed by the Friedman ANOVA test with pairwise comparisons. Bodyweight

and BMI significantly improved, indicating improvement of nutritional status. Femoral BMD in the men's group showed a significant reduction after 1 year. After the second year, it returned to a value like that of the pretransplant status. In women, the three measurements were insignificantly different (p trend > 0.05). In the men's group, levels of 25OHVD and Ca increased progressively throughout the period with a significant reduction of PTH (especially in the first year), and ALP enzyme. However, insignificant changes in their levels were noticed in the women group. The prevalence of VD-D (defined by 25OHVD < 25 nmol/L) is presented in Figure 4. A progressive reduction of the VD_D was noticed, especially in the men's group, indicating sufficient VD supplementation. Despite supplementation, Mg serum level showed progressive reduction ($p < 0.05$) in men and women ($p = 0.74$). Percentages of study participants with osteopenia and osteoporosis at the lumbar spine and femoral neck throughout the study period are presented in Figure 5. Longitudinal changes in the 25OHVD, and PTH, as well as BMD at the lumbar spine and femoral neck, are shown in Figure 6.

Table 2. Bone mineral density and related biochemical parameters before, 1 year, and 2 years after the heart transplant.

Variables	Male (n = 60)			p-Value †	Female (n = 15)			p-Value †
	Pre-Transplant Mean ± SD	1-Y Post-Transplant Mean ± SD	2-Y Post-Transplant Mean ± SD		Pre-Transplant Mean ± SD	1-Y Post-Transplant Mean ± SD	2-Y Post-Transplant Mean ± SD	
Weight (kg)	70.08 ± 13.24 ^{a,*}	76.95 ± 14.95 ^{b,*}	89.03 ± 12.40 ^{b,*}	<0.001	52.41 ± 12.56 ^{a}	61.89 ± 17.61 ^{b}	64.35 ± 18.97 ^{b}	0.015
BMI (kg/m^2)	24.75 ± 4.43 ^{a}	26.85 ± 5.37 ^{b,*}	27.21 ± 5.15 ^{b}	0.011	21.46 ± 5.20 ^{a}	24.86 ± 6.52 ^{b}	26.27 ± 7.27 ^{b}	0.021
DEXA parameters								
BMD lumbar spine (gm/cm^2)	1.07 ± 0.16 ^{a,*}	1.02 ± 0.17 ^{a,*}	0.99 ± 0.27 ^{a,*}	0.231	0.95 ± 0.11 ^{a}	0.93 ± 0.09 ^{a}	1.09 ± 0.17 ^{a}	0.247
Lumbar spine Z-score	−0.28 ± 1.49 ^{a}	−0.66 ± 1.39 ^{b,*}	−0.50 ± 1.47 ^{a}	0.019	−0.12 ± 0.99 ^{a}	−1.02 ± 0.59 ^{b}	−0.80 ± 1.10 ^{a}	0.016
BMD femoral neck (gm/cm^2)	0.88 ± 0.33 ^{a}	0.75 ± 0.35 ^{b,*}	0.87 ± 0.25 ^{a}	0.012	0.91 ± 0.12 ^{a}	0.66 ± 0.38 ^{a}	0.90 ± 0.13 ^{a}	0.268
Femoral neck Z-score	−0.09 ± 1.33 ^{a,*}	−0.56 ± 1.22 ^{b,*}	−0.47 ± 1.18 ^{a,*}	0.106	−0.10 ± 0.61 ^{a}	−0.78 ± 0.75 ^{b}	−0.36 ± 0.62 ^{a}	0.007
Biochemical parameters								
25OHVD (nmol/L)	22.07 ± 22.02 ^{a}	54.79 ± 31.13 ^{b,*}	70.05 ± 27.58 ^{b,*}	<0.001	27.20 ± 20.13 ^{a}	37.80 ± 24.36 ^{a}	58.20 ± 27.69 ^{a}	0.143
Vitamin D_3 (nmol/L)	17.07 ± 14.36 ^{a}	40.44 ± 25.43 ^{b,*}	64.00 ± 25.06 ^{c}	<0.001	17.80 ± 18.83 ^{a}	32.47 ± 19.65 ^{a}	51.21 ± 25.19 ^{a}	0.145
Intact parathyroid h (pmol/L)	77.40 ± 53.88 ^{a,*}	30.85 ± 39.41 ^{b,*}	86.09 ± 35.36 ^{c}	0.013	98.67 ± 55.93 ^{a}	102.80 ± 18.10 ^{a}	126.25 ± 61.1 ^{b}	0.015
ALP (U/L)	103.70 ± 42.42 ^{a,*}	94.55 ± 42.85 ^{a,*}	81.71 ± 29.10 ^{b}	0.026	89.00 ± 40.14 ^{a}	65.00 ± 12.63 ^{a}	63.80 ± 4.55 ^{a}	0.449
Calcium (mmol/L)	2.18 ± 0.14 ^{a,*}	2.26 ± 0.11 ^{b,*}	2.27 ± 0.9 ^{b,*}	0.024	2.25 ± 0.12 ^{a}	2.17 ± 0.04 ^{a}	2.02 ± 0.50 ^{a}	0.449
Phosphate (mmol/L)	1.10 ± 0.30 ^{a,*}	1.12 ± 0.18 ^{a,*}	1.07 ± 0.16 ^{a}	0.314	1.00 ± 0.44 ^{a}	1.09 ± 0.25 ^{a}	1.01 ± 0.34 ^{a}	0.531
Mg (mmol/L)	0.88 ± 0.12 ^{a,*}	0.72 ± 0.10 ^{b}	0.64 ± 0.18 ^{b}	<0.001	0.91 ± 0.11 ^{a}	0.71 ± 0.16 ^{a}	0.62 ± 0.05 ^{a}	0.074
Creatinine (umol/L)	95.85 ± 35.06 ^{a,*}	108.25 ± 52.63 ^{a,*}	95.67 ± 23.88 ^{b}	0.819	79.00 ± 23.36 ^{a}	80.40 ± 31.30 ^{a}	79.00 ± 24.22 ^{a}	0.437
Urea (mmol/L)	10.00 ± 6.33 ^{a,*}	7.30 ± 3.04 ^{b,*}	9.01 ± 10.55 ^{b}	0.030	7.60 ± 3.21 ^{a}	7.28 ± 2.82 ^{a}	6.38 ± 1.63 ^{a}	0.819
Sodium (mmol/L)	135.10 ± 7.40 ^{a,*}	141.3 ± 2.92 ^{a}	140.02 ± 3.44 ^{a}	0.051	139.86 ± 9.21 ^{a}	140.43 ± 2.23 ^{a}	145.05 ± 4.34 ^{a}	0.869
Potassium (mmol/L)	4.07 ± 0.64 ^{a,*}	4.11 ± 0.35 ^{a,*}	4.10 ± 0.15 ^{a}	0.600	4.21 ± 0.58 ^{a}	4.31 ± 0.40 ^{a}	4.19 ± 0.39 ^{a}	0.725
Chloride (mmol/L)	95.45 ± 7.57 ^{a,*}	104.45 ± 3.44 ^{b}	99.33 ± 4.90 ^{a}	0.031	104.14 ± 8.21 ^{a}	105.57 ± 2.37 ^{a}	102.90 ± 4.32 ^{a}	0.625

† p-values of the three related samples by Friedman's two-way analysis of variance by ranks. Values with different superscripts (^{a} and ^{b}) mean significant vs. pretransplant phase; * Significant versus the related samples in the women group by Mann–Whitney U test; BMI is body mass index; DEXA is dual-energy X-ray absorptiometry; BMD is bone mineral density; 25OHVD is 25 hydroxyvitamin D; ALP is alkaline phosphatase enzyme; Mg is magnesium.

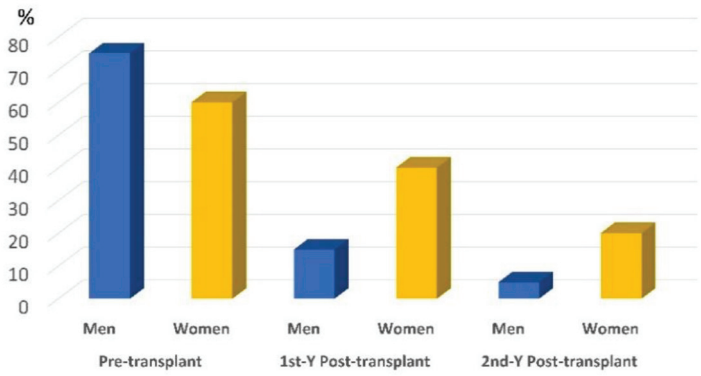

Figure 4. Prevalence of VD deficiency defined by 25OHVD < 25 nmol/L in men and women's groups throughout the study period.

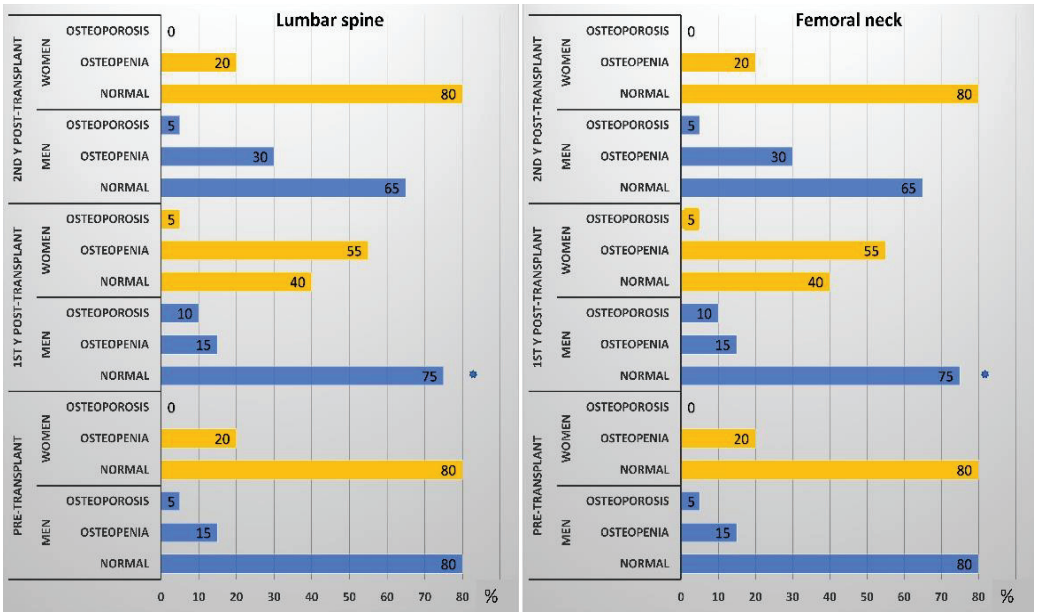

Figure 5. Prevalence of osteopenia and osteoporosis in men and women groups throughout the study period (* means significantly different between men and women by Mann–Whitney U test).

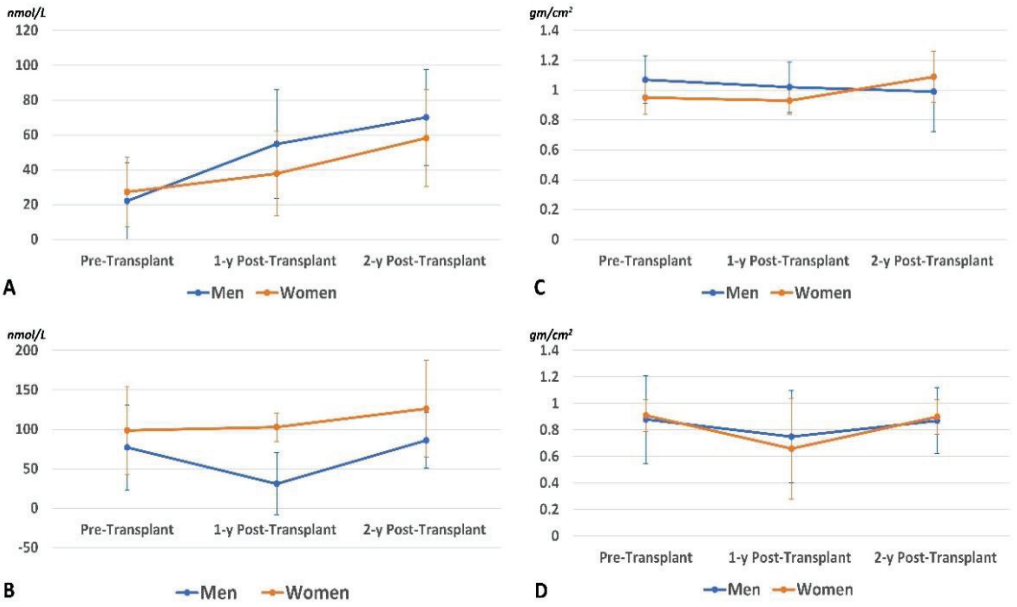

Figure 6. Longitudinal changes in the 25OHVD level (**A**), PTH level (**B**), BMD at the lumbar spine (**C**), and femoral neck (**D**).

Table 3 further reports the changes in study parameters in both study groups. Pre-transplant body BMI was insignificantly different between group I and group II. However, at the post-transplant assessment points, the BMI was significantly higher in the VD-sufficient group (group II). Besides, longitudinal changes showed a progressive increase of BMI with time, especially in the VD-deficient group. Group I had significantly lower VD_3, 25OHVD, and calcium levels. In Group I (VD-D group), significant reductions in BMD parameters were detected after the first year which improved at the second year to be insignificantly different from the pretransplant levels. In Group II, insignificant changes were reported in the three-time points. VD_3 level progressively increased with time due to the supplementation, while the 25OHVD level significantly increased in group I rather than group II. Similarly, levels of iPTH, ALP, calcium, and magnesium showed significant changes in group I rather than group II.

Table 3. Bone mineral density and related biochemical parameters before, 1 year, and 2 years after the heart transplant between study groups.

Variables	Group I; 25OHVD < 25 nmol/L (n = 54)			p-Value †	Group II; 25OHVD ≥ 25 nmol/L (n = 21)			p-Value †
	Pre-Transplant Mean ± SD	1-y Post-Transplant Mean ± SD	2-y Post-Transplant Mean ± SD		Pre-Transplant Mean ± SD	1-y Post-Transplant Mean ± SD	2-y Post-Transplant Mean ± SD	
Weight (kg)	64.55 ± 9.22 [a]	68.67 ± 12.04 [b,*]	70.89 ± 9.67 [b,*]	0.035	74.57 ± 26.57 [a]	83.39 ± 17.79 [b]	86.99 ± 19.35 [b]	0.057
BMI (kg/m^2)	23.42 ± 3.63 [a]	24.92 ± 4.58 [b,*]	26.21 ± 5.02 [b,*]	0.037	27.60 ± 7.51 [a]	31.17 ± 4.56 [a]	32.67 ± 3.47 [a]	0.063
DEXA parameters								
BMD lumbar spine (gm/cm^2)	1.04 ± 0.14 [a]	1.01 ± 0.16 [a]	0.98 ± 0.26 [a]	0.179	1.09 ± 0.15 [a]	0.83 ± 0.11 [a]	1.01 ± 0.16 [a]	0.207
Lumbar spine Z-score	−0.19 ± 1.43 [a]	−0.69 ± 1.28 [b]	−0.45 ± 1.20 [a]	0.002	−0.40 ± 1.37 [a]	−0.84 ± 1.33 [a]	−0.86 ± 1.86 [a]	0.368
BMD femoral neck (gm/cm^2)	0.86 ± 0.33 [a]	0.71 ± 0.34 [b]	0.85 ± 0.25 [b]	0.001	0.96 ± 0.17 [a]	0.79 ± 0.38 [a]	0.97 ± 0.13 [a]	0.867
Femoral neck Z-score	−0.06 ± 1.09 [a]	−0.73 ± 0.96 [b]	−0.49 ± 1.00 [a]	0.001	−0.17 ± 1.58 [a]	−0.27 ± 1.52 [a]	−0.34 ± 1.35 [a]	0.867
Biochemical parameters								
25OHVD (nmol/L)	11.52 ± 6.49 [a,*]	48.49 ± 27.11 [b]	64.00 ± 22.23 [b]	<0.001	52.86 ± 16.41 [a]	58.86 ± 38.52 [a]	77.14 ± 38.33 [a]	0.368
Vitamin D_3 (nmol/L)	10.97 ± 5.84 [a,*]	41.43 ± 24.84 [b]	57.67 ± 16.67 [b]	<0.001	33.29 ± 19.35 [a]	32.19 ± 22.96 [a]	76.14 ± 38.06 [b]	0.032
Intact parathyroid h (pmol/L)	63.50 ± 51.29 [a]	23.89 ± 39.54 [a]	91.64 ± 43.51 [b]	0.002	83.86 ± 58.24 [a]	37.86 ± 35.92 [a]	100.48 ± 45.78 [a]	0.368
ALP (U/L)	91.28 ± 35.60 [a]	79.39 ± 26.70 [a]	71.01 ± 13.56 [a]	0.056	125.14 ± 48.61 [a]	112.43 ± 59.71 [a]	96.43 ± 42.96 [a]	0.066
Calcium (mmol/L)	2.16 ± 0.11 [a,*]	2.26 ± 0.99 [b]	2.25 ± 0.93 [b]	0.016	2.27 ± 0.17 [a]	2.18 ± 0.12 [a]	2.15 ± 0.45 [a]	0.565
Phosphate (mmol/L)	1.14 ± 0.33 [a]	1.11 ± 0.22 [a]	1.05 ± 0.17 [a]	0.454	1.10 ± 0.27 [a]	1.14 ± 0.25 [a]	1.11 ± 0.30 [a]	0.867
Mg (mmol/L)	0.88 ± 0.10 [a]	0.72 ± 0.12 [b]	0.62 ± 0.17 [b]	<0.001	0.90 ± 0.14 [a]	0.70 ± 0.10 [a]	0.68 ± 0.11 [a]	0.066
Creatinine (umol/L)	85.94 ± 28.05 [a]	105.83 ± 55.69 [a]	89.47 ± 25.01 [a]	0.486	109.29 ± 41.92 [a]	94.57 ± 32.35 [a]	99.86 ± 22.70 [a]	0.156
Urea (mmol/L)	8.06 ± 4.25 [a]	7.31 ± 3.28 [b]	5.93 ± 1.86 [b]	0.076	13.29 ± 7.97 [a]	7.26 ± 2.04 [a]	15.06 ± 16.70 [a]	0.102
Sodium (mmol/L)	134.50 ± 6.79 [a]	141.44 ± 2.79 [a]	143.12 ± 4.14 [a]	0.251	139.71 ± 7.50 [a]	140.43 ± 2.70 [a]	143.15 ± 3.36 [a]	0.867
Potassium (mmol/L)	4.04 ± 0.54 [a]	4.18 ± 0.33 [a]	4.07 ± 0.19 [a]	0.600	4.29 ± 0.75 [a]	4.01 ± 0.42 [a]	4.09 ± 0.24 [a]	0.867
Chloride (mmol/L)	95.72 ± 7.64 [a]	104.83 ± 3.49 [b]	98.29 ± 5.91 [a]	0.131	98.29 ± 5.47 [a]	104.14 ± 2.85 [a]	102.97 ± 3.34 [a]	0.867

† p-values of the three related samples by Friedman's two-way analysis of variance by ranks. Values with different superscripts ([a] and [b]) mean significant vs. pretransplant phase; * Significant versus the related samples in Group II by Mann–Whitney U test; BMI is body mass index; DEXA is dual-energy X-ray absorptiometry; BMD is bone mineral density; 25OHVD is 25 hydroxyvitamin D; ALP is alkaline phosphatase enzyme; Mg is magnesium.

3.3. Survival Analysis Based on VD and BMD

Notably, in Group I with VD-D, Cox's regression analysis showed that for each additional unit of BMI, the hazard increases by about 33% (Table 4). This was not the case in Group II. Besides, in both groups, for each additional unit of 25OHVD or VD_3, the hazard ratio (HR) showed insignificant changes (Table 4). Furthermore, the age and BMD parameters had insignificant impacts.

Table 4. Multivariable Cox proportional hazards regression model based on pretransplant variables.

Variables	Group I (25OHVD < 25 nmol/L)				Group II (25OHVD ≥ 25 nmol/L)			
	HR	95% CI		p-Value	HR	95% CI		p-Value
		Lower	Upper			Lower	Upper	
Age	0.962	0.888	1.042	0.345	0.869	0.696	1.086	0.218
BMI	1.327	1.015	1.733	0.038	1.145	1.756	1.734	0.524
Normal BMD at LS	Reference				Reference			
Osteopenia at LS	0.342	0.000	77,594.19	0.865	0.008	0.000	-	0.995
Osteoporosis at LS	0.000	0.000	-	0.995	0.000	0.000	-	0.995
Normal BMD at FN	Reference				Reference			
Osteopenia at FN	0.538	0.000	123,625.63	0.922	0.004	0.000	-	0.994
Osteoporosis at FN	0.000	0.000	-	0.996	0.382	0.000	-	0.999
VD_3 serum level	0.916	0.712	1.179	0.497	0.958	0.868	1.058	0.394
25OHVD serum level	0.930	0.681	1.270	0.648	0.345	0.650	1.163	0.345

HR is hazard ratio; BMI is body mass index; BMD is bone mineral density; LS is lumbar spine; FN id femoral neck; 25OHVD is 25 hydroxyvitamin D; VD_3 is vitamin D_3.

4. Discussion

This study investigated changes in the VD levels and BMD in HT patients for two years and analyzed the association of VD status (by using an arbitrary cutoff value of 25OHVD, i.e., 25 nmol/L), and BMD with all causes-mortality in vitamin D-deficient and -sufficient groups of HT Saudi recipients. Most of our study participants were on VD supplementation at least by a maintenance dose of 10,000 IU/day. This was successful in the reduction of the percentage of the VD deficiency in both men and women's groups (Figure 2). Besides, means of the 25OHVD and VD_3 serum levels were significantly increased progressively in the men's group and Group I, while the rise of their levels in the women's group and group II were insignificant. Indicating that the benefit of VD supplementation is prominent in those with VD-D. Our baseline percentage of the VD-D was much higher than that reported by Stein et al. [24] where severe deficiency (25OHD <25 nmol/L) was found in 16% of heart transplant patients. Moreover, a Slovenian cohort of HT recipients showed 21.3% with severe VD deficiency and 54.7% with mild-to-moderate VD-D. However, these patients were on VD_3 supplementation in a dose of 2000 IU/day and Alfacalcidol of 0.5 µg/day [25]. This frequently reported phenomenon is critical, since the VD-D is linked to post-HT bone loss and fracture possibility, sarcopenia, and may aggravate the immunosuppressive action of corticosteroids or calcineurin inhibitors [26]. Besides, it is associated with periodontal disease and gingival inflammation in HT recipients which impair the nutritional intake [27]. The improvement of VD status after HT especially in the VD- and Ca-supplemented men was in line with a previous report by Gilfraguas et al. [28]. Supplementation in addition to relief of hepatic congestion and improvement of general condition with more mobility and sunlight exposure were the causes of VD status improvement [29]. Unfortunately, this was not the case in the women's group especially in Saudi Arabia where indoor lifestyle and extensive body covering are the traditions.

Bone metabolism and VD status are closely related. Low 25OHVD levels can negatively impact bone turnover biochemical markers. The PTH as an indicator of bone resorption is usually investigated. Baseline measurements of PTH in our sample indicated higher serum levels of iPTH and low normal Ca levels together with low 25OHVD (Tables 2 and 3). This secondary hyperparathyroidism was improved in the men's group and the deficient groups after the first year then relapsed later, while in Group II and women, insignificant changes were detected in the first year and a significant increase in iPTH (with insignificant reduction of Ca level) were detected by the second year. This finding was consistent with previous reports [27,29]. The increase in the 25OHVD level leads to normalization of serum calcium and phosphate levels, nevertheless, serum iPTH level remained high, especially in the women's group and in the second year in the men's group, indicating a status of persistent hyperparathyroidism in the HT recipients. This persistent secondary hyperparathyroidism occurred in both deficient and sufficient groups in the second year. This finding was consistent with previous reports about HT [30,31], renal transplant adults [32], and up to 50% of children's kidney transplants [33]. Persistent secondary hyperparathyroidism may then lead to autonomous hyperplasia of parathyroid glands. Besides, perioperative administration of large amounts of citrate during blood transfusion leading to precipitation of calcium resulting in hypocalcemia-induced hyperparathyroidism. This persistent hyperparathyroidism or tertiary hyperparathyroidism is usually reported after successful renal transplantation. PTH levels usually decline significantly within the first 3–6 months after kidney transplantation due to the reduction of the functional mass of parathyroid glands [34]. Persistent hyperparathyroidism despite normalization of renal functions, and overall survival was reported in 25% of kidney transplant recipients 1-year after the procedure. Medical management and even parathyroidectomy may be required in these cases [35–40]. Moreover, it may cause serious consequences such as hypercalcemia, organ calcification, hypophosphatemia, and hypercalciuria [34,41].

Despite VD and Ca supplementation, a significant reduction in femoral neck BMD after 1 year was noticed especially in group I, while all remaining measurements were insignificantly different from the pretransplant status, especially in Group II and women. These findings were consistent with previous reports [13,42] about both lung and heart recipients. Compared to pretransplant status, Caffarelli et al. [42] found an increase in the incidence rate of vertebral fractures in the first period post-transplantation (9.6% vs. 25.7%). These vertebral fractures were predicted only by the history of any fracture, while in lung transplant recipients, vertebral fractures were predicted by age, BMD at the femur neck, and history of fracture [43]. The transplantation-associated abnormalities in bone metabolism are generally similar regardless of the transplanted organ, pre-existent low BMD, and previous treatment. Typically, bone loss occurs in the first year after the organ transplant, because of immunosuppressive medications, and the long period of immobilization. Supplementation with VD, Ca, and Mg was not sufficient in the improvement of BMD in the HT population. At least in part, tertiary hyperparathyroidism may be the underlying mechanism. In another hand, Calcitriol (1,25(OH)2VD) supplementation in a dose of 0.5–0.75 μg/day for 12 or 24 months in addition to calcium 600 mg/day in comparison with calcium 600 mg/day produced improvement in the femoral neck (but not at lumbar spine) in the calcitriol groups at 12 months [44]. In another trial, Calcidiol (25OHVD) in a dose of 32,000 IU/week showed a mild improvement of about 4.9% only at the lumbar spine in HT patients [45]

In our study ALP, and urea significantly decreased in the male group rather than the women's group (Table 2). In the pretransplant phase, congestive hepatopathy and even liver cirrhosis may be evident resulting in impaired hepatic functions such as protein and lipid biosynthesis and decreased ability for detoxification of toxic metabolites. Besides, secretions of hepatic enzymes show an abnormal pattern such as rises in alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) [46]. Post-transplant relief of hepatic congestion greatly correct this abnormal pattern. Moreover, Przybyłowski et al. [10] reported that vitamin D was correlated with kidney functions in heart transplant patients, i.e., improvement of VD status was associated with improvement of renal functions.

Interestingly, Cox's regression analysis showed that in Group I, for each additional unite of 25OHVD, the hazard for all-cause mortality decreases by 7% (HR = 0.930; 95%CI: 0.681–1.270, p = 0.648), while in Group II, for each additional unite of 25OHVD, the hazard decreases by 65.5% (HR = 0.345; 95%CI: 0.650–1.163, p = 0.345). However, these findings were statistically insignificant (Table 4). The current study finding was in line with Zittermann et al. [18] who reported no effect of 4000 IU/day oral vitamin D supplementation in reduction of mortality in patients with advanced heart failure. After 3 years there was no beneficial latency impact of VD supplementation on all-cause mortality in the same study participants [47]. In a meta-analysis of randomized clinical trials, with >83,000 participants, VD supplementation failed in reducing the risks of major adverse cardiovascular events, stroke, myocardial infarction, cardiovascular disease mortality, or all-cause mortality [48]. In children undergoing hematopoietic stem cell transplant, there was no significant difference in overall survival for those with pretransplant VD deficiency, or sufficiency or optimal level (p = 0.51) [49]. In a renal transplant study, Cox regression analysis showed no significant prediction between 3-month 25OHVD or 3-month $1,25(OH)_2VD$ levels and mortality (HR = 0.97; 95% CI: 0.93–1.02, p = 0.27 for 1 25OHVD unit increase, and for 10 units increase it was 0.86; 95% CI: 0.70–1.06, p = 0.16) [50]. On the other hand, Zittermann et al. [17] found that low postoperative levels of $1,25(OH)_2VD$ were associated with high 1-year mortality in HT recipients. Furthermore, in a large cohort of kidney transplant recipients, survival was better in recipients with sufficient vitamin D which was measured 10 weeks post-transplant [51]. However, we used a different indicator of the VD status (i.e., 25OHVD), and we used the pretransplant level. Another report from patients with chronic heart failure showed about a 14% reduction of all-cause mortality with a 2.7-fold increment in the 25OHVD Level (95% CI: 1–26%; p =

0.04) [52]. Pediatric reports also stated that VD-D was associated with lower survival on a short-term basis after hematopoietic stem cell transplantation in children [53].

This study's findings indicate a significant increase in the HR of all-cause mortality in the VD-deficient group rather than the VD-sufficient group (HR = 1.327; 95% CI: 1.015–1.733, $p = 0.038$). Independent of the VD status, a systematic review showed that pretransplant BMI was associated with increased risk of mortality in those with BMI above 30 Kg/m^2 (10% increase in HR) and those above 35 kg/m^2 (by about 24%) [54]. Moreover, Doumouras et al. [55] added the low BMI to the obesity as independent factors for increased mortality in HT recipients. While Nagendran et al. [56] excluded BMI up to 35 kg/m^2 from the factors that worse mortality risk. Our sample's pretransplant BMI was at the range of normal BMI in Group I, and the overweightedness in Group II. In the general population, obesity may affect the association between VD and cardiac disorders. However, VD supplementation failed to reduce the incidence rate of cardiovascular diseases and mortality [57]. In the HT population, the current study tested the BMI and VD levels in the same Cox's regression model and resulted in a significant effect of BMI with an insignificant effect of the VD levels. This indicates that the mortality-increasing effect of the BMI is independent of VD.

5. Conclusions

This study tracked the changes of the VD3, 25OHVD, and BMD in VD-supplemented heart transplant recipients for 2 years. Further, it investigated the association of 25OHVD, and BMD with all-cause mortality, based on an arbitrary cutoff value of 25OHVD equal 25 nmo/L. Supplementation with VD_3 10,000 IU daily dose, Ca 1200 mg/day, and Mg 200 mg/day were being effective in elevating the serum level of 25OHVD especially in vitamin D deficient HT recipients; however, no significant impact was detected on the preservation of the BMD at measured sites, or on correction of tertiary hyperparathyroidism. Interestingly, the 25OHVD failed to ameliorate the all-cause mortality hazard ratio.

6. Limitations

The main limitation of this study was the lack of a placebo-controlled group. Instead, we used the comparison of VD deficient (Group I) vs. VD sufficient (Group II). The limited number in the women's group is also a considerable limitation that may affect the obtained results. However, the number of female candidates is usually less than males in many centers all over the world. Another important limitation is the missing of about 21.8% of our participants at the end of the study; either by death (15.6%) or by no-show (6.25%). Missing data are usually common in longitudinal studies. Besides, we did not measure the 1,25(OH)2VD levels and considered the commonly used indicator for VD status which is 25OHVD.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Isoform GC1f of the Vitamin D Binding Protein Is Associated with Bronchiectasis Severity

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Abstract: Vitamin D modulates immune responses and its deficiency has been observed in more than 60% of bronchiectasis patients. Vitamin D binding protein (DBP) is coded by the GC gene, is involved in the transport of vitamin D, and includes a number of isoforms based on single nucleotide polymorphisms (SNPs) in the coding region at rs7041 and rs4855. We evaluated the possible clinical impact of DBP polymorphisms and isoforms in an observational, cross-sectional study conducted in 116 bronchiectasis patients, who were genetically characterized for rs4588 and rs7041 SNPs. Results showed that the GC1f isoform (rs7041/rs4588 A/G) correlated with a more severe disease (18.9% vs. 6.3%, $p = 0.038$), a higher incidence of chronic infections (63.6% vs. 42%, $p = 0.041$), and a lower BACI score (0.0 (0.0, 2.5) vs. 3.0 (0.0, 3.0), $p = 0.035$). Moreover, blood concentration of vitamin D was higher in patients carrying GC1s (median (IQR): 20.5 (14.3, 29.7 vs. 15.8 (7.6, 22.4), $p = 0.037$)). Patients carrying GC1f isoform have a more severe disease, more chronic infections and lower asthmatic comorbidity in comparison to those without the GC1f isoform. Presence of the GC1s isoform (rs7041/rs4588 C/G) seems to be associated to a milder clinical phenotype with increased vitamin D levels and lower comorbidities score.

Keywords: bronchiectasis; vitamin D; DBP; rehabilitation

1. Introduction

Bronchiectasis is a chronic respiratory disease characterized by abnormal dilation of bronchi with patients experiencing daily cough, sputum production and frequent exacerbations [1]. Pulmonary infections often become chronic leading to a vicious circle of airway inflammation [2]. Host–pathogen interaction is, nowadays, a hot topic in bronchiectasis investigations, and the identification of treatable traits is one of the main interests of both scientists and clinicians [3].

Among candidate treatable traits in bronchiectasis, vitamin D and its pathway seem to be among the most promising. Vitamin D is involved in pulmonary immunity and its deficiency has been observed in more than 60% of bronchiectasis patients [4,5] and correlates to disease and radiological severity, increased airway inflammation and poor quality of life [4,5]. Vitamin D binding protein (DBP) is involved in the transport of vitamin D, expressed in

different tissues, and produced also by neutrophils. Other functions of this protein include macrophage activation after conversion to macrophage-activating factor (DBP-MAF) by enzymes released from lymphocytes, modulation of neutrophils and monocytes chemotaxis through the increased production of C5-derived peptides and actin scavenging [6–9].

DBP is coded by the single copy *GC* gene (NCBI GENE ID2638) [6]. A great number of genetic variants have been identified both in the intronic and exonic portion of this gene. Isoforms of this protein were isolated through functional studies, and now we know that they are based on single nucleotide polymorphisms (SNPs) in the coding region at rs4855 and rs7041. SNPs and isoforms of DBP have been associated with asthma and COPD [6,10–12]. No data are available to date in bronchiectasis. Vitamin D regulates more than 900 genes and it is involved in both innate and adaptive immunity. Polymorphisms in the *GC* gene can be involved in both vitamin D bioavailability and have also direct effects on immunity in lungs and, therefore, on the patient's disease state [13,14].

For these reasons, the aim of this work was to evaluate the impact of DBP polymorphisms and isoforms on bronchiectasis patients' characteristics.

2. Materials and Methods

2.1. Study Design and Population

This observational, cross-sectional study enrolled consecutive adults (aged ≥18 years) with bronchiectasis referring to the Bronchiectasis Program of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, between March 2017 and March 2019. Patients with clinically (daily sputum production) and radiologically significant bronchiectasis (at least one lobe involvement on chest CT) enrolled during clinical stability (at least 1 month apart from the last exacerbation and antibiotic course). Patients with cystic fibrosis or traction bronchiectasis due to pulmonary fibrosis were excluded along with patients under supplementation with vitamin D. Informed consent was obtained from all the subjects prior to inclusion in the study. The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the institutional review board of Institute (Comitato Etico Milano Area 2, #255_2020, 8 April 2020)

2.2. Study Procedures

Peripheral blood samples were collected from all bronchiectasis patients. Patients underwent clinical, radiological, microbiological, quality of life and functional evaluation during routine visits. We carried out 25-hydroxy vitamin D blood test, immunological functionality and white blood cells count as per clinical practice.

2.3. GC Single Nucleotide Polymorphisms (SNPs) Genotyping

GC SNPs (rs4588 and rs7041) were evaluated by allelic discrimination real-time PCR using pre-designed TaqMan® probes (C___8278879_10, and C___3133594_30 assays respectively, ThermoFisher Scientific, Waltham, MA, USA) on DNA extracted from peripheral blood using the QIAsymphony platform as per the manufacturer's instructions. The PCR consisted of a hot start at 95 °C for 10 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Fluorescence detection (VIC and FAM fluorophores) takes place at a temperature of 60 °C. All assays were performed in 10 µL reactions, using TaqMan Genotyping Master Mix and 20 ng of DNA on 96-well plates using a CFX96 instrument (Bio-Rad, Hercules, CA, USA). Control samples representing all possible genotypes and a negative control were included in each reaction [15].

Polymorphisms were considered throughout the manuscript as: SNPs genotype, allele, isoform and isoforms' phenotype. Isoforms genotype in the two loci are provided in Table 1.

Table 1. Isoform genotype in rs7041 and rs4588.

Isoform	*GC* rs7041	*GC* rs4588
GC1f	A	G
GC1s	C	G
GC2	A	T

2.4. Clinical Evaluation

Disease severity was assessed through both Bronchiectasis Severity Index (BSI) and FACED score (evaluating FEV_1, Age, Chronic infection with Pseudomonas, Radiological Extension and Dyspnea) [16,17]. A modified Reiff score was used to assess radiological severity of bronchiectasis. It rates the number of involved lobes (with the lingula considered to be a separate lobe) and the degree of dilatation (range: 1–18) [18]. All bacteriology was performed on spontaneous sputum samples as previously described [19]. Murray-Washington criteria for sputum quality were used in all cases, with all samples having less than 10 squamous cells and more than 25 leukocytes per low-power microscope field. Chronic infection is defined as 2 isolation of the same bacteria at least 3 months apart over 12 period [20]. Quality of life was assessed through the Quality of life -Bronchiectasis (QoL-B) questionnaire [21]. Asthma was diagnosed according to the latest international guidelines (Global Initiative for Asthma. Pocket Guide for Asthma Management and Prevention. Available online: https://ginasthma.org/pocket-guide-for-asthma-management-and-prevention accessed on 2 April 2021).

2.5. Statistical Analysis

Variables were collected in an ad hoc electronic form. Qualitative variables were summarized with absolute and relative (percentage) frequencies, whereas quantitative variables with medians (interquartile ranges, IQR). Differences between groups were statistically assessed with chi-squared or Fisher exact tests when appropriate for qualitative variables, whereas with Mann–Whitney tests for quantitative non-parametric variables. A two-tailed *p*-value was considered statistically significant when less than 0.05. Analyses were performed considering genotype, allele, isoform and isoform phenotype for the considered SNPs. The statistical software SPSS version 25 (IBM, Armonk, NY, USA) was used for all statistical computations.

3. Results

We included 116 bronchiectasis patients (78 (67.2%) female, age median (IQR) 62.0 (48.8, 72.0)) in the study. Vitamin D concentration in blood was median (IQR) 20.4 (13.0, 28.4) ng/mL. A full description of clinical characteristics of the study population is reported in Table S1 of supplementary file.

Frequency of DBP isoforms and isoform phenotypes is reported in Table 2. GC1s isoform was found in 95 (81.9%) of the enrolled patients, GC2 in 55 (47.4%) and GC1f in 37 (31.9%).

Table 2. Isoforms of vitamin D binding protein (DBP) in bronchiectasis patients.

Isoform	Genotype (rs7041/rs4588)	Bronchiectasis *n* (%)
GC1s	C/G	95 (81.9)%
GC1f	A/G	37 (31.9)%
GC2	A/T	55 (47.4)%
Isoform Phenotype	**Genotype (rs7041/rs4588)**	**Bronchiectasis *n* (%)**
GC1f-GC1f	A/G-A/G	0 (0)%
GC1s-GC1f	C/G-A/G	24 (20.7)%
GC1s-GC1s	C/G-C/G	37 (31.9)%
GC1s-GC2	C/G-A/T	34 (29.3)%
GC1f-GC2	A/G-A/T	13 (11.2)%
GC2-GC2	A/T-A/T	8 (6.9)%

Full description of rs7041 and rs4588 alleles and genotypes are reported in Table S2 of supplementary file.

3.1. GC Isoforms and Clinical Characteristics

3.1.1. GC1f Isoform

A significant difference in disease severity measured with FACED score was found between GC1f and the other isoforms (FACED severe, *n* (%): GC1f 7 (18.9%) vs. other isoforms 5 (6.3%), $p = 0.038$).

Chronic infection was higher in patients with GC1f isoform 12 (63.6%) compared to the others 29 (42%), ($p = 0.041$), although no differences in terms of chronic infection by specific bacteria were found in the study groups.

Patients with GC1f isoform showed higher values at QoL-B emotional section [median (IQR): GC1f 83.3 (75–100) compared to the other isoforms 75 (50–89.6), ($p = 0.019$), along with a lower asthma prevalence as comorbidity [GC1f 2 (5.4%) vs. other isoforms 18 (22.8%), $p = 0.032$].

Full comparison of the study groups is reported in Table 3.

Table 3. Comparison of clinical characteristics of patients with GC1f isoform vs. others.

Demography				
		GC1f (*N* = 37)	**Other Isoforms (*N* = 79)**	***p*-Value**
Sex (Female)		26 (70.3%)	52 (65.8%)	0.634
Age		61.0 (53.0, 71.0)	63.0 (48.0, 72.0)	0.528
BMI		22.0 (19.0, 25.0)	21.7 (19.0, 24.2)	0.902
Radiology				
Reiff score		4.0 (3.0, 6.0)	4.0 (3.0, 4.5)	0.615
Disease Severity				
BSI score		7.0 (4.0, 10.0)	6.0 (3.5, 9.0)	0.318
BSI risk classes	mild	11 (29.7%)	29 (36.7%)	
	moderate	12 (32.4%)	26 (32.9%)	0.675
	severe	14 (37.8%)	24 (30.4%)	
BSI Moderate-severe		26 (70.3%)	50 (63.3%)	0.461
BSI Severe		14 (37.8%)	24 (30.4%)	0.425
FACED score		2.0 (1.0, 4.0)	2.0 (1.0, 4.0)	0.669
FACED risk classes	mild	21 (56.8%)	42 (53.2%)	
	moderate	9 (24.3%)	32 (40.5%)	0.055
	severe	7 (18.9%)	5 (6.3%)	
FACED Moderate–severe		16 (43.2%)	37 (46.8%)	0.717
FACED Severe		7 (18.9%)	5 (6.3%)	0.038
Clinical Status				
Exacerbation in the previous year		2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	0.887
Hospitalization (at least one previous year)		7 (19.4%)	6 (7.6%)	0.063
Comorbidities				
BACI		0.0 (0.0, 3.0)	0.0 (0.0, 3.0)	0.505
Osteoporosis		2 (5.4%)	6 (7.6%)	0.664
Depression		2 (5.4%)	10 (12.7%)	0.232
Anxiety		1 (2.7%)	4 (5.1%)	0.56
Asthma		2 (5.4%)	18 (22.8%)	0.021
Lung Function				
FEV1%		79.5 (69.8, 102.0)	84.5 (69.2, 98.5)	0.602
FEV1% < 50%		6 (16.7%)	9 (11.5%)	0.451
FEV1% < 35%		3 (8.3%)	5 (6.4%)	0.709

Table 3. *Cont.*

Demography			
	GC1f (*N* = 37)	**Other Isoforms (*N* = 79)**	***p*-Value**
Standard Microbiology			
Chronic infection	21 (57.6%)	29 (36.7%)	0.041
Chronic *P. aeruginosa*	12 (36.4%)	18 (26.1%)	0.287
Chronic *H. influenzae*	2 (6.1%)	4 (5.8%)	0.958
Chronic *MSSA*	4 (12.1%)	4 (5.8%)	0.266
Chronic *A. xylosoxidans*	1 (3.0%)	1 (1.4%)	0.59
Chronic Others	2 (2.9%)	2 (6.1%)	0.441
Aetiology			
Idiopathic	17 (45.9%)	46 (58.2%)	0.477
Primary Ciliary Dyskinesia	5 (13.5%)	5 (6.3%)	
Primary immunodeficiency	4 (10.8%)	9 (11.4%)	
Post Infective	3 (8.1%)	3 (3.8%)	
Secondary Immunodeficiency	4 (10.8%)	2 (2.5%)	
Others *	4 (10.8%)	14 (17.8%)	
QoL-B Questionnaire			
Physical section	66.7 (43.4, 86.7)	60.0 (41.3, 80.0)	0.459
Role section	70.0 (53.3, 93.3)	66.7 (49.2, 86.7)	0.502
Vitality section	61.2 (44.4, 77.8)	55.6 (33.3, 66.7)	0.232
Emotion section	83.3 (75.0, 100.0)	75.0 (50.0, 85.4)	0.019
Social section	75.0 (39.6, 91.7)	58.3 (41.7, 83.3)	0.452
Treatment Burden section	77.8 (58.4, 77.8)	66.7 (55.6, 77.8)	0.095
Health section	44.4 (33.3, 66.7)	37.5 (16.7, 58.3)	0.25
Respiration section	74.1 (66.7, 81.5)	74.1 (58.3, 81.5)	0.451
Vitamin D			
Vitamin D (ng/mL)	20.2 (11.0, 26.0)	20.4 (14.8, 29.1)	0.224

* Other includes COPD, Connective Tissue Diseases, Alpha1-antitrypsin deficiency, ABPA, Asthma, CFTR-RD, Aspiration. BSI: Bronchiectasis severity index; BACI: Bronchiectasis Aetiology and Co-Morbidity Index; FEV1: Forced expiratory volume first second.

3.1.2. GC1s Isoform

Patients with GC1s were similar to the other isoforms in all clinical characteristics but vitamin D peripheric blood levels were higher in the group carrying this isoform GC1s 20.5 (14.3, 29.7) vs. others 15.8 (7.6, 22.4), (p = 0.037). Moreover, BACI score was significantly lower in GC1s: 0.0 (0.0, 2.5) vs. other isoforms: 3.0 (0.0, 3.0), (p = 0.035), although no difference in the comorbidities that may be associated to vitamin D deficiency was found (Table 4).

3.1.3. GC2 Isoform

Patients with GC2 showed a lower rate of hospitalization in the year prior enrolment (GC2 2 (3.6%) vs. other isoforms 11 (18.0%), p = 0.015) along with a decreased incidence of osteoporosis (GC2 1 (1.8%) vs. other isoforms 7 (11.5%), p = 0.04) (Table S3 of the supplementary file).

3.2. GC Isoform Phenotypes and Clinical Characteristics

Hospitalization rate was increased in patients with GC1s-GC1f and GC2-GC2 in comparison to GC1f-GC2 (GC1s-GC2 0 (0.0%) vs. GC2-GC2 2 (25.0%), p = 0.003; GC1s-GC1f 7 (29.2%) vs. GC1s-GC2 0 (0.0%), p = 0.001). Moreover, asthmatic comorbidity was less frequent in patients with GC1s-GC1f 1(4.2%) vs. GC2-GC2 4 (50.0%), (p = 0.002). Full comparison of the study groups is reported in Table S4 of supplementary file.

Table 4. Comparison of clinical characteristics of patients with GC1s isoform vs. others.

Demography				
		GC1s (*N* = 95)	**Other Isoforms (*N* = 21)**	***p*-Value**
Sex (Female)		64 (67.4%)	14 (66.7%)	0.951
Age		62.0 (48.0, 72.0)	62.0 (49.0, 71.0)	0.991
BMI		21.6 (19.0, 24.2)	22.0 (19.0, 26.0)	0.397
Radiology				
Reiff score		4.0 (3.0, 5.5)	4.0 (3.0, 6.0)	0.746
Disease Severity				
BSI score		6.0 (3.5, 9.0)	6.0 (4.0, 10.0)	0.793
BSI risk classes	mild	33 (34.7%)	7 (33.3%)	
	moderate	31 (32.6%)	7 (33.3%)	0.993
	severe	31 (32.6%)	7 (33.3%)	
BSI Moderate-severe		62 (65.3%)	14 (66.7%)	0.903
BSI Severe		31 (32.6%)	7 (33.3%)	0.951
FACED score		2.0 (1.0, 4.0)	3.0 (2.0, 4.0)	0.3
FACED risk classes	mild	53 (55.8%)	10 (47.6%)	
	moderate	33 (34.7%)	8 (38.1%)	0.722
	severe	9 (9.5%)	3 (14.3%)	
FACED Moderate-severe		42 (44.2%)	11 (52.4%)	0.496
FACED Severe		9 (9.5%)	3 (14.3%)	0.512
Clinical Status				
Exacerbation in the previous year		2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	0.449
Hospitalization (at least one previous year)		11 (11.6%)	2 (10.0%)	0.839
Comorbidities				
BACI		0.0 (0.0, 2.5)	3.0 (0.0, 3.0)	0.035
Osteoporosis		8 (8.4%)	0 (0.0%)	0.168
Depression		12 (12.6%)	0 (0.0%)	0.085
Anxiety		90 (94.7%)	21 (100.0%)	0.282
Asthma		15 (15.8%)	5 (23.8%)	0.379
Lung Function				
FEV1%		84.0 (70.0, 101.0)	83.0 (56.0, 88.0)	0.233
FEV1% < 50%		11 (11.8%)	4 (19.0%)	0.377
FEV1% < 35%		5 (5.4%)	3 (14.3%)	0.149
Standard Microbiology				
Chronic infection		41 (48.2%)	9 (52.9%)	0.723
Chronic *P. aeruginosa*		24 (28.2%)	6 (35.3%)	0.56
Chronic *H. influenzae*		6 (7.1%)	0 (0.0%)	0.259
Chronic *MSSA*		8 (9.4%)	0 (0.0%)	0.188
Chronic *A. xylosoxidans*		1 (1.2%)	1 (5.9%)	0.201
Chronic Others		3 (3.5%)	1 (5.9%)	0.648
Aetiology				
Idiopathic		47 (49.5%)	16 (76.2%)	
Primary Ciliary Dyskinesia		10 (10.5%)	0 (0.0%)	
Primary Immunodeficiency		9 (9.5%)	4 (19.0%)	
Post Infective		6 (6.3%)	0 (0.0%)	0.739
Secondary Immunodeficiency		5 (5.3%)	1 (4.8%)	
Others *		18(18.9%)	0 (0%)	

Table 4. *Cont.*

Demography			
	GC1s (*N* = 95)	**Other Isoforms (*N* = 21)**	***p*-Value**
QoL-B Questionnaire			
Physical section	63.4 (41.3, 80.0)	55.6 (40.0, 70.0)	0.608
Role section	66.7 (53.3, 86.7)	73.3 (56.6, 90.0)	0.873
Vitality section	55.6 (33.3, 66.7)	55.6 (44.4, 77.8)	0.724
Emotion section	75.0 (56.2, 91.7)	75.0 (62.5, 95.8)	0.684
Social section	58.3 (41.7, 83.3)	83.3 (62.5, 87.5)	0.147
Treatment Burden section	66.7 (55.6, 77.8)	66.7 (55.6, 77.8)	0.855
Health section	37.5 (22.9, 58.3)	44.4 (23.6, 58.4)	0.995
Respiration section	74.1 (58.5, 82.8)	74.1 (63.0, 77.8)	0.478
Vitamin D			
Vitamin D (ng/mL)	20.5 (14.3, 29.7)	15.8 (7.6, 22.4)	0.037

* Other includes COPD, Connective Tissue Diseases, Alpha1-antitrypsin deficiency, ABPA, Asthma, CFTR-RD, Aspiration. BSI: Bronchiectasis severity index; BACI: Bronchiectasis Aetiology and Co-Morbidity Index; FEV1: Forced expiratory volume first second.

4. Discussion

The most important finding of the present study is that bronchiectasis patients with the GC1f isoform have a more severe disease, more frequency of chronic infections and lower asthmatic comorbidity in comparison to those without the GC1f isoform. Moreover, the GC1s isoform seems to be associated to a milder phenotype with increased vitamin D levels and lower comorbidities score (BACI).

Notably, the link between GC1f isoform and clinical/biological data is mainly based on observational data and correlations, not proving any mechanistic rationale.

The effect of *GC* isoforms on chronic respiratory diseases is various. Studies on asthmatic patients reported GC2 isoform to be associated with asthma susceptibility and to an allergic-like immune response [12,22]. Experiences in COPD reported a decreased disease risk with the GC2 variant, whereas the same isoform was associated with increased risk of bronchiectasis in these patients and SNPs in rs7041 were related to a1-antitrypsin deficiency. The isoform GC1f was reported instead as risk factor in this respiratory disease [22].

Vitamin D in our cohort resulted in being 20.5 ng/mL (median level), higher than in other bronchiectasis patients reported by Chalmers and colleagues [4] (24.7 nmol/L, approximately 9.9 ng/mL) and slightly higher than that presented by Ferri et al., with 17.3 ng/mL [5]. We can speculate that the difference between our cohort and the Scottish one should be caused by a diversity in sun exposure of the two countries. We found an increase of vitamin D levels in patients with GC1s isoform compared to those without this isoform. Data in literature reporting vitamin D serum levels in association with GC isoforms are contradictory. The isoform associated with the lowest levels of vitamin D is GC2, and GC2-GC2 phenotype; however, GC1s is usually associated with an intermediate value of vitamin D [23]. The molecular mechanism underneath the association of low vitamin D blood levels are still unknown [23].

GC1f prevalence seemed related to a severer clinical profile in the disease cohort. Bronchiectasis is a chronic respiratory disease that was associated with chronic infection

and inflammation in lungs. Among all the genes activated by the vitamin D pathway, we can find some involved in both innate and adaptative immunity [22]. DBP itself seems to be involved in C5 modulation that leads a modification in neutrophils and monocytes chemotaxis [6]. The association of vitamin D pathway and DBP with immunity reported in literature should explain some of our findings, even if further studies should be needed in order to understand the effect of SNPs in *GC* in relation to local inflammation and infection in lungs.

The radiological severity evaluated through the Reiff score was not associated with the GC1f isoform. This is not surprising in view of the fact that the Reiff score was not originally derived in a population of non-cystic fibrosis bronchiectasis and because it might not capture the complexity of the radiological manifestations of the disease (e.g., bronchial wall thickness, sputum plug, etc.). As a consequence of this, in clinical practice discrepancies are often observed between the radiological and the clinical severity due to the presence of several confounders and it being a very complex disease.

Furthermore, it seems that the presence of isoform GC1f does not affect the number of exacerbations, disease duration, or patient's BMI. Thus the presence and absence of the isoform GC1f might not be closely related to disease progression.

Poor data in literature associated GC1s with respiratory diseases. GC1s-GC1s have been associated in literature with high levels of DBP in sputum of COPD patients and with higher pulmonary obstruction in this disease [24]. Another study reported GC1s variant significantly more frequent in non-smoker controls. Researchers speculated that GC1s may have a role in the detoxification of substances found in smoke [25].

Both rs4588 and rs7041, polymorphisms responsible for the three isoforms, are located in the exon 11 of the *GC* gene. [6]. These modifications in the amino acid conformation of the protein should lead to functional difference including half-life, affinity to the substrate, cell transit time and others that the scientific community has not fully disclosed yet [6].

These functional differences among isoforms may be involved in the associations we found with clinical data in bronchiectasis.

Strength and Limitations

This study has some limitations. Firstly, this is a monocentric study reporting data from Italy, a south-European country that has a natural higher exposure to sunlight, as a consequence, a bias in vitamin D levels evaluation should have been introduced and this could be one of the reasons explaining why we did not find differences in vitamin D levels among isoforms. Secondly, data regarding reversibility testing, asthma treatments, or pack/years history were not collected, and clinical data on exacerbation rate, antibiotic use, and BMI were missing. All this information would have allowed us to define the role of GC1f across different subpopulations of bronchiectasis patients more precisely.

All these caveats notwithstanding, the study has some potentially interesting clinical implications. Bronchiectasis has a large heterogeneity in symptoms, aetiology, disease severity and biological characteristics, and the identification of a new biomarker could help in the stratification of patients and might contribute to the development of a personalized approach to the disease.

As mentioned above, further studies investigating inflammation and bacterial colonization of lungs, as well as cytological assessment and DBP quantification in association with polymorphisms in the *GC* gene, should be carried out in the future in order to develop a deeper knowledge of the clinical associations of these genetic factors to bronchiectasis. Moreover, these data on clinical associations in the bronchiectasis cohort should be confirmed in a larger and more generalizable population.

5. Conclusions

In conclusion, we demonstrated an increased disease severity, increased chronic infection in adult patients with the GC1f isoform of DBP, along with an association of

the GC1s isoform with a milder phenotype with increased vitamin D levels and a lower comorbidities score. These results may represent a step towards the identification of a new biomarker that should help in the stratification of patients and may contribute to the development of a personalized approach to bronchiectasis treatment.

Supplementary Materials: The following are available online at https://www.mdpi.com/2227-9059/9/11/1573/s1, Table S1: Clinical characteristics of the study population presented as median (IQR) or *n* (%), Table S2: SNPs in *GC* gene in bronchiectasis patients, Table S3: Comparison of clinical characteristics of patients with GC2 isoform vs. other isoforms, Table S4: Comparison of clinical characteristics of patients among isoform phenotypes.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy concerns.

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Review

Current Overview on Therapeutic Potential of Vitamin D in Inflammatory Lung Diseases

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Abstract: Inflammatory lung disorders (ILDs) are one of the world's major reasons for fatalities and sickness, impacting millions of individuals of all ages and constituting a severe and pervasive health hazard. Asthma, lung cancer, bronchiectasis, pulmonary fibrosis acute respiratory distress syndrome, and COPD all include inflammation as a significant component. Microbe invasions, as well as the damage and even death of host cells, can cause and sustain inflammation. To counteract the negative consequences of irritants, the airways are equipped with cellular and host defense immunological systems that block the cellular entrance of these irritants or eliminate them from airway regions by triggering the immune system. Failure to activate the host defense system will trigger chronic inflammatory cataracts, leading to permanent lung damage. This damage makes the lungs more susceptible to various respiratory diseases. There are certain restrictions of the available therapy for lung illnesses. Vitamins are nutritional molecules that are required for optimal health but are not produced by the human body. Cholecalciferol (Vitamin D) is classified as a vitamin, although it is a hormone. Vitamin D is thought to perform a function in bone and calcium homeostasis. Recent research has found that vitamin D can perform a variety of cellular processes, including cellular proliferation; differentiation; wound repair; healing; and regulatory systems, such as the immune response, immunological, and inflammation. The actions of vitamin D on inflammatory cells are dissected in this review, as well as their clinical significance in respiratory illnesses.

Keywords: inflammatory lung disorders; cholecalciferol; mechanism; metabolic pathways; treatment

1. Introduction

The majority of the world's population is affected by pulmonary system inflammation, which is both a serious public health issue and a substantial financial burden. Approximately 510 million individuals are thought to be affected by these diseases globally. Inflammatory lung disorders (ILDs) are one of the world's major reasons for fatalities and sickness, impacting millions of individuals of all ages and constituting a severe and pervasive health hazard. During the onset and progression of many lung diseases, structural aberrations in several sections of the lung, including the nasal passages and airways,

are evident. In both small and large airways, this comprises airway remodeling, parenchymal fibrosis, and epithelial lesions. Additionally, cell proliferation, excessive mucus formation, enhanced collagen accumulation, and the stimulation of molecular pathways such as oxidative stress and inflammation all contribute to the loss of elasticity. Asthma, lung cancer, bronchiectasis, pulmonary fibrosis acute respiratory distress syndrome, and COPD all include inflammation as a significant component [1,2]. Microbe invasions, as well as the damage and even death of host cells, can cause and sustain inflammation. Tobacco smoke; professional sensitizers (coal dust asbestos and silica); ecological toxins both exterior (particulate matter and exhausts of diesel) and interior (second- and third-hand smoke and lead particles); biofuel fumes (charcoal, dung, and wood-burning smoke); irritants (cockroach allergens and house dust mites); and microorganisms (viruses and bacteria), as well as malnutrition and a deficiency of vitamins, can all trigger ILDs. To counteract the negative consequences of irritants, the airways are equipped with cellular and host defense immunological systems that block the cellular entrance of these irritants or eliminate them from airway regions by triggering the immune system. Failure to activate the host defense system will trigger chronic inflammatory cataracts, leading to permanent lung damage. This damage makes the lungs more susceptible to various respiratory diseases [3,4].

Bronchodilators and anti-inflammatory drugs are commonly used in the current therapeutic strategy for pulmonary disorders [5]. The phosphodiesterase-4 inhibitors, methylxanthines, orally as well as an inhaled corticosteroid, and monoclonal antibodies are the anti-inflammatory agents used in the management of ILDs [6]. The muscarinic antagonists and β2 agonists are two types of bronchodilators used in the management of pulmonary disorders [7]. There are certain restrictions to using steroids and bronchodilators. Prolonged use of anti-inflammatory steroids has been linked to glaucoma, the development of cataracts and progression of diabetes, elevated body mass, and GIT hemorrhaging, among the other side effects. Furthermore, patients with severe asthma and COPD may develop steroid resistance [8]. As a result, using steroids as anti-inflammatory medicines in certain illnesses may not be a smart idea. Likewise, the long-term use of bronchodilators has been linked to complications such as convulsions, elevated heart beats, and osteoporosis. Additionally, the usage of methylxanthines is prohibited due to their limited therapeutic index and inability to be utilized long-term [9]. As a result, it is critical to find novel therapeutic agents that are regarded as safe, have few or no adverse effects, and have a broad therapeutic index to successfully treat patients and enhance their overall health [10].

Vitamins are nutritional molecules that are required for optimal health but are not produced by the human body. Cholecalciferol (Vitamin D) is classified as a vitamin, although it is a hormone. Vitamin D is thought to perform a function in bone and calcium homeostasis. Recent research has found that vitamin D can perform a variety of cellular processes, including cellular proliferation; differentiation; wound repair; healing; and regulatory systems, such as the immune response, immunological, and inflammation [11,12]. The actions of vitamin D on inflammatory cells are dissected in this review, as well as their clinical significance in respiratory illnesses.

2. Method of Literature Search

We conducted a literature search using thesaurus terms and keywords in the PubMed, Embase, Science direct, Scopus, and Springer Library databases from database inception to 26 August 2020; the following search terms were used: vitamin D, asthma, lung cancer, bronchiectasis, pulmonary fibrosis, acute respiratory distress syndrome, and COPD. There were no language limitations. If data were not accessible, we contacted the authors of the papers. Additionally, we carefully reviewed the included articles' references for the most recent reviews. We began with a preliminary screening of the titles and abstracts, followed by a full-text review. Exclusions included case reports, case series, duplicate reports, comments, and author reactions.

3. The Metabolism of Vitamin D

The prohormone vitamin D must be converted to physiologically active molecules that link to nuclear receptors to regulate a variety of biological activities. The conventional and alternative vitamin D metabolic routes, as well as vitamin D metabolism's hormonal control, are discussed in this section (Figure 1) [13,14].

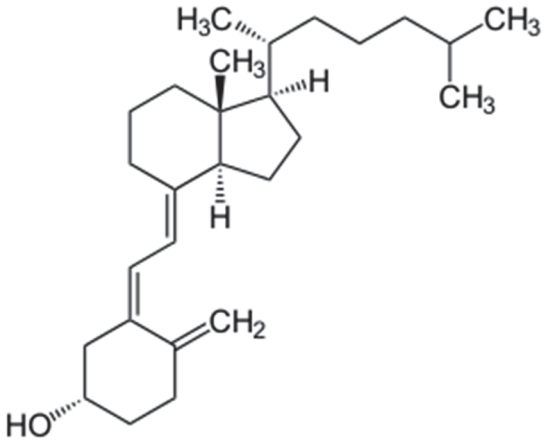

Figure 1. Structure of Vitamin D_3.

4. Conventional Metabolic Pathway

Cholecalciferol: vitamin D_3 and ergocalciferol: vitamin D_2 are both vitamin D isoforms. In plant fungi and yeasts, in the presence of UV light, ergosterol is converted into vitamin D_2, which is consumed through a diet rich in plant-based foods like mushrooms. UV light in the epidermis of human skin converts 7-dehydrocholesterol to vitamin D_3, which may also be found in animal sources like cod liver oil. Vitamin D from the food or in the epidermis of the skin attaches to a protein that binds to vitamin D (VDBP) in the bloodstream and is then transported to the hepatic system. Vitamin D 25-hydroxylase (CYP27A1 and CYP2R1) in the hepatic system converts vitamin D to the calcidiol that is 25(OH)D, which is the most common circulating type of vitamin D in the blood [15,16]. Calcidiol is converted to 1,25-calcitriol in the proximal tubule of the kidney by the hydroxylase of 25(OH)D1 that is a physiologically active type of vitamin D. After entering into the bloodstream and then comparing it to VDBP, calcitriol is transported towards target organs, including the kidneys, gut, and bone, where vitamin D is expected to improve the calcium and phosphate uptake, mobilization, and reabsorption. Since being generated, 24-hydroxylase of 25(OH)D, as that is the main calcitriol downregulating enzyme catalyzing hydroxylation at C-23 and C-24 of both calcidiol and calcitriol, closely regulates their levels. Calcitriol binds to VDRs in target tissues, triggering the nongenomic and genetic control of downwind signaling pathways, also with a wide range of biological activities [17]. Calcitriol interacts with cytosolic VDR in the genomic route, causing it to be phosphorylated, heterodimerized with the retinoid-X receptor (RXR), and then translocated to the nucleus. The composite of calcitriol–VDR–RXR attaches at the promoter region of the vitamin D response element and its target genes and activates transcriptional coactivators to control target gene RNA production and, therefore, a range of activities, including calcium and phosphate metabolism. In hepatic stellate cells, the autophagy adaptor protein p62/SQSTM1 has been shown to perform an important function in heterodimerization and transportation of the RXR–VDR composite to target genes by effectively connecting to RXR, as well as VDR [18]. Calcitriol attaches to VDR, which is bonded to the membrane, also recognized as the 1,25D membrane-associated rapid-response steroid-binding protein, in the nongenomic pathway, and this interplay causes slight alterations in the signaling of the cell, such as Ca^{2+} and MAPK signaling via direct peptide–peptide interactions with signaling molecules, a present intracellularly associated with certain phenotypic functions [19].

5. Alternative Metabolic Pathway

A new vitamin D metabolism route involving CYP11A1 has just been discovered. Due to the discovery of CYP11A1 expression in GIT and the epidermis of the skin, it has come out as a novel metabolizing enzyme of calcitriol. 17(OH)D, 22(OH)D, and 20(OH)D are the most common vitamin D metabolites produced by CYP11A1. CYP11A1 hydroxylates these to produce 17,20,23 (OH)3D, 17,20(OH)2D, 20,22(OH)2D, and 20,23(OH)2D [20]. The anti-inflammation effects, as well as differentiation and antiproliferation of the CYP11A1 metabolites, are equivalent to or greater than those of calcitriol in the epidermis of the skin. Importantly, CYP11A1 metabolites have been found to act as biased agonists of VDR [21].

6. Vitamin D Metabolism and Hormonal Regulation

Calcitriol has a unique negative feedback process that closely controls the metabolism of vitamin D. The inactivating enzyme of vitamin D, CYP24A1, is one of the calcitriol–VDR–RXR complex's strongest transcriptional targets. Around 250-bp and 150-bp upstream of the transcriptional initiation codon in the gene promoter of CYP24A1, two VDREs cause a significant stimulation of CYP24A1 by vitamin D, which could also promote CYP24A1 expression by attracting RNA polymerase II and histone H4 acetyl transferases to a region 50–70 kb downstream of the CYP24A1 gene. As a result, in the kidney calcitriol-driven CYP24A1, its expression may closely control the amounts of calcitriol and calcidiol [22,23]. Furthermore, calcitriol suppresses CYP27B1 production in the kidney by a complicated process, including epigenetic changes to the promoter region. Vitamin D metabolism is controlled by two hormones, FGF-23 and PTH, which, together, perform significant functions in regulating calcium and phosphate homeostasis, in addition to the negative feedback controlled by calcitriol. The parathyroid gland secretes PTH in accordance with a reduced blood Ca^{+2} quantity, which is traced by receptors of Ca^{+2}-sensing located on parathyroid cells. PTH increases calcitriol synthesis by stimulating renal CYP27B1 expression through methods such as enhanced regulation of the nuclear orphan receptor, the transcription dependent on NR4A2 or enhanced cAMP-dependent transcription [24]. While increasing the calcitriol can cause its breakdown by boosting CYP24A1 expression, PTH can maintain the calcitriol levels by causing CYP24A1 mRNA destruction in the kidney via the stimulation of the cAMP–PKA pathway. As a negative feedback mechanism, the elevated calcium levels caused by persistent calcitriol production can inhibit PTH production by interacting with CaSRs in the parathyroid gland. In reaction to elevated plasma phosphate and calcitriol levels, osteocytes and osteoblasts release FGF-23 [25]. Via interacting with FGF receptor–Klotho complexes in the plasma membrane, FGF-23 promotes phosphate excretion by suppressing sodium–phosphate cotransporter 2 expression, which is found at the apical membranes of PCT. FGF-23 also lowers the blood calcitriol levels by blocking the CYP27B1 expression while increasing the CYP24A1 expression in the renal gland; however, the mechanisms are unknown (Figure 2) [26].

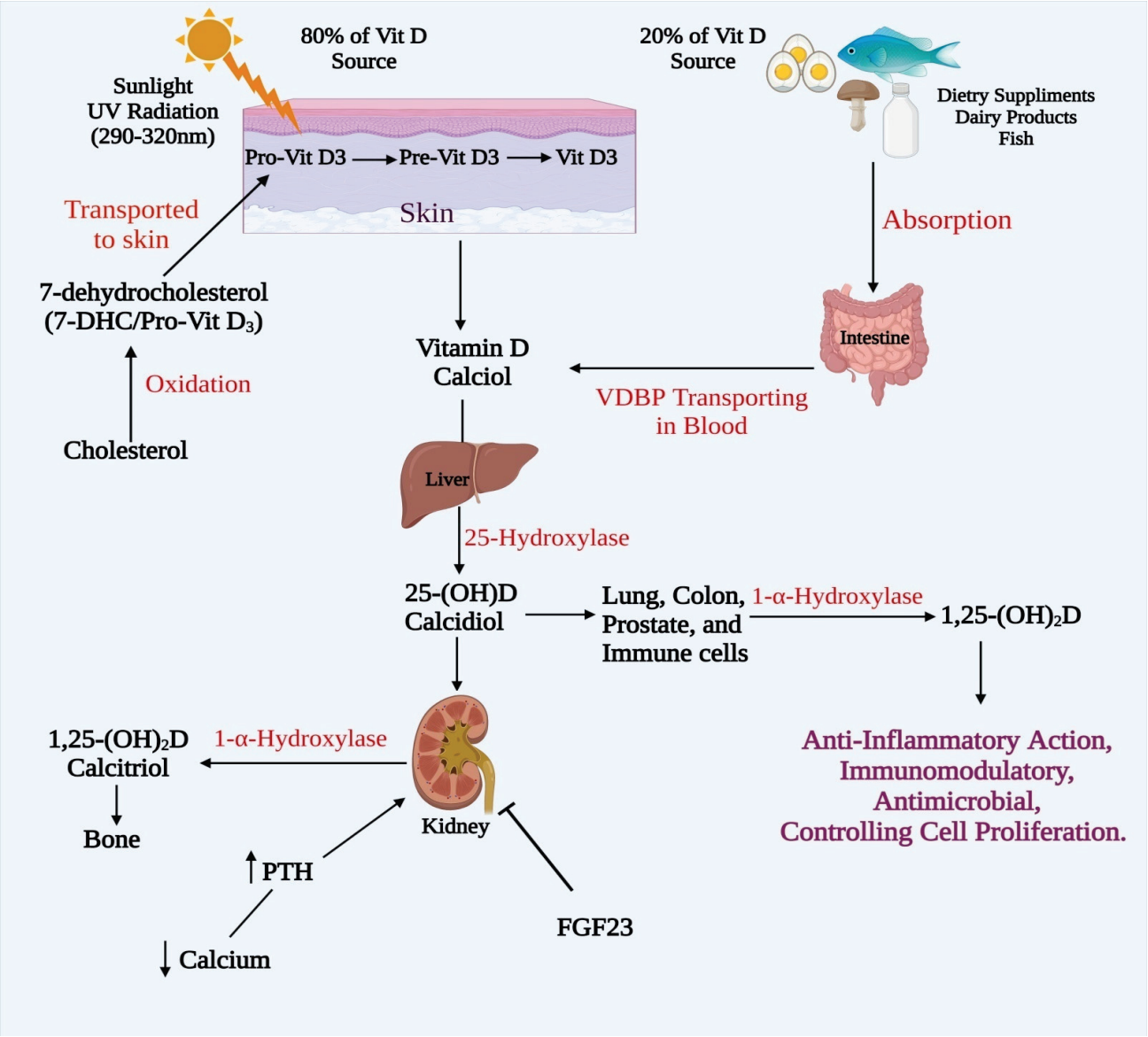

Figure 2. Pathway of calcitriol metabolism.

7. Vitamin D Consumption and Status

The majority of individuals in the United States take less vitamin D than is advised. According to a 2015 to 2016 National Health and Nutrition Examination Survey (NHANES) analysis, the average daily vitamin D intake from foods and beverages was 5.1 micrograms (204 international units) for men, 4.2 micrograms (168 international units) for women, and 4.9 micrograms (196 international units) for children aged 2–19 years. Indeed, according to the 2013–2016 NHANES data, 92% of males, more than 97% of women, and 94% of persons aged 1 year and older consumed less than the EAR of 10 mcg (400 IU) of vitamin D through foods and drinks [27]. Certain individuals had supplements with extremely high levels of vitamin D. Between 2013 and 2014, an estimated 3.2 percent of the adult population in the United States consumed vitamin D supplements containing 100 mcg (4000 IU) or more of vitamin D. Based on the vitamin D consumption through foods, drinks, and even dietary supplements, one may predict a sizable fraction of the US population to be vitamin D-deficient [28]. Comparing vitamin D intakes to the serum 25(OH)D levels, on the other hand, is difficult. One explanation is that sun exposure

influences the vitamin D status, and hence, the blood 25(OH)D levels are frequently greater than would be anticipated just from vitamin D food intakes [29].

8. Inflammatory Lung Diseases

Inflammatory lung disease is a broad term for a group of diseases characterized by lung inflammation and an elevated neutrophil number. Acute and chronic inflammatory respiratory problems are both related with airway inflammation and are affected by a mix of ecological, genomic, and epigenetic factors [30].

8.1. Inflammation Mechanism in Lung Diseases

Inflammation is the body's natural defensive mechanism for removing infections, toxins, and injured cells while also starting the process of healing. Pathogens or chemical exposure, allergens, contaminants, and irritants are the most common causes of airway inflammation. Toll-like receptors recognize pathogen-shared molecular processes and trigger inflammatory cells mediators such as TNF-α, chemokines, and cytokines such as interleukin 8 (IL-8) and NF-κB, which then generate growth factors to begin the corrective actions [31]. TNF-α increases the production of endothelial cell adhesion molecules in lung capillaries, while IL-8 elicits neutrophils [32]. Furthermore, several of the recognized target inflammatory proteins, including vascular cell adhesion molecule-1, cytosolic phospholipase A2, cyclooxygenase-2, intercellular adhesion molecule-1, and matrix metalloproteinase-9 (MMP-9), have been linked to airway inflammation in response to varied sensations. Severe inflammation in the lungs, which is responsible for supplying oxygen to all of the body's organs, can be fatal. Pulmonary homeostasis requires a precise balance of inflammatory and anti-inflammatory factors. As a result, in the therapy of patients with pulmonary inflammation, a clear assessment of the inflammatory processes is critical [33–35]. Tissue microenvironment, stress, energy, illness, neighborhood, and seasonal variations are some of the variables that influence inflammatory reactions. In particular, contact among epithelial cells and neutrophils is possible during inflammatory reactions in which the tissue microenvironment has a direct impact on the signaling pathways and causes immune cells to rush to the damaged tissue. For many illnesses, distinct inflammatory responses have been identified, and the amplification of the inflammatory reaction that develops with age is recognized as a significant component that contributes to inflammation [36].

8.2. The Anti-Inflammatory Property of Vitamin D

Macrophages, an important cell type in the innate immune system, have been found to express VDR. When *Mycobacterium tuberculosis* activates the TLR1/2 heterodimer in macrophages, it causes the overexpression of VDR and CYP27B1, which culminates in the production of the antimicrobial peptide cathelicidin and the death of internalized *M. tuberculosis*. The differentiation of macrophages induced by TLR2/1 is linked to the antimicrobial pathway dependent on the vitamin D in this process, and IL-15 is involved. When the CYP27B1 levels rise, more 1,25(OH)2D3 is produced, which triggers VDR and causes target gene transcription through calcitriol responsive ingredients enshrined in the regulatory regions of the target genes of 1,25(OH)2D3 [37,38]. TLR signaling may be regulated by 1,25(OH)2D3 by modulating miR-155 in macrophages, which provides a unique negative feedback regulatory system for vitamin D to modulate the innate immune response. Vitamin D is considered to be a spontaneous endoplasmic reticulum stress reducer that can selectively inhibit IFN-γ-stimulated macrophages' main effector activities. VDR was shown to inhibit gene transcription in the vicinity of 1,25(OH)2D3 by shifting the deoxyribonucleic acid-bound nuclear factor of stimulated T-cell, suppressing inflammatory cytokine production [39]. The most powerful antigen-presenting cells are dendritic cells (DCs). In a VDR-dependent way, calcitriol suppresses the proliferation, development and immunomodulatory ability of human DCs, characterized as tolerogenic characteristics, according to several studies. Declined costimulatory molecule surface ex-

pressions (CD86, CD80, and CD40) and major histocompatibility complex II, upregulation of inhibitory immunoglobulin-like transcript 3 molecules, and increased release of IL-10 and chemokine (C–C motif) ligand 22 are among the molecular mechanisms influencing the attenuation of tolerogenic properties of DCs by 1,25(OH)2D3 [40,41].

8.3. Vitamin D Dysregulation in Inflammatory Lung Diseases

Vitamin D insufficiency has been related to numerous chronic illnesses in vulnerable populations exposed to airborne particles. The bioactive version of vitamin D, calcitriol, is important for immunological modulation [42]. Many researchers have discussed the incidence and causes of calcitriol insufficiency in calcitriol, the function of vitamin D in COPD, and the vitamin D route connecting airways and inflammatory processes in COPD, among other topics [43]. Furthermore, new research has linked calcitriol deficiency in nutrition to the pathophysiology of chronic respiratory illnesses, including asthma and COPD. In vulnerable US populations residing in urban areas, the third National Health and Nutrition Examination Survey research found a significant connection between the blood levels of calcidiol and lung function, as measured by FVC and FEV1 [44]. Based on the dietary evaluation of the vitamin D status, another cross-sectional research in hospitalized adult COPD patients in Spain found similar results on reduced vitamin D consumption [45,46]. However, there was no link between the blood calcidiol levels (dietary vitamin D consumption) and lung function in spirometrically characterized COPD patients in UK population. In the general UK population, vitamin D is not a major predictor of adult lung function or COPD, according to this study. VDBP has immunomodulatory properties in the lungs and is linked to macrophage stimulation and neutrophil chemotactics [47,48]. The functional significance of the VDBP gene's polymorphism relationship with COPD has been demonstrated in several studies. Additionally, demographic research has highlighted the connection among the reduced vitamin D blood levels and steroids used for chronic asthmatics and COPD patients, as well as individuals with a decreased glucocorticoid response. Furthermore, the impact of dietary vitamin D supplements on lung functions and lung immune–inflammatory cellular control via epigenetic chromatin changes with tobacco smoke and biological pollutants is less well-characterized [49,50].

9. Vitamin D-Based Therapy in Asthma

Asthma is the most common noncommunicable illness, impacting millions of people's health. Asthma is ranked 16th globally in terms of illnesses. Asthma affects about 300 million individuals worldwide, with an additional 100 million people predicted to get the disease by 2025. It is a multigenic inflammatory disease characterized by hyperresponsiveness of the lungs. Dyspnea, wheeze, chest discomfort, and coughing are all indications of asthma. Asthma sufferers encounter a variety of signs ranging from mild to serious, which might happen daily or only rarely. Asthma attacks can occur as the symptoms grow severe. Asthma usually begins in childhood, although it may strike anybody at any age [51,52]. Adult-onset asthma refers to asthma that develops in adults, particularly women. Hereditary, race, sex, lifestyle, and medical problems are some of the risk variables for asthma. Although asthma fatality, comorbidities, and incidences are all greater in low–middle-income nations, there are substantial regional differences in asthma fatalities, comorbidities, and incidence. In asthma, several different types of cells contribute, including epithelial cells, neutrophils, eosinophils, macrophages, mast cells, and T lymphocytes [53]. Asthma is connected to T-helper cell type 2 activity. There is now no asthma-preventive approach, although treatment and action plans can help to minimize the asthma intensity and episodes. Asthma care necessitates the constant monitoring, medication, and avoidance of triggers [54]. Although there has been improvement in the asthma treatment techniques in current years, there is still room for improvement in terms of early illness detection, patient awareness and case-by-case disease prevention. The leukotrienes regulators inhaled long-acting β2-agonists, mast cell stabilizers, and

corticosteroids and are now often used to alleviate inflammation and dilate airways. Even though Vitamin D performs vital functions in asthma relief, there is a paucity of tangible data due to various limitations in observational research [55]. Exogenous vitamin D therapy may assist enhance glucocorticoid responsiveness in some genetically susceptible asthma patients. NF-κB is a key controller of adaptive and innate immunity, and clinical investigations have indicated that 1,25(OH)2D2 reduces NF-κB expression, although the specific method of regulation remains unknown. Due to a lack of strong proof, the significance of Vitamin D in asthma is unknown; however, numerous cross-sectional studies have shown a link between asthma and vitamin D. Reduced concentrations of blood vitamin D have been associated with increased hospitalization and severity, as well as reduced lung performances in adolescents with asthma, according to the controlled studies [56,57]. Vitamin D supplementation has been shown in certain clinical studies to lessen the intensity of asthma. Vitamin D has an effect on pregnant women and children with asthma, according to the research. The results of the clinical research show that the supplementation of vitamin D during pregnancy is vital for preventing asthma in newborns. Increased vitamin D consumption during early pregnancy is a prenatal approach for preventing asthma, according to the researchers. Vitamin D can be utilized to lower asthma fatality and morbidity according to the research findings [58]. Vitamin D supplementation reduces the blood IL-17A levels while increasing IL-10 levels in asthma patients, according to the experimental research. IL-17 is an inflammatory cytokine that is implicated in asthma allergies and has a vital function in bacterial defense. In a mouse model of asthma, for example, vitamin D treatment suppresses airway inflammation, reduces IL4 levels, hinders T-cell recruitment, and reduces the inflammatory process (Table 1) [59].

Table 1. Studies on the investigation of vitamin D role in combating asthma.

Study Type	Study Design	Parameter Examined	Findings	Ref
Clinical Trial	At 3 US centers, Placebo controlled randomized double-blind trial in woman, Placebo (n = 436) v/s 4000 IU/d vitamin D (n = 447), administered parentally.	Through the age of three, parents reported having asthma or recurrent wheeze, according to a physician's diagnosis; Level of calcitriol in pregnant woman 3rd trimester.	Supplementing with 4400 IU/d vitamin D vs. 400 IU/d vitamin D substantially enhanced vitamin D levels in pregnant mothers at risk of getting an asthmatic child. At age three, their children had a 6.1 percent declined asthma incidence and recurrent wheeze, although this did not reach statistical significance.	[60]
	Placebo-controlled, randomized double-blind trial, Physician diagnosed children between ages 5–13 years with moderate to severe asthma, Vitamin D_3 60,000 IU/month for v/s Placebo.	Asthma exacerbation	At 6 months, the D3 treated group had a significantly higher reduction in asthma severity as per GINA standards.	[61]
Cross-sectional Studies	5110 Physician diagnosed asthma patients of age between 50–84 years treated with vitamin D supplement.	Asthma exacerbation.	Asthmatic individuals with vitamin d Deficiency are more inclined to seek emergency medical attention for their asthma and have poor asthma management.	[62]
Preclinical Studies	BALB/c mice	Measurement of in vivo AHR; cytokine production and proliferative reactions to OVA;inflammatory cells.	Suppresses AHR and airway cellular response; Reduces the severity of asthma by reducing mediator release.	[63]
	Sprague–Dawley rats; Vitamin D 100 ng/mL	Airway remodeling; Asthma exacerbation.	Vitamin D treatment reduced airway remodeling in asthma patients by inhibiting the Wnt/β-catenin signaling pathway.	[57]

10. Vitamin D Based Therapy in COPD

COPD is among the leading reasons for death globally, and it is expected to be the third-largest reason for fatality by 2030. It is classified as an inflammatory disease of the

pulmonary system by the GOLD guidelines. COPD is a severe pulmonary illness marked by continuous respiration difficulties, restricted airflow, and abnormal inflammation. Breathing difficulties and inadequate airflow to the lungs are caused by inflammation in the bronchial tube linings (obstructive bronchiolitis) and breathlessness owing to alveolar sac damage (emphysema) [64,65]. The discovery of this vitamin's numerous novel and important functions has been made possible by its deficiency. Its lack has been related to different ailments, like carcinoma, autoimmune disorders, and respiratory illnesses. Intriguingly, declined calcitriol levels have been associated with significant problems in individuals with respiratory illnesses, particularly COPD. Deficient vitamin D has been associated with the advancement of pathological conditions such as inflammatory regulation, severe oxidative stress, increased protease expression, weakened host defense, and lung airway remodeling [66]. The vitamin D (25-OH) levels in a typical adult person vary from 30 to 100 ng/mL. Deficient vitamin D (less than 20 ng/mL) in the human body can be characterized by reduced vitamin D supplementation, lack of sunshine exposure, and vitamin D malabsorption, all of which contribute to the development of COPD symptoms. Vitamin D treatment lowers the incidence of exacerbations in COPD patients who are woefully deficient in the vitamin [67]. Due to an upsurge in the synthesis of numerous matrix metalloproteinases (MMP12, MMP9, and MMP2), emphysema occurs rapidly in VDR knockout mice, resulting in inequity in the protease/antiprotease expression. Vitamin D also suppresses the TGF-b1-signaling pathway, which is linked to COPD fibrosis. Targeting oxidative stress that is important in the progression of COPD and might be a promising treatment target. Vit-D and its analogs have a possible function in activating Nrf2, according to numerous studies [68]. Vitamin D was also related to oxidative treatment. Vitamin D boosts the expression of Nrf2, which boosts alveolar macrophage phagocytosis in COPD patients. There is a growing body of data from medical studies that vitamin D administration and helps to reduce COPD exacerbation. In a double-blinded placebo-controlled randomized clinical study, for example, it was discovered that oral vitamin D treatment reduces COPD aggravation and enhances FEV1 in serious COPD patients. Another clinical trial found that vitamin D treatment enhances the respiratory performance in COPD patients with calcitriol deficiency [69]. The supplementation of vitamin D in the diet enhances COPD patients' quality of life. At present, theophylline, long-acting beta2-agonists (LABA), and inhaled corticosteroids (ICS) are the most often utilized medications to treat individuals with COPD. The major function mechanisms of these medications are to eradicate inflammation and to relax the bronchial tube to lower the airway resistance. Interestingly, vitamin D sufficiency is probably advantageous in guarding against airway and systemic inflammatory illnesses. Many researchers have explored that vitamin D administration can enhance COPD symptoms. According to earlier research, vitamin D might raise the blood levels of the previously listed medications and improve patients' lung function [70].

Thus, vitamin D treatment in COPD patients with deficient vitamin D may be a useful and effective therapeutic strategy to avoid the disease from deteriorating. COPD exacerbation is connected to a high incidence of deficient vitamin D, although low vitamin D blood concentrations are not linked to susceptibility [71]. Vitamin D supplementation can help avoid COPD aggravation. As a result, an additional study is aimed at understanding the significance of vitamin D in COPD patients, as definitive data from many interventional trials are lacking (Table 2).

Table 2. Studies on the investigation of the role of vitamin D in combating COPD.

Study Type	Study Design	Parameter Examined	Findings	Ref
Clinical Studies	Multi-center, randomized, double-blind, placebo-controlled intervention trial, age > 40 years, 16,800 IU vitamin D3 (n = 120) v/s placebo (n = 120) weekly and orally.	COPD exacerbation, Total lung capacity, and maximum respiratory mouth pressure.		[72]
	Randomized clinical study with a double-blind placebo control, 88 severe COPD patients, placebo receive 100,000 IU vitamin D monthly for six months.	FEV1, COPD exacerbation.	Improved FEV1, Reduces COPD exacerbation.	[40]
	Controlled, randomized, double-blind trial, 50–58 year patents, 200,000 IU followed by 100,000 IU vitamin D monthly for 1.1 years (n = 226) v/s placebo (n = 216).	FEV1, COPD exacerbation.	Only smokers benefitted from vitamin D supplementation, particularly those with vitamin D insufficiency or COPD.	[73]
	Multi-center, randomized, double-blind, placebo-controlled intervention trial, Vitamin D_3 (n = 122) v/s Placebo (n = 118)	COPD exacerbation.	Vitamin D_3 supplementation reduced the severity of COPD exacerbations in those with mild to severe COPD.	[74]

11. Vitamin D Based Therapy in Lung Cancer

Lung cancer is the commonest reason of carcinoma-related death in both sexes across the globe. Non-small-cell lung cancer (NSCLC), which contributes to 85% of all cases of pulmonary carcinoma, and small-cell lung cancer (SCLC), which accounts for 15% of the cases, are the two primary types of lung cancer. The carcinoma of squamous cells, adenocarcinoma, and lung carcinoma of the large cells are the three types of NSCLC [75,76]. While smoking tobacco is the leading cause of lung cancer, an approximated 25% of instances occur among nonsmokers, most commonly in the form of adenocarcinomas. Vitamin D has been widely investigated in a variety of cancer situations, and there is compelling proof that it is antineoplastic [77].

Vitamin D's anticancer properties are considered to be triggered by calcitriol interacting with the VDR. Lung cancer cell growth is inhibited, apoptosis is promoted, and angiogenesis is reduced through these methods. A recent in vitro study found that calcitriol caused G0/G1 cell cycle blockage by downregulating cyclins that facilitate cell cycle entrance into the S phase. 1,25(OH)2D inhibits angiogenesis by inhibiting the production of VEGF, which is reported to stimulate endothelial cell excitation, recruitment, and multiplication. In lung cancer cells treated with 1,25(OH)2D, parathyroid hormone-related protein (PTHrP), MMP-9, and MMP-2 expression and synthesis are similarly decreased [78,79]. Since PTHrP and MMPs are both important factors in carcinoma progression, this might be a major mechanism. In a mouse model of lung cancer, the researchers discovered that supplementing with 1,25(OH)2D reduced the tumor occurrence and dramatically reduced tumor multiplication in a dose-dependent way [80]. In vivo, 1,25(OH)2D can reduce pulmonary carcinoma cell metastasis development, implying that sufficient vitamin D levels can inhibit pulmonary carcinoma etiology. While the research to date shows that the vitamin D level influences the start of tumor development, additional clinical research is required to establish if vitamin D can inhibit tumorigenesis (Table 3) [81].

Table 3. Studies on the investigation of the old vitamin D role in combating lung cancer.

Study Type	Study Design	Parameter Examined	Findings	Ref
Clinical Studies	Double-blind, randomized trial, Vitamin D 1200 IU/d (n = 77) v/s placebo (n = 78)	Overall survival and relapse-free survival.	Patients with early-stage lung adenocarcinoma may benefit from vitamin D therapy.	[82]
Preclinical Studies	A/J Mouse model, Vitamin D_3 (2.5 or 5 microgram/Kg diet)	Tumor incidence and tumor cell differentiation.	Reduces incidence of the tumor as well as having combating potential against lung carcinogenesis.	[83]
	Mouse model of N-nitroso-tris-chloroethyl urea; Vitamin D_3 2000 IU/Kg.	The premalignant tumors progressing of Carcinoma	Reduces proliferation, development of premalignant lesion, swelling of squamous cell carcinoma of the lung.	[84]
In vitro studies	NCI-H1975 and A549 tumor cells	Metastasis, tumor cell apoptosis.	The tumor cell growth, infiltration, and metastasis are inhibited, while tumor cell apoptosis is promoted.	[85]

12. Vitamin D Based Therapy in Pulmonary and Cystic Fibrosis

Idiopathic pulmonary fibrosis (IPF) or pulmonary fibrosis is a long-term lung fibrotic infection characterized by a mix of hereditary and ecological variables. IPF can also be caused by cystic fibrosis. Cystic fibrosis (CF) is a fatal autosomal-recessive genetic condition that shortens the lifespan. It mostly affects the lungs, pancreas, and other organs, resulting in blockage and inflammation. A polymorphism in the CF transmembrane conductance regulator (CFTR) gene causes it [81]. The FDA-approved medications for IPF include pirfenidone and nintedanib; however, neither of these drugs is very successful. Furthermore, vitamin D insufficiency has been associated with pulmonary fibrosis and the deterioration of lung function, although the basic concept is unclear. According to studies, vitamin D insufficiency affects 90% of CF patients. Vitamin D deficiency is common in CF patients with pancreatic insufficiency owing to fat malabsorption. Vitamin D supplements are commonly given to children with CF at a young age; however, too much vitamin D might cause respiratory difficulties [86]. Supplemental vitamin D doses and a routine review of the vitamin concentrations must be performed for people to maintain adequate levels. Lung transplantation, which demands an appropriate amount of vitamin D, can enhance the comfort and duration of life in certain CF patients. Concurrently, it was discovered that vitamin D reduces the occurrence of transplant rejection. Vitamin D is unquestionably important along the course of CF [87]. It is worth noting that vitamin D has a positive physiological action on the morbidities and the intricacy that comes with this fundamental condition. As a result, more research is needed to confirm the undisputed potential for alleviating fibrosis complications. Vitamin D supplementation was advised in CF patients with *P. aeruginosa* infection in a scientific investigation that had anti-inflammatory properties by lowering the level of IL-23 and IL-17A [88]. Vitamin D therapies were tested on paraquat (PQ)-induced paralysis. Male C57/BL6 mice with lung fibrosis had fewer leukocytes in their BALF and lower levels of MMP-9, TGF-β, IL-17, and IL-6. Vitamin D treatment in mice may reduce bleomycin-induced lung fibrosis, according to the research. Vitamin D combined with DNA-damaging chemicals might be utilized to treat pulmonary fibrosis. RAS can be triggered by a lack of vitamin D [89]. RAS overexpression has been linked to pulmonary fibrosis. A study found that blood samples from CF patients with high 25(OH)D levels are strongly linked to pulmonary function, although further research is needed to confirm this. According to another study, increasing the vitamin D blood levels can reduce respiratory complications [90]. Ultimately, there are some signs that vitamin D may be a viable therapeutic strategy for CF and IPF, but further large-scale research is needed. Personalized therapy and

pharmacogenomics research has some validity for illness therapy on a case-by-case basis (Table 4).

Table 4. Studies on the investigation of the vitamin D role in combating pulmonary and cystic fibrosis.

Study Type	Study Design	Parameter Examined	Findings	Ref
Clinical Studies	Randomized open-labeled intervention, 16 Cystic fibrosis patients receive Vitamin D_3 35,000 IU/week for age < 16 years or 50,000 IU/week for age > 16 years for 3 months	T cell activation, myeloid dendritic cells.	In people with CF, vitamin D has a wide range of immunomodulatory effects	[91]
	Multicenter, randomized, double-blind, placebo-controlled intervention trial, 23 CF patients chronically affected withP. aeruginosa receive 1000 IU/d for 3 months *v/s* Placebo orally.	Quantification of IL-17A and IL-23.	Vitamin D had an anti-inflammatory impact, lowering the levels of IL-17A and IL-23 in CF patients' airways. Vitamin D supplementation is recommended for CF patients.	[17]
Preclinical Studies	C57/BL6 male mice, Vitamin D I.P. daily at a dose of 5 μg/kg.	Leucocyte count, estimation of inflammatory mediators.	Vitamin D decreases leucocyte count; reduces the level of MMP-9, TGF-β IL-17, and IL-6; beneficial effect in PF treatment	[19]
	C57/BL6 mice treated with bleomycin, Vitamin D 1 μg/kg/day between 3rd day–13th days.	Level of hydroxyproline, Masson Trichrome staining and level of mRNA α-SMA, col3a1 and col1a1.	Up-regulation of mRNA of VDR level, Vitamin D hasthe potential of combating IPF.	[92]
In vitro Studies	Human myofibroblasts, Alveolar epithelial cells type II	DNA damaging	In the vicinity of a DNA damaging chemical in PF, vitamin D had an unexpectedly negative effect.	[93]

13. Vitamin D Based Therapy in Pulmonary Infection including COVID-19

13.1. Pneumonia

Pneumonia is a serious pulmonary illness that has a high fatality rate. It leads to pus and fluid in filled alveoli, resulting in inflammation of the alveoli and adjacent tissue, resulting in a sluggish inhalation of oxygen. Indications such as sputum cough, high temperature, muscular tiredness, breathlessness, and others are seen as consequences. Bacteria (such as *Pseudomonas aeruginosa, Haemophilus influenzae, Streptococcus pneumonia,* and *Staphylococcus aureus*); viruses (such as Coronaviruses, Respiratory Syncytial Virus, Influenza A and B, and others); fungi; and parasites can all cause pneumonia [94,95]. As per the World Health Organization, it is the leading cause of mortality among children globally, with an estimated 1.4 million child fatalities each year. In 2017, there were over 808,694 child fatalities confirmed, accounting for 15% of all child deaths under the age of five. Calcitriol has been associated with pneumonia in several studies. Inadequate vitamin D levels can cause pneumonia in newborns and hospitalization in individuals with serious instances of pneumonia. A decreased amount of calcidiol in the blood of the placental cord has been related to an increased risk of pneumonia in infancy [96,97]. As a result, we may deduce that vitamin D insufficiency in mothers may lead to vitamin D deficit in children; therefore, women should consume enough amounts of vitamin D throughout pregnancy, either through nutrition or supplementation. Pneumonia is typically classified as either hospital-acquired or community-acquired, but individuals with vitamin D deficiency are more likely to develop severe community-acquired pneumonia (CAP), resulting in prolonged stays in clinics and critical care units. Patients admitted to the hospital with vitamin D deficiency and acute ischemic stroke are more prone to developing stroke-

associated pneumonia [98]. As the vitamin D level falls, the risk of pneumonia rises. Many researchers have found a link between pneumonia and vitamin D deficiency; however, these investigations yielded ambiguous results.

13.2. Tuberculosis

After HIV/AIDS, tuberculosis (TB) is the second-most well-known infectious illness, accounting for more than 2 million fatalities worldwide each year. A mild respiratory infection occurs during the early stages of tuberculosis, and a failure in identification and management at this point allows the infection to spread easily through sneezing and coughing [99]. Regardless of the reality that just 10% of the global population with latent tuberculosis will manifest as the aggressive form of the illness, determining which individuals will advance through the disease and will preserve their immunological control or resolve it becomes a critical question with significant health implications [100]. People with tuberculosis are frequently impoverished, which causes the immune response to deteriorating. As a result, dietary supplements that include both micro- and macronutrients may be helpful. Vitamins have long-been thought to be important immune enhancers. Vitamin D has been found to have antimycobacterial effects in recent research. The level of vitamin D is among the extrusive risk deciding variables. Studies on high-risk individuals have looked at several host factors that lead to comprehensive knowledge of the active TB pathway. Periodic variations that cause a decrease in type B UV radiation, lack of sunshine exposure, and dietary insufficiency have all been linked to vitamin D deficiency. Two recent studies have confirmed a substantial link between the seasonal changes in vitamin D blood levels and the prevalence of tuberculosis. A meta-analysis research found a link between poor vitamin D levels and an elevated chance of tuberculosis. Vitamin D supplementation, as a result, might be a powerful tool for reducing the risk of tuberculosis, maintaining the immunity of the host, and improving the antituberculosus therapy efficacy, and it is a hot topic in the current study [101]. Vitamin D's immunostimulatory properties (which include the activation of innate antibacterial activity or actions, as well as anti-inflammatory pathways) have also helped it gain widespread interest. Infection with Mycobacterium tuberculosis causes cellular injury by increasing the production of MMP-10, MMP-1, and MMP-7 in macrophages. MMP-9 has been linked to the intensity of the tuberculosis and the development of TB granulomas [102]. MMP-9 has been related to tuberculosis severity and the formation of tuberculoma granulomas. In epithelial cells infected with Mtb, tissue regulators of MMPs (TIMP) were shown to be reduced. Vitamin D therapy of PBMCs decreases the MMP-10, MMP-1, and MMP-7 expression while increasing the TIMP-1 expression, indicating its critical function in preventing cellular injury and providing symptom relief while having an infection [103]. Additionally, vitamin D deficiency enhances patients' vulnerability to tuberculosis, as well as the likelihood of the illness progressing from dormant to aggressive, supporting its use as a prophylactic in patients with latent TB [104].

13.3. COVID-19

The new coronavirus illness (COVID-19), which was first discovered in late 2019 in Wuhan, China, is still spreading throughout the world, infecting more than 100 million people and killing approximately 2.4 million people in 221 nations around the globe. Due to the pandemic's fast spread and very substantial fatality rates, particularly among vulnerable groups, controlling and preventing it has proven difficult [105,106]. The infection has now quickly spread to nearly every part of the globe, claiming many lives, hurting economies, and jeopardizing recent medical achievements. Several cellular mechanisms have been recognized in associated to a virus of COVID-19, including RIG-I and MDA5 host–recognition evasion, the disruption of M–protein-mediated type-1 IFN induction, dipeptidyl peptidase-4 receptor binding, and papain-like protease-mediated replication [107]. Human DPP-4/CD26 is manifested to interplay with the S1 domain of the COVID-19 spike glycoprotein, representing that it might be a vital virulence factor

in COVID-19 infection. When the vitamin D deficiency is corrected, the DPP-4/CD26 receptor expression is dramatically decreased in vivo [108]. There is also proof that managing calcitriol levels may lessen some of the unfavorable downwind immunological complications associated with the infection of COVID-19, such as an IL-6 surge, postponed IF-γ reactions, and an adverse prognostic indicator in individuals with acute illness pneumonia, even those with COVID-19 infectious disease [9,109]. Recent studies have shown some of the mechanisms through which calcitriol reduces the incidences of viral infections (Figure 3).

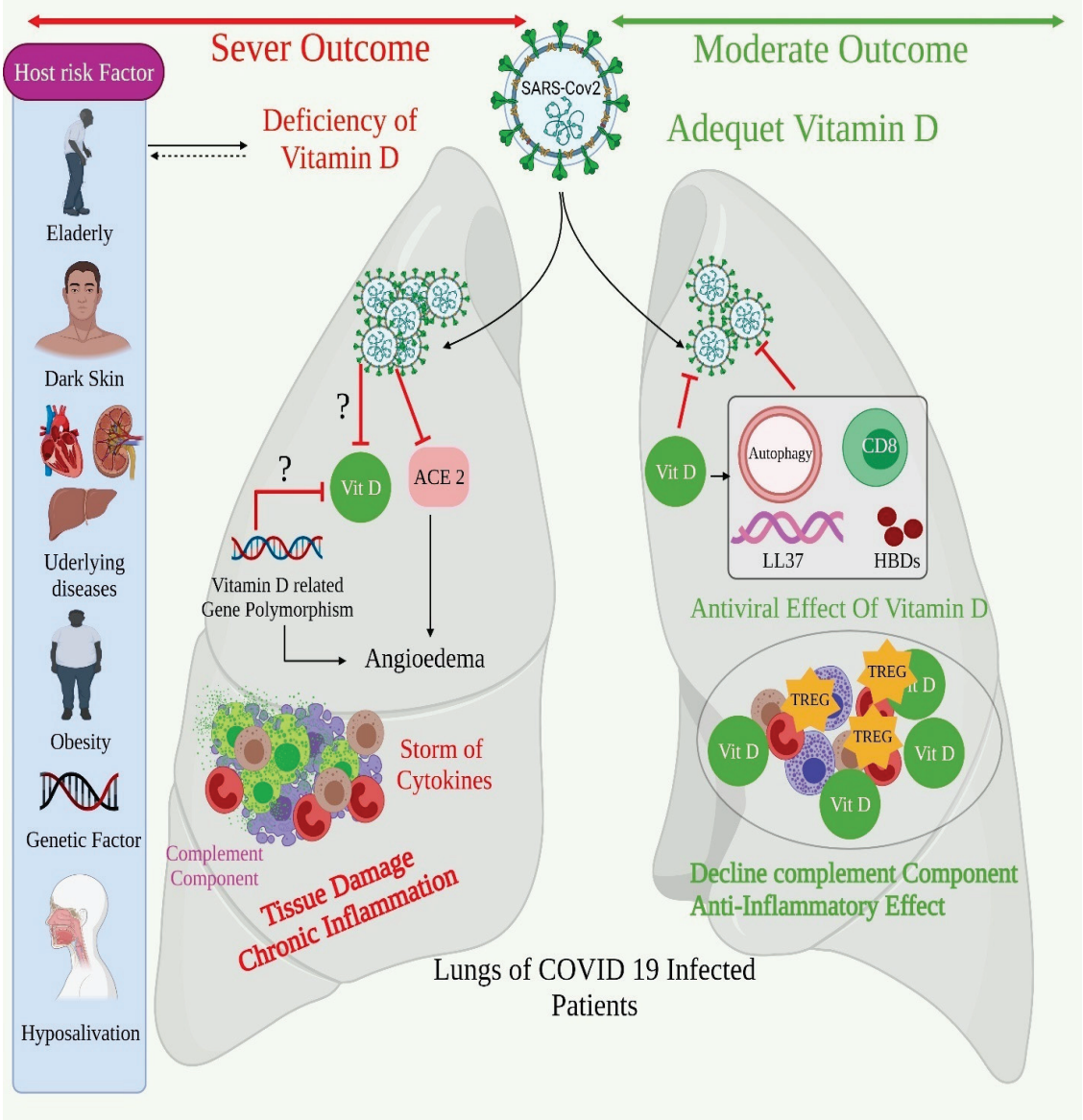

Figure 3. Mechanism of vitamin D in combating COVID-19.

Vitamin D minimizes the incidences of viral infection and death in a variety of ways. Vitamin D works through three mechanisms to lower the chance of catching a cold: cellular natural immunity, adaptive immunity, and the physical barrier [110]. Many in vitro investigations have shown that vitamin D performs an important function in local "respiratory homeostasis", either by promoting the production of antiviral proteins or by interacting with pulmonary virus multiplication. Some retrospective investigations revealed a link between COVID-19 and vitamin D instances and outcomes, whereas others found no link when influencing the factors that were taken into account [59,111]. There is,

however, the inadequate information to link vitamin D levels to COVID-19 intensity and death (Table 5).

Table 5. Studies on the investigation of the vitamin D role in combating COVID-19.

Study Type	Study Design	Parameter Examined	Findings	Ref
Clinical Trials	Multi center, open-label, randomized controlled trial, Vitamin D 50,000 IU daily orally to 260 COVID19 Patients of age $\geq$ 65 years.	All Causes of mortality.	Vitamin D supplementation at high doses might be an efficient, well-tolerated, and quickly available therapy for COVID-19.	[112]
	Household cluster-randomized with a planned pragmatic, double-blinded trial, 2700 subjects 1:1 ratio vitamin D 3200 IU/d *v/s* Placebo.	The likelihood of hospitalization and/or fatality among newly diagnosed people.	Lowering hospitalization and/or death rates in recently diagnosed patients, as well as avoiding infection within their intimate infected persons	[113]
	Open-label randomized parallel pilot, double-blinded trial, 76 COVID-19 hospitalized patients.	ICU admissions and fatalities rate	The use of calcifediol has been shown to minimize the requirement for ICU care in individuals who require hospitalization. COVID-19	[109]
	Randomized Multicenter clinical trials, 69 COVID-19-positive patients, 5000 IU/d (n = 36); 1000 IU/d (n = 33) for two weeks orally.	Gustatory sensory loss and cough recovery	The time it takes for patients to recover from gustatory sensory loss and cough is reduced by taking 5000 IU of vitamin D3 daily for two weeks.	[114]
	65 hospitalized COVID-19 positive patients of age between 63–89 years.	Commodities, Type of respiratory involvement, laboratory parameters (vitamin, C-reactive protein, D, D-dimer), Pulmonary parameters (PaO_2/FiO_2, $PaCO_2$, PaO_2, and SO_2)	Vitamin D insufficiency is linked to more serious respiratory involvement, a lengthier illness period, and a higher chance of mortality.	[112]

14. Excessive Use of Vitamin D Pharmaceutical Formulations

Vitamin D is a necessary prohormone that is required for the maintenance of healthy bones and calcium levels. Vitamin D insufficiency results in hypocalcemia and bone mineralization abnormalities. Vitamin D has become a common alternative due to the rising awareness of vitamin D insufficiency and associated health issues, and its use has expanded significantly. Increased vitamin D supplementation by the general population and an increasing number of therapeutic dosage prescriptions (including extremely high doses) without medical supervision may result in an increased risk of exogenous hypervitaminosis D, commonly known as vitamin D toxicity [115].

Hypervitaminosis D, along with hypercalcemia, occurs as a result of uncontrolled usage of megadoses of vitamin D or vitamin D metabolites. Hypervitaminosis D may develop in some clinical circumstances as a result of the use of vitamin D analogs (exogenous vitamin D toxicity). Hypervitaminosis D, in conjunction with hypercalcemia, may also be a symptom of excessive 1,25(OH)2D production in granulomatous diseases, lymphomas, and idiopathic infantile hypercalcemia (IIH). Exogenous vitamin D toxicity is often induced by the chronic usage of megadoses of vitamin D, not by excessive sun exposure or a diverse diet. The human body can control the amount of previtamin D (tachysterol and lumisterol) generated by ultraviolet B light in the skin. The vitamin D levels in a varied diet are often low, and the vitamin D fortification of food items is minimal [116]. Exogenous vitamin D toxicity is defined by very increased 25(OH)D levels (>150 ng/mL), severe hypercalcemia and hypercalciuria, and extremely low or undetectable parathyroid hormone (PTH) activity. The earliest quantifiable signs of vitamin D poisoning are hypercalciuria and hypercalcemia. In individuals with vitamin D toxicity, the 1,25(OH)2D concentration may be within the reference range, slightly raised, or decreased (less commonly) when a high calcium level in the serum reduces the PTH activity. 1,25(OH)2D is suppressed by inhibiting 1-hydroxylase activity and increasing the 24-hydroxylase activity [117].

15. Conclusions

Vitamin D is an essential vitamin that influences the physiological activities, such as cellular differentiation and proliferation, host defense, immunological regulation, and inflammation, in addition to the usual bone and calcium homeostasis functions. Deficient vitamin D has been associated with different problems related to health, such as bone softening and skeletal abnormalities, as well as immunological disorders. A vitamin D deficit is also linked to inflammatory lung diseases, such as COVID-19, asthma, lung cancer, COPD, pulmonary and cystic fibrosis, pneumonia, and TB putting people with a vitamin D deficiency at a greater chance of developing respiratory diseases with substantial morbidity and fatality. As a result, the interrelationships among vitamin D and respiratory illnesses have sparked a new age of attention in supplementing as a cost-effective way to enhance world health. Furthermore, the clinical usage of vitamin D would aid in immunological sustenance, which would be an exciting future prospect.

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Vitamin D Prevents High Glucose-Induced Lipid Droplets Accumulation in Cultured Endothelial Cells: The Role of Thioredoxin Interacting Protein

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Abstract: Vitamin D (VitD) exerts protective effects on the endothelium, which is fundamental for vascular integrity, partly by inhibiting free radical formation. We found that VitD prevents high glucose-induced Thioredoxin Interacting Protein (TXNIP) upregulation. Increased amounts of TXNIP are responsible for the accumulation of reactive oxygen species and, as a consequence, of lipid droplets. This is associated with increased amounts of triglycerides as the result of increased lipogenesis and reduced fatty acid oxidation. Remarkably, VitD rebalances the redox equilibrium, restores normal lipid content, and prevents the accumulation of lipid droplets. Our results highlight TXNIP as one of the targets of VitD in high glucose-cultured endothelial cells and shed some light on the protective effect of VitD on the endothelium.

Keywords: D-glucose; endothelial cell; vitamin D; oxidative stress; lipid metabolism

1. Introduction

Vascular endothelial cells (EC) form a quiescent monolayer that coats the inner lumen of all vessels and retains critical functions that are essential to preserve the integrity of the vasculature and, consequently, health [1]. They act as a metabolic interface between the blood and tissues and ensure optimal nutrient and oxygen delivery to all of the tissues [2]. EC are steadily exposed to glucose, which is taken up from the blood, mainly through the glucose transporter 1 (GLUT1). Glucose is partially utilized for endothelial metabolic needs and is also delivered to the surrounding cells and tissues. Rather than shunting glucose to oxidative phosphorylation to maximize adenosine triphosphate (ATP) production, EC rely on glycolysis, which takes place in the cytosol and does not demand oxygen [3]. As a consequence, oxygen is saved to be delivered to the parenchymal tissues, protecting the EC against the accumulation of Reactive Oxygen Species (ROS), which are typically produced during oxidative phosphorylation [4]. Additionally, the high extent of Fatty Acid β-Oxidation (FAO) in quiescent EC makes important contributions to the maintenance of the redox balance [5]. Therefore, EC sustain different metabolic pathways to protect themselves against oxidative stress, which is the root of endothelial dysfunction [6]. It is avowed that high glucose levels are detrimental for the endothelium both in the large vessels as well as in the microvasculature [2]. When fasting, the EC are exposed to about 5 mmol/L (5 mM) D-glucose, a concentration that increases post-prandially and that remains below 7.8 mmol/L (7.8 mM) in healthy people. The failure to contain post-prandial glucose spikes as well as the chronic increase of glucose in uncontrolled diabetic patients ultimately generate endothelial dysfunction [2], partly through the increased endothelial production of free radicals [7,8], and partly through conducted through metabolic reprogramming [9]. High extracellular glucose boosts glucose uptake and metabolism through

different pathways, i.e., the polyol pathway, which promotes oxidative stress by consuming Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH), and glycolytic side branches, such as the hexosamine and the pentose phosphate pathways [2,10]. Moreover, it also results in the activation of Protein-kinase C (PKC) because diacyl glycerol accumulates in response to high intracellular glucose concentrations [11]. As a result of endothelial-altered metabolism caused by high glucose, advanced glycation end products (AGE), which activate the inflammatory response, are generated. Both PKC activation and AGE formation are implicated in the promotion of oxidative stress in EC that have been exposed to high glucose levels [12]. Far less is known about fatty acid metabolism in EC. FAO plays an important role in endothelial homeostasis both in vitro and in vivo [13]. It sustains nucleotide synthesis, fuels the tricarboxylic acid cycle and, as mentioned above, contributes to redox homeostasis via the synthesis of NADPH [14]. A particular light has been shed on Carnitine Palmitoyltransferase 1A (CPT1A), a crucial enzyme that converts long-chain acyl-CoAs into long-chain acyl-carnitines, which then enter the mitochondria where FAO takes place [15]. Silencing *CPT1A* in the EC triggers oxidative stress and the overexpression of genes that are involved in controlling redox balance [14].

Remarkably, EC can generate lipid droplets and dynamic cytosolic fat storage compartments [16]. This means that EC can store neutral lipids, which then provide fatty acids to be metabolized in the mitochondria or to be transported to nearby tissues [16]. It is also emerging that lipid droplets are critical components of the cellular stress response, as they protect against lipotoxicity [16]. Lipid supplementation for 24h to cultured EC results in the reversible accumulation of lipid droplets, and similar results were observed in the aortic endothelium of hypertriglyceridemic mice [17].

Beyond its essential role in bone health, Vitamin D (VitD) exerts protective effects on the endothelium. Indeed, VitD deficiency is related to endothelial dysfunction, partially because of the downregulation of the VitD Receptor (VDR) [18]. Consistently, VitD supplementation in VitD-deficient diabetic patients improved endothelial function [19], and a recent systematic review and metanalysis of randomized clinical trials demonstrated that VitD supplementation decreases circulating inflammatory cytokines in patients with altered glucose tolerance [20]. However, another study reported no significant effects of VitD supplementation on endothelial dysfunction [21]. The results that have been obtained in vitro sustain a beneficial effect of VitD in the EC. Indeed, in Human Umbilical Vein Endothelial Cells (HUVEC), 1,25$(OH)_2D_3$ (calcitriol), the most active metabolite of VitD, prevents leptin-induced endothelial dysfunction in a VDR-dependent fashion [22]. Moreover, in HUVEC treated with acetoacetate in order to mimic ketosis, VitD inhibits ROS formation and monocyte adhesion [19]. Our study sought to address some fundamental questions on the response of HUVEC to high D-glucose and on the potential protective role of VitD. Initially, we investigated the levels of some pro- and antioxidant proteins. Then, we studied the contribution of oxidative stress in reprogramming lipid metabolism. Finally, we focused on the effects of VitD in protecting HUVEC from oxidative stress, metabolic derangements, and lipid droplet accumulation.

2. Materials and Methods

2.1. Cell Culture

HUVEC were purchased from the American Type Culture Collection (ATCC, Manassas, WV, USA), cultured in medium M199 (Euroclone, Milano, Italy) containing 10% Fetal Bovine Serum (FBS) (Euroclone), 1 mM L-Glutamine (Euroclone), 1 mM Sodium Pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 1 mM Penicillin-Streptomycin (Euroclone), 5 U/mL Heparin (Sigma-Aldrich), and 150 µg/mL Endothelial Cell Growth Supplement (Sigma-Aldrich) on collagen-coated dishes (50 µg/mL) (Sigma-Aldrich). The cells were routinely tested for the expression of endothelial markers and were used for 6–7 passages. D-glucose (Sigma-Aldrich) was used at the concentrations of 11.1 mM and 30 mM, and L-glucose (Sigma-Aldrich) was used as a control of osmolarity at the concentration of 30 mM. After testing 1α,25-Dihydroxyvitamin D_3 (VitD)

(Sigma-Aldrich) cytotoxicity in a dose-dependent fashion by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (the data are available at the following link https://dataverse.unimi.it/dataverse/biomedicines/ accessed on 30 September 2021), VitD was used at the concentration of 20 nM. Thioredoxin Interacting Protein (TXNIP) was inhibited using small interfering RNAs (siRNAs). Subconfluent cells were transfected with siRNAs targeting *TXNIP* (20 nmol, 5′-AAGCCGTTAGGATCCTGGCT-3′ (Qiagen, Hilden, Germany)). All the non-silenced samples were transfected with a scrambled non-silencing sequence (NS) (20 nmol, Qiagen, cat. n° 1027310) that was used as a control, which produced the same results as the non-transfected samples (data not shown). Lipofectamine RNAiMAX was used as a transfection reagent (Invitrogen, Carlsbad, CA, USA) and was used according to the manufacturer's recommendations. After 6h, the siRNA transfection medium was replaced with a culture medium with the addition of either 11.1 mM or 30 mM of D-glucose in the presence or not of 20 nM of VitD. In some experiments, N-acetylcysteine (NAC, 5 mM) (Sigma-Aldrich) was used as antioxidant [23], in combination or not with high glucose. All the experiments were performed in triplicate.

2.2. ROS Production

For ROS detection, confluent HUVEC were cultured in a 96-well black plate (Greiner Bio-One, Kremsmunster, Austria) and, at the end of the experiments, they were incubated for 30 min with 10 mM 2′-7′-dichlorofluorescein diacetate (DCFH) solution (Sigma-Aldrich). The DCFH dye emission was monitored at 535 nm (excitation λ = 484 nm) using the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). Then, the cells were fixed in Phosphate Buffered Saline (PBS) containing 3% paraformaldehyde (PFA) and 2% sucrose (pH 7.6) for 30 min and, after extensive washing, the cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI), which was used to stain the nuclei (1:10,000). DAPI florescence ($\lambda_{ex/em}$ = 350/470 nm) was monitored using the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) and was used to normalize the DCFH dye emission [7]. The results are the mean of three independent experiments performed in triplicate ± SD.

2.3. Triglyceride Quantification

Triglycerides were quantified using a Triglyceride Quantification Kit (Sigma-Aldrich) according to the manufacturer's recommendations. Briefly, triglycerides are broken down into free fatty acids and glycerol, which is then oxidized to generate a fluorescent product ($\lambda_{ex/em}$ = 535/587 nm). Fluorescence was monitored using the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific). Prior to the extraction of the triglycerides, the cells were trypsinized. An aliquot was stained with 0.4% trypan blue solution and were counted using a Luna Automated Cell Counter (Logos Biosystems, Anyang-si, Gyeonggi-do, Korea). The fluorescent results were normalized on the cell number. The results are the mean of three independent experiments that were performed in triplicate ± SD.

2.4. Staining of Neutral Lipids

HUVEC were seeded to detect lipids as well as to perform the MTT assay under the same experimental conditions. Oil Red O Staining was used to detect neutral lipids. After the 24h treatments, the cells were washed three times with PBS, fixed in PFA 10% for 30 min at room temperature, washed once again with PBS, and then stained with 60% filtered Oil Red O stock solution (Sigma-Aldrich) for 20 min. After extensive washing, the Oil Red O was solubilized in 100% isopropanol, was quantified by measuring the absorbance at 500 nm, and was normalized to the cell number by MTT assay [24,25] after image acquisition using FLoid Cell Imaging Station (Thermo Fisher Scientific). To further confirm the results, staining with BODIPY 493/503 was performed (see dataverse at the following link https://dataverse.unimi.it/dataverse/biomedicines/ accessed on 30

September 2021). The results are the mean of three independent experiments performed in triplicate ± SD.

2.5. Western Blot Analysis

HUVEC were lysed in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (Sigma-Aldrich), 1% NP40, 0.25% sodium deoxycholate (Sigma-Aldrich), protease inhibitors (10 μg/mL Leupeptin, 10 μg/mL Aprotinin and 1 mM Phenylmethylsulfonyl fluoride, PMSF) (Sigma-Aldrich), and phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium vanadate, 5 mM sodium phosphate) (Sigma-Aldrich). Lysates (40 μg/lane) were separated on SDS–PAGE and were transferred to nitrocellulose sheets using the Trans-Blot Turbo Transfer System (Biorad, Hercules, CA, USA.). Western Blot analysis was performed using antibodies against TXNIP (Thermo Fisher Scientific, cat. n° 40–3700, 1:250), Sirtuin 1 (SIRT1) (Thermo Fisher Scientific, cat. n° PA5–17074, 1:1000), Sirtuin 2 (SIRT2) (Millipore, Vimodrone, Italy, cat. n° 09–843, 1:4000), Superoxide-dismutase 2 (SOD2) (BD Transduction Laboratories, Milano, Italy, cat. n° 611580, 1:1000), Heat Shock Protein 70 kilodaltons (HSP70) (Santa Cruz Biotechnology, Dallas, TX, USA, cat. n° sc-1060, 1:200), EDF1 (Aviva Biosciences, San Diego, CA, USA, cat. n° ARP37729_T100, 1:500), PPARγ(Santa Cruz, cat. n° sc-7196, 1:200), and CPT1A (Thermo Fisher Scientific, cat. n° 15184-1-AP, 1:1000). Actin (Santa Cruz, cat. n° sc-1616, 1:200) was used as the equal loading control. After extensive washing, secondary antibodies labelled with horseradish peroxidase (GE Healthcare, Waukesha, WI, USA) were used. Immunoreactive proteins were detected by the SuperSignal Chemiluminescence Kit (Thermo Fisher Scientific). A representative blot is shown. The densitometric analysis was performed using Image J Lab software (Biorad). The results are the mean of three independent experiments ± SD.

2.6. Fatty Acid Oxidation

FAO, the primary metabolic pathway for the degradation of fatty acids, was monitored by Fatty Acid Oxidation assay (Abcam, Cambridge, UK) in living cells. The cells were seeded in a 96-well black plate (Greiner Bio-One), and upon treatment with high glucose for 24 h, they were rinsed twice with pre-warmed Fatty Acid-Free medium followed by the addition of pre-warmed Fatty Acid Measurement Medium. Extracellular O_2 Consumption Reagent (Abcam) was added into all the wells except for the blank control well. The FAO activator carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 0.625 μM) was used as the positive control. Finally, the wells were sealed with pre-warmed high-sensitivity mineral oil. Subsequently, the 96-well black plate was placed into the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific), which had been pre-set to 37 °C. The fluorescent signal ($\lambda_{ex/em}$ = 380/650 nm) was measured every 2 min for 180 min. To simplify the procedure, the results were expressed in a box plot graph. The results are the mean of three independent experiments performed in triplicate ± SD.

2.7. Statistical Analysis

Data are reported as means ± standard deviation (SD). The data were normally distributed, and they were analyzed using the two-way repeated measures ANOVA. The *p*-values deriving from multiple pairwise comparisons were corrected using the Bonferroni method. Statistical significance was defined for *p*-value < 0.05. Concerning the figures, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results

3.1. TXNIP Is Upregulated in HUVEC Exposed to High Glucose

To get insights into the mechanisms that are involved in high glucose-triggered oxidative stress, we analysed the levels of some of the proteins that are implicated in the control of redox balance. HUVEC were cultured in media containing physiological (5.5 mM, CTR) or high glucose (11.1 mM and 30 mM) concentrations for 24 h. L-glucose (30

mM) was used as a control for osmolarity. As shown in Figure 1, we found a substantial increase in the total amounts of TXNIP. When the cells were cultured at the highest D-glucose concentration (30 mM), we also observed the significant downregulation of SIRT1, the most evolutionarily conserved member of the sirtuin family, which exerts beneficial effects on the endothelium. HSP70, PON2, SIRT2, and SOD2 were not modulated. L-glucose exerted no effects (Figure 1).

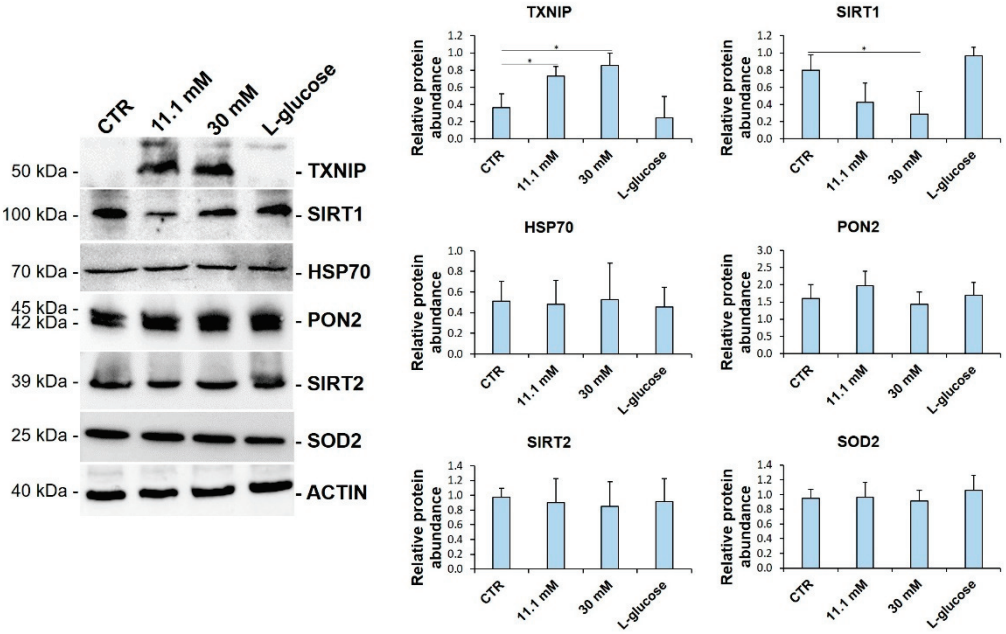

Figure 1. High glucose upregulates TXNIP and downregulates SIRT1 in HUVEC. Western blot (left panel) was performed on cell lysates using specific antibodies against TXNIP, SIRT1, HSP70, PON2, SIRT2, and SOD2. Actin was used as an equal loading control. A representative blot is shown. Densitometric analysis (right panel) was performed using Image J Lab software on three different blots, and the results are the mean of three independent experiments ± SD. * $p < 0.05$.

3.2. TXNIP Upregulation Accounts for ROS Accumulation in HUVEC Cultured in High Glucose

In this paper, we focused on the role of TXNIP upregulation in high glucose-treated HUVEC. For this purpose, we transiently silenced the cells with specific siRNAs targeting *TXNIP*. Then, HUVEC were cultured for 24 h in medium containing physiological (5.5 mM, CTR), high-extracellular D-glucose (11.1 mM and 30 mM) or L-glucose (30 mM) as a control. Figure 2A shows that *TXNIP* silencing prevents high glucose-induced TXNIP increase. Moreover, upon *TXNIP* silencing, high glucose-induced ROS production was dampened by the same amount after the administration of the antioxidant NAC (5 mM), the precursor of glutathione that is widely used as an antioxidant (Figure 2B).

3.3. VitD Prevents TXNIP Upregulation in HUVEC Cultured in High Glucose

Since (i) TXNIP was initially characterized as a target of VitD and since (ii) VitD exerts a protective effect upon metabolic challenge in HUVEC, we anticipated that VitD might affect the levels of TXNIP found in HUVEC cultured in high glucose conditions. Therefore, we exposed HUVEC to media containing high levels of glucose in the presence or in the absence of VitD (20 nM) for 24 h. VitD counters high glucose-induced TXNIP upregulation and ROS accumulation (Figure 3A,B).

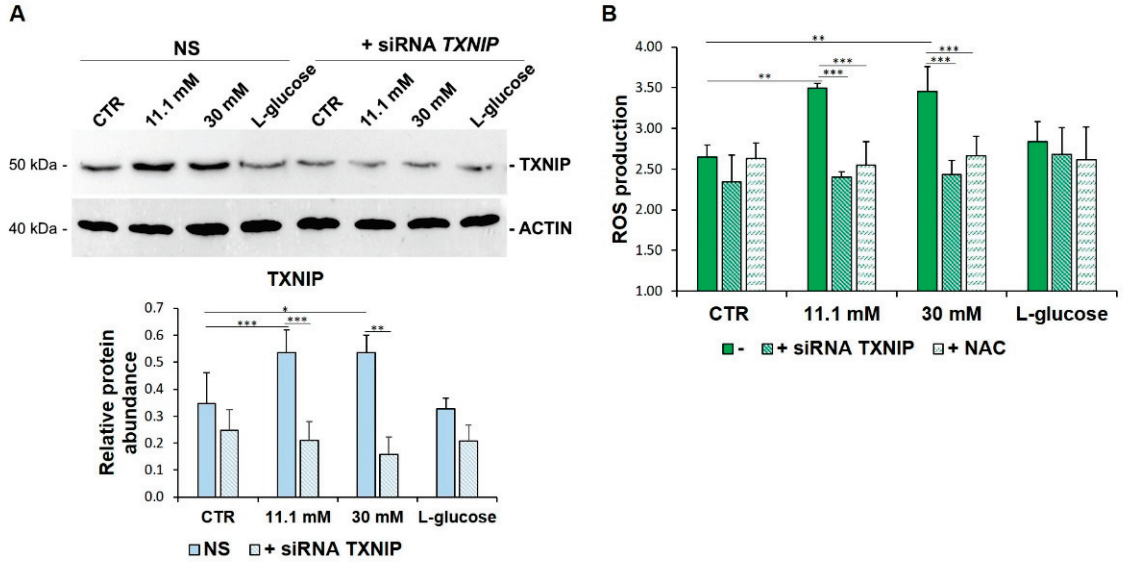

Figure 2. TXNIP upregulation is responsible for ROS accumulation in HUVEC cultured in high glucose levels. (**A**) HUVEC were cultured in medium containing 5 mM (CTR), 11.1 mM or 30 mM of D-glucose after *TXNIP* silencing. A scrambled non-silencing sequence (NS) was used as a control for silencing. Western blot (upper panel) was performed on cell lysates using specific antibodies against TXNIP. Actin was used an equal loading control. A representative blot is shown. Densitometric analysis (lower panel) was performed using Image J Lab software on three different blots, and the results are the mean of three independent experiments ± SD. (**B**) HUVEC were cultured in medium containing 5 mM (CTR), 11.1 mM or 30 mM of D-glucose (-) and either after *TXNIP* silencing or the addition of NAC 5 mM. L-glucose (30 mM) was used as a control of osmolarity. ROS production was evaluated by DCFH, as described in the methods. The results are the mean of three experiments performed in triplicate ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

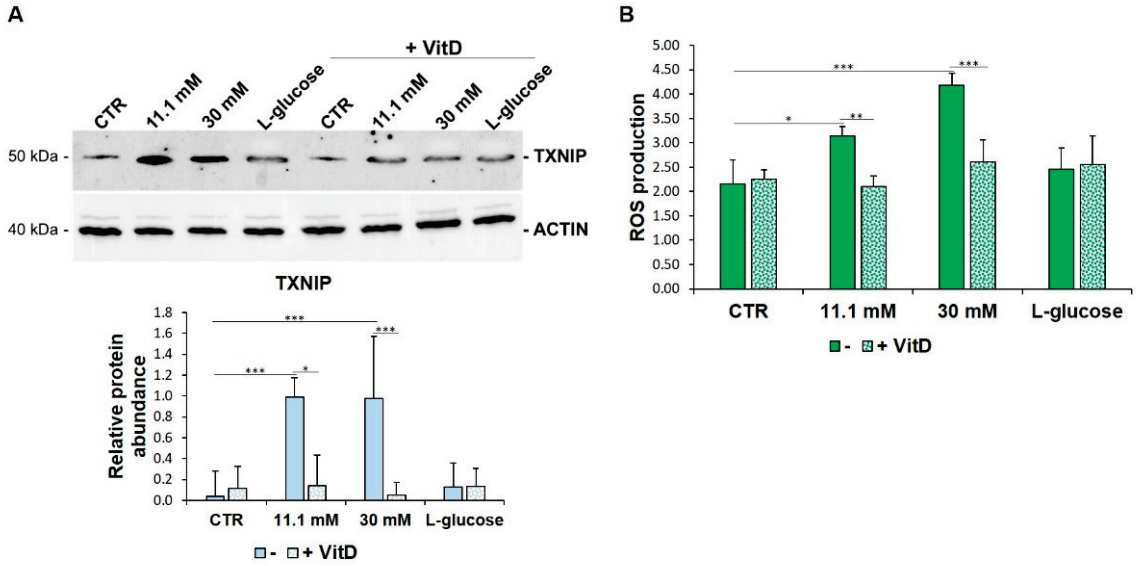

Figure 3. VitD prevents TXNIP upregulation and ROS accumulation in HUVEC cultured in high glucose conditions. (**A**) Western blot (upper panel) was performed on cell lysates using specific antibodies against TXNIP. Actin was used as an equal loading control. A representative blot is shown. Densitometric analysis (lower panel) was performed using Image J Lab software on three different blots, and the results are the mean of three independent experiments ± SD. (**B**) HUVEC were cultured in medium containing 5 mM (CTR), 11.1 mM, or 30 mM of D-glucose in the absence (-) or in the presence of VitD 20 nM. L-glucose (30 mM) was used as a control of osmolarity. ROS production was evaluated by DCFH, as described in the methods. The results are the mean of three experiments performed triplicate ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.4. VitD Hinders Lipid Droplets Formation in HUVEC Exposed to High Glucose

In several cell types, the accumulation of lipid droplets storing triglycerides is interpreted as an adaptive response to stress. To test whether HUVEC behave in a similar manner, we initially evaluated the amounts of triglycerides in HUVEC that had been cultured in media containing high levels of glucose. Control and L-glucose-cultured cells contain a certain amount of triglycerides that is dose-dependently increased upon exposure to 11.1 mM and 30 mM of D-glucose for 24 h. Moreover, the silencing of *TXNIP* as well as the treatment with VitD reduced the triglyceride amounts to normal physiological levels (Figure 4A). We then stained HUVEC, exposed to high levels of glucose for 24 h, with Oil Red O to detect neutral lipids to analyse the potential role of TXNIP in driving the accumulation of lipids. Moreover, since treatment with VitD downregulates TXNIP, we also treated the cells with VitD. It is noteworthy that, at baseline, HUVEC contain lipid droplets and that high D-glucose levels increase their number. Interestingly, the high glucose-induced deposition of lipids is dampened by *TXNIP* silencing as well as by the addition of VitD (Figure 4B).

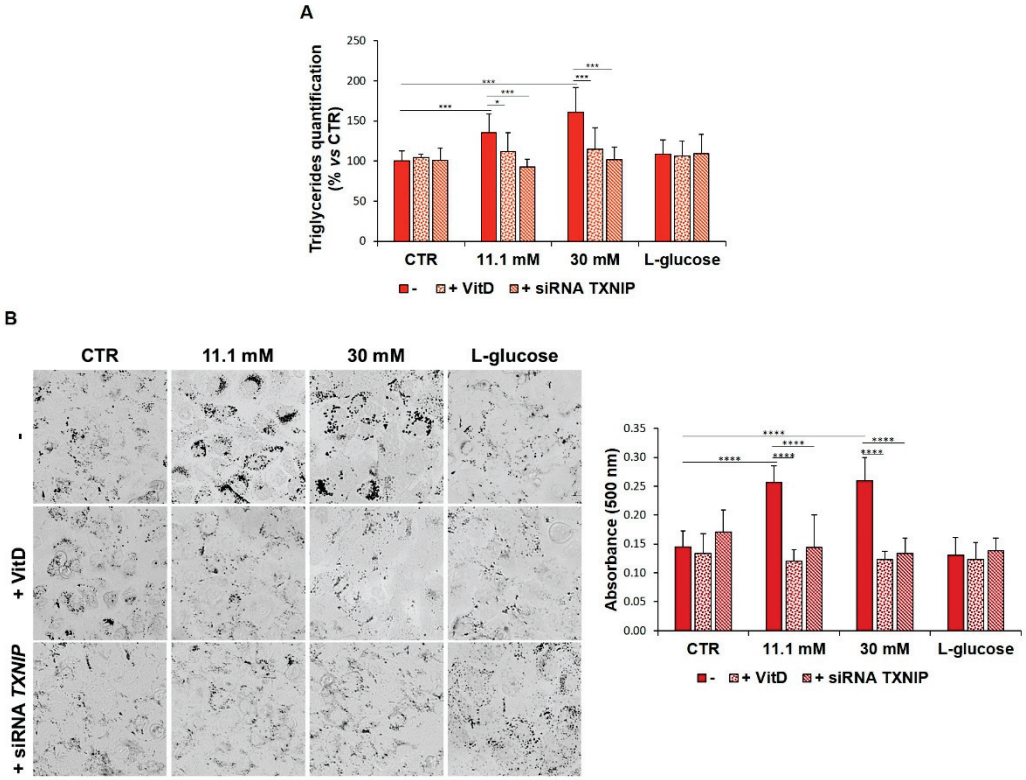

Figure 4. VitD and *TXNIP* silencing prevent high glucose-induced accumulation of triglycerides and lipid droplets. HUVEC were cultured in medium containing 5 mM (CTR), 11.1, or 30 mM of D-glucose (-) and either after *TXNIP* silencing or after the administration of 20 nM VitD. L-glucose (30 mM) was used as a control of osmolarity. (**A**) Triglyceride accumulation was measured by the Triglyceride Quantification Kit, as described in the methods. (**B**) The cells were stained with Oil Red O, and after the image acquisition using the FLoid Cell Imaging Station (Thermo Fisher Scientific) (left panel), the cells were solubilized, and the triglycerides were quantified by measuring the absorbance at 500 nm using the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) (right panel). The results are the mean of three experiments that were repeated in triplicate ± SD. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

3.5. VitD Corrects High Glucose-Induced Imbalance of Lipid Metabolism in HUVEC

To shed some light on the pathways leading to the deposition of triglycerides, we analysed some key markers that have been found to be involved in lipid metabolism. First, we focused our attention on some molecules that are involved in lipogenesis. We analysed the modulation of PPARγ and its transcriptional coactivator EDF1, both of

which are required for lipogenesis. In high glucose level-cultured cells, Western blot revealed a significant upregulation of both EDF1 and PPARγ, which was averted by VitD (Figure 5A) and *TXNIP* silencing (Figure 5B). Secondly, we analysed the expression of CPT1A, an enzyme that is located on the mitochondrial membrane and that is involved in the transport of fatty acids into the mitochondria to undergo β-oxidation. The total amount of CPT1A was reduced in the cells that had been cultured in high glucose-containing media, and VitD rescued it to normal levels, as did *TXNIP* silencing (Figure 5A,B). This result is in accordance with the decreased β-oxidation rate that was measured in the high glucose-cultured cells, which was recovered in the presence of VitD and after *TXNIP* silencing (Figure 5D).

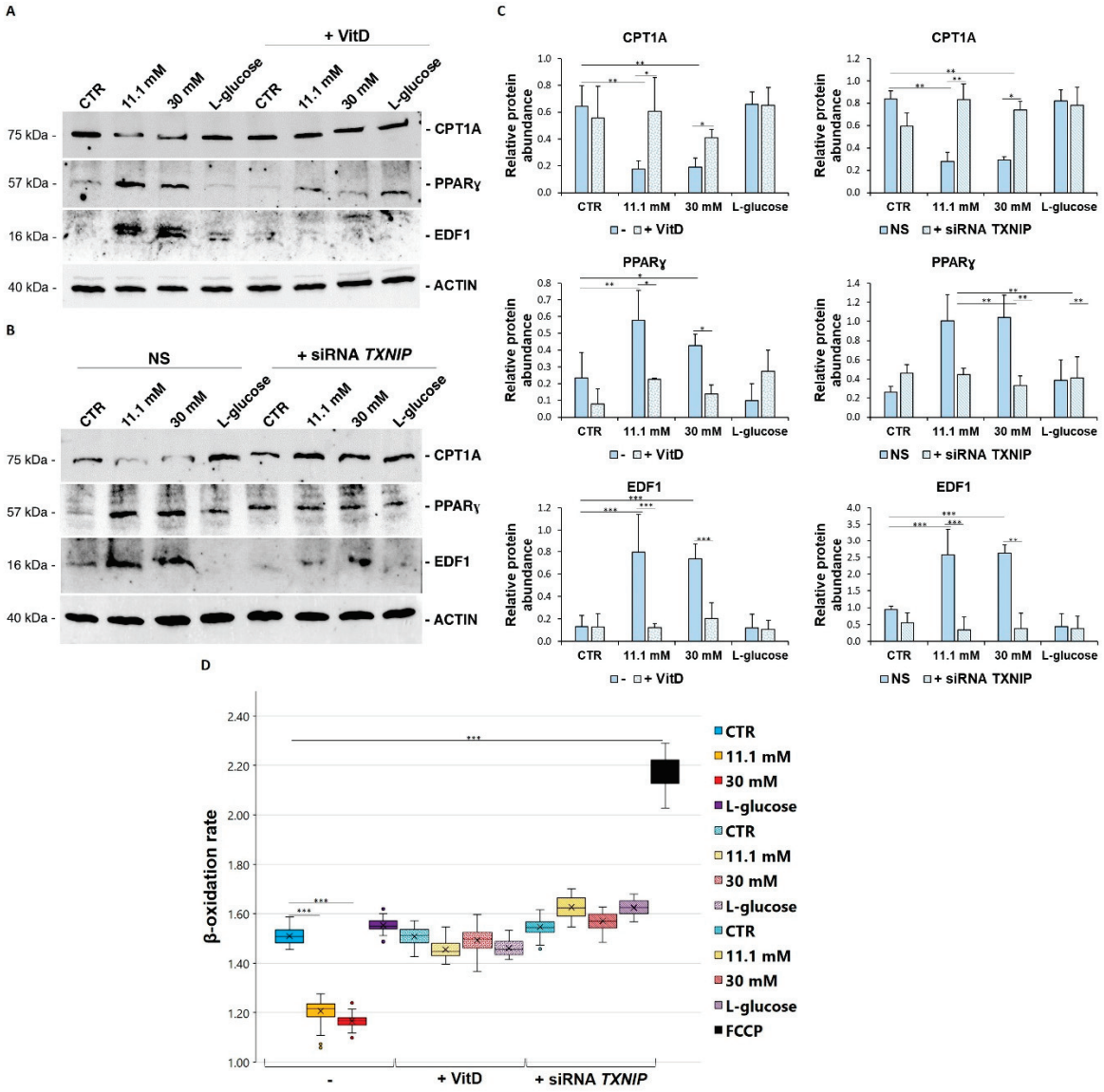

Figure 5. VitD and *TXNIP* silencing restore lipid metabolism in HUVEC cultured in high glucose. HUVEC were cultured in medium containing 5 mM (CTR), 11.1, or 30 mM of D-glucose (-) and either after *TXNIP* silencing or after the administration of 20 nM VitD. L-glucose (30 mM) was used as a control of osmolarity. (**A**,**B**) Western blot was performed on cell lysates using specific antibodies against CPT1A, PPARγ, and EDF1. Actin was used as an equal loading control. A representative blot is shown. (**C**) Densitometric analysis was performed using Image J Lab software on three different blots, and the results are the mean of three independent experiments ± SD. (**D**) The β-oxidation rate was measured using a fatty acid oxidation assay kit, as described in the methods. The results are the mean of three experiments that were conducted in triplicate ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

Observational data have consistently established low serum concentrations of VitD in patients with Type 2 Diabetes Mellitus (T2D). Of note, it seems that the duration of diabetes rather than glycemic control is associated with VitD deficiency [26]. Remarkably, observational studies as well as preclinical data support that low VitD correlates with increased risk of hypertension, atherosclerosis, metabolic disorders, and low-grade inflammation, all of which are conditions that share endothelial dysfunction as a common element [27–29]. Accordingly, through the high-frequency ultrasonographic imaging of the brachial artery to assess endothelium-dependent flow-mediated vasodilation, it was demonstrated that VitD improves endothelial function in VitD-deficient subjects [30] and in patients with T2D and with low serum levels of VitD [31]. These findings prompted us to investigate the effects of VitD on HUVEC. VitD has been reported to protect HUVEC from hydrogen peroxide-induced oxidative stress by inhibiting superoxide formation as well as to improve antioxidant defenses in HUVEC that have been treated with high concentrations of ketone bodies [19,32]. Here, we show that VitD downregulates TXNIP and, accordingly, mimics the effects of *TXNIP* silencing in HUVEC that have been cultured in high levels of glucose by preventing oxidative stress and by correcting lipid metabolism and storage in lipid droplets.

Despite originally being isolated as a VitD-upregulated protein [33,34], TXNIP is differently regulated by VitD in various cell types [35]. Cell specificity in the modulation of the response to VitD is described, and the downregulation of TXNIP after exposure to VitD seems to occur in cells harboring wild type p53 [35], as HUVEC do. Elevated TXNIP is implicated in the pathogenesis of various complex diseases, including diabetes and neurologic and inflammatory disorders [36]. This is not surprising since TXNIP regulates lipid and glucose metabolism both dependently and independently from the inhibition of thioredoxin (TRX) [36]. Focusing on the endothelium, TXNIP is overexpressed in the vascular EC of many vessels in hypertensive rats and contributes to oxidative stress and endothelial dysfunction in hypertension [7,37,38]. It is upregulated in the aortic endothelium of diabetic rats and in human aortic EC that have been cultured in high levels of glucose, associated with dysfunction in both cases [38]. Interesting results were obtained in endothelial TXNIP knockout mice under metabolic stress since the aorta was protected from damage through antioxidant and anti-inflammatory mechanisms [39]. Additionally, long non-coding RNAs (lnc), which disrupt the stability of the target protein, are involved in the regulation of TXNIP. Indeed, a recent report showed that high glucose-treated HUVEC downregulate lnc-SNHG15, which reduces TXNIP expression by enhancing its ubiquitination [40], thus mitigating high glucose-induced endothelial dysfunction. Our results are in keeping with the increasing evidence pointing to upregulated TXNIP as a player in endothelial dysfunction in response to high glucose levels. Indeed, we found that the downregulation of *TXNIP* by specific siRNAs reduces oxidative stress and the accumulation of triglycerides in lipid droplets in HUVEC.

Lipid droplets are intracellular organelles that store neutral lipids and have been detected in many eukaryotic cell types and have been interpreted as an adaptation mechanism under metabolic stress [16,17]. Interestingly, they are very abundant in the EC lining of mammalian atheromas and in cultured EC that have been exposed to hypercholesterolemic serum [41]. A seminal paper showed the prompt formation of lipid droplets in intact murine aortic EC in vivo and ex vivo after a load of fatty acid [17]. This study suggests that beyond being an energy resource, endothelial lipid droplets represent a defense mechanism against lipotoxicity. Here, we show that 24 h culture in media containing high levels of glucose results in lipid droplet accumulation in HUVEC. To get insights into the involved mechanisms, we evaluated the amounts of PPARγ and its transcriptional coactivator EDF1. PPARγ is a ligand-activated transcription factor that is able to exert a broad spectrum of biological functions, including fatty acid handling and storage [42]. EDF1 is a low molecular weight protein that shuttles between the cytosol and the nucleus in response to environmental challenge [43] and is induced in

HUVEC that have been exposed to oxidative stress [25]. When nuclear, it functions as a transcriptional coactivator for PPARγ [24,44]. HUVEC cultured in the presence of high glucose upregulate both PPARγ and EDF1. We hypothesize that the activation of the EDF1/PPARγ axis might fuel fatty acid synthesis in HUVEC. A similar conclusion was reached in HUVEC that had been cultured in a medium containing low levels of magnesium [25], thus suggesting that lipid accumulation is a common feature in EC exposed to metabolic stress. Moreover, culture in high glucose downregulates CPT1A, which consequently impairs lipid transport to the mitochondria. Accordingly, FAO is reduced in HUVEC that have been exposed to high amounts of glucose. We propose that the formation of lipid droplets in response to high amounts of glucose results from an imbalance between the synthesis and oxidation of fatty acids.

Whether lipid droplet-derived fatty acids are used as substrates for energy metabolism or for protection against lipoperoxidation in our experimental model remains to be elucidated. Other interesting aspects that we plan to investigate are the dynamics and the fate of these organelles. Moreover, a topic that we only mentioned briefly but that deserves more attention is the reason why SIRT1 appeared to be downregulated in our experimental setting. For this purpose, we recall that SIRT1 exerts beneficial effects on the endothelium, and consistently, antidiabetic drugs, anti-oxidants, and anti-inflammatory agents increase its amounts [45,46].

In conclusion, we identified TXNIP as one of the targets of VitD in HUVEC cultured in media containing high amounts of glucose. Therefore, VitD might represent a serviceable tool that can be used to control redox equilibrium with the aim of limiting or, at least, of delaying the onset of high glucose-induced endothelial dysfunction.

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Data Availability Statement: Data are available in a publicly accessible repository. The data presented in this study are openly available in Dataverse at the following link: https://dataverse.unimi.it/dataverse/biomedicines/ accessed on 30 September 2021.

Conflicts of Interest: The authors declare no conflict of interest.

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Tipping the Balance: Vitamin D Inadequacy in Children Impacts the Major Gut Bacterial Phyla

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Abstract: Vitamin D inadequacy appears to be on the rise globally, and it has been linked to an increased risk of osteoporosis, as well as metabolic, cardiovascular, and autoimmune diseases. Vitamin D concentrations are partially determined by genetic factors. Specific single nucleotide polymorphisms (SNPs) in genes involved in vitamin D transport, metabolism, or binding have been found to be associated with its serum concentration, and these SNPs differ among ethnicities. Vitamin D has also been suggested to be a regulator of the gut microbiota and vitamin D deficiency as the possible cause of gut microbial dysbiosis and inflammation. This pilot study aims to fill the gap in our understanding of the prevalence, cause, and implications of vitamin D inadequacy in a pediatric population residing in Qatar. Blood and fecal samples were collected from healthy subjects aged 4–14 years. Blood was used to measure serum metabolite of vitamin D, 25-hydroxycholecalciferol 25(OH)D. To evaluate the composition of the gut microbiota, fecal samples were subjected to 16S rRNA gene sequencing. High levels of vitamin D deficiency/insufficiency were observed in our cohort with 97% of the subjects falling into the inadequate category (with serum 25(OH)D < 75 nmol/L). The CT genotype in rs12512631, an SNP in the *GC* gene, was associated with low serum levels of vitamin D (ANOVA, $p = 0.0356$) and was abundant in deficient compared to non-deficient subjects. Overall gut microbial community structure was significantly different between the deficient (D) and non-deficient (ND) groups (Bray Curtis dissimilarity $p = 0.049$), with deficient subjects also displaying reduced gut microbial diversity. Significant differences were observed among the two major gut phyla, *Firmicutes* (F) and *Bacteroidetes* (B), where deficient subjects displayed a higher B/F ratio ($p = 0.0097$) compared to ND. Vitamin D deficient children also demonstrated gut enterotypes dominated by the genus *Prevotella* as opposed to *Bacteroides*. Our findings suggest that pediatric vitamin D inadequacy significantly impacts the gut microbiota. We also highlight the importance of considering host genetics and baseline gut microbiome composition in interpreting the clinical outcomes related to vitamin D deficiency as well as designing better personalized strategies for therapeutic interventions.

Keywords: pediatric vitamin D deficiency; host genetics; gut microbiota; Qatar; *Bacteroidetes* to *Firmicutes* ratio

1. Introduction

Vitamin D and its metabolites play a crucial role in early life, including bone growth [1] and development of the immune system [2]. Recent epidemiologic reports linking low vitamin D levels in children to diabetes, metabolic syndrome, asthma, dermatitis, and anemia have piqued interest in pediatric vitamin D investigations [3]. Even though the cutoff of vitamin D deficiency and insufficiency varies between institutions, with some recommending levels above 75 nmol/L as sufficient or normal [4], the consensus is that ideal 25(OH)D levels should be greater than 50 nmol/L [5]. This limit was based on data indicating levels less than 50 nmol/L are linked to a variety of disease outcomes [6].

In the Middle East, low levels of serum vitamin D have been seen in people of all ages and genders [7], and vitamin D inadequacy (below 75 nmol/L) has been widely reported in the GCC (Gulf cooperative council) populations, particularly among children [8,9]. In a study of 331 Saudi children aged 6 to 17 years, 71.6% had vitamin D levels below 50 nmol/L [10]. Similarly, in a cohort of 293 adolescent girls (11–18 years) living in the United Arab Emirates, the authors reported vitamin D deficiency (<27.5 nmol/L) in 78.8% of the study subjects [11]. In Qatar, 61% (11–16 years old) adolescents, 29% (5–10 years old) children, 9.5% (below 5 years old) children demonstrated serum 25(OH)D levels <75 nmol/L, along with delayed milestones, gastroenteritis, fractures, and rickets [12].

Vitamin D levels are affected by skin pigmentation, sun protection, latitude, age, and exposure to sunlight [13]. Studies have also shown that genetic factors play a significant role in determining the serum 25(OH)D levels in various populations such as Caucasians, African American, Arabs, and Asian [14–17]. These studies have shown that single nucleotide polymorphism (SNPs) in several candidate genes involved in vitamin D metabolism are associated with its low levels and that the allele frequencies of some of these SNPs vary between populations [14,18]. Despite the widespread prevalence of vitamin D deficiency in children living in Qatar [12,19], there has yet to be a study examining whether a genetic susceptibility to vitamin D deficiency exists in this multi-ethnic population.

The link between vitamin D and the composition of the gut microbiome is well established [20,21]. More than 2000 species of bacteria comprise the gut microbiota and are distributed throughout the gastrointestinal tract (GIT) [22]. The gut microbiota is responsible for a variety of tasks, including nutrition metabolism, vitamins synthesis, and short chain fatty acid (SCFA) generation, tight junction barrier function and modulation, antimicrobial agent release, and immunological regulation [23–25]. Several studies suggest that both vitamin D status and supplementation has an impact on the composition of the gut microbiome [21,26–28]. Vitamin D intake was found to be inversely associated with *Prevotella* abundance and positively associated with *Bacteroides* abundance in a cross-sectional study of healthy subjects [29]. Another study conducted with healthy, but overweight or obese individuals found a higher abundance of genus *Coprococcus* and a lower abundance of genus *Ruminococcus* in the study participants with sufficient serum 25(OH)D (>75 nmol/L) as compared to those with lower serum 25(OH)D (<50 nmol/L) [30]. Vitamin D supplementation dramatically boosted the gut microbial diversity and abundance of probiotic taxa like *Akkermansia* and *Bifidobacterium*, according to data from our recent study on a cohort of healthy females [21]. Dynamic shifts of genera *Bacteroides* and *Prevotella* were also noted, indicating a change in intestinal enterotypes following supplementation [21]. The evidence presented above supports the idea that vitamin D metabolism is inextricably linked to human gut microbiota composition. A recent study done in older men also showed that the gut microbiome influences the level of active vitamin D and its metabolism [20], wherein the phylum Firmicutes was positively linked to increased amounts of the active form of vitamin D (1,25(OH)2D) [20]. Such studies, however, are lacking in the pediatric population despite the vital role of vitamin D in early stages of life.

The goal of this study is to gain better understating of the association between vitamin D levels, host genetics, and gut microbiota composition in a pediatric population living in Qatar. Improving our knowledge of this complex interaction is crucial for delineating the

determinants of the vitamin D status as well as for stratifying subjects for supplementation and early personalized interventions.

2. Methods

2.1. Study Participants and Design

The approval for the study was obtained from Sidra Medicine IRB (1708012909). The study followed the latest iteration of the Declaration of Helsinki as well as the ICH Guidelines for Good Clinical Practice (CPMP/ICH/135/95), published in July 1996. Children who visited Sidra (Pediatric clinics and the Emergency department) were evaluated for study eligibility and enrolled. Before being included in the study, all participants underwent a physical examination and gave their informed consent. All the participants were in good health and had no underlying disorders or chronic conditions. They had not taken vitamin D in the previous 6 months and had not been exposed to antibiotics in the previous month. All participants were requested to fill out a questionnaire about their current and previous medical histories, supplementation information, dietary habits, sun exposure, and other pertinent information.

2.2. Vitamin D Serum Measurement

Around 3.5 mL of blood was collected in a serum separator tube (SST). Vitamin D serum levels were tested at the Sidra Medicine Pathology laboratory using in a two-step competitive binding immunoenzymatic assay. In the initial incubation, samples were added to a reaction vessel with a vitamin D-binding protein (VDBP) releasing agent and paramagnetic particles coated with a sheep monoclonal anti-25(OH) vitamin D antibody; 25(OH) vitamin D is released from VDBP and binds to the immobilized monoclonal anti-25(OH) vitamin D in the solid phase. Subsequently, a 25(OH) vitamin D analogue, alkaline phosphatase conjugate, was added, which competes for binding to the immobilized monoclonal anti-25(OH) vitamin D. After a second incubation, materials bound to the solid phase were held in a magnetic field while unbound materials were washed away. Then, the chemiluminescent substrate Lumi-Phos* 530 (Lumigen, Southfield, MI, USA) was added to the vessel, and light generated by the reaction was measured with a luminometer. The light production was inversely proportional to the concentration of 25(OH) vitamin D in the sample. The amount of analytes in the sample was determined from a stored, multipoint calibration curve. Although there is no unanimity on the required serum levels of 25(OH)D, the Endocrine Society has defined the levels below a threshold of 50 nmol/L (or 20 ng/mL) as vitamin D deficient [31]. Furthermore, various expert bodies and societies have established 50 nmol/L as the "vitamin D requirement of almost all normal healthy adults," using bone health as the primary criterion. In their "Dietary Reference Intakes" the Institute of Medicine (IOM, Washington, DC, USA) proposes a threshold level of 50 nmol/L [32]. According to some evidence, a 25(OH)D level greater than 50 nmol/L may be necessary for effective risk reduction for a variety of outcomes [33], which is also supported by the main pediatric societies such as American Academy of Pediatrics (AAP), European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) and the Committee on Nutrition of the Spanish Association of Pediatrics (AEP) [34]. Based on the above criteria, the participants were classified as either deficient (those with serum levels of 25(OH)D below <50 nmol/L) or non-deficient (those with serum levels of 25(OH)D above >50 nmol/L) [35,36].

2.3. Genotyping

Based on an exhaustive literature review, we chose 37 SNPs from 6 candidate genes that met the following criteria: (1) biological significance in vitamin D metabolism, transport, or degradation; and (2) previous GWAS evidence of a substantial association. [37]. The selected genes were group-specific component (*GC*, coding for vitamin D binding protein VDBP), vitamin D receptor (*VDR*), vitamin D 25-hydroxylase (*CYP2R1*), 1-alpha-hydroxylase (*CYP27B1*), vitamin D 24-hydroxylase (*CYP24A1*), and 7-dehydrocholesterol

reductase (*DHCR7*/*NADSYN1*). The roles of these selected genes in the vitamin D metabolic cascade are shown in Figure 1. Genotyping was performed by high-throughput quantitative PCR (qPCR), using the Fluidigm Biomark HD platform (Fluidigm Corporation, South San Francisco, CA, USA), as previously described [38]. Samples were run in duplicate. Genotyping calls were assessed based on the allele discrimination plots and single amplification plots. The genotyping calls were saved as .csv files and processed for further investigation. The replicates of SNP rs757343 produced discordant calls and were thus removed from the analysis. Hardy–Weinberg equilibrium (HWE) for each SNP was tested using the chi-square test. SNPs rs10877012 and rs7041 did not meet HWE, and SNP rs2882679 gave one genotype only; thus, they were also discarded from further analysis. The chi-square test was used to test the association with D/ND classification. An ANOVA test was performed to test the association between genotypes and vitamin D levels. p-value < 0.05 was considered significant for all statistical assessments.

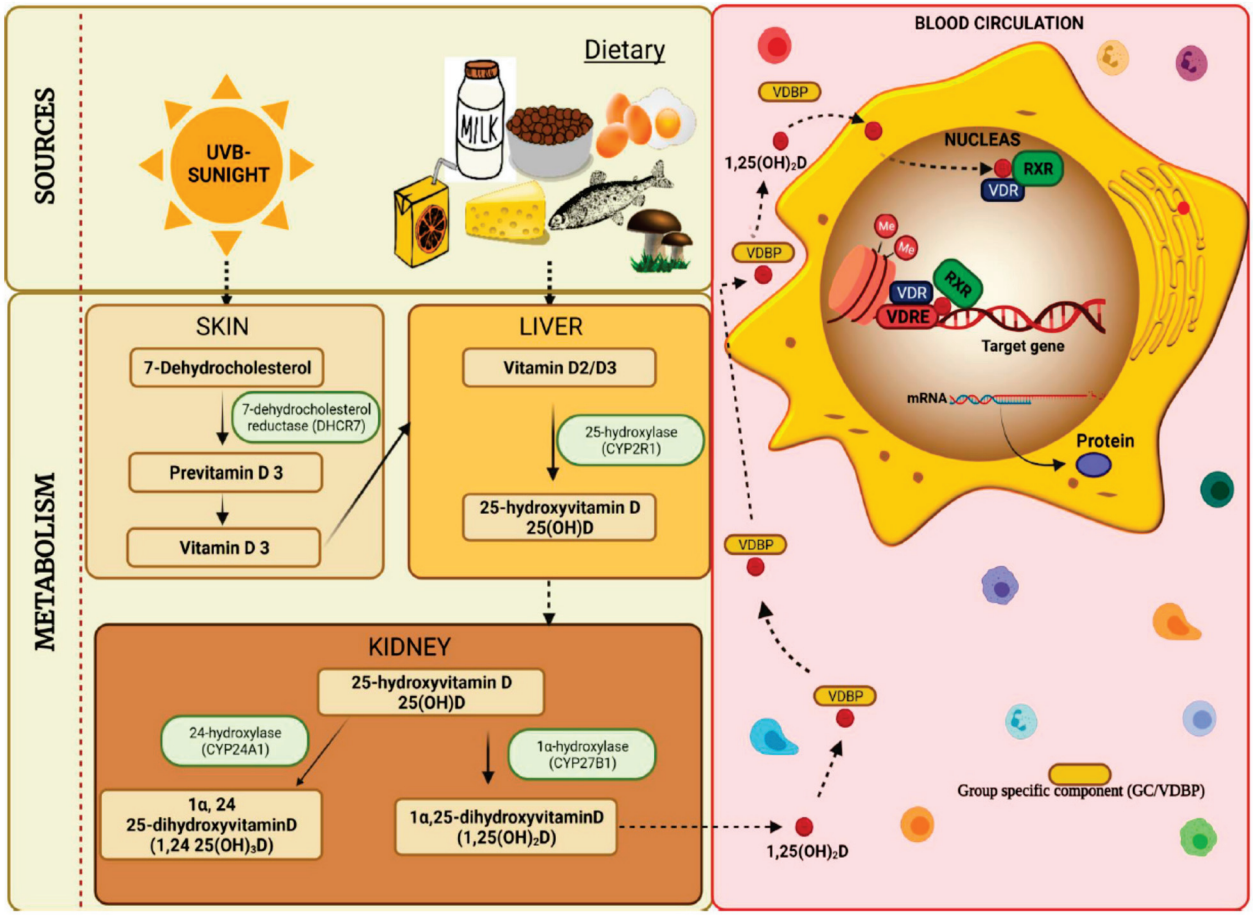

Figure 1. The chosen candidate genes' involvement in the vitamin D cascade are displayed. Vitamin D is mostly obtained through sunlight or food sources in humans. UVB light from the sun penetrates the skin and converts 7-dehydrocholesterol (7DHC) to pre-vitamin D, which is rapidly transformed to vitamin D. *DHCR7/NADSYN1* removes 7DHC from the vitamin D pathway. D is hydroxylated in the liver to 25(OH)D3, primarily by *CYP2R1*. Following that, 25(OH)D is delivered to the kidney by vitamin D binding protein (VDBP), which is encoded by *GC*, where it is converted by *CYP27B1* to its active form, 1,25(OH)2D. Finally, *CYP24A1* catabolizes both 25(OH)D and 1,25(OH)2D to calcitroic acid, which is physiologically inactive and water soluble. The active form of vitamin D bound to VDBP is delivered in blood to its target cells, where it acts as a ligand for the vitamin D receptor (*VDR*), a nuclear transcription factor that regulates transcription and translation of messenger RNA, leading to the synthesis of vitamin D-dependent proteins.

2.4. Microbial DNA Extraction from Stool Samples

A portion (400 mg) of the stool sample was transferred to the OMNIgene GUT kit (DNA Genotek Inc, Ottawa, Canada). Microbial DNA was extracted using the QIAamp Fast DNA Stool Mini Kit. In a 2 mL tube, 200 mg of fecal sample was combined with 0.5 mL of InhibitEX buffer and vortexed until well homogenized. Thereafter, the samples were combined with 0.2 g of sterile zirconia/silica beads (diameter, 0.1 mm; Biospec Product, ROTH, Karlsruhe, Germany), vortexed, and incubated at 70 °C for 10 min to finish the lysis. The supernatant (600 mL) was transferred into a 2.0 mL microcentrifuge tube containing 25 mL proteinase K. The subsequent steps were carried out as per the instruction of the QIAamp DNA stool MiniKit. The eluted DNA samples (50 µL) were stored at −20 °C until library preparation.

2.5. Bacterial 16S rRNA PCR Amplification and High Throughput Sequencing

Polymerase chain reaction (PCR) was used to amplify the 16S rRNA variable regions V3 and V4 using the Illumina suggested amplicon primers with adapters.

Forward:

5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG ′3.

Reverse:

5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC ′3.

In a 25 µL reaction mixture, 5 µL of each forward and reverse primer (1 µM), 2.5 µL of template DNA, and 12.5 µL of 1× Phusion Hot start Master Mix (Thermo scientific, Waltham, MA, USA) were combined. The amplifications were carried out using a Thermo Scientific Veriti 96-well thermal cycler using the following program: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, with a final elongation at 72 °C for 5 min used for the amplifications. Amplicons were then purified according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol on 27 January 2020 (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). Samples were multiplexed using the Nextera XT Index kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The amplicons were then pooled to achieve an equimolar library concentration. Illumina MiSeq platform (Illumina, San Diego, CA, USA), at the Sidra research facility, was used for sequencing of the final pooled product using a MiSeq Reagent Kit v3 (paired-end 2 × 300 bp).

2.6. 16S Sequence Data Processing and Analysis

Fast QC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used to assess the sequencing quality on 2 October 2020. The Quantitative Insights into Microbial Ecology (QIIME2; version 2019.4.0) software package [39,40] was used to input the demultiplexed sequencing data. Even though the overall distribution was consistent, samples 24, 26, 38, and 48 had poor sampling depth and were excluded from the final analysis. The rarefaction curves tapered phylogenetically, showing that the complete microbial population was sufficiently represented, and the samples were rarefied at a depth of ≥3531. The data were denoised with DADA2 [41]. Taxonomic classification was performed utilizing Silva [42] for QIIME2 classifier (version silva-132-99-515-806). The data were then imported into R (RStudio v 1.4 with R v 4.1) [43] in a Biological Observation Matrix (biom.) format, before further evaluation with the Phyloseq package [44].

Observed species, Chao1 [45] Shannon [46], and inverse Simpson (InvSimpson) [47] indices were used to quantify alpha diversity in RStudio using the R package "vegan" (v2.5–6) [48]. Weighted Unifrac, Unweighted Unifrac, Bray–Curtis, and Jaccard distance metrics were used to measure beta diversity. Bray–Curtis dissimilarity was employed to establish significance, and CCA was utilized as an ordination approach. We also used Wilcoxon or Kruskal–Wallis nonparametric statistical tests, followed by Dunn post hoc analysis when needed. Bonferroni correction was used to compute the false discovery rate (FDR), with a p-value of 0.05 considered significant for all tests.

Metagenome functional contents were analyzed using the (phylogenetic investigation of communities by reconstruction of unobserved states) PICRUSt software package (v1.0.0) to predict gene contents and metagenomic functional information [49]. The statistical STAMP [50] was used to do the statistical analysis, and significant pathways (p-value 0.05, CI 99 percent) were exported and utilized to construct the results.

2.7. Statistical Analysis

The analysis by group of the continuous variables was performed using a Mann–Whitney–Wilcoxon test, while the association between two categorical variables was performed using a chi-square test (Table 1). A machine learning technique called L0L2-regularized regression [51], implemented in the R package L0Learn, was applied for the analysis of multivariate data. This method allows one to estimate the coefficients of regression and to select the best subset of variables in one single procedure. The unimportant variables are automatically estimated by zero. In addition, we used the R Package randomForest [52] to run a random forest regression to facilitate meaningful comparisons of predictor variables.

Table 1. Baseline characteristic of the study participants.

	Vitamin D Status		
	Deficient (below 20 ng/mL or 50 nmol/L)	**Non-Deficient (above 20 ng/mL or 50 nmol/L)**	***p*-Values**
Number	61	27	n/a
Age (years)	9.02 ± 3.23	8 ± 2.69	0.14
BMI, mean ± SD	19.4 ± 5.65	15.8 ± 4.08	0.049
BMI z-score, mean ± SD	0.357 ± 1.65	0.054 ± 1.73	0.54
25(OH)D levels	36.1 ± 8.52	62.6 ± 11.0	9.3×10^{-14}
Gender, ***n*** **(%)**			
Male	31 (50.82%)	14 (51.85%)	1.00
Female	30 (49.18%)	13 (48.15%)	
Ethnicity, ***n*** **(%)**			
Arab	46 (75%)	16 (59%)	0.2
non-Arab	15(25%)	11 (40%)	
Average Daily Exposure to Sun			
Less than 1/2 h, *n* (%)	10 (16%)	1 (0.03%)	
1/2 h to 1 h, *n* (%)	28 (45%)	22 (81.4%)	0.781
More than 1 h, *n* (%)	23 (37%)	4 (14.8%)	
Consumption of Fish			
Daily, *n* (%)	2 (0.03%)	1 (0.03%)	
Weekly, *n* (%)	26 (42.6%)	15 (55%)	0.7104
Monthly, *n* (%)	22 (36%)	7 (25%)	
None, *n* (%)	11 (18%)	4 (14.8%)	
Consumption of Dairy Products, ***n*** **(%)**			
YES	58 (95%)	25 (92.5%)	1.00
NO	3 (4.9%)	2 (7.4%)	
History of Vitamin D Deficiency, ***n*** **(%)**			
YES	14 (22.95%)	8 (29.63%)	0.5619
NO	47 (77.05%)	19 (70.37)	

Abbreviation: BMI, body mass index. Chi-square test was used for categorical variables, and Mann–Whitney test for comparing continuous variables.

3. Results

A total of 112 subjects between 4 and 14 years old attending the Sidra Pediatric clinics and Emergency department was assessed for eligibility and consented to participate in the study. Subjects were excluded if they had chronic diseases, took antibiotics in the last month or were on vitamin D supplementation for the last six months before enrolment,

failed to provide blood samples, and/or if their serum vitamin D status was unavailable. A total of 88 subjects met the above criteria (Table 1), but only 64 provided a stool sample and were included for gut microbiota profiling.

Based on the above information about vitamin D classification in the methods section and taking serum 25(OH)D of 50 nmol/L (20 ng/mL) as a point of reference, our data suggest 69% of the subjects fell into the vitamin D deficient category (Table 1), with a mean 25(OH)D of 36.13 nmol/L. There was a fair representation of male and female subjects in both the deficient and non-deficient cohorts. Most of the subjects belonging to both the deficient (75%) and non-deficient (59%) groups were of Arab origin (Supplementary Table S6). Average daily exposure to sun was above half hour in 82% of the deficient and 96% of the non-deficient subjects. After carrying out the group analysis, sun exposure was determined as a significant predictor variable.

3.1. Gut Microbial Composition and Diversity Are Altered in Vitamin D Deficient Children

The analysis of 16S rRNA gene sequencing data of stool samples (n = 63, one sample did not yield any PCR product) showed a significant decrease in bacterial diversity at the genus level in deficient (D) subjects compared to the non-deficient (ND) subjects as measured by the alpha diversity indices, observed species (p_{Observed} = 0.039), and Chao1 (p_{Chao1} = 0.047) (Figure 2a). Shannon and Simpson metrics for alpha diversity were borderline and failed to reach the significance level (p_{Shannon} = 0.052, p_{Simpson} = 0.057). The CCA profile based on the Bray–Curtis distance (Figure 2b) indicated that the two groups had significantly different microbiota community structures ($p = 9 \times 10^{-3}$). Interestingly, lower α-diversity and richness together with significantly different β-diversity as observed in the vitamin D deficient subjects as opposed to the non-deficient ones in our cohort were also seen in children with Crohn's disease [53], inflammatory bowel disease [54], infections [55], diabetes [56], obesity [57], and others [58] when compared to healthy controls.

A total of 98–99% of the 16S rRNA gene sequences in both D and ND groups belonged to four major phyla: *Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria* (Figure 2). In general, a healthy pediatric gut microbiota is dominated by *Firmicutes*, followed by *Bacteroidetes*, with these two major phyla representing more than 90% of the total gut bacteria [59,60]. Our results showed that the bacterial community composition was altered in the D group, with significantly greater abundance of *Bacteroidetes* (p = 0.019) and lower abundance of *Firmicutes* (Figure 3a). As such, the ratio of *Bacteroidetes* to *Firmicutes* (B/F $p = 9.7\times 10^{-3}$) was significantly higher in the D vs. ND group (Figure 3b). The B/F (or contrarily *Firmicutes/Bacteroidetes* (F/B)) ratio is known to play a role in maintaining intestinal homeostasis [61]. At the genus level, we observed that the D group had a significantly higher relative abundance of *Prevotella 9* ($p = 6.6 \times 10^{-12}$) and a significantly lower relative abundance of *Bacteroides* ($p = 9.9 \times 10^{-6}$) and *Alistipes* (p = 0.0069) compared with the ND group (Figure 3c,d and Supplementary Figure S1). *Prevotella 9* and *Bacteroides* were the top two most abundant genera in our cohort (both belonging to phylum Bacteroidetes) and are also suggested to be two of the main enterotypes used to classify gut microbiota samples [62], often inferred by the *Bacteroides-to-Prevotella* Ratio (B/P) [63]. We found that the D group had a significantly lower B/P ratio compared to the ND group (Figure 3e). Considering the major taxonomic alteration at the phylum and genus levels, we then looked at the community shift at the species level; however, no significant shift was observed (data not shown). The abundance of several species of the genera *Bacteroides* and *Alistipes*, such as *Alistipes finegoldii*, *Alistipes* sp. *AL1*, *Alistipes* sp. *N15 MGS-157*, *Bacteroides caceae, Bacteroides eggerthii,* and *Bacteroides plebeius*, were higher in the ND subjects, whereas species such as *Prevotella dislens*, *Prevotella bivia*, *Bacteroides massillensis*, *Alistipes marseille,* and *Alistipes indistinctus* were higher in deficient subjects (Supplementary Figures S2 and S3).

Therefore, our data indicate that the vitamin D deficient subjects had imbalanced gut microbial communities with a high B/F ratio, and a tendency towards a *Prevotella*-dominated enterotype.

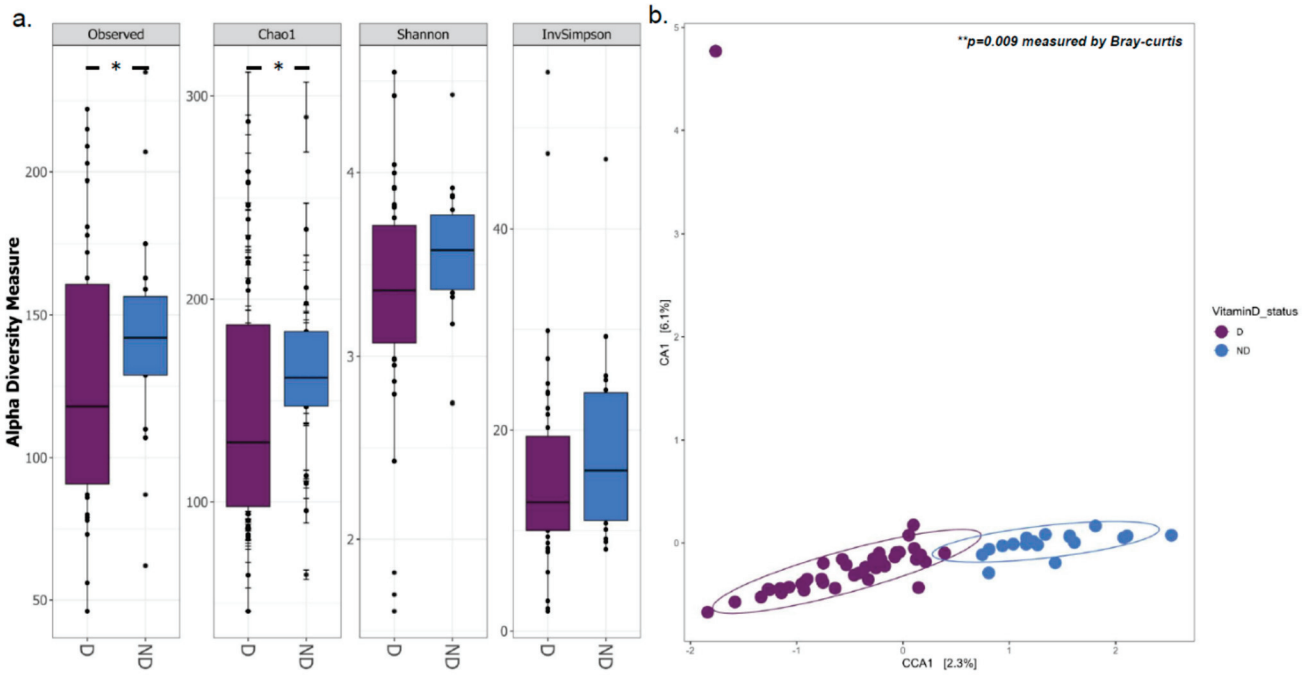

Figure 2. Alpha and beta diversity plot comparisons for the vitamin D deficient (D) and non-deficient (ND) groups. (**a**) Observed species, Chao1, Shannon, and Inverse Simpson boxplots representing the alpha-diversity indices. The horizontal line inside the box indicates the median, while the boxes reflect the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively). Whiskers reflect the lowest and greatest values from the first and third quartiles that are within 1.5 times the IQR, respectively. The Wilcoxon test with FDR–Bonferroni corrected *p* values was used to determine statistical significance. * $p < 0.05$; (**b**) CCA plot showing the beta diversity measure ** $p < 0.01$ deep purple: deficient samples, blue: non-deficient. Each dot represents an individual sample. The figure was generated using RStudio v 1.4 with R v 4.1.

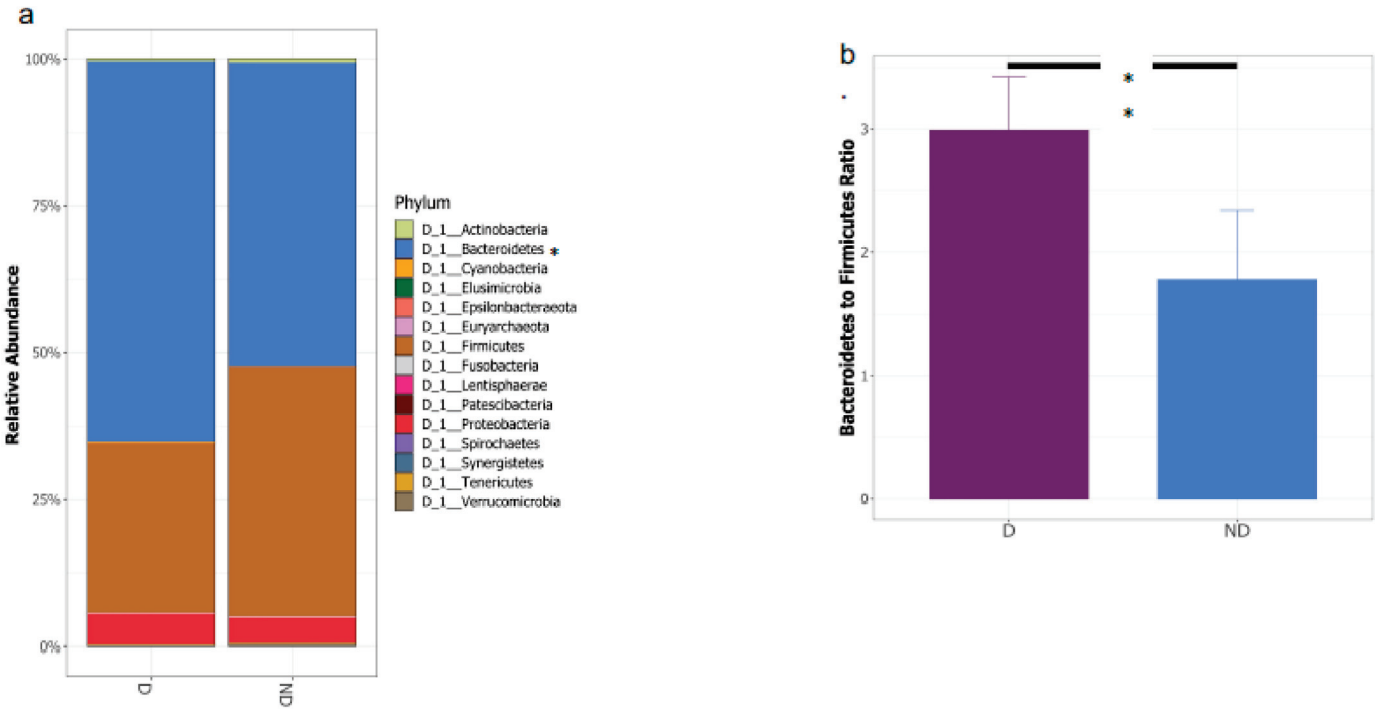

Figure 3. *Cont.*

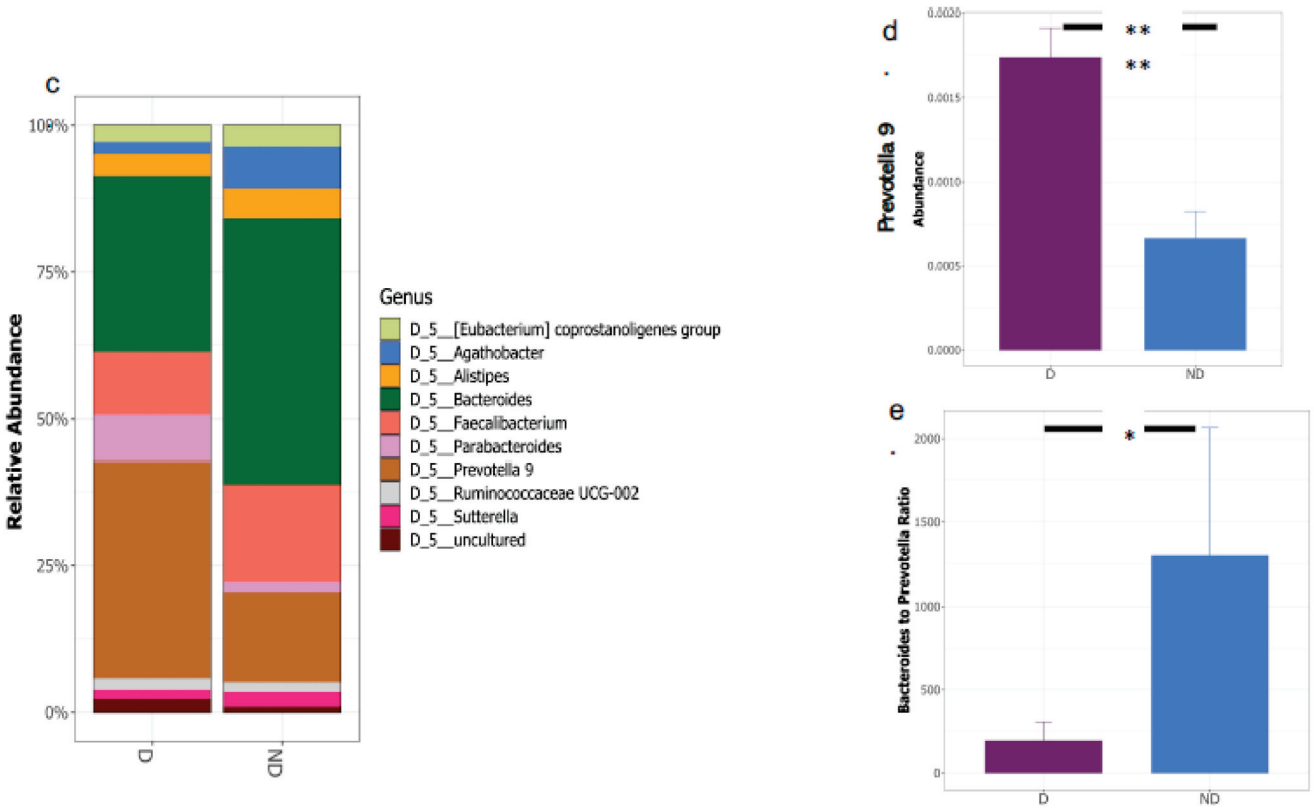

Figure 3. Comparison of the gut microbiota composition in vitamin D deficient (D) and non-deficient (ND) groups at the phylum level. (**a**) Relative abundance of different bacterial phyla in the deficient and non-deficient groups. The relative abundance of the major bacterial phyla, Firmicutes and Bacteroidetes, showed variation between the two groups. The abundance of Phylum Bacteroidetes was significantly elevated in the D group as compared to ND (Wilcoxon test with false discovery rate (FDR)–Bonferroni corrected, * $p < 0.05$). The figure was generated using RStudio v 1.4 with R v 4.1. (**b**) Comparison of the ratio of Bacteroidetes to Firmicutes in vitamin D deficient (D) and non-deficient (ND) groups (Wilcoxon test with false discovery rate (FDR)–Bonferroni corrected, * $p < 0.05$). The figure was generated using RStudio v 1.4 with R v 4.1. Comparison of the gut microbiota composition in vitamin D deficient (D) and non-deficient (ND) groups at the genus level. (**c**) Relative abundance of different bacterial genus in the deficient and non-deficient groups. The relative abundance of the major bacterial genera, *Prevotella* and *Bacteroides*, showed variation between the two groups. (**d**) Abundance of genus *Prevotella 9* was significantly elevated in the D group as compared to ND. (**e**) Comparison of the ratio of *Bacteroides* to *Prevotella* in vitamin D deficient (D) and non-deficient groups (ND) groups (Wilcoxon test with false discovery rate (FDR)–Bonferroni corrected, * $p < 0.05$ and ** $p < 0.01$; **** $p < 0.0001$. The figure was generated using RStudio v 1.4 with R v 4.1.

3.2. Differential Functional Gut Microbiome Pathways in Children with Vitamin D Deficiency

To investigate the effect of vitamin D status on gut microbial function, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) was performed to predict functional abundances based on the 16S marker gene sequences. This meta-analysis identified 81 pathways that were significantly different between the two groups (Supplementary Figure S4). In deficient subjects, 57 differential pathways were more abundant than that in non-deficient controls. Notably, the gut microbiome of deficient subjects was enriched in the orthologs related to lipopolysaccharide (LPS) biosynthesis and LPS biosynthesis proteins along with pathways related to primary immunodeficiencies, type II diabetes mellitus, when compared with non-deficient subjects. On other hand pathways related to fatty acid metabolism, glycerolipid metabolism, valine, leucine, and isoleucine metabolism were enriched in the ND group (Figure 4). Among other notable

differences, reduction in xylene/dioxin degradation and increased metabolism of drugs and xenobiotics was also observed in the deficient subjects.

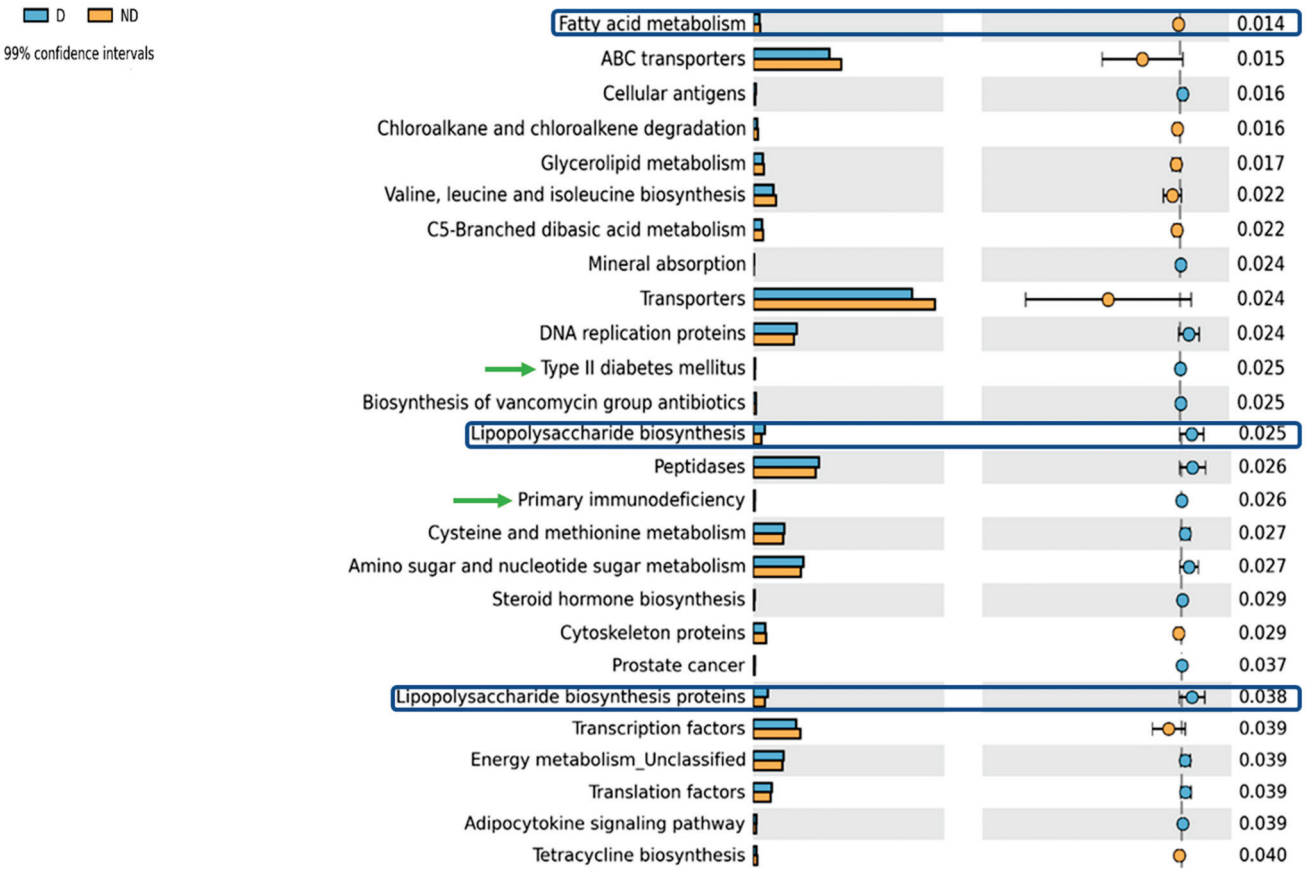

Figure 4. PICRUSt was used to infer gut microbiota functions using the 16S rRNA gene sequence data from vitamin D deficient (D) and non-deficient (ND) groups. The differences in projected functions of genes involved in fatty acid metabolism, as well as lipopolysaccharide biosynthesis and lipopolysaccharide biosynthesis proteins, are highlighted.

3.3. SNP Selection and Genotyping

The allele frequencies of the SNPs genotyped in this study were comparable to the frequencies reported for the HapMap project [64]. The details of the selected genes and SNPs are provided in (Supplementary Tables S1 and S2). Three SNPs (rs10877012 in the CYP27B1 gene, rs7041 in GC gene, and rs2882679 in GC) were excluded based on the HWE test ($p < 0.05$) (Supplementary Table S3). Upon the removal of SNPs that failed the quality control methods, 34 SNPs were used for further analysis. A chi-square test was used to test the association of SNP genotype frequencies with D/ND classification, and an ANOVA test was performed to test the association between genotypes and vitamin D level (Supplementary Table S4). The CT genotype (SNP rs12512631 in the GC gene) was found to be associated with low levels of serum 25(OH)D and was highly abundant in the deficient compared to non-deficient subjects (Figure 5a,b). The GC gene is 42.5 kb long with 13 exons and is found on chromosome 4q11–q13 [65]. Vitamin D actions are considerably facilitated by the GC gene, for the vitamin D binding protein (VDBP), which transports vitamin D metabolites to multiple sites of action [65]. Polymorphism in VDBP can result in varying affinity for the active vitamin D (1,25(OH)2 D) metabolite [65,66]. The SNP rs12512631 is in the downstream 3′ region of the GC gene and has been linked to circulating 25(OH)D levels [67]. Upon correlating the two groups of data, we found that the overall microbial abundance was significantly different between the three genotypes of several SNPs included in the study (Supplementary Table S5) along with the SNP of interest

rs12512631 (Figure 5c); however, these differences were not observed in relation with other notable taxonomic alteration such as the B/F ratio, abundance of *Prevotella* or *Bacteroides*, or the diversity measures (Supplementary Figure S5). We also performed regression analysis to investigate the effect of various variables on the vitamin D levels. Several variables in our study, notably age, ethnicity, B/F ratio, BMI z scores, sun exposure, dairy consumption, and rs12512631 genotypes, were found to be highly predictive of vitamin D levels. The random forest test was conducted to determine variables of importance, and BMI z scores, B/F ratio, and age were among the most predictive variables of vitamin D levels (Figure 6). Similarly, in the multivariable regression (Supplementary Table S7), age, B/F ratio, exposure to sunlight, and BMI z scores were found to be strongest predictors of circulating 25(OH)D levels among other variables.

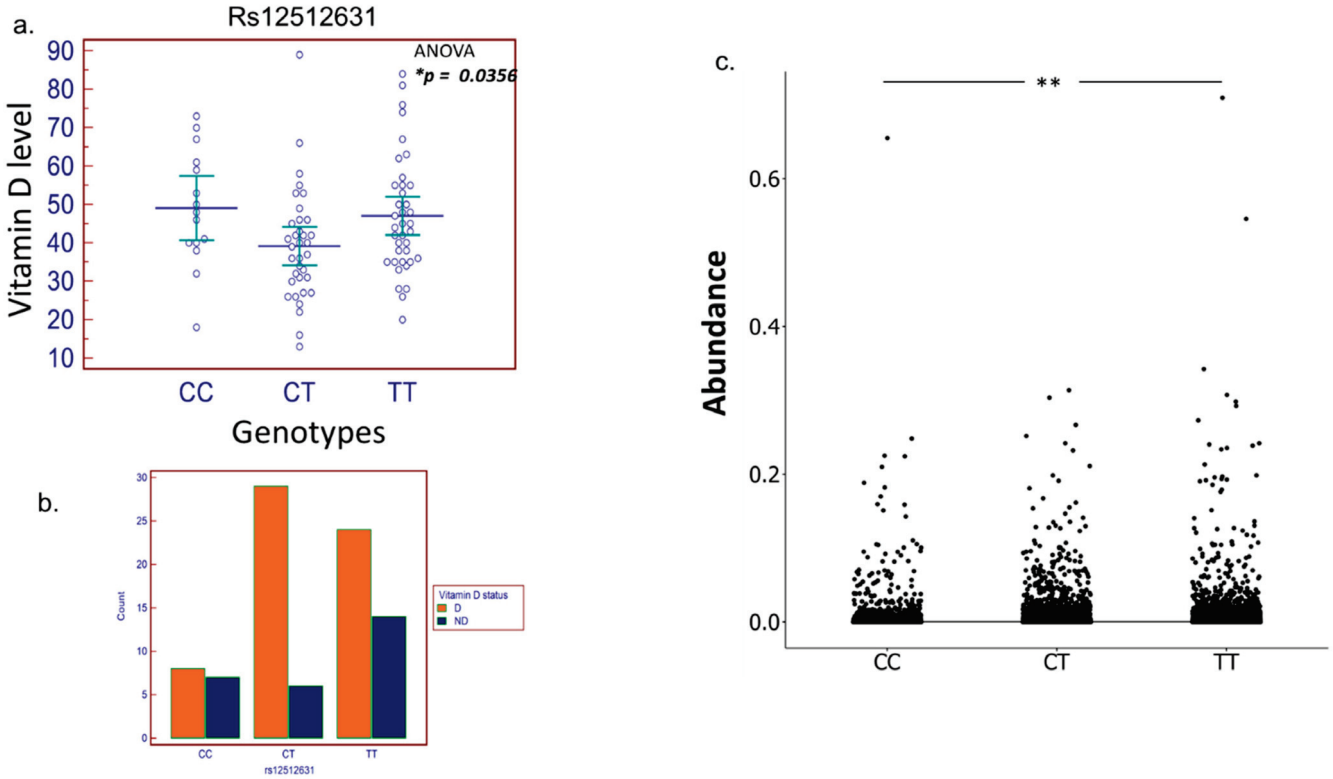

Figure 5. (**a**) The CT genotype (SNP rs12512631 in the GC gene) was associated with low levels of serum vitamin D and (**b**) was highly abundant in the deficient compared to non-deficient subjects (ANOVA * $p < 0.05$). (**c**) Overall microbial abundance was significantly different between the three genotypes of SNP rs12512631 (Kruskal–Wallis followed by post hoc Dunn's test with false discovery rate (FDR)–Bonferroni corrected, ** $p < 0.01$).

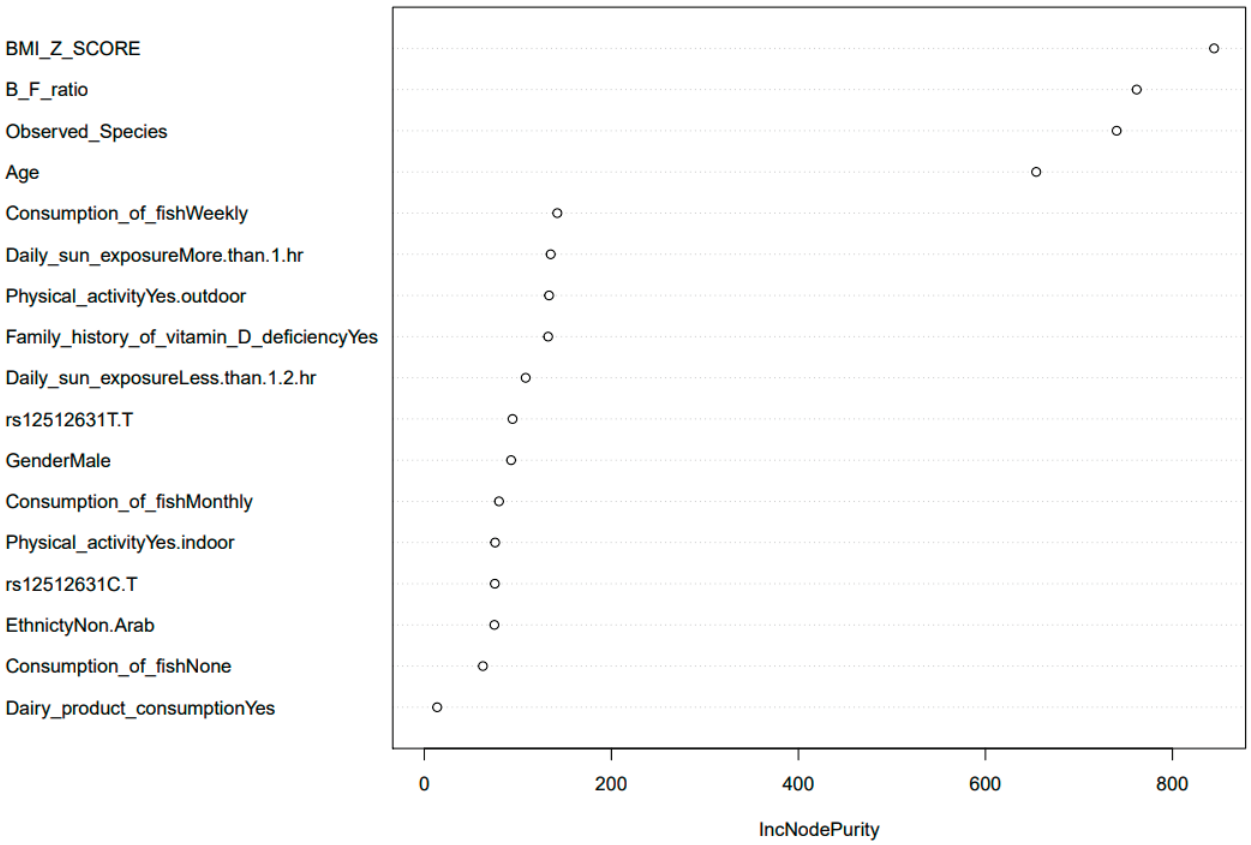

Figure 6. Random forest analysis used to determine the variables that had the largest contribution in determining the vitamin D levels. Inc Node Purity was used as a measure of "variable importance".

4. Discussion

Despite ample sunshine, approximately 80% of the population throughout the GCC region, including Qatar, suffers from vitamin D deficiency [9]. However, the information about the vitamin D status in the pediatric populations from Qatar remains scarce. High levels of deficiency were observed in a study conducted among the young adults in Qatar, mostly in the age group of 11–16 years, and among these, vitamin D deficient children had significantly higher rates of rickets, fractures, gastroenteritis, and delayed milestones [19]. Our study included children aged 4 to 14 years old, and we observed a high prevalence of vitamin D insufficiency/deficiency, with nearly 97 percent of the study participants demonstrating serum 25(OH)D levels below the sufficient level (75 nmol/L) [32].

Studies have shown associations between low vitamin D serum levels and conditions that may predispose patients to chronic diseases, such as obesity [68], metabolic syndrome [69], and cardiometabolic diseases [70]. Inadequate vitamin D levels during childhood and adolescence has also been linked to an increased prevalence of respiratory tract infections [71–73] and pediatric autoimmune-related disease [74]. This shows that vitamin D inadequacy in childhood may have a role in the progression of a variety of disorders and could help explain why hypertension, metabolic syndrome, hyperglycemia, obesity, diabetes, and cardiovascular disease are all on the rise in Qatar.

The 25(OH)D concentration has high heritability (28–80%) [14]. GWAS studies have identified several common genetic variants in genes related to the vitamin D pathway, the metabolism that influences 25(OH)D concentrations, and the risk of insufficiency [37]. Vitamin D-binding protein (VDBP) is the primary carrier of vitamin D metabolites in the blood, including 25(OH)D and 1,25(OH)2D, and is encoded by a group-specific component (GC) located on chromosome 4 (4 q11–13) [75]. VDBP affects the bioavailability of vitamin D metabolites by transporting them to the target tissues and thus may also influence disease risk associated with these metabolites [76]. GC variants have been linked to susceptibility to

various diseases in the past [77], and recent GWAS studies have shown that allelic variation in GC contributes to changes in affinity of VDBP for the vitamin D metabolites as well as the serum concentrations of 25(OH)D [37,76,78,79]. Our data suggest that the CT genotype of rs12512631 (GC gene) was significantly associated with low levels of vitamin D in children and was highly prevalent in the vitamin D deficient subjects. Previous studies have shown that maternal rs12512631 (GC gene) genotypes had a significant impact on the association between 25(OH)D and the infant's birth weight [80]. Additionally, rs12512631 was found to be linked to 25(OH)D levels in both children and adults [14], with a stronger impact in younger than older adults [81]. The reason behind the age-dependent association needs to be elucidated; lifetime environmental and/or body composition changes might also contribute to explain such a phenomenon. Studies have also highlighted the physiological importance of rs12512631 by demonstrating its associations with melanoma and prostate and colorectal cancers [65,82,83]. The rs12512631 SNP is found in the 3′ untranslated region of GC [65]. While the exact consequences of rs12512631 on the function of VDBP are unknown, it could alter the gene expression via disruption of miRNA binding, post-transcriptional processing, and 3′-cleavage/polyadenylation; however, large-scale genomic studies with a bigger cohort size are required to examine the full functional impact of this SNP in pediatric subjects.

As for the other SNPs, it should be highlighted that there might be other loci, different from the ones tested in our study, that might have a role in the association with vitamin D in the pediatric population of Qatar. Additionally, it is possible that the sample size of our cohort did not carry enough power to detect association.

The gut microbiota is a diverse community of microorganisms that exist in symbiotic relationship with their host and perform essential functions such as digestion, intestinal homeostasis, and the maturation and education of the immune system [83]. Like any ecosystem, a healthy, immune resilient, and stable gut relies on high microbiota richness and biodiversity [84]. Low diversity on other hand is commonly found is several disease states such as obesity [85,86], type 2 diabetes [87], and IBD [88]. The gut microbiota diversity of healthy pre-adolescent children aged 6–12 years has been shown to be substantially higher than that of healthy adults living in the same environment [89]. Our vitamin D deficient children's gut microbiome showed a considerably low alpha diversity (observed species and Chao 1). The same low-diversity dysbiotic states were observed in our recent interventional study with vitamin D deficient adult subjects, where supplementation with vitamin D resulted in an increase in both the richness and diversity of gut microbiota [21]. The presence of VDRs in the gastrointestinal tract could be one of the methods by which vitamin D influences microbial composition and diversity, particularly through immune response control, modulation, and preservation of gut epithelial integrity [90]. Antimicrobial peptides such as cathelicidin and defensin are also produced by VDRs and (1,25(OH)2D), the active bioavailable form of vitamin D [91,92]. These antimicrobial peptides are important for maintaining microbial equilibrium.

Vitamin D is a fat-soluble molecule, and in order for it to be absorbed, it must be made water-soluble in the intestines [93]. This is accomplished by emulsification of vitamin D in the intestinal lumen, through the action of bile acids, forming small droplets which are incorporated into micelles—complex aggregates facilitating its absorption by the intestinal cells [94]. Without proper functioning of this mechanism, the relative bioavailability of vitamin D will be low. A favorable gut microbial environment produces beneficial metabolites such as SCFAs, bile acids driving increased micelle formation/assembly and regulation of intestinal barrier integrity, which allows the proper absorption of vitamin D. On the other hand, alteration in human bile acid pool composition induced by changes in the gut microbiota may influence the absorption and bioavailability of vitamin D. This suggests a central role for the gut microbiome and its metabolites in the mechanism of vitamin D absorption. However, there is a paucity of human studies in this field and there is a great need for research to elucidate the exact mechanisms to support this hypothesis.

About 90% of the intestinal bacteria can be assigned towards two major bacterial phyla, the "Firmicutes" and "Bacteroidetes". Composition-wise, Firmicutes (~60%) generally overtakes Bacteroidetes (~20%) in the healthy adult human gut [95]. In comparison to adults, the average healthy child's gut community has significantly more Firmicutes and less Bacteroidetes [89]. In our study, vitamin D deficient children exhibited an overabundance of Bacteroidetes, resulting in a significantly higher Bacteroidetes/Firmicutes (B/F) ratio compared to non-deficient children. The B/F ratio is commonly considered to have a key role in maintaining proper intestinal homeostasis, and disbalance in the ratio is often regarded as a general determinant for gut dysbiosis [61]. Microbial dysbiosis associated with inflammatory gastrointestinal diseases such as IBD is characterized by an increase in the abundance of the phylum Bacteroidetes and a decrease in Firmicutes, resulting in a higher B/F ratio [61,96,97]. Interestingly vitamin D deficiency is highly prevalent in IBD patients (as high as 90% in some cases) and was found to be significantly associated with disease activity [98]. In addition, many studies have confirmed the close relation between vitamin D deficiency and the development of insulin dysregulation/T2DM, increased length of respiratory infections, and mortality in patients with common immunodeficiencies [99–101]. Dysbiosis of the gut microbiota caused by vitamin D deficiency could be a possible reason for increased vulnerability to inflammatory and immune-mediated illnesses. Bacteroidetes are gram-negative bacteria that can induce activation of macrophages via the LPS present on their surface, triggering proinflammatory cascade and systemic inflammation that can cause infection or diseases under certain conditions [102]. In support of this observation, our PICRUSt analysis revealed that the gut microbiome involved biosynthesis pathways related to LPS biosynthesis that were elevated in vitamin D deficient children.

Wu *et al.* classified fecal communities into two enterotypes based on the abundance of *Bacteroides* and *Prevotella* and discovered that vitamin D intake was strongly positively associated with the *Bacteroides* enterotype and negatively to the abundance of the *Prevotella* enterotype [29]. Subsequently, studies have shown that vitamin D supplementation resulted in an increased abundance of *Bacteroides* and a decrease in *Prevotella* in both human and animals [21,103]. In line with the above data, we found overabundance of *Prevotella* enterotypes in the gut of vitamin D deficient children, resulting in a significantly higher *Prevotella* to *Bacteroides* ratio. Individual genes, the external environment, and eating habits all influence the prevalence of one enterotype over another, resulting in uniquely distinct host microbiomes [104]. These finding could indeed be very promising in the field of personalized medicine, as individual stratification based on these two microbial enterotypes (i.e., the dominance of *Prevotella* or *Bacteroides*) could help predict responses to dietary supplements or medications [105–107].

A high prevalence of vitamin D deficiency/insufficiency in the subjects enrolled in the study suggests the need for urgent preventative actions. Several predictors of vitamin D levels have been reported in the past (e.g., age [108], sex, BMI, skin color and protection, vitamin D supplementation, season, latitude) [4,109–112] that allow for the detection of populations at risk, which may benefit from attentive prevention. The strongest predictors of vitamin D levels in our study were BMI z scores, age, and the B/F ratio. Evidence suggests that obese children are more prone to vitamin D deficiency than non-obese children [113], and the degree of deficiency is directly related to adiposity [113]. Our univariate analysis showed that vitamin D deficient children showed higher BMI compared to non-deficient (Table 1, Supplementary Figure S6), suggesting that overweight children are at significantly greater risk of vitamin D inadequacy. Additionally, the multivariate regression analysis demonstrated that BMI z scores were inversely related to vitamin D levels; these data were consistent with numerous studies demonstrating that BMI z scores are strong predictors of vitamin D levels, especially in children [114–119]. This reduction in the levels of serum 25(OH)D coinciding with an increase in BMI z scores may be due to volumetric dilution of vitamin D in the large adipose stores or excess sequestration of vitamin D in fat, leading to decreased bioavailability [115]. Earlier research showed that subcutaneous synthesis of vitamin D declines with age [120]; our study shows a negative

association of age with vitamin D levels, suggesting that older children are more susceptible to vitamin D deficiency.

Milk and milk products contribute nearly half of the dietary vitamin D intake in many countries [121,122]. Our data indicate that dairy consumption is a significant predictor of vitamin D status, and people who consumed dairy are less likely to suffer from low vitamin D levels. The duration of exposure to sunlight played an important role, with children who were exposed to sunlight for more than 1 h daily showing a positive association with higher vitamin D levels, whereas children who received less than 30 min of sunlight showed a negative association. In addition, children with Arab ethnicities and those with family history of vitamin D deficiency were more likely to suffer from low vitamin D levels.

Interestingly, we also found significant predictive potential of B/F ratio and rs12512631 genotypes on the vitamin D status; these are novel findings and considering the importance of these variables, one may consider genotyping for the risk allele and to predict the population prone to vitamin D deficiency, gut microbiota targeted therapeutic intervention such as the use of probiotics/prebiotics in combination with higher consumption of dairy products can be recommended for the risk population. However, replication studies are warranted to confirm our findings in cohorts of bigger sizes.

Altogether, our results confirm that the gut microbial diversity, B/F ratio, and *Prevotella* driven gut enterotypes appear to be general features distinguishing deficient and non-deficient gut microbiota in pediatric subjects. A myriad of factors may impact microbial communities, including host genetics. Thus, based on the promising results, we expanded our efforts to examine the interactions between genetic variation and the gut microbiota. We were able to identify significant associations between specific SNPs and the overall microbiome composition. All the three genotypes of the SNP of interest, rs12512631, were identified to be significantly associated with vitamin D levels and showed a significant difference in the overall microbial abundance; however, no relation was observed with other biomarkers such as B/F ratio, diversity indices, or P/B ratio. We believe the reason for this could be the smaller cohort size, and studies with larger cohorts are needed to delineate the association.

Our findings underscore the need to consider host genetics and baseline gut microbiota makeup in interpreting vitamin D status and designing better personalized strategies for therapeutic interventions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/biomedicines10020278/s1. Table S1. The Basic Characteristics of the Candidate Genes and SNPs selected; Table S2. SNPs ID and related sequences; Table S3. Allele frequencies of the SNPs assessed in this study. HWE: Hardy-Weinberg equilibrium; NA: not available. All alleles in the table below are reported in the Forward orientation; Table S4. Association of genotypes with D/ND and SD/MD/ND (Chi-square test) and with vitamin D level (ANOVA); Table S5. Association of variants of different SNPs selected in the study with overall all microbial abundance using Kruskal-Wallis followed by post hoc Dunn's test (with p-adjusted with Bonferroni); Table S6. Ethnicities of the non-Arab participants in the study; Table S7. Predictor variables of serum 25-hydroxy vitamin D levels and their coefficient; Figure S1: (A) Abundance of genus Bacteroides (B) genus Alistipes in Vitamin D deficient(D) and non-deficient groups (ND) groups. (Wilcoxon test with false discovery rate (FDR)-Bonferroni corrected * $p < 0.05$ and *** $p < 0.001$). The figure was generated using (RStudio v 1.4 with R v 4.1). Figure S2: Differences in Abundance of top 20 species between the Vitamin D deficient(D) and non-deficient groups (ND) groups. Figure S3: Differences in Abundance of top 20 species between the Vitamin D deficient(D) and non-deficient groups (ND) groups. Figure S4: This meta-analysis identified 81 pathways that were significantly different between the two groups. Figure S5: Correlation of alpha diversity measures and the three genotypes of SNP of interest rs12512631; Figure S6: Significant differences in the BMI of Deficient and non-deficient kids.

Author Contributions: Conceptualization, S.A.K.; Preparation of the published work, specifically writing the initial draft, conducting a research and investigation process, specifically performing the experiments, or data/evidence collection, produce metadata, and maintain research data, P.S.; Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data. Implementation of the computer code and supporting algorithms, A.R.; Produce metadata, scrub data and maintain research data M.S. and D.E.; Analysis or synthesize of gentyping data, S.T.; Application of statistical, mathematical, computational, or other formal techniques to analyze the study data, M.E.; Patient recruitment, preparation and creation of the published work, specifically critical review, commentary or revision—including pre- or post-publication stages, A.K.A.; A.M.; I.A.; S.U.; M.A.H.; Management and coordination responsibility for the research activity planning and execution. Acquisition of the financial support for the project leading to this publication. Development or design of methodology; Oversight and leadership responsibility for the research activity planning and execution, S.A.K.; All authors have read and agreed to the published version of the manuscript.

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Vitamin D Counteracts Lipid Accumulation, Augments Free Fatty Acid-Induced ABCA1 and CPT-1A Expression While Reducing CD36 and C/EBPβ Protein Levels in Monocyte-Derived Macrophages

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Abstract: The biologically active form of vitamin D, calcitriol (VD3), has received great attention for its extraskeletal effects, such as a protective role on the cardiovascular system. The aim of the present work is to test the capacity of VD3 to affect lipid metabolism and fatty acid accumulation in an in vitro model of monocyte (THP-1)-derived macrophages. Cells were treated for 24 h with oleic/palmitic acid (500 μM, 2:1 ratio) and different VD3 concentrations (0.1, 1, 10, 50 and 100 nM). Lipid accumulation was quantified spectrophotometrically (excitation: 544 nm, emission: 590 nm). C/EBPβ, PPAR-γ1, CD36, CPT-1A, and ABCA1 protein levels were assessed by ELISA kits at different time-points (1, 2, 4, 8, and 24 h). VD3 at 50 and 100 nM significantly reduced fatty acids accumulation in macrophages by 27% and 32%, respectively. In addition, tested at 50 nM, VD3 decreased CD36, PPAR-γ1, and C/EBPβ, while it increased ABCA1 and CPT-1A protein levels in free fatty acid-exposed cells. In conclusion, VD3 reduced fatty acid accumulation in THP-1-derived macrophages exposed to lipid excess. The anti-atherogenic effect of VD3 could be ascribable to the regulation of proteins involved in lipid transport and clearance.

Keywords: calcitriol; fatty acids; foam cells; C/EBPβ; PPAR-γ1; ABCA1; CPT-1A

1. Introduction

Vitamin D (VD) is a fat-soluble vitamin whose chemical structure derives from cyclopentanoperhydrophenanthrene, the same structural root of steroid hormones [1]. The human body produces VD in the skin by converting 7-dehydrocholesterol to pre-VD upon exposure to ultraviolet-B (UVB) sunlight (290–320 nm wavelength), which undergoes non-enzymatic isomerization to produce VD [1–3]. VD is then converted in the liver by 1 or 4 cytochrome P50 microsomal enzymes to the most stable form of VD (i.e., 25(OH)D3) in the circulation, which is finally converted into the active form, calcitriol (1,25(OH)2D3) by the kidneys and other tissues such as colon, lung, breast, prostate and immune cells. [4–7]. VD can be obtained from vegetal dietary sources in the form of ergocalciferol (e.g., mushrooms, yeasts, and fortified foods) but, above all, from animal foods in the form of cholecalciferol (e.g., fish, egg yolk, meat, offal, and fortified foods) [8,9]. Dietary sources of VD and vitamin tablet supplements are particularly important for populations who live in areas with little sunlight (e.g., Nordic countries) and groups who have higher requirements such as children, pregnant women, the elderly, and patients with certain diseases such as osteoporosis and chronic kidney disease. The VD status in humans is based on the serum concentration of 25(OH)D3 as it reflects intake and is more stable than the active form (1,25(OH)2D3). An interesting cross-sectional study of subjects from the general population

in Denmark demonstrated a 20% prevalence of subjects with insufficient serum VD levels in the winter months [10]. The lower levels of VD in the winter months are attributed to low levels of incoming sunlight and clothing (due to cold weather), and VD levels are not completely replenished by the dietary intake of, especially, seafood.

An adequate status of VD is crucial for the normal activity of many organs and tissues. In this regard, the Endocrine Society Clinical Practice Guideline has set the optimal VD serum level at over 30 ng/mL (75 nmol/L). Serum levels below 20 ng/mL (50 nmol/L) reflect a deficiency that could lead to rickets in childhood or osteomalacia and a higher risk of osteoporosis in adults [7,11,12]. In addition, a moderate and severe deficiency appears to increase the risk for atherosclerosis, metabolic syndrome, CVDs [13–18], and mortality for CVDs [19,20]. VD affects the redox balance, platelet aggregation, and regulation of the innate and adaptive immune system by preventing infections and autoimmune disorders. [21–30]. Furtherly, VD has been shown to lower total cholesterol, triglycerides, and LDL-cholesterol and to increase HDL-cholesterol in plasma, thus playing an important role in lipid metabolism and atherosclerosis [28].

It is widely recognized that the hallmark of atherosclerosis is an accumulation of lipids in macrophages and foam cells, which are located in a pro-inflammatory microenvironment of the intima in arteries [31]. The oxidation and modification of the lipid structure represent relevant changes in terms of CVD risk [32]. In addition, the profile of sphingolipids and phosphatidylcholines contained in LDL impacts the susceptibility of their aggregation, which confers a higher risk of future CVD events [33]. Oxidation of LDL (ox-LDL) leads to the activation of the adaptive immune response, mediated by B- and T-cells that enhance the progression of atherosclerotic plaques [34]. In addition, ox-LDL has a higher affinity to plaque macrophage scavenger receptors, resulting in an increased intracellular lipid accumulation [35]. Moreover, the modification of LDL induces the inflammatory process through NLRP3 in macrophages and the consequent detrimental effect through apoptosis and necrotic core development [36]. Additionally, alterations of lipoproteins are responsible for calcium deposits in the arterial intima, which determine a negative effect on hemodynamic pressure and alter viscosity and elasticity of blood vessels, contributing to plaque progression [37].

Numerous are the potential mechanisms implicated in lipid accumulation. Among them, peroxisome proliferator-activated receptor gamma (PPAR-γ) represents the main regulator of fatty acids storage and mobilization of lipids. However, PPAR-γ interacts with other proteins involved in lipid metabolism (Figure 1). For example, the cluster of differentiation 36 (CD36) [38], devoted to the uptake of lipids, ox-LDL, and their accumulation [39]; CCAAT enhancer-binding protein beta (C/EBPβ), which regulates the progression of foam cell formation and the inflammatory response induced by lipid accumulation [40]; carnitine palmitoyltransferase 1A (CPT-1A) [41], involved in FA oxidation (FAO) [42]; ATP-binding cassette transporter 1 (ABCA1) [43], devoted to maintaining cellular cholesterol homeostasis and preventing foam cell formation through the removal of cellular lipids [44,45]. In addition, ABCA1 has been recognized to exert an anti-inflammatory activity [46].

However, the mechanism through which VD is able to impede the development or progression of atherosclerosis is unknown. We hypothesized that VD could play an important role by reducing lipid accumulation through the regulation of the genes involved in lipid metabolism. Based on this hypothesis, the aim of the present study is to evaluate the effect of the active form of VD on the lipid accumulation in monocyte-derived macrophages THP-1, as a cellular model of atherogenesis, by evaluating the main key proteins involved in the uptake, accumulation, metabolism, and efflux of lipids such as PPAR-γ1, CD36, C/EBPβ, CPT-1A, and ABCA1.

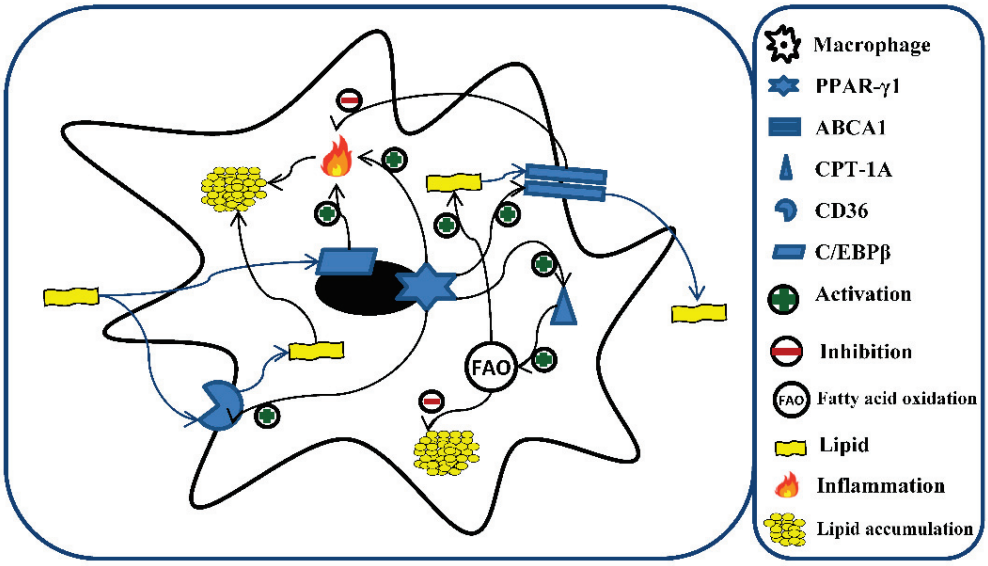

Figure 1. Role of C/EBPβ, PPAR-γ1, CD36, CPT-1A, and ABCA1 in lipid metabolism and fatty acid oxidation (FAO) in macrophages. The excess of lipids is responsible for the induction of different genes involved in the metabolism of fatty acids and the inflammatory process. A central role is represented by PPAR-γ1, which orchestrates the storage and mobilization of lipids through the activation of CD36, CPT-1A, and ABCA1. Additionally, lipids lead to the activation of CD36 and C/EBPβ, which promote intracellular lipid accumulation through the enhancement of lipid uptake and inflammatory response, respectively. Moreover, the activation of CPT-1A, and ABCA1 by the excess of lipids counteract the accumulation of the latter through the stimulation of lipid efflux and increment of fatty acid oxidation, respectively. C/EBPβ (CCAAT enhancer-binding protein beta); PPAR- γ (peroxisome proliferator-activated receptor gamma); CD36 (cluster of differentiation 36); CPT-1A (carnitine palmitoyltransferase 1A); ABCA1 (ATP-binding cassette transporter 1).

2. Materials and Methods

2.1. Chemicals and Reagents

Fetal bovine serum (FBS; CAS No. F4135), Hanks' balanced salt solution (CAS No. H6648), phorbol-12-myristate-13-acetate (PMA; ≥99% TLC; CAS No. P8139), bovine serum albumin (BSA; CAS No. A9418), palmitic acid (≥99% GC; CAS No. P0500), oleic acid (≥99% GC; CAS No. O1008), standard of VD3 (calcitriol; ≥99% HPLC; CAS No. D1530-10UG), Trypan Blue (CAS No. T8154), hydrochloric acid (37%; CAS No. 320331), methanol (≥99.9% HPLC; CAS No. 34860), ethanol (EtOH; ≥99.8% HPLC; CAS No. 51976), pluronic F127 (CAS No. P2443) and Nile Red (≥97%; HPLC CAS No. 19123) were obtained from Merck (Darmstadt, Germany). RPMI-1640 medium (CAS No. 21875091), sodium pyruvate (CAS No. 11360070), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; CAS No. 15630106), gentamicin (CAS No. 15710064), and trypsin–EDTA (CAS No. 25200056) were purchased from Life Technologies (Monza Brianza, Italy). Enzyme-linked immunosorbent assay (ELISA) kits related to PPAR- γ, C/EBP-β, CD36, CPT-1A, and ABCA1 were purchased from MyBioSource (San Diego, CA, USA), while ultrapure water was from a Milli-Q apparatus (Millipore, Milford, MA, USA).

2.2. Preparation of Calcitriol Stock Solution

Lyophilized VD3 (Figure 2) standard (10 μg) was dissolved in EtOH in order to prepare a stock solution. The final concentration of EtOH in the medium for cell culture was 0.0025%. The VD3 stock solution was aliquoted and stored at −20 °C until use.

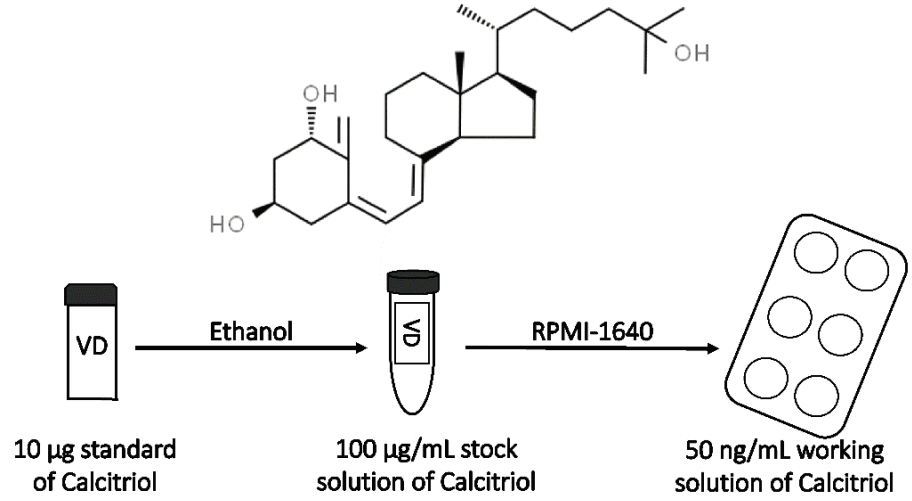

Figure 2. Preparation of calcitriol working solution and its chemical structure.

2.3. Preparation of Fatty Acid Solution and Its Control

The FFA stock solution was prepared in EtOH by using oleic and palmitic acid in a 2:1 ratio with a final concentration of 0.2 M. The mix of FFA and their ratio were chosen based on previous experiments, where the cytotoxicity of palmitic acid (saturated fatty acid) is blunted in the mixture with oleic acid (mono-unsaturated fatty acid) in order to reproduce a more in vivo situation as oleic and palmitic acids are abundant in human plasma [47,48]. The solution was stored in a dark tube at −20 °C. On the day of use, FFA stock solution was added to a Hanks solution with 10% BSA in order to obtain a water-soluble solution (FFA/BSA solution) with an FFA final concentration of 5 mM. The latter solution was prepared through an incubation period of 30 min at 37 °C with occasional shaking. The final concentration of FFA in the cell culture medium was 500 µM, while the negative control cell culture medium contained an equal volume of EtOH instead of the FFA solution. The final EtOH and BSA concentrations in the cell media were 0.1% each.

2.4. THP-1 Cell Culture

The THP-1 cell line (human monocytic leukemia) was obtained from the American Type Culture Collection (Manassas, VA, USA). The cell culture medium was RPMI-1640 supplemented with 10% of heat-inactivated FBS, 1% sodium pyruvate, 1% HEPES, and 0.1% gentamicin. Every third day during cell growth, the THP-1 cells were subcultured by withdrawing a volume of medium containing cells from the culture flask and adding complete fresh medium to obtain the appropriate seeding density of 3×10^5 cells/mL. The THP-1 cell line maintains monocytic characteristics for over 14 months of continuous growth [49]. In our study, a maximum period of 2 months was adopted for cell growth, which corresponds to a passage number between 5 and 15.

2.5. Viability Assay

The Trypan Blue dye exclusion assay was used to assess the toxicity of the compounds tested on THP-1-derived macrophages. The cell count was performed by using a TC20TM automated cell counter and dual-chamber cell counting slides (BIORAD, Segrate, Milan, Italy). The cells were resuspended in fresh complete medium in order to reach 4×10^5 cells/mL as the final concentration, and 1 mL of cell suspension (4×10^5 cells) was added into each well of a 12-well plate. Monocytes were differentiated into macrophages and incubated with VD3 at different concentrations (from 0.01 to 100 µM) in the presence of FFA/BSA solution (500 µM) for 24 h. Afterward, macrophages were trypsinized and resuspended in their supernatants (in order to also include dead cells), and viability was analyzed by the Trypan Blue dye exclusion assay. Each compound and concentration was assessed in triplicate over three independent experiments (Table 1). Triton X-100 (0.1%)

was used as positive control. The absolute viability is expressed as the percentage of viable cells out of all counted cells.

Table 1. Cell Viability.

Condition	VD3 Concentration (nM)	% of Cell Viability *
Control	-	90.7 ± 1.5
Triton X-100	-	20 ± 6.2 **
FFA	-	92.1 ± 2.2
FFA + VD3	0.1	91.3 ± 2.6
FFA + VD3	1	90.9 ± 1.8
FFA + VD3	10	93.4 ± 3.3
FFA + VD3	50	92.5 ± 2.4
FFA + VD3	100	90.3 ± 1.3

Legend: Results derived from three independent experiments, in which each concentration was tested in triplicate. Data are reported as the mean ± standard deviation. * The total number of cells was not different between the exposure groups (i.e., 4×10^5 cells/well, corresponding to a plating efficiency higher than 90%). ** $p \leq 0.01$ compared with control. VD3: calcitriol; FFA: free fatty acids.

2.6. Lipid Accumulation Assay in Macrophages

THP-1 cells were maintained in complete RPMI-1640 medium at 37 °C and 5% CO_2. Once a proper cell number was reached, the differentiation into macrophages was induced by exposure to 5 ng/mL PMA for 72 h. Undifferentiated cells were removed by washing with Hanks' solution since THP-1-derived macrophages became adherent to the culture flask surface. Afterward, in order to collect the differentiated macrophages, cells were trypsinized by incubating with 3 mL trypsin (0.05%)–EDTA (0.53 mM) at 37 °C and 5% CO_2 for 2 min. The addition of 2 mL of complete RPMI-1640 medium allowed us to inactivate the trypsin. Falcon tubes were used to collect and centrifuge the cells (mod. Eppendorf 5804R Centrifuge, Milan, Italy) at 250× *g* for 5 min. Then, the cells were quantified by using a TC20TM automated cell counter and resuspended in fresh complete medium in order to reach 2.5×10^5 cells/mL as the final concentration. The adhesion of macrophages was carried out by using a black 96-well plate and by adding 200 µL of cell suspension (equivalent to 5×10^4 cells) into each well. Cells were incubated at 37 °C and 5% CO_2 for 24 h. Then, the medium was replaced with new RPMI-1640 containing 500 µM of FFA and VD3 at different concentrations, and macrophages were incubated for 24 h at 37 °C and 5% CO_2. The concentrations of VD3 were 0.01, 0.1, 10, 50 and 100 µM. The Nile Red dye was used to measure the intracellular lipid accumulation [47], which is extensively fluorescent in lipid-rich environments. In detail, macrophages were washed with Hanks' solution and then stained using 0.5 µg/mL of Nile Red dissolved in Hanks' solution supplemented with 0.01% Pluronic F127 and 0.01% Pluronic F127 and maintained in incubation for 15 min at 37 °C and 5% CO_2. Cells were washed twice with Hanks' solution (200 µL) and soaked in 100 µL of new Hanks' solution prior to the fluorescence analysis. For the quantification, a fluorescence spectrophotometer (mod. F200 Infinite, TECAN, Milan, Italy) was used and the fluorescence determined (excitation: 544 nm, emission: 590 nm). Each concentration of VD3 was derived from 3 independent experiments in which each concentration was tested 7 times.

2.7. Protein Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

In order to collect a sufficient number of cells, THP-1-derived macrophages were seeded in 6-well plates (1×10^6 cells/well) instead of 96-well plates (5×10^4 cells/well). In conformity with the manufacturer's protocol, the supernatant of cell culture was collected for PPAR-γ1, CD36, and ABCA1 analyses; cell extract was used for C/EBPβ and CPT-1A analysis. We used cell culture supernatants for PPAR-γ1, CD36, and ABCA1 measurements because it was within the manufacturer's specification of the assays and because soluble CD36 and ABCA1 in plasma are used as biomarkers of cardiovascular diseases in humans [50,51]. The supernatant was centrifuged at 500× *g* for 10 min at 4 °C. Protein extraction was performed by placing cells on ice, washing with cold PBS, and incubating for 30 min on ice with an extraction buffer. The cell lysate was centrifuged at 4500× *g*

for 20 min at 4 °C. Aliquots of supernatants and cell lysate were stored at −80 °C until analysis. ELISA kits were used to assess the protein levels of C/EBPβ, PPAR-γ1, CD36, CPT-1A, and ABCA1 (Cat No. were MBS2709324, MBS263089, MBS2020315, MBS166979, and MBS267210, respectively; MyBioSource, Inc. San Diego, CA, USA). The analyses were conducted in triplicate, and the results were derived from three independent experiments.

2.8. Data Analysis

The effect of VD3 supplementation on cell viability, the lipid accumulation process, and the protein levels of C/EBPβ, PPAR-γ1, CD36, CPT-1A, and ABCA1 was evaluated by one-way ANOVA using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). Differences between treatments were identified by the least significant difference (LSD) test by setting the level of statistical significance at $p < 0.01$. Results are reported as means ± standard deviation (SD).

3. Results

3.1. Effect of VD3 on Cell Viability

Table 1 presents the effect of the different VD3 concentrations (0.1–100 nM) tested for 24 h in the presence of FFA/BSA solution (500 μM) on the cellular viability measured by Trypan Blue exclusion assay. The control condition is represented by cells in their normal growth medium without VD3 and FFA. VD3 + FFA did not reduce the cell viability, which remained higher than 90% ($p > 0.05$), while, as expected, the addition of Triton X-100 (0.1%) induced a significant reduction (−77.9%, $p < 0.0001$).

3.2. Effect of VD3 on Lipid Accumulation in THP-1 Derived Macrophages

Figure 3 depicts the effect of the supplementation with VD3 on lipid accumulation in THP-1-derived macrophages. Exposure to 500 μM of FFA significantly increased ($p < 0.01$) the lipid accumulation in macrophages compared to cells without treatment (No FFA). Treatment with 50 and 100 nM of VD3 significantly lowered ($p < 0.01$) lipid accumulation in macrophages compared to the positive control (FFA exposure only). In particular, the size of the effect was similar, −27% and −32%, respectively, for VD3 at 50 and 100 nM. Since the effect on lipid accumulation was comparable at 50 and 100 nM, the experiments on gene expression were performed by using the lowest concentration.

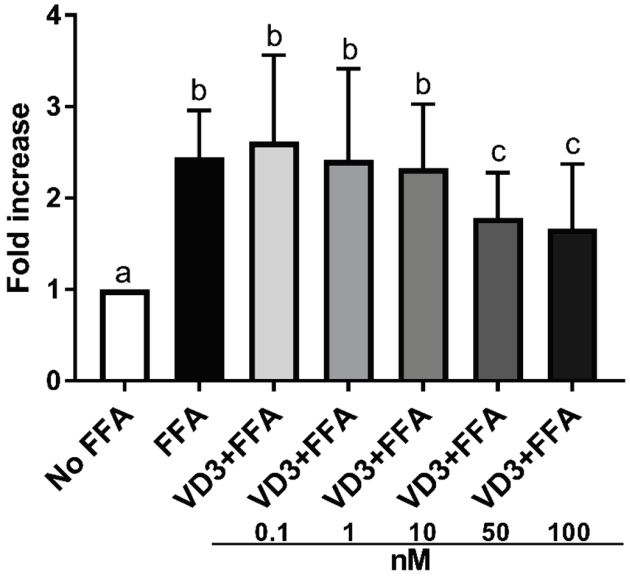

Figure 3. Effect of different concentrations (0.1–100 nM) of VD3 on cell lipid accumulation. Data are reported as a fold increase and mean ± standard deviation. [a,b,c] Bar graphs with different letters are significantly different ($p \leq 0.01$). No FFA: no free fatty acids (control); FFA: free fatty acids (500 μM); VD3: calcitriol + free fatty acids (500 μM).

3.3. Effect of VD3 on PPAR-γ1 Protein Levels

The results of the protein expression kinetic of PPAR-γ1 after the administration of VD3 at a concentration of 50 nM are depicted in Figure 4. A statistically significant increase ($p < 0.01$) in PPAR-γ1 protein levels was documented at 2 and 24 h following the incubation with FFA compared to No FFA (+65.3% and +54.4%, respectively). The treatment with VD3 (VD3 + FFA) induced an increase in PPAR-γ1 levels at 2 h compared to the negative control (+57%; $p < 0.01$), while the exposure for 24 h determined a reduction in PPAR-γ1 protein level (−33.4%; $p < 0.01$). No difference was found when considering the negative control (No FFA) or the other time points analyzed.

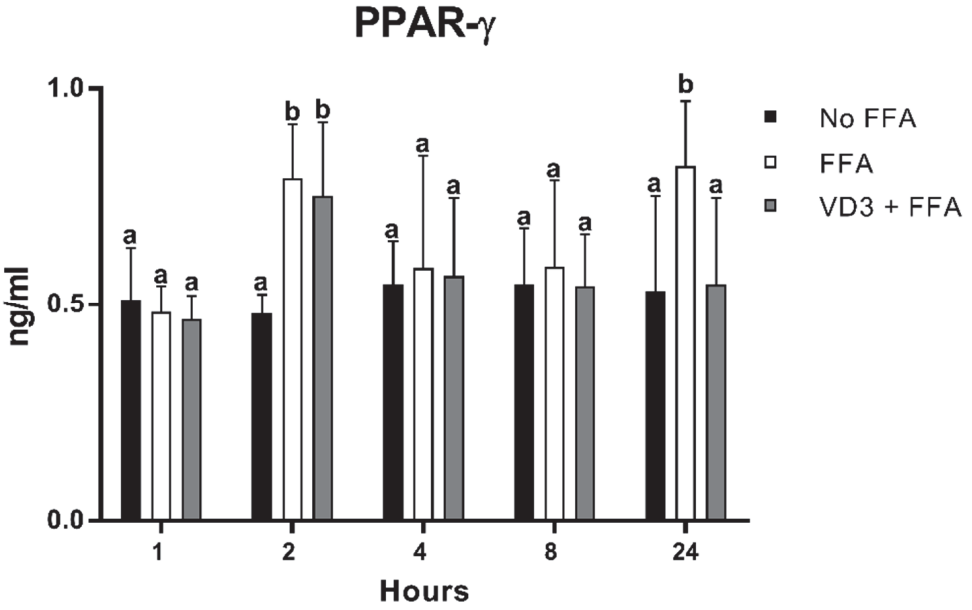

Figure 4. Effect of VD3 supplementation on PPAR-γ1 protein levels. Data are reported as mean ± standard deviation. [a,b] Bar graphs with different letters are significantly different ($p \leq 0.01$). No FFA: no free fatty acids (control); FFA: free fatty acids (500 μM); VD3: calcitriol (50 nM).

3.4. Effect of VD3 on CD-36 Protein Levels

Figure 5 depicts the result of FFA and VD3 treatments on the expression of CD-36 in macrophages at different time points. There was a statistically significant increase of CD-36 protein expression after 1 h of FFA incubation compared to the negative control (+45.2%; $p < 0.01$), while, at the same time point, the treatment with VD3 in the presence of FFA was able to counteract the increase of CD-36 protein levels induced by FFA administration (−29.1%; $p < 0.01$). No statistically significant difference was observed between the negative control and the VD3 + FFA. No difference was documented for the other time points analyzed.

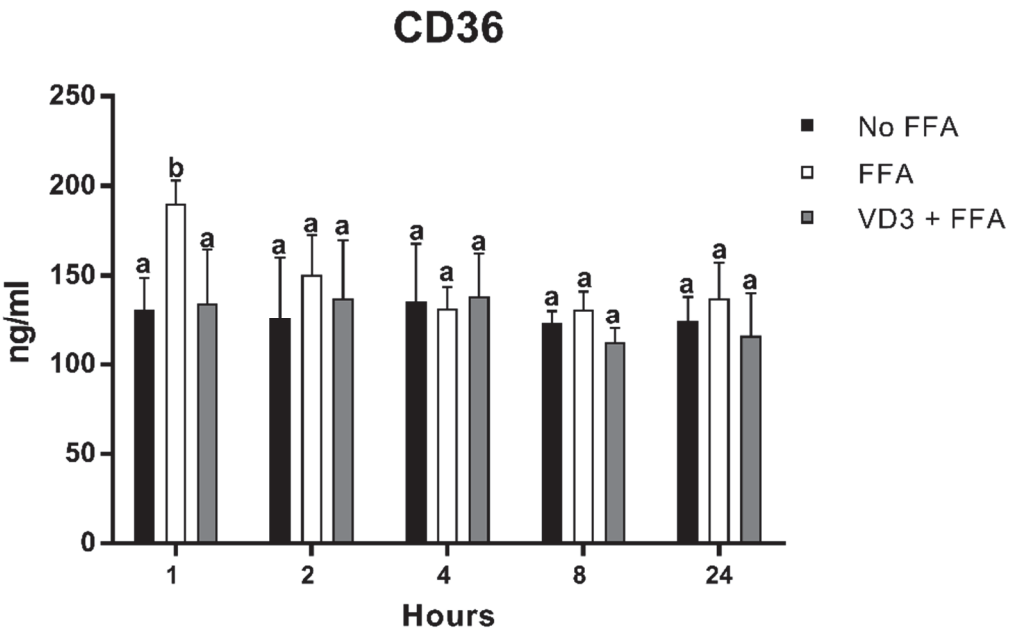

Figure 5. Effect of VD3 supplementation on CD36 protein levels. Data are reported as mean ± standard deviation. [a,b] Bar graphs with different letters are significantly different ($p \leq 0.01$). No FFA: no free fatty acids (control); FFA: free fatty acids (500 μM); VD3: calcitriol (50 nM).

3.5. Effect of VD3 on C/EBPβ Protein Levels

Figure 6 shows the protein levels of C/EBPβ at different time points after the treatment with VD3 (50 nM) and FFA (500 μM). A statistically significant increase in C/EBPβ protein levels was documented following cell incubation with FFA (positive control) compared to negative control (No FFA) at 2 h (+65.2%; $p < 0.01$) and 4 h (+23.9%; $p < 0.01$). The treatment with VD3 inhibited the FFA-induced increase in C/EBPβ protein level at both 2 h (−35.9%; $p < 0.01$) and 4 h (−16.1%; $p < 0.01$) compared to only FFA. No difference was documented with respect to No FFA.

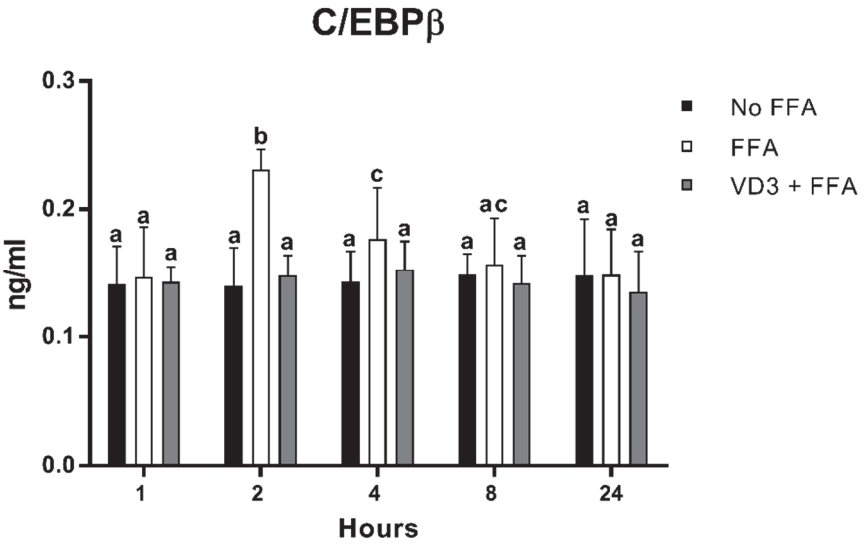

Figure 6. Effect of VD3 supplementation on C/EBPβ cell protein levels. Data are reported as mean ± standard deviation. [a,b,c] Bar graphs with different letters are significantly different ($p \leq 0.01$). No FFA: no free fatty acids (control); FFA: free fatty acids (500 μM); VD3: calcitriol (50 nM).

3.6. Effect of VD3 on CPT-1A Protein Levels

The results of FFA and VD3 administration on CPT-1A protein expression kinetics are shown in Figure 7. The earliest statistically significant modification occurred after 2 h of treatment with VD3, which was an increase of CPT-1A protein levels compared to both negative control and the FFA condition (+96.7% and +71.4%, respectively; $p < 0.01$). No significant difference was documented between the negative control and the positive control at the same time point. After 4 h, the incubation with FFA increased the CPT-1A protein levels, similar to the condition of VD3 + FFA (not statistically different) but significantly higher than the negative control (+48%; $p < 0.01$). However, at 8 and 24 h, the protein levels of CPT-1A after FFA treatment returned to baseline. The addition of VD3 (VD3 + FFA) was able to maintain significantly higher levels of CPT-1A at 8 and 24 h compared to the negative control (+35.5% and + 18.8%, respectively; $p < 0.01$).

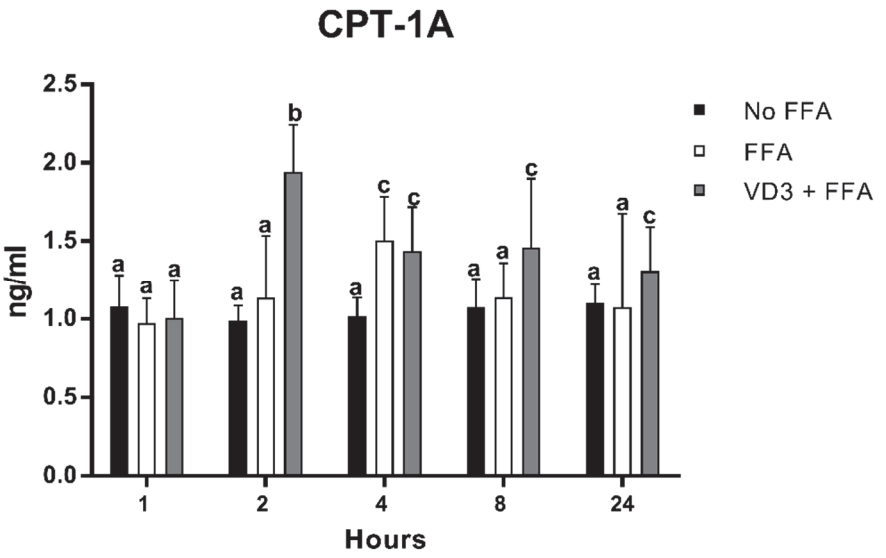

Figure 7. Effect of VD3 supplementation on PPAR-γ1 protein levels. Data are reported as mean ± standard deviation. [a,b,c] Bar graphs with different letters are significantly different ($p \leq 0.01$). No FFA: no free fatty acids (control); FFA: free fatty acids (500 μM); VD3: calcitriol (50 nM).

3.7. Effect of VD3 on ABCA1 Protein Levels

The results of the protein expression kinetic of ABCA1 after VD3 and FFA treatments are shown in Figure 8. THP-1-derived macrophages incubated with FFA + VD3 had significantly higher protein levels of ABCA1 compared to the positive control (only FFA) at 4 h (+72.5%; $p < 0.01$), 8 h (+26.8%; $p < 0.01$), and 24 h (+33.6%; $p < 0.01$). The treatment with FFA alone was able to increase ABCA1 in a significant way compared to the negative control only at 8 h (+46.5%; $p < 0.01$) and 24 h (+54.7%; $p < 0.01$), although to a lower extent compared to the addition of VD3.

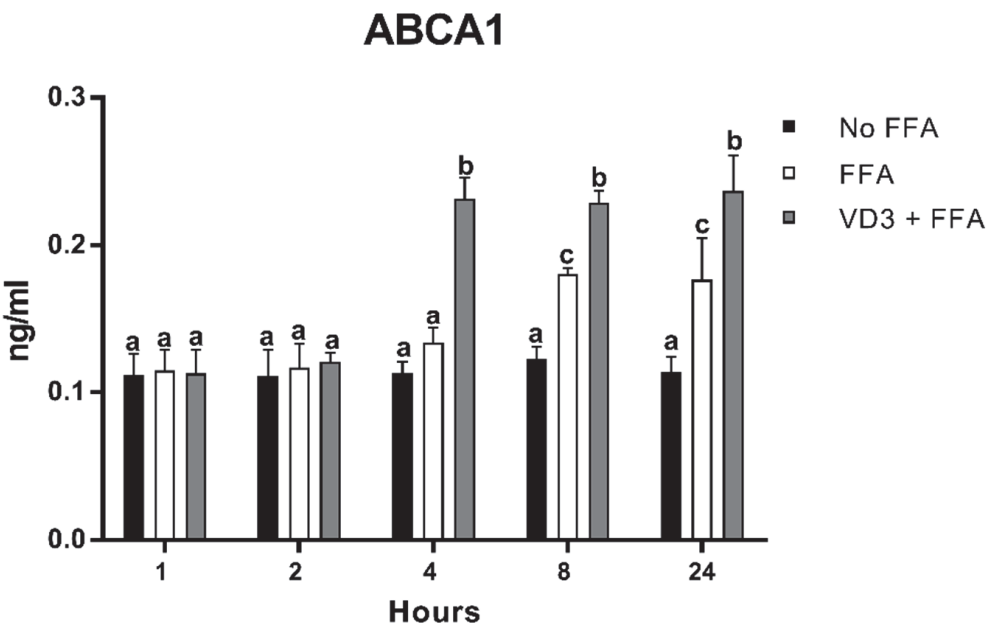

Figure 8. Effect of VD3 supplementation on ABCA1 protein levels. Data are reported as mean ± standard deviation. [a,b,c] Bar graphs with different letters are significantly different ($p \leq 0.01$). No FFA: no free fatty acids (control); FFA: free fatty acids (500 μM); VD3: calcitriol (50 nM).

4. Discussion

In the present study, we document the capacity of VD3 to mitigate the accumulation of lipids in THP-1-derived macrophages exposed to FFA. This effect seems to be attributed to an upregulation of several proteins involved in the metabolism of fatty acids, such as C/EBPβ, PPAR-γ1, CD36, CPT-1A, and ABCA1, supporting the potential anti-atherogenic activity of VD3.

The role of VD in the modulation of lipid metabolism has been evaluated in different studies [52–56]. The results seem to support the contribution of VD in counteracting lipid accumulation in THP-1-derived macrophages and other cell lines in line with our findings. For example, Riek and colleagues [52] showed that VD3 supplementation (100 nM for 5 days) prevented foam cell formation through the reduction of cholesterol deposition and the enhancement of cholesterol efflux in human monocyte-derived macrophages from adults with type 2 diabetes mellitus (T2DM). Moreover, the same authors [53] documented that the deletion of the vitamin D receptor (VDR) in mice accelerated the atherosclerotic process, probably due to an increase of lipid-laden macrophages in atheroma as well as higher cholesterol uptake and deposition in foam cells. Yin and coworkers [54] observed lower levels of lipid accumulation in THP-1-derived macrophages incubated with oxidized LDL (ox-LDL; 50 μg/mL for 48 h) and VD3 (10 nM for 24 h) compared to the same model, but without VD3 and only treated with ox-LDL. In addition, the use of VDR antagonists determined the inhibition of cholesterol efflux, supporting the anti-atherogenic role of VD3 through the interaction with its ubiquitous receptor. The reduction in lipid droplet accumulation has also been shown in a murine model of 3T3-L1 preadipocytes [55]. Li et al. demonstrated that VD3 treatment was able to counteract the storage of lipids by 79% compared to the control in 3T3-L1 cells, although they used higher concentrations (1 μM) and times of exposure (up to 60 h) compared to our experimental conditions. Finally, Chang and colleagues [56] documented a reduction in lipid accumulation and an increase in lipolysis after treating 3T3-L1 adipocytes with 100 nM VD3 for 24 h.

The role of VD3 in the modulation of atherosclerosis is not well understood. The main mechanisms potentially involved in this effect include the up- or downregulation of the expression of genes embroiled in the lipid uptake and efflux. Among transcriptional pathways, PPAR-γ1 has been recognized to play a pivotal role in the control of FA

metabolism. FA and FA-derived compounds are strong ligands for PPAR γ1 [57]. In fact, in our experimental conditions, we found that macrophages incubated with FFA increased PPAR-γ1 levels after 2 and 24 h. The addition of VD3 was able to inhibit PPAR-γ1, but this effect was only at 24 h. This lack of PPAR-γ1 overexpression could be due to a resolution of intracellular lipid accumulation, in line with what was previously published [58]. The effect of VD3 on PPAR-γ1 and lipid accumulation has been investigated in different in vitro models, with results in line with our findings [55,59–61]. For example, Oh and colleagues [60] demonstrated the capacity of VD3 (100 nM) to inhibit the expression of PPAR-γ1 in macrophages and to reduce cholesterol uptake and foam cell formation. Scrimieri and coworkers [59] showed that VD3 (20 nM) downregulated PPAR-γ1 and decreased intracellular fat storage accumulation, induced by 30 mM D-glucose for 24 h, in endothelial cells. Likewise, Li et al. [55] demonstrated the prevention of lipid droplet accumulation in mouse 3T3-L1 preadipocytes following VD3 treatment (1 μM, 60 h), attributing the anti-adipogenic effect to the capacity to decrease the expression of PPAR-γ1.

An overexpression of PPAR-γ1 leads to an upregulation of CD-36, a protein transporter recognized to promote the cellular uptake of ox-LDL and FFA. We have found that FFA increased CD36 expression at 1 h from their administration; however, this effect was only temporary, suggesting that it was strictly dependent on FFA availability. The addition of VD3 was able to inhibit CD36 overexpression, which results in major control of FFA uptake into the cell. Similar findings were also reported by other authors. For example, Szeto and colleagues [62] demonstrated that $LDLR^{-/-}VDR^{-/-}$ mice supplemented with a high fat–high cholesterol diet for 8–12 weeks experienced higher lipid accumulation in macrophages due to an overexpression of CD36, compared with $LDLR^{-/-}$ mice. Similarly, Oh et al. [53] found that the bone marrow transplant of VDR into KODMAC mice (in which VDR was inactivated in myeloid cells) decreased foam cell formation and suppressed atherosclerosis. In addition, the authors observed that macrophages treated with 100 nM of VD3 expressed lower levels of CD36 and reduced cholesterol uptake. In a previous study [60], the same authors documented that VD3 supplementation downregulated CD36 by preventing cholesterol uptake and counteracting foam cell formation in isolated macrophages from diabetic patients. Conversely, Alizadeh et al. [63] observed higher levels of CD36 expression in the aorta of adult male VD/diabetic rats after 4-week supplementation with higher doses of VD3 (5000 IU/kg).

The intracellular concentration of FAs brings an overexpression of not only PPAR-γ but also of other genes involved in the lipid metabolism, including those related to the inflammatory response, such as C/EBPβ, and the beta-oxidation, such as CPT-1A, as documented by their temporary upregulation (at 2 and 4 h for C/EBPβ and at 4 h for CPT-1A). The incubation of VD3 with FFA blunted the overexpression of C/EBPβ, while it maintained upregulated CPT-1A from 2 h up to 24 h compared to control, suggesting that VD3 stimulates the lipid metabolism by reducing intracellular lipid accumulation and increasing the oxidation process; meanwhile, VD3 was also able to blunt the inflammatory response at the levels of macrophages. To the best of our knowledge, the role of VD3 on C/EBPβ and CPT-1A has been poorly investigated, in particular at the levels of macrophages, but the results seem to be in line with our observations [64–67]. Specifically, Blumberg et al. [64] reported that the treatment with VD3 (1–100 nM) in the preadipocytes 3T3-L1 cell line reduced the expression of C/EBPβ and upregulated the C/EBPβ corepressor, determining the blocks of adipogenesis and the reduction of lipid accumulation. Chang and colleagues [67] showed significantly higher levels of CPT-1 in muscle cells treated with VD3 (100 nM; 24 h). Scrimieri and coworkers [59] observed an upregulation of CPT-1A induced by VD3 (20 nM) in endothelial cells incubated with a high-glucose concentration (30 mM d-glucose for 24 h). The effect of VD3 on CPT-1A has also been investigated in animal models, showing similar findings following the co-administration of a high-fat/high-sugar diet and VD3 [68,69].

The cellular uptake of FFA and their accumulation, together with the upregulation of PPAR-γ, is reported to induce the transcription of different cell membrane proteins

involved in the lipid efflux, such as ATP-binding cassette transporter ABCA1 [45]. Here, we documented that FFA increased in ABCA1 protein levels at 8 and 24 h; at the same time points, the supplementation with VD3 augmented the ABCA1 protein levels, also stimulating an early expression at 4 h compared to the positive control (only FFA administration). This increase in ABCA1 protein levels could represent another anti-atherogenic potential mechanism of VD3, consisting of a major cellular lipid efflux and a lower intracellular lipid level. In addition, several studies have reported that ABCA1 upregulation is recognized to have an anti-inflammatory function in a diverse range of diseases where inflammation is an underlying pathogenic mechanism [70,71]. These results, together with those reported for C/EBPβ, seem to also support the potential anti-inflammatory activity of VD3. Coherently with our findings, Yin and colleagues [38] found that VD3 (10 nM) led to an upregulation, at 12 and 24 h, of ABCA1 expression in THP-1-derived macrophages that had been stimulated with ox-LDL (50 μg/mL, 48 h) prior to the VD3 supplementation. This delay in ABCA1 activation between our and their study could be attributed to a difference in VD3 concentrations. The same authors found that Yucatan microswine fed for 48 weeks with a high cholesterol vitamin D-deficient (0 IU/d) diet had decreased ABCA1 liver levels, while the supplementation with vitamin D (3000 IU/d) increased HDL plasma levels and reduced liver cholesterol accumulation. Another recent study documented that the supplementation with VD3 (1 μM; at 6 and 24 h) stimulated ABCA1 expression in murine dermal fibroblasts and immortalized human epidermal keratinocytes (HaCaT cells) [72].

The present study has certain limitations. First, the statistical interaction between VD3 and FFA has not been assessed, which would have been possible by including a group with only VD3-treated cells. This does not allow us to verify whether VD3 alone is able to upregulate these genes in normal conditions (without stimulation) or if the effects are attributable exclusively to a stimulus (e.g., when administering FFA). Second, we made the decision to analyze only selected proteins involved in lipid metabolism instead of providing an overview of all genes potentially involved in such modulation. Third, the expression of proteins was assessed in cell extracts or cell culture supernatants. This was done according to the specification of the assay kits. However, a more elaborate analysis could include measurements of mRNA levels of the corresponding proteins or even protein levels by Western blot or immunocytochemistry. This would provide information regarding the mRNA-protein correspondence. Fourth, there was a lack of quantification of the specific lipids entered, accumulated, and leaked from the cells, which could sustain the upregulation observed. Finally, there was an absence of markers related to inflammation (e.g., interleukins, TNF-α, INF-γ) or oxidative stress (e.g., ox-LDL, malondialdehyde) that would be able to substantiate our findings.

5. Conclusions

In conclusion, based on our results, VD3 has a beneficial effect on lipid metabolism by affecting the production of different proteins involved in the cellular uptake, transport, oxidation, and efflux of lipids in macrophages. Thus, the maintenance of an optimal vitamin D nutritional status could represent a relevant strategy to reduce the risk of CVDs. PPAR-γ1 seems to represent a key regulator in the modulation of lipid metabolism due to its direct interaction with the other proteins involved, such as C/EBPβ, CD36, CPT-1A, and ABCA1. However, since we cannot exclude that VD3 may also influence lipid metabolism through the up/downregulation of other potential target genes, further mechanistic studies are encouraged to corroborate the actual findings and explore new potential metabolic pathways, including those related to oxidative stress and inflammation, in order to better elucidate the biological role of VD3. In this regard, the combination with an integrated multi-omic approach (e.g., lipidomic, metabolomic, and transcriptomic) would allow better comprehension of the signaling pathways involved in this complex landscape.

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Review

The Pathogenesis of Cardiac Arrhythmias in Vitamin D Deficiency

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Abstract: The global prevalence of vitamin D deficiency is more than 20%, and the main causes include insufficient intake, reduced absorption, abnormal metabolism, or resistance to its effects. The levels of serum vitamin D appear to influence cardiovascular risk, and the mechanism involved is linked to the transient outward current and the ultrarapid delayed rectifier K+ current densities, activated through the nuclear vitamin D receptor and Akt pathway. A significant number of studies have correlated vitamin D deficiency with an increased risk of developing cardiac arrhythmias and sudden cardiac death. For this reason, the purpose of this review is to analyze the relation between vitamin D deficiency and the pathogenesis of cardiac arrhythmias. Atrial fibrillation, increased QT interval, and QT dispersion were the most common findings associated with vitamin D deficiency. Due to the heterogeneity among existing studies, further research is necessary to confirm the existing data and to analyze its relationship with other types of arrhythmias.

Keywords: arrhythmias; atrial fibrillation; vitamin D deficiency; hypovitaminosis; pathogenesis; sudden cardiac death; ventricular repolarization

1. Introduction

Vitamin D represents a fat-soluble steroid hormone with two main forms: D_2 and D_3, ergocalciferol and cholecalciferol, respectively [1]. Exposure to sunlight initiates its synthesis in the skin, but, before it can be functional, it undergoes two processes of hydroxylation: first in the liver to 25(OH)D3 (calcidiol) and then in the kidney to 1,25-dihydroxycholecalciferol (calcitriol)—the active vitamin D hormone [1,2]. This last process is controlled by the parathyroid hormone (PTH) and the serum level of calcium, phosphate, and $1,25(OH)_2D$ [1,2]. Vitamin D has many functions, playing an important role in the absorption of calcium and phosphorus, resorption, mineralization and maturation of bone, as well as tubular reabsorption of calcium and its analogues [1]. It can also be used in

the treatment of various diseases-like skin conditions, renal osteodystrophy, hypoparathyroidism, cancer, and even COVID-19 [3,4]. Since its synthesis is directly linked to sunlight, it can be influenced by the pigment of the skin, aging, barriers (sunscreen, light passing through glass or plastic; staying indoor, dressing habits), air pollution, latitude, altitude, season, and time of day the sun exposure takes place [1]. The main dietary intake source for vitamin D is fortified food, especially milk [1,5]. Egg yolks, fish oils, butter, and liver are also known natural sources [5].

Insufficient intake, reduced absorption, abnormal metabolism, or resistance to its effects can cause vitamin D deficiency [1,2,5]. The global prevalence of this condition (serum level <30 nmol/L) is more than 20% [5]. A sufficient level amounts to 50–100 nmol/L, and values ≤30–50 nmol/L count as hypovitaminosis [5]. Vitamin D deficiency can cause, depending on age, rickets (children), osteomalacia, and osteoporosis (adults) [5]. The drug categories suspected to affect vitamin D levels include antidepressants, antihypertensives, corticosteroids, chemotherapeutic agents, anti-epileptics, and oral antidiabetics (metformin), among others [6]. Excess serum vitamin D levels (>150–250 ng/mL) are also dangerous with possible outcomes such as gastrointestinal disorders (anorexia, nausea, constipation, or diarrhea), hypercalcemia, hypovolemia, cardiac arrhythmias, suppression of parathyroid hormone, and neuropsychiatric disorders [5].

The levels of serum vitamin D appear to influence cardiovascular risk, with the cardioprotective role of calcitriol proven in murine models [7]. The mechanism involved in ventricular myocytes is an increase in the fast transient outward current and the ultrarapid delayed rectifier K+ current densities, activated through the nuclear vitamin D receptor and Akt pathway [7]. A significant number of studies have correlated vitamin D deficiency with an increased risk of developing heart arrhythmias, arterial hypertension, diabetes mellitus, and sudden cardiac death [8,9].

Lower serum of calcitriol levels are linked to cardiac disease, more specifically associated with increased coronary arteries calcifications, as well as with atrial fibrillation (AF) and heart failure, while downregulation of CYP27B1 (1-hydroxylase) is associated with cardiovascular disease severity [10]. However, single-cell sequencing shows minimal CYP27B1 mRNA expression in heart cells [11], which suggests that the effects of CYP21B1 are mediated by serum vitamin D and are not direct.

For this reason, the purpose of this review is to analyze the relation between vitamin D deficiency and the pathogenesis of cardiac arrhythmias.

2. Pathophysiology of Arrhythmias and Conduction Disorders

Data emerging in recent years has strongly linked vitamin D deficiency to cardiovascular disease [12,13]. Aside from its essential role in calcium and bone homeostasis, vitamin D is involved in the physiological processes of various tissues and organs, including the heart [12], via nuclear vitamin D receptor (VDR) activation and transcriptional control of various genes [14]. VDRs were found in cardiomyocytes and cardiac fibroblasts, which are the cells involved in cardiac remodeling processes [15]. Several studies suggested that vitamin D exhibits antioxidant properties and counteracts RAS [16] and other inflammatory pathways [17]. Moreover, a heart-failure (HF) rabbit model by Hanafi et al. showed that the vitamin D metabolite 1,25- dihydroxyvitamin D reduces action potential duration and increases left atrium (LA) contractility, possibly via modulation of calcium release [18].

Furthermore, vitamin D exerts anti-inflammatory effects by counteracting NF-κB signaling in epicardial adipocytes, which can delay the progression of atherosclerosis, coronary remodeling, and ischemic heart disease [19], as well as potentially acting directly on cardiac myeloid cells, which express the vitamin D receptor at a higher level compared to other heart cells [20].

Already established as a vital element in the correct functioning of the cardiovascular system, low vitamin D levels were linked to several diseases, including hypertension [21], peripheral vascular disease [22], coronary artery disease [23], and heart failure [24,25]. In developing hypertension, Forman et al. reported that a low level of vitamin D causes an

abnormally active renin–angiotensin–aldosterone system, with an increased aldosterone level and consecutive hypokalemia, increasing susceptibility to developing ventricular arrhythmias [26].

In patients with vitamin D deficiency, the dispersion of the P-wave and the left intra- and inter-atrial electromechanical delay are increased [8]. Paricalcitol restored the levels of myocardial vitamin D receptors and prolonged action potentials in obstructed rats, protecting against myocardial remodeling associated with increased arrhythmogenic risk induced by the decrease in the expression of these receptors [27]. An increased concentration of parathyroid hormone might cause arrhythmias in ischemic heart disease [28]. Low vitamin D levels, poor calcium intake, and renal calcium leak caused by sensitivity to salt and aging generate chronic moderate increases in parathyroid hormone, inducing weight gain, insulin resistance, high blood pressure, left ventricular hypertrophy, and a possible elevation in acute phase reactants [28]. Close monitoring of the parathyroid hormone is of great importance, with decreased levels of the hormone causing hypocalcemia and increased renal phosphate reabsorption [29]. With the parathyroid hormone closely involved in regulating phosphorus levels in the organism, existing evidence suggests that an increased phosphorus level is associated with aortic calcifications, carotid atherosclerosis, and increased ventricular mass, as well as overall increased cardiovascular morbidity and mortality [30–32]. Results from the Atherosclerosis Risk in Communities (ARIC) Study have also established a correlation between phosphorus and calcium-phosphorous compound levels and cardiac arrhythmias, with increased levels being associated with a greater risk of AF [33].

Another effect of vitamin D deficiency is the prolongation of the corrected QT interval (QTc) [34]. The implications of vitamin D levels in sudden cardiac death were also investigated: over-supplementation of vitamin D in broilers had a negative outcome, whereas lower 25(OH)D associated with higher parathyroid hormone levels appeared to be independently associated with sudden cardiac death in cardiovascular disease-free older adults [35].

In a series of both in vitro and in vivo studies [36–38], vitamin D was also proved to have anticoagulant effects by increasing thrombomodulin expression and reducing tissue factor (TF) expression via VDR on monocytes and endothelial cells [39]. TF is involved in the activation of Factor X and thrombin, both targets of anticoagulant therapeutic agents used to treat AF; therefore, vitamin D may have an influence on LA thrombus formation even under anticoagulant therapy [40].

Despite the availability of an efficient treatment, more than 3.7 million patients die each year worldwide from cardiac arrhythmias [41,42], a number that exceeds the total number of deaths from all cancers in the Western world. Nevertheless, it is still debatable whether the trigger is an asymptomatic clinical profile, a delayed diagnosis, ineffective care, uncertain pathophysiology, or all of the above.

Cardiac arrhythmias present with ambiguous symptoms, from completely asymptomatic to a wide range of symptoms and signs, including palpitations, chest pain, shortness of breath, anxiety, fatigue, lightheadedness or dizziness, blurry vision, and profuse sweating [41,42]. The most frequent symptom of arrhythmia is the sensation of an abnormal heartbeat, called palpitations. Since the risk of myocardial infarction, stroke, sudden cardiac death, syncope, and embolic events is significantly higher in patients with arrhythmias, the clinical profile can be modified by the occurrence of the specific complications, requiring a tailored therapy.

The etiopathogenesis of cardiac arrhythmias is complex, and the most frequent associations include high blood pressure, ischemic heart disease, valvulopathies, congestive heart failure, diabetes, hypoglycemia, hyperthyroidism, electrolyte imbalance, infections, stroke, certain medications, excess of coffee, alcohol abuse, smoking, substance abuse disorder, certain dietary and herbal supplements, psychological stress, and sleep apnea [41,42].

Rare causes of cardiac arrhythmias include fatty acid oxidation disorders, atrial standstill, autoimmune disorders (sarcoidosis, systemic lupus erythematosus, scleroderma, type

1 diabetes, Graves' disease, rheumatoid arthritis, ankylosing spondylitis, psoriasis, and celiac disease), and genetic conditions called familial arrhythmia (Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, long QT syndrome, short QT syndrome, Timothy syndrome, Wolff–Parkinson–White syndrome, arrhythmogenic right ventricular dysplasia, idiopathic ventricular fibrillation, and Mahaim syndrome) [43,44].

The complex interacting patterns of ion current activation and inactivation that underpins the action potential production and propagation across successive areas of the heart is fundamentally disrupted in most arrhythmias [45]. Changes in certain genes' coding-specific ion channels, however, result in well-defined arrhythmic situations, thus providing practical clinical insights into how they could generate arrhythmic tendencies [45].

Changes in the activity of ionic channels or transport systems have a vital role at cellular level. Modifications in electrolyte composition, acidosis or alkalosis, autonomic and hormonal influences, membrane active metabolites, drugs, and poisons are all responsible for these changes. Other factors, such as route shape, anisotropic conduction, and cell coupling resistances, are particularly important in conduction disruptions [46].

3. Atrial Fibrillation

Atrial fibrillation is the most common cardiac arrhythmia, affecting an estimated 46.2 million people worldwide in 2016 [47], representing a leading cause of mortality and morbidity, such as thromboembolic stroke and congestive heart failure, even in patients with no prior cardiovascular disease [48,49].

Several mechanisms have been proposed as the explanation of AF development and perpetuation in patients with vitamin D deficit, involving proliferative and proinflammatory actions of renin–angiotensin system (RAS) and catecholamine excess, interstitial fibrosis in the left atrium, and various electrical anomalies that cause fibrillatory conductions [50–58]. Both in vivo and in vitro studies exist, which are detailed below.

Although catecholamine excess, activation of RAS, and consequent cascade activation of various neurohumoral agents leading to atrial oxidative stress and inflammation are demonstrated promoters of AF [50–52], the precise pathogenesis of AF fibrillation has yet to be determined. AF development relies on an intricate process involving structural and electrical remodeling of the left atrium (LA) [53,54]. Multiple theoretical models involved an increase in atrial interstitial fibrosis as a substrate of AF [55–57], as it increases with age [58] and causes electrical conduction abnormalities through the atria, thus creating favorable terrain upon which certain triggers could initiate AF [55,56]. RAS components (including angiotensin II and aldosterone) and profibrotic cytokines, such as transforming growth factor-b1, appear to contribute to these remodeling processes through their proliferative and proinflammatory actions [59]. Although numerous models, both animal [60–62] and human [50,58,59], have demonstrated this purported link between interstitial fibrosis and increased AF susceptibility, the mechanisms that generate AF are still under debate [63,64]. Multiple wavelet [57,65], mother rotor [66,67], and focal source [68] mechanisms have all been proposed and demonstrated on animal models; however, a general consensus has yet to be reached.

Considering the associations between vitamin D and AF risk factors and key pathogenetic factors, several recent studies investigated a possible link between low vitamin D levels and AF [40,69–75]. The main summarized studies investigating the relationship between AF and vitamin D levels are presented in Table 1.

Table 1. The relationship between vitamin D and AF risk factors.

Author (Year)	Study Design and Participants' Characteristics	Parameters Investigated	Outcome
Çakır et al. [40] (2020)	observational study 201 patients suffering from AF (133 female) following treatment with continuous non-vitamin K antagonist oral anticoagulant	thrombus occurrence	low 25(OH)D levels associated with dense spontaneous echo contrast and LA thrombus occurrence
Chan et al. [69] (2017)	case-control study 156 patients with AF 1019 control all female	SNPs of vitamin D mechanistic pathways and 25(OH)D levels in serum	genetically deprived vitamin D exposure constitutes a predisposition for a higher risk of AF in patients with coronary artery disease
Canpolat et al. [70] (2017)	prospective study 48 patients (41.7% female) suffering from lone paroxysmal AF 48 healthy controls	LA fibrosis	lower 25(OH)D levels are significantly linked to more considerable LA fibrosis and may play a role in relapse after cryoablation
Albert et al. [73] (2021)	randomized clinical trial 25,119 patients without preexisting cardiovascular disease (incl. AF) and cancer; aged 50 and higher	6272 subjects: 460 mg/d eicosapentaenoic acid + 380 mg/d of docosahexaenoic acid + 2000 IU/d vitamin D3 6270 subjects: eicosapentaenoic and docosahexaenoic acid + placebo 6281 subjects: vitamin D3 + placebo 6296 subjects: 2 placebos	over a median follow-up of 5 years, no significant differences in the occurrence of AF
Turin et al. [76] (2018)	retrospective study 47,062 patients with documented 25(OH)D levels	incidence of AF in patients with ACEI −/+ ARB treatment vs. patients not following treatment with ACEI or ARB	use of ACEI/ARB links to less AF events (attenuated in patients taking 25(OH)D) vitamin D deficiency not statistically significant associated with AF incident
Yang et al. [20] (2018)	observational study 20,788 female patients diagnosed with osteoporosis	implication of osteoporosis treatment in the occurrence of AF	different risk for AF associated with diverse osteoporosis treatment; vitamin D could have beneficial effects in patients suffering from osteoporosis
Chen et al. [77] (2014)	observational study 162 patients with nonvalvular persistent AF; without any other cardiovascular disease 160 healthy controls	25(OH)D serum levels	low vitamin D levels associated with AF occurrence in Chinese adults with no other vascular risk factors
Özsin et al. [78] (2018)	prospective randomized clinical trial 50 patients with postoperative atrial fibrillation (66% male) 50 patients without postoperative atrial fibrillation (74% male)	AF occurrence until discharge, immediate measurement of 25(OH)D serum levels after the event	lower levels of 25(OH)D could be one of the reasons for postoperative atrial fibrillation and are an independent predictor for this event
Kara and Yasim [79] (2020)	randomized controlled, blinded, and parallel-arm trial 116 patients with vitamin D deficiency or insufficiency who had coronary artery bypass grafting: 58 patients with oral vitamin D supplementation 48 h before procedure = treatment group and 58 patients without any vitamin D supplementation = control	occurrence of postoperative atrial fibrillation until discharge	significant prevention of postoperative atrial fibrillation with short-term preoperative supplementation of vitamin D
Cerit et al. [80] (2018)	randomized, blinded clinical trial 328 consecutive patients with on-pump coronary artery bypass grafting 80 patients with vitamin D insufficiency and 56 patients with vitamin D deficiency; treatment group: 68 patients with oral vitamin D 48 h before surgery; control group: 68 patients without oral vitamin D	occurrence of postoperative atrial fibrillation until discharge	preoperative vitamin D supplementation strongly associated with the prevention of occurrence of postoperative atrial fibrillation in patients suffering from vitamin D deficiency
Skuladottir et al. [81] (2016)	randomized, double-blind, placebo-controlled clinical trial 118 patients undergoing coronary artery bypass grafting and/or valvular repair surgery with available preoperatively and postoperatively (the third day after) plasma samples of vitamin D2 and vitamin D3	occurrence of postoperative atrial fibrillation	no association for plasma levels of total 25(OH)D and $25(OH)D_3$; higher levels of $25(OH)D_2$ linked with higher occurrence of postoperative atrial fibrillation
Yaman et al. [82] (2020)	retrospective study 52 patients with AF and rhythm control strategy scheduled for medical or electrical cardioversion	recurrence of atrial fibrillation after cardioversion and vitamin D levels	increased risk of AF recurrence associated with lower vitamin D levels
Tamez et al. [83] (2012)	randomized trial 196 patients suffering from chronic kidney disease, left ventricular hypertrophy (mild to moderate) with preserved ejection fraction receiving either 2 μg of oral paricalcitol or placebo for 48 weeks	two-dimensional echocardiography and levels of brain natriuretic peptide	patients receiving an analogue of vitamin D presented reduced left atrial volume index and an attenuated rise in brain natriuretic peptide

A 2017 matched case-control study by Chan et al. investigated the connection between genetic vitamin D deficiency and AF risk [69]. A cohort of 1175 Chinese cardiac patients underwent genotyping and had their serum 25-hydroxivitamin D measured. Out of twelve single nucleotide polymorphisms (SNPs) investigated, four involved in vitamin D pathways such as vitamin D binding protein (VBP) were associated with low serum 25-hydroxivitamin D [25(OH)D] and strongly correlated with the presence of AF [69]. Measuring life-long exposure to vitamin D by calculating a multi-loci genetic risk score (GRS) for the investigated SNPs helped mitigate the variability of 25(OH)D levels caused by any external factors, which strengthens the link between vitamin D and AF. However, the study still faces a few shortcomings that might limit its generalizability, such as limited ethnic variability and the high-risk nature of the patients, all of whom had stable coronary disease. The design of the study also might enable recall and selection bias to influence the results, but by measuring a genetic exposure to low vitamin D levels, instead of only a baseline vitamin D level, the findings may indeed show a significant link between vitamin D and AF [69].

Canpolat et al. conducted a prospective study comparing LA fibrosis between 48 patients with symptomatic lone paroxysmal AF who underwent cryoballoon-based catheter ablation and 48 healthy subjects using DE-MRI scans [70]. Results showed that serum 25(OH)D levels were significantly lower in patients with lone paroxysmal AF compared to the control group and in patients with moderate-severe LA fibrosis compared to those with mild-moderate LA fibrosis. Moreover, the extent of the LA fibrosis was correlated to AF recurrence during the follow-up period, further confirming the importance of interstitial fibrosis as a mechanism of AF [70].

A recent prospective study by Çakir et al. analyzed the implications of vitamin D levels in the development of thromboembolic complications in patients with AF [40]. A group of 201 patients with AF undergoing non-vitamin K antagonist oral anticoagulant therapy (NOAC) were assigned into two groups based on the presence or absence of a LA thrombus. The study found that plasma levels of 25(OH)D were significantly lower in patients with LA thrombus and AF and independently associated with LA thrombus occurrence; therefore, they may predict thrombotic events in patients with AF despite NOAC treatment [40].

Vitamin D levels in the body are, nevertheless, greatly influenced by several environmental and intrinsic factors, such as lifestyle, diet, seasonal variation, and disease activity [74], which makes it difficult to appreciate the involvement of vitamin D in AF pathogenesis and its therapeutic impact in preventing AF in the general population [73,74]. Meta-analyses by Huang et al. and Zhang et al., respectively [71,72], revealed conflicting results concerning the relationship between vitamin D and the onset of AF, especially when excluding case-control studies. Nonetheless, the variability of individual 25(OH)D levels does not fully counteract the supposed beneficial effects of vitamin D demonstrated in experimental studies. A large retrospective study by Turin et al. investigated the link between vitamin D deficiency and AF in relation to RAS modulation using angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blocker (ARB) drugs [76]. The study concluded that, although there was no significant association between levels of 25(OH)D and AF, findings consistent with previous meta-analyses, vitamin D deficiency appears to attenuate the protective effect of RAS inhibition, thus indicating the need for further investigation of the possible benefits to vitamin D supplementation in specific clinical contexts [76].

A randomized controlled study (RCT) conducted in 2021 by Albert et al. investigated the potential use of vitamin D and marine omega-3 fatty acids in the primary prevention of incident AF [73]. Healthy participants were randomly assigned into groups and received either omega-3 and placebo, vitamin D and placebo, or vitamin D, omega-3 or 2 placebos, over a period of roughly three years and were reevaluated yearly using questionnaires [73]. The trial showed no statistical evidence of an effect of either the omega-3 fatty acid or vitamin D supplementation regarding AF. These findings, therefore, do not support the sup-

plementation of vitamin D as a primary prevention of AF. The large number of participants and the use of questionnaires as a means of data collection may cause an underdetection of AF events, limiting the possibility of observing the smaller effects on AF occurrence [73]. Although large-scale use in AF prophylactic use may not be justified, the experimental benefits of vitamin D supplementation and the demonstrated interaction with the other factors involved in AF mechanisms [76] implies a need for further investigation of possible therapeutic involvement of vitamin D in cardiovascular disease.

In a nationwide Taiwanese study of women treated for osteoporosis with either bisphosphonates or vitamin D, Yang et al. identified a significant increase in AF risk in the bisphosphonate group relative to the untreated control group and a protective effect of vitamin D supplementation relative to controls. Both treated groups had a higher prevalence of hypertension, ischemic heart disease, and cerebrovascular disease compared to controls, with the above-mentioned diseases being risk factors for AF. Subsequently, the protective effects of vitamin D are likely of a greater magnitude than estimated. While the paper is strictly an epidemiological study, the authors mention the beneficial effects of vitamin D supplementation on blood pressure and lipid profile as mediators of its protective effect against AF [20].

In this context, the AF-preventing effects of vitamin D seem even more remarkable considering the link between ischemic heart disease and AF, in that ischemia can induce reentry circuits, ectopic foci, and neuronal remodeling [84]. There is evidence that vitamin D is more directly involved in the above-mentioned ischemia-fibrillation vicious circle, as vitamin D deficiency favors left atrial thrombogenesis in patients with non-valvular AF treated with direct anticoagulants. However, in this study, the left atrial thrombus group had a lower left ventricular ejection fraction and an increased left atrial diameter, which could act as confounding factors [40]. Nonetheless, left atrial enlargement is itself influenced by vitamin D, as will be discussed later. Regarding the mechanisms involved, the authors mention antagonism of renin–angiotensin signaling, as well as the direct effects on the coagulation cascade (upregulation of thrombomodulin), as contributors to the inverse relation between serum calcidiol and atrial thrombus formation [40].

Another observation of the above-mentioned study is that the left atrial thrombus group had, in addition to a significantly lower level of serum vitamin D, a higher C-reactive protein concentration [40]. This is consistent with the anti-inflammatory effects of vitamin D, which counteracts NF-κB signaling in epicardial adipocytes, thereby delaying the progression of atherosclerosis, coronary remodeling, and ischemic heart disease [19], as well as potentially acting directly on cardiac myeloid cells, which express the vitamin D receptor at a higher level compared to other heart cells [11].

Comparing Chinese patients with nonvalvular AF and no other associated cardiovascular condition with healthy controls, patients with a serum calcidiol level below 20 ng/L (threshold for vitamin D deficiency) are twice as likely to have AF compared to those with calcidiol levels over 30 ng/L (threshold for hypovitaminosis). Similar to the study of Çakır et al. [40], the AF group had, in addition to a lower calcidiol concentration, a higher left atrial diameter and an increased serum C-reactive protein [77]. The authors identify left atrial diameter, pulmonary arterial systolic pressure, and C-reactive protein as predictors of calcidiol levels in AF patients, but they do not analyze multicollinearity and do not assess the extent to which these predictors influence one another [77,85].

Consistent with its AF-preventing role [18], the risk of AF after coronary artery bypass grafting (CABG) is significantly increased in vitamin D-deficient patients [78]. Unlike in previous studies, the differences between the postoperative AF and no-AF group regarding C-reactive protein, left atrial diameter, and serum lipids were not significant, thereby strengthening the likelihood of a direct association between serum vitamin D and AF risk [78]. Moreover, the risk of post-CABG AF is approximately halved by preoperative vitamin D supplementation in patients with preexisting vitamin D deficiency [79,80].

However, in the milder situation of vitamin D insufficiency, only Kara and Yasim [79] identified a protective role for vitamin D supplementation, which can be related to the

significantly higher doses used by the former (300.000 IU in vitamin D deficiency, 150.000 IU in insufficiency) compared to Cerit et al. (50.000 IU in both groups) [80]. The thresholds for deficiency and insufficiency are consistent between the two papers and the same as those of Chen et al. [77].

Regarding AF risk after any kind of cardiac surgery, a meta-analysis has identified a deleterious effect of vitamin D deficiency [86]. However, the trial of Skuladottir et al. shows contrasting results (i.e., a higher calcitriol level in the AF group), which are, nonetheless, not statistically significant. This outcome can be attributed to confounding factors, in that patients in the AF group are older, less likely to smoke, and have a greater intake of fish [81]. It is noteworthy that the trial differentiates between the two components of calcitriol, namely dihydroxyergocalciferol (of dietary origin) and dihydroxycholecalciferol (of endogenous origin) [81]. The association between postoperative AF and calcitriol is only significant for the former: the fact that it is the minor form of vitamin D in serum, and that it is of dietary origin, further suggests confounding factors as the source of the contradictory results of Skuladottir et al. [81,86].

Additionally, lower serum calcitriol is a risk factor for AF recurrence after cardioversion. However, left atrial diameter is itself positively correlated with AF recurrence risk and negatively with calcitriol levels [82]: while left atrial enlargement is itself a risk factor for AF recurrence after radiofrequency ablation [87], vitamin D supplementation has beneficial effects on left atrial volume, at least in a subset of patients with chronic kidney disease and left ventricular hypertrophy [83]. It is thus unclear whether left atrial enlargement is a confounding or mediating factor.

In the case of catheter ablation, vitamin D deficiency is strongly associated with moderate-severe (Utah III or greater) atrial fibrosis [70], which is itself associated with recurrence [88]. Regarding AF recurrence, serum calcitriol is a highly significant predictor in a univariate Cox regression model but is just above the significance threshold ($p = 0.053$) in a multivariate model. However, these results should be interpreted with care, considering the small sample size ($n = 48$) for a five predictor multivariate regression and the lack of assumption testing [70].

Current evidence exists that correlates low serum vitamin D levels with atrial fibrillation. Nevertheless, further research is needed to better assess the relationship between vitamin D levels and atrial fibrillation given the fact that many of the included studies obtained contradictory results.

4. Ventricular Repolarization and Vitamin D Deficiency

Apart from increasing the risk of developing atrial fibrillation, the impact of low vitamin D levels on the dynamic of ventricular repolarization has also been analyzed. More recent research conducted on pediatric patients has also shown a correlation between low vitamin D levels and changes in QT interval, QT interval correction (QTc), QT dispersion (QTd), JT interval, JT interval correction (JTc), Tpeak-to-Tend interval (Tp-e), and Tp-e/QTc [89]. These studies were, more specifically, aimed at the impact of low vitamin D levels on ventricular repolarization and the occurrence of cardiac arrhythmias [90,91].

Bekdas et al. conducted a study on 67 children and adolescents who had similar characteristics and were not suffering from any diseases, dividing them into "Sufficiency", "Insufficiency," and "Deficiency" groups, based on serum vitamin D levels [89]. The study investigated the variation of several parameters between the three groups, such as QTc, QTd, JT, JTc, Tp-e, Tp-e/QT, Tp-e/QTc, Tp-e/JT, and Tp-e/JTc. Additionally, the team revealed that patients with insufficient vitamin D levels had a higher pulse, compared to children and adolescents belonging to the "Sufficiency" group (101 $\pm$ 18.7 vs. 81.9 $\pm$ 13.4) [89]. Nevertheless, significant differences were noticed when comparing the groups with sufficient and deficient vitamin D levels. Thus, Tp-e notably increased in the "Deficiency" group (86.2 $\pm$ 10.6 vs. 71.6 $\pm$ 6.7), as well as Tp-e/QT, Tp-e/QTc, Tp-e/JT, and Tp-e/JTc [89]. The research also assessed the differences between patients with vitamin D insufficiency and deficiency, concluding that those in the "Deficiency" group had a

prolonged Tp-e (86.2 ± 10.6 vs. 74.4 ± 9.2), a lower QTc (402.6 ± 21.1 vs. 426.3 ± 25.1) and, consequently, a higher Tp-e/QTc ratio (0.21 ± 0.02 vs. 0.17 ± 0.01) [89]. However, the study found no considerable distinctions in the values of certain measured parameters between the "Sufficiency" and "Deficiency" groups, such as QTc and JT (p = 0.25 and p = 0.75, respectively) [89].

Bagrul and Atik conducted a similar study, including 150 adolescents, in which they measured their vitamin D levels and organized them into vitamin D "Sufficient", "Insufficient," and "Deficient" [90]. Moreover, QTc, QT dispersion (QTd), JT dispersion (JTd), Tp-e, Tp-e/JTpeak, and Tp-e/QTc were measured [90]. The study concluded that adolescents with deficient and insufficient vitamin D levels had a prolonged Tp-e, when compared to patients from the "Sufficient" group. Consequently, these patients had an increased Tp-e/JTpeak ratio. Similarly, patients with vitamin D deficiency had a significantly increased JTd when compared to both other groups, as well as a higher QTd [90]. Additionally, the study also recorded an increased Tp-e/QTc ratio in patients with deficient and insufficient vitamin D levels [90].

Another study on patients with type 2 diabetes investigated the relationship between 25-hydroxyvitamin D deficiency and QT interval duration and dispersion [91]. Yetkin et al. included 253 diabetic patients and 170 healthy controls without prior cardiovascular disease or known history of cardiac arrhythmias and assessed the frequency of vitamin D deficiency in the two groups, as well as prolonged QT intervals. The study concluded that diabetic patients with prolonged QTc and a higher QTc dispersion were more frequently vitamin D deficient [91]. Thus, 65.4% of the patients with type 2 diabetes were suffering from a vitamin D deficiency, as well as 64.7% of the total volunteers. However, a prolonged QTc interval was observed in 26.8% of the diabetic patients, in comparison with only 4.1% in the healthy group. Nevertheless, advanced age, higher HbA1c levels, and a longer disease duration were also less strongly associated with increased QTc intervals [91]. The main studies assessing the relationship between vitamin D levels and ventricular repolarization are summarized in Table 2.

Table 2. The relationship between vitamin D levels and ventricular repolarization.

Author (Year)	Study Design and Participants' Characteristics	Parameters Investigated	Outcome
Bekdas et al. [89] (2021)	Observational study 67 children and adolescents with the following vitamin D levels: - sufficient: 44 - insufficient: 13 - deficient: 10	QRS QTmin,max,av Pulse QTc,d,dc JT, JTc Tp-e Tp-e/QT Tp-e/QTc Tp-e/JT Tp-e/JTc	Vitamin D deficient: prolonged Tp-e, Tp-e/QT, Tp-e/QTc, Tp-e/JT, and Tp-e/JTc. Vitamin D insufficient: lower QTmax and QTmin, increased pulse, and JTc.
Bagrul and Atik [90] (2019)	Observational study 150 adolescents with the following vitamin D levels: - sufficient: 50 - insufficient: 50 - deficient: 50	QT QTc,d Tp-e Tp-e/QTc JT JTd Tp-e/JTpreak	Vitamin D deficient: prolonged Tp-e, increased Tp-e/QTc ratio, increased Tp-e/JTpeak ratio, higher QTd, and JTd Vitamin D insufficient: prolonged Tp-e, increased Tp-e/QTc ratio, increased Tp-e/JTpeak ratio, lower QTd, and JTd when compared to the "Deficiency group".
Yetkin et al. [91] (2015)	Observational study 423 patients among which: - 253 patients with type 2 diabetes - 170 healthy volunteers	QTc QTd HbA1c	QTc and QTd prolongation were associated with type 2 diabetes mellitus, advanced age, a longer duration of the disease, and higher HbA1c levels.

There is a significant correlation between vitamin D deficiency and disturbances in ventricular repolarization; numerous studies show notable differences between QT interval durations in patients with normal or low vitamin D levels [89–91]. Although the relationship between vitamin D and heart arrhythmias is established, further research is

needed in order to better understand the impact that vitamin D has on the pathogenesis of cardiac arrhythmias.

5. Conclusions

The occurrence of atrial arrhythmias, mainly atrial fibrillation, together with significant changes in ventricular repolarization, represented by a prolonged QTc interval, are positively associated with vitamin D deficiency. Due to the heterogeneity among existing studies, further research is necessary to confirm the existing data and to analyze other types of arrhythmias.

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