CLASSIC AND PLEIOTROPIC ACTIONS OF VITAMIN D

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CLASSIC AND PLEIOTROPIC ACTIONS OF VITAMIN D

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The vitamin D is widely advertised as a solution for a large spectrum of diseases and health issues. Growing number of pharmaceuticals and supplements containing vitamin D, increasing availability of them in pharmacies, stores, online distribution and, sometimes, an intrusive commercial publicity campaigns have raised great interest, and have triggered reasonable controversies and fears. The self-administration of high doses of vitamin D has also appeared major concern in society. There is an increasing number of dilemmas regarding side effects including nephrocalcinosis, urinary stone disease, drug interactions and other adversity. On the other hand, it is recognized that vitamin D deficiency is a global health problem with potential negative consequences on health, welfare and morbidity during growth and adulthood, and therefore influencing health care services worldwide. According to current published reports, the vitamin D deficiency is regarded a significant risk factor for several civilization diseases including cancer, cardiovascular diseases, hypertension, autoimmune and metabolic disorders, infectious diseases and many other chronic conditions. Thus, it is essential to discuss vividly, and share scientific reports and evidence demonstrating both the safety issues and the significance of vitamin D for health of children, adolescents, middle-aged men and women, professionally active individuals, and seniors.

This eBook is a collection of articles presented at the 3rd International Conference "Vitamin D - Minimum, Maximum, Optimum" (EVIDAS 2017) held in Warsaw (Poland) on September 22–23, 2017. EVIDAS (European Vitamin D Association) is a scientific society focused on vitamin D and its meaning for human health.

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EDITORIAL
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Editorial: Classic and Pleiotropic Actions of Vitamin D

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Editorial on the Research Topic

Classic and Pleiotropic Actions of Vitamin D

Vitamin D deficiency is the world-wide health problem. Vitamin D deficiency has been associated with a wide variety of acute and chronic diseases including infectious diseases, autoimmune diseases including multiple sclerosis, type 1 diabetes and rheumatoid arthritis, cardiovascular heart disease and stroke, type 2 diabetes, depression and neurocognitive dysfunction, and several fatal malignancies and cancer (1, 2). It is also recognized that vitamin D deficiency is associated with increased mortality and negative birth outcomes (3–7). This recognition has not only led to a marked increase in the sales figures of supplements and prescriptions containing vitamin D, but has also resulted in innovative vitamin D food fortification programs, including those implemented most recently in India (8, 9). These continue to cause concerns about children and adults ingesting too much vitamin D that could potentially lead to toxicity including hypercalcemia, nephrocalcinosis, nephrolithiasis, or cardiovascular calcification.

The International "Vitamin D- minimum, maximum, optimum" (co-organized by the European Vitamin D Association, EVIDAS) has over the past several years served as a forum for scientists, clinicians and health care professionals to discuss various aspects of vitamin D related to health and disease. The 3rd International Conference "Vitamin D- minimum, maximum, optimum" (EVIDAS 2017) held in Warsaw (Poland) in September 22–23, 2017 (www.witaminad.waw.pl) was attended by scientists and health care professionals who discussed a broad spectrum of topics and controversies on vitamin D which were, at least partly, published in the journal Frontiers in Endocrinology. The "Classic and Pleiotropic Actions of Vitamin D" research topic, edited in Frontiers in Endocrinology, also welcomed "insights from outside," i.e., 4 topical papers submitted by authors who did not present their work at the EVIDAS 2017 conference. These articles have been labeled below as "invited papers."

The article by Carol L. Wagner and Bruce W. Hollis (invited paper) reviewed on what is known about the roles of vitamin D status during pregnancy for both mother and the developing fetus (Wagner and Hollis). The recognized leaders in research on vitamin D supplementation during pregnancy reported that pregnant women receiving 4,000 IU/d of vitamin D3 throughout their pregnancy achieved 25-hydroxyvitamin D [25(OH)D] concentrations above 40 ng/ml (100 nmol/l) without any evidence of toxicity, as demonstrated by the maintenance of a normal calcaemia and no significant changes in 24-h urinary calcium excretion. The authors emphasized that benefits of the vitamin D supplementation program was associated with a reduced risk of preeclampsia and prematurity as well as other essential benefits, including better fetal neurodevelopmental parameters, improved lung maturation and respiratory function. This paper reviewed the results

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from several relevant clinical trials, and thereby highlighted that trial results should rather be analyzed with respect to achievable serum 25(OH)D concentrations, not just the vitamin D dose. Properly designed clinical trials have demonstrated—according to the published review—that higher maternal 25(OH)D concentrations reduced the risk of postnatal asthma, preeclampsia, preterm birth, and a variety of other gestational complications (Wagner and Hollis).

The trends toward changes in 25(OH)D levels and alterations of markers of calcium-phosphate metabolism were investigated over the 30 years (1981–2011) in a population of newborns and infants who were suspected of having a disorder in calcium-phosphate metabolism ($n = 3163$; mean age 9.0 ± 3.0 months) (Wójcik et al.). In neonates and infants, the 25(OH)D as low as <10 ng/ml (<25 nmol/l) was found in 4.5% of patients ($n = 163$), 10-20 ng/ml (25–50 nmol/l) was noted in 14.7% ($n = 465$), 20–30 ng/ml (50–75 nmol/l) in 23.9% ($n = 756$) and 30–50 ng/ml (75–125 nmol/l) in 35.9% ($n = 1136$). The mean 25(OH)D concentration was 37.5 ± 24.5 ng/ml (93.8 ± 61.2 nmol/l). In subjects with 25(OH)D concentration <10 ng/ml (<25 nmol/l), normal calcaemia (2.25–2.65 mmol/l) was found in 83.4% of neonates and infants ($n = 136$). Eighty-one subjects had 25(OH)D concentrations above 100 ng/ml (250 nmol/l), with co-existing serum calcium ranging from 2.6 mmol/l up to 4.38 mmol/l (mean Ca = 2.69 mmol/l). Hypocalcaemia (Ca < 2.25 mmol/l) was observed in 0.54% only. Of the total studied population, 13.8% of infants had calcium levels > 2.65 mmol/l ($n = 435$). In general, the average values of calcium-phosphate markers were within the age-adjusted reference range. The highest mean 25(OH)D concentration of 51.8 ng/ml ± 38.8 (129.5 ± 97.0 nmol/l) was noted in years 1981–1999 ($n = 305$). The lowest 25(OH)D value was observed in years 2010–2011 (29.0 ng/ml ± 13.6; 72.5 ± 34.0 nmol/l; $n = 412$). The trend to a decline in 25(OH)D concentration throughout the studied period appeared significant (r=-0.29, $p < 0.0001$) (Wójcik et al.).

Associations between vitamin D status and obesity have been extensively studied during the last decade, however, conflicting results have been reported. The carbohydrate and lipid metabolism parameters in overweight children and adolescents in relation to vitamin D insufficiency were analyzed by a research group from Russian Federation (Zakharova et al.). They confirmed a high proportion of vitamin D insufficiency in overweight and obese Russian children and adolescents (escalating along with the severity of obesity). In this comprehensive study, the authors reported on the relationships and interplay between the principal adipokines responsible for fat metabolism (leptin, adiponectin, resistin) and vitamin D metabolism (Zakharova et al.).

A complex endocrine pathology was reported in an invited paper from Ukraine, while the study pointed out essential vitamin D issues as a serious healthcare problem in Ukraine following the historical nuclear disaster in Chernobyl in 1980s (Komisarenko et al.). The 25(OH)D and markers of immune function in response to vitamin D intervention were investigated in adult patients with type 1 and type 2 diabetes mellitus (T1DM and T2DM, respectively) and coincident autoimmune thyroiditis (AIT). It was reported that patients with combined endocrine disorders (DM and AIT) with vitamin D deficiency/insufficiency had significantly increased concentrations of Th1-type cytokines and reduced concentrations of Th2-type cytokines (IL-4 and IL-5), IL-10, and IL-17. The results of the Ukrainian study showed that vitamin D3 supplementation in patients with T1DM and T2DM may reduce the activity of the inflammatory Th1-type cytokines, and increase the levels of Th2-type cytokines (Komisarenko et al.).

In a paper, authored by 35 contributors from Europe and North America, the rationale and plans for vitamin D food fortification were reviewed with a suggestions for action (Pilz et al.). The rationale for vitamin D food fortification includes a large proportion of worldwide populations with low 25(OH)D concentrations and the mounting evidence from observational and clinical trials, that higher 25(OH)D concentrations and vitamin D intake are associated with several non-skeletal health benefits such as reduced mortality rates, respiratory tract infections, asthma exacerbations and pregnancy complications. The results of a systematic voluntary food fortification program in Finland in 2003 were reviewed. Fat spreads and fluid milk products could be fortified. The mean overall increase in serum 25(OH)D concentration from 2000 to 2011 attributed to food fortification was 2.4 ng/ml (6 nmol/l). The suggested goal in this review was to bring everyone up to >20 ng/ml (>50 nmol/l). The mean dietary vitamin D intake in Finland in 2011 was 14 micrograms daily for men and 12 micrograms daily for women (10). As of April 30, 2019, this paper was viewed more than 11,000 times.

A study in Poland found that long-term acenocoumarol treatment due to recurrent venous thromboembolism, atrial fibrillation, or mechanical heart valve prostheses was significantly inversely correlated with serum 25(OH)D concentration (Sawicka-Powierza et al.). The authors of this interesting research concluded that acenocoumarol treatment, being today a largely used anticoagulation therapy, might be responsible for a decrease of 25(OH)D concentration. They acknowledged, however, that other reasons for the inverse correlation could not be ruled out (Sawicka-Powierza et al.).

Vitamin D supplementation guidelines were prepared for the general population and groups at risk of vitamin D deficiency in Poland by an expert panel of 28 vitamin D researchers, national specialist consultants and representatives of scientific societies (Rusinska et al.). In general, the guidelines recommended 800 to 2,000 IU/d vitamin D for those aged 11 to 75 years of age, lower doses for the younger, and higher ones for older or those at risk of vitamin D deficiency. However, about 15–30 min long sun exposure daily between 10 a.m. and 3 p.m. from May to September (depending on skin type, time of day and season) could provide some of the vitamin D, at least for the latitudes similar to Poland. Based on the available body of evidence cited in this review, the 25(OH)D of 30–50 ng/ml (75–125 nmol/l) was identified as the optimal concentration, with the potentially toxic concentrations >100 ng/ml (>250 nmol/l). The recommendations were based on beneficial skeletal and non-skeletal effects, derived largely from observational studies. The lack of a strong support from RCTs including vitamin D was attributed to trial design problems, in a way, and was discussed as

a limitation in this guidance paper (Rusinska et al.). As of April 30, 2019, this paper had 33,261 total views.

The topic of vitamin D toxicity (VDT) was discussed from a clinical perspective by Marcinowska-Suchowierska et al.. The main hallmarks of VDT are hypercalciuria, hypercalcemia, suppressed parathyroid hormone, and 25(OH)D concentrations above 150 ng/ml (375 nmol/l). The most likely hypothesis to explain VDT is that high 25(OH)D concentrations saturate the vitamin D binding protein, thus increasing the bioavailability of 1,25(OH)2D for the target cell nucleus. In addition high concentrations of 25(OH)D can stimulate transcription through the VDR. The symptoms of VDT may include neuropsychiatric manifestations such as difficulty in concentration, confusion, depression, gastrointestinal symptoms such as vomiting, abdominal pain, constipation, cardiovascular manifestations such as hypertension, renal symptoms such as dehydration. Various aspects of VDT treatment were given in this article. The most common cause of VDT appears to be accidental overdosing of vitamin D due to a neglect, unawareness and/or manufacturing error (Marcinowska-Suchowierska).

A 12-week study in Gdansk, Poland, dealt with the role of vitamin D supplementation and exercise on blood cholesterol in elderly women (Prusik et al.). The study was conducted from the mid-October till mid-January. For those who were supplemented with vitamin D3 (4000 IU/d), no changes in lipid profile were observed. However, in those who were supplemented (mean serum 25(OH)D increased from 21 ng/ml to 38 ng/ml; from 52.5 nmol/ to 95 nmol/l) and did Nordic walking for about an hour three times per week, a decrease in total cholesterol, low-density lipoprotein cholesterol and triglycerides of about 10% was observed. Meanwhile in the control group, the high-density lipoprotein cholesterol increased (Prusik et al.).

An observational study (invited paper) found no relationship between maternal and cord blood vitamin D status and anthropometric measurements in term-born neonates at birth (Wierzejska et al.). A total of 94 mother-infant pairs were studied. On the other hand, a study in India of women with a mean serum 25(OH)D concentration of 18 ng/ml (45 nmol/l) at time of birth found that "fetal femur length and birth length were significantly shorter in mothers with low 25(OH)D ($P < 0.01$)" (11). A study in Finland involving 723 mother-child pairs concluded: "A sufficient maternal vitamin D status, specified as 25(OH)D above 50 nmol/L (20 ng/ml), may be a threshold above which the physiological requirements of pregnancy are achieved" (12).

A cross-sectional study of serum 25(OH)D concentrations in relation to activities of daily living (ADL) was conducted among octogenarians—residents of Vilnius city (Lithuania) from January 2017 to February 2018 (Alekna et al.). No association was found between seasonal variations of blood sampling and serum 25(OH)D concentration. Functional status of the oldest old subjects was evaluated based on bathing, dressing, toileting, transferring, continence, and feeding. Those in the lowest ADL category had 25(OH)D concentration near 7 ng/ml (17.5 nmol/l), those in the category 5: approx. 15 ng/ml (37.5 nmol/l), and those in the category 6: 20 ng/ml (50 nmol/l). The regression coefficient for 25(OH)D concentration vs. ADL category was 0.2 ($p = 0.01$). As highlighted by the authors, it was not possible in this study to determine whether ADL status was a cause or an effect of serum 25(OH)D concentration (Alekna et al.).

An interesting issue addressing the personalized individual response of the transcriptome on vitamin D supplementation was discussed by Carlberg. Before and 24 h after a vitamin D3 bolus, the chromatin and RNA were prepared from peripheral blood mononuclear cells for epigenome- and transcriptome-wide analysis. The study subjects showed a specific personalized response to vitamin D and could be stratified into high, mid and low responders. Comparable principles of vitamin D signaling were identified *in vivo* and *in vitro* concerning target gene responses as well as changes in chromatin accessibility (Carlberg).

A study conducted in Brazil (invited paper) found that the frequency of the TaqI (C allele) vitamin D receptor was significantly lower in the controlled ovarian stimulation groups than in the control groups, and that follicle number, but not oocyte number, was lower in patients with TaqI polymorphic (TC/CC) genotypes (Reginatto et al.). The same group also reported that the frequency of the TaqI CC genotype was higher in polycystic ovary syndrome (PCOS) group, while the CT genotype was the most frequent in controls (13). These studies seem to underscore the importance of serum 25(OH)D concentration for reproductive health in humans, particularly among women intending to become pregnant.

Vitamin D receptor (VDR) gene polymorphic variants (ApaI rs7975232, BsmI rs1544410, TaqI rs731236, and Cdx2 rs11568820) were investigated by Belorussian and Lithuanian research group in the context of the risk of postmenopausal osteoporosis (PMO) (Marozik et al.). Patients with osteoporosis were three times more likely to carry the rs1544410 G/G genotype, when compared to controls. The rs7975232, rs1544410 and rs731236 variants were in a strong direct linkage disequilibrium ($P < 0.0001$), suggesting that risk alleles of these markers are preferably inherited jointly. For the bearers of C-G-C haplotype (consisting of rs7975232, rs1544410 and rs731236 unfavorable alleles), the risk of PMO was significantly higher (OR = 4.7, 95% CI 2.8 to 8.1, $p < 0.0001$) compared to controls. This haplotype appeared to be significantly over-represented in PMO group compared to all other haplotypes (Marozik et al.).

Further, the associations between vitamin D status and VDR gene polymorphisms were also investigated in the context of metabolic syndrome (MS) and its markers in middle-aged Russian women, and this was reported by Karonova et al.. The 25(OH)D) concentrations and four VDR gene polymorphisms rs1544410 (BsmI), rs7975232 (ApaI), rs731236 (TaqI) and rs2228570 (FokI) as well as metabolic syndrome (MS) parameters were investigated in 697 women aged between 30 to 55 years. Cases with vitamin D deficiency showed an increased risk of abdominal obesity (AO) [CI 95% 2.23; 1.15–4.30] and low high-density lipoprotein cholesterol (HDL-C) [CI95% 2.60; 1.04–6.49], compared to subjects with normal 25(OH)D concentration. An impaired glucose tolerance (IGT) and T2DM risk were present only when 25(OH)D concentration was < 39.0 nmol/l (15.6 ng/ml), however, the risk of MS did not differ between adequate vitamin D status subjects and the insufficient/deficient ones ($p>0.05$). T allele

carriers (A) of rs7975232 had higher total cholesterol and low-density lipoprotein cholesterol levels compared with the GG (aa) genotypes. Similarly, GG (BB) genotype carriers of rs1544410 had higher triglyceride levels than subjects with A (b) allele carriers. However, VDR gene polymorphisms did not seem to be associated with an increased risk of MS. Karonova and colleagues concluded that vitamin D deficiency, rs7975232 and rs1544410 VDR gene variants were associated with MS parameters in Russian middle-aged women (Karonova et al.).

Shymanskyi et al. conducted studies in female Wistar rats evaluating the effect of vitamin D in improving glucocorticoid-induced changes in bone marrow cells related to bone remodeling (Shymanskyi et al.). They observed that prednisolone-induced abnormalities in glucocorticoid and RANKL/RANK/OPG signaling pathways were associated with the impairments of vitamin D auto/paracrine system in bone marrow cells, and could be ameliorated by vitamin D supplementation (Shymanskyi et al.).

It is well-documented that secondary hyperparathyroidism is a major complication affecting bone metabolism in patients with chronic kidney disease. Various strategies have been developed to prevent and treat secondary hyperparathyroidism. One of the management strategies is to provide patients with 1,25-dihydroxyvitamin D3 or one of its active analogs with, in order to enhance intestinal calcium absorption and to decrease the expression and production of parathyroid hormone (PTH). The other strategy is to use a calcimimetic that is recognized by the calcium sensor resulting in a decrease in the signal that stimulates PTH expression and production. Zawierucha et al. reported a study whereby they treated 131 patients with hemodialysis and uncontrolled PTH secretion for 12 months, using intravenous paricalcitol, oral cinacalcet or a combination of both. Interestingly, they demonstrated that intravenous paricalcitol had a significant effect on the reduction of PTH activity whereas the combination of paricalcitol and cinacalcet had no additional benefit.

SUMMARY

A large-scale debate about non-calcemic action and the extra-skeletal health benefits of vitamin D continues, although limitations in vitamin D research and study design, vitamin D controversies and negative results are also extensively discussed in the current literature. The current research topic in Frontiers in Endocrinology may actually cover the needs, and may provide important input into the content of a well-managed scientific exchange of views. The series of the international conference "Vitamin D—minimum, maximum, optimum" (EVIDAS) provide a unique forum targeting basic scientists, clinical researchers, physicians and other health care professionals to discuss these dilemmas, controversies, pros and cons, as well as to evaluate most recent advances and updated information regarding vitamin D and health. The 4th International "Vitamin D- minimum, maximum, optimum" (EVIDAS 2019) conference which is scheduled in Warsaw, Poland, for October 11-12, 2019 will follow its basic concept to offer an opportunity for scientists and clinicians to present new developments, updated and critical information on the physiology, pathophysiology and health-related issues of vitamin D.

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REVIEW
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The Implications of Vitamin D Status During Pregnancy on Mother and her Developing Child

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Pregnancy is a time of tremendous growth and physiological changes for mother and her developing fetus with lifelong implications for the child. The concert of actions that must occur so mother does not reject the foreign tissue of the fetus is substantial. There must be exquisite balance between maternal tolerance to these foreign proteins of paternal origin but also immune surveillance and function such that the mother is not immunocompromised. When this process goes awry, the mother may experience such pregnancy complications as preeclampsia and infections. Vitamin D deficiency affects these processes. Controversy continues with regard to the optimal daily intake of vitamin D, when sunlight exposure should be taken into account, and how to define sufficiency during such vulnerable and critical periods of development. The importance of vitamin D supplementation during pregnancy in preventing some of the health risks to the mother and fetus appears linked to achieving 25(OH)D concentrations >40 ng/mL, the beginning point of the plateau where conversion of the vitamin D metabolite 25(OH)D, the pre-hormone, to $1,25(OH)_2D$, the active hormone, is optimized. Throughout pregnancy, the delivery of adequate vitamin D substrate—through sunlight or supplement—is required to protect both mother and fetus, and when in sufficient supply, favorably impacts the epigenome of the fetus, and in turn, long term health. There is a growing need for future research endeavors to focus not only on critical period(s) from pre-conception through pregnancy, but throughout life to prevent certain epigenetic changes that adversely affect health. There is urgency based on emerging research to correct deficiency and maintain optimal vitamin D status. The impact of vitamin D and its metabolites on genetic signaling during pregnancy in both mother and fetus is an area of great activity and still in its early stages. While vitamin D repletion during pregnancy minimizes the risk of certain adverse outcomes (e.g., preterm birth, asthma, preeclampsia, and gestational diabetes), the mechanisms of how these processes occur are not fully understood. As we intensify our research efforts in these areas. it is only a matter of time that such mechanisms will be defined.

Keywords: vitamin D, cholecalciferol, pregnancy, fetal development, health effects, immune mediator, genetic effects, developmental origins of later disease

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INTRODUCTION

Now three decades later, the established doctrine of the "Barker Hypothesis," first described by David Barker in 1986, noting a connection between neonatal growth restriction and small for gestational age status with the risk of heart disease later in adult life (1) has become mainstream thinking, but when first proposed was a startling revelation. The theory that diseases that manifest in adulthood actually began during the perinatal period and that the very nutrients and environmental milieu—good and bad—affects our ability to prevent or even fight disease is a daunting idea. It is essential that we understand how early-life exposures can affect later health. One example often given to highlight the effect of perinatal factors is perinatal thyroid function, which is absolutely essential for early-life brain development and maturation. In this example, iodine deficiency and its consequences certainly qualify as an instance of the Barker hypothesis applied. A critical feature of diseases that support the Barker hypothesis is nutritional irreversibility; beyond specific critical points in the developmental cascade, despite full nutrient availability and repletion, the earlier nutritional alteration or deficiency's effect cannot be mitigated.

Perhaps a more publicized nutrient deficiency during the perinatal period is folate deficiency resulting in spina bifida, a neural tube defect that can have catastrophic and certainly life-long sequelae (2). In the 1960's, Hibbard and Smithells established a registry of infants with malformations born in Liverpool, England (3), which included infants with the spina bifida. Through careful study of the infant families, these investigators were able to establish a link between spina bifida and a nutrient-poor diet. Smithells et al went on to define the lack of folic acid, specifically, in the early perinatal period as a contributing factor in the development of spinal bifida (4), a truly remarkable association. The provision of folate beyond the neural tube formation period during gestation does not correct the defect; for those who are folate deficient and with certain genetic risk factors, the provision of adequate folate must occur during the critical period of neural tube formation. As will be discussed later, it appears that with vitamin D deficiency is similar to folate deficiency in that there is a critical period when vitamin D deficiency is more deleterious—for example, placentation can be altered, leading to adverse pregnancy outcomes.

In the past, only the phenotypic result could be observed as a consequence of any given nutritional deficiency, including thyroxine and folate deficiencies. Other nutrient deficiencies, which may impact the long-term health status of the fetus and child, are not so easily discernible by phenotypic changes along. Vitamin D deficiency, long known to be linked to bone mineralization and the development of rickets in its extreme form, was not known until recently to affect not only bone metabolism, but also immune function. Our understanding of nutrient interactions with genes is just beginning to be deciphered. Presently, we have the capabilities of not only observing the phenotype but also the genetic changes contributing to any given phenotype. The focus of this review is to shed light on the potential impact of dietary vitamin D during the prenatal and perinatal periods as a contributor to maternal/infant afflictions, and thus its role in instituting examples of the Barker hypothesis at both the genetic and phenotypic levels. These afflictions include but are not limited to autoimmune disorders, complications of pregnancy, immune function and respiratory disease. We will not be discussing vitamin D metabolism in this text as that information is readily available elsewhere (5, 6).

WHAT IS A "NORMAL" CIRCULATING 25(OH)D CONCENTRATION IN HUMANS?

Because the parent compound vitamin D or cholecalciferol has a brief half-life of 12–24 h, it is its metabolite 25-hydroxy-vitamin D or 25(OH)D with its 2–3 weeks-half-life that is used as the indicator of vitamin D status, defining a "normal" circulating concentration of 25(OH)D in humans remains controversial. For instance, while the most recent Institute of Medicine (IOM) concludes that a circulating concentration of 20 ng/mL is adequate to meet human physiological requirements (7), the Endocrine Society suggests that a 25(OH)D concentration of at least 30 ng/mL is linked with better health outcomes (8). The difference is that the IOM recommendation was based exclusively on skeletal integrity data and did not include extraskeletal and immune function vitamin D data (7), whereas the Endocrine Society used both skeletal studies and other studies surrounding vitamin D's immune effects, and included studies that included observational and clinical trials of subjects with various cancers, immune dysfunction, and pregnancy complications, and their associations with vitamin D deficiency (8).

Given the fact that vitamin D is the only preprohormone in the body that is made following sunlight (UV-B) exposure to the skin/epidermis with little contribution from Western and vegetarian diets, defining what is "normal" should include those who regularly have exposure to sunlight. Thus, to define "normal" circulating 25(OH)D status in humans in a meaningful manner, vitamin D sufficiency should be based on 25(OH)D concentrations in "healthy subjects" who are, in fact, exposed to the sun: sunbathers, fieldworkers, and indigenous people living and functioning in environments to which they are native. To reach a circulating concentration of 150 nmol/L (60 ng/mL), which is the concentration of individuals with full access to sunlight living in a sunrich environment achieve, and is the concentration achieved by the sun-exposed lifeguards (5), a dietary intake of 4,000–6,000 IU/d in adults, including the pregnant woman is required (6). Just to be clear, a daily intake of 10,000 vitamin D IU/day in an adult is deemed to be safe (8).

The current RDA of 400–600 IU vitamin D/day unless augmented with sunlight exposure does not achieve this in most adults (9). In fact, the 400 IU/d dose that is recommended by the IOM for older children and adults also is recommended for breastfeeding neonates a few days after birth (7, 10). When taking a dietary supplement of vitamin D containing 400 IU, on a per kilogram basis, the reference newborn infant weighing 3 kg receives ∼133 IU/kg and the reference 60 kg pregnant woman receives 6.7 IU/kg. Based on pharmacokinetics, unless the pregnant woman has access to sunlight exposure,

her circulating 25(OH)D concentration, which is the gage of her vitamin D status, will be around 37.5–62.5 nmol/L (15–25 ng/mL) whereas the newborn infant receiving 400 IU/day will have achieved circulating 25(OH)D concentrations around 100 nmol/L (40 ng/mL) (11, 12). Because of differences in various factors such as vitamin D binding protein genotype, body mass index, latitude, sunlight exposure, and season, the amount of vitamin D taken orally will result in a wide variety of circulating 25(OH) concentrations among individuals (6). Thus, at this juncture, the only way to know for certain what an individual's vitamin D status is would be to measure that individual's total circulating 25(OH)D concentration.

RELEVANT DATA SURROUNDING VITAMIN D DEFICIENCY DURING PREGNANCY: ANIMAL MODELS AND HUMAN STUDIES

Placental Function

Observational studies have linked vitamin D deficiency with preeclampsia in humans (13). Preeclampsia is a condition that complicates up to 10% of pregnancies, 3% with severe, early-onset with potential life-threatening consequences; and is characterized by abnormal placentation and vasculitis in the mother that leads to hypertension, proteinuria and often abnormal liver function affecting growth and development often necessitating early delivery of the fetus, and remains the leading cause of premature delivery (14). The condition can progress to eclampsia or maternal seizures, associated with significant morbidity and mortality. To date, there is no preventive or treatment measure for this condition other than delivery of the fetus. While the etiology of this serious pregnancy affliction remains unknown and without definitive treatment other than delivery of the fetus, there are studies that suggest there is a derangement in placentation and placental function early-on in pregnancy that manifests weeks later. To support this premise are animal studies and observational studies in humans.

Liu et al. (15) using a pregnant mouse preeclampsia model, studied the effect of low vitamin D status on the risk of preeclampsia. Female BL6 mice raised on vitamin D-sufficient or deficient diets were mated with vitamin D-sufficient BL6 males. The resulting pregnant mice were either allowed to deliver and monitored for blood pressure (BP) or euthanized prior to delivery for analysis of serum, placental/kidney tissues, and fetuses. Vitamin D-deficient pregnant mice exhibited both elevated systolic and arterial pressure that continued through pregnancy until 7 days postpartum, returning to baseline at 14 days postpartum. Analysis of maternal kidney samples showed an association between increased renin and the angiotensin II receptor mRNA expression and vitamin D deficiency. Histological analysis of deficient placentas showed decreased vascular diameter within the labyrinth region. Re-supplementation of vitamin D post-conception partially reversed the effects of vitamin D deficiency. Overall, these data provide evidence that low vitamin D status may predispose pregnant women to dysregulated placental development and elevated blood pressure.

The findings of Liu et al. (15) were supported by a recent study by our group involving healthy pregnant women enrolled in a vitamin D supplementation trial. We analyzed placental tissue from 43 women who had participated in a vitamin D supplementation trial. Women had been randomized to 400 IU or 4,400 IU of vitamin D_3 (cholecalciferol, the parent compound) per day during pregnancy (16). Within 1 h of delivery, placental mRNA was isolated, and analyzed by quantitative PCR for mRNA expression associated with both angiogenesis and vitamin D metabolism. Based on our earlier work where the conversion of 25(OH)D to 1,25(OH)2D was optimized at 25(OH)D concentrations of at least 100 nmol/L (40 ng/mL), the mRNA expression was analyzed on the basis of those women who had achieved this threshold within 1 month of delivery vs. those who had not (11). Soluble FMS-like tyrosine kinase 1 (sFlt-1) and vascular endothelial growth factor (VEGF) gene expression were significantly downregulated in women with circulating 25(OH)D $\geq$100 nmol/L compared to those with a concentration $<$100 nmol/L (11). This novel finding suggests that early vitamin D status plays a role in placentation and that this is a critical nutrient threshold concentration. It appears that the impact of maternal vitamin D_3 supplementation on gene transcription in the placenta, and placentation itself, may be through the balance of such antiangiogenic factors (17, 18).

Consistent with the premise that derangement early-on in placentation and placental growth and function is the finding of incomplete human extra villous trophoblast invasion of the decidua and maternal spiral arteries in pre-eclamptic placentas (19). It is hypothesized that suboptimal vitamin D action on placental tissues leads to extra-villous invasion and altered placentation (20). Chan et al. (20) demonstrated that vitamin D significantly increased extra-villous trophoblast invasion. This was accomplished by increased promatrix metalloproteinases (MMP's), supporting the role of vitamin D in extra-villous trophoblast invasion and preeclampsia. Further studies by this research group have shown that vitamin D is an important component during pregnancy of anti-inflammatory immune responses in the placenta (21). Finally, consistent with the work of Schulz et al. (16), Ma et al. showed that vitamin D supplementation prevents placental ischemia-induced endothelial dysfunction through downregulation of placental soluble FMS-like tyrosine kinase-1, which has been implicated in the pre-eclamptic state (22).

Neurodevelopment

Vitamin D is a potent neurosteroid which mediates numerous actions in the brain. Localization studies have shown the vitamin D activating enzyme CYP27B1 and catabolic enzyme CYP24A1 in neural cells of the cerebral cortex and cerebellar Purkinje cells, suggesting that the brain is capable of vitamin D metabolism at the local level (23). Eyles et al. (24), in their landmark studies, demonstrated that rats born to vitamin D-deficient mothers had profound alterations in the brain at birth. The cortex was longer but not wider, the lateral ventricles were enlarged, the cortex was proportionally thinner and there was more cell proliferation throughout the brain. There also were reductions in brain content of nerve growth factor and glial cell line-derived

neurotrophic factor and reduced expression of $p75^{NTR}$, the low-affinity neurotrophic receptor (24).

Further studies by the Eyles group (25) demonstrated vitamin D-deficiency *in utero* resulted in embryos and pups having significantly less apoptotic cells and more mitotic cells. Hawes et al went on to show more recently that targeted gene arrays specific for apoptosis and cell cycle genes were associated with specific transcriptomic deregulation in the vitamin D-deficient group (26). In this animal model, the investigators also showed an association between vitamin D deficiency during pregnancy and a reduction in fetal crown-rump length, head size, and lateral ventricle volume (26). Brain-derived neurotrophic factor, transforming growth factor-β_1, and forkhead box protein gene expression also were altered. Further, there was a reduction in Foxp2 immunoreactive cells in the developing cortex associated with vitamin D deficiency. In the substantia nigra, both brain-specific tyrosine hydroxylase gene expression and localization were reduced (26). Taken together, these changes have significant implications for structural and functional alterations in neurodevelopment.

Through various animal models, certain associations have been noted that link vitamin D status with brain development. Vuillermot et al. (27), utilizing a murine model of autism, demonstrated that vitamin D treatment during pregnancy attenuated and/or prevented neurodevelopment disorders following maternal inflammation during pregnancy. The study further demonstrated that prenatal administration of $1,25(OH)_2D$, the active vitamin D hormone, abolished all behavioral defects in polyriboinosinic-polyribocytidylic acid (poly[I:C])-treated juvenile mice with autism (27). Other findings in rodent models have linked maternal vitamin D-deficiency during pregnancy with later spatial learning deficits in the offspring (28) and fertility dysfunction in female mice offspring thought to be due to deleterious effects on the neuroendocrine axis (29).

Extending the findings from animal models to the human realm, Dr. John Cannell in 2008 first proposed the association between vitamin D and Autism Spectrum Disorders (ASD) (30). Included in ASD are autistic disorder, Asperger's Syndrome, Rett's Syndrome, Childhood Disintegrative Disorder, and Pervasive Development Disorders. Epidemiological studies as well as animal models have suggested a potential role for vitamin D deficiency in the development of ASD (27, 31).

Lung Maturation and Function

The link between vitamin D deficiency and abnormal development is not limited to the brain. It is known that vitamin D deficiency has risen in the past few decades around the globe (32) and with this rise in deficiency is the concomitant rise in autoimmunity and asthma (33, 34). Vitamin D is known to affect certain genes in the developing lung that are upregulated; and these same genes (for example, matrix mellallopeptidase 9; NF-k light polypeptide gene enhancer in B cells inhibitor; epidermal growth factor receptor; E1A binding protein p300), are linked to the later development of asthma (35).

Zosky, et al, using a relevant BALB/c mouse model of vitamin D deficiency, studied somatic growth, lung function and lung structure at 2 weeks of age (36). It was determined that volume dependence of lung mechanics was altered by vitamin D deficiency, suggesting altered tissue structure. In this model, the primary histological difference between vitamin D-deficient and replete groups manifested in differences in lung volume, supporting the link between vitamin D deficiency and lung development. The same group subsequently published another that described the effects of *in utero* vitamin D deficiency on airway smooth muscle mass and function (35). Using a mouse model, this team showed that there was differential expression of certain gene pathways involved in embryonic organ development, pattern formation, branching morphogenesis, wingless/Int, and inflammation in vitamin D deficient mice, and included genes upregulated in individuals with asthma (e.g., matrix metallo-peptidase 9, NF-κ light polypeptide gene enhancer in B cells inhibitor, an epidermal growth factor receptor and E1A binding protein p300) (35). Vitamin D deficiency in this model also was associated with increased airway smooth muscle mass (ASM) and baseline airway resistance as well as altered lung function (36). Collectively, these data suggest that vitamin D deficiency states during pregnancy are associated with alteration in lung structure and function and increased inflammation, contributing to asthma, which manifests well after birth, and further suggests epigenetic mechanisms of action of vitamin D.

One of the most interesting animal studies comes from Yurt, et al., who investigated the effect of vitamin D supplementation on lung morphology (37). Specifically, using an *in vivo* rat model, the investigators determined the effects of perinatal vitamin D deficiency on overall pulmonary function and tracheal contraction as a functional marker of airway contractility. One month before pregnancy, rat dams were randomized to either a no cholecalciferol-added or a 250, 500, or 1,000 IU/kg cholecalciferol-added diet, which was continued throughout pregnancy and lactation. At postnatal day 21, offspring plasma 25(OH)D concentrations and pulmonary function (as measured by whole body plethysmography and tracheal contraction response to acetylcholine) were determined. In a dose-dependent manner, compared with the 250 and 500 IU/kg vitamin D-supplemented groups, the no cholecalciferol-supplemented group, following a methacholine challenge, showed a significant increase in airway resistance. What was particularly interesting was that the vitamin D deficiency-mediated increase in tracheal contractility was only blocked by supplementation with the higher maternal dose of 500 IU/kg cholecalciferol. Therefore, in addition to altering alveolar epithelial-mesenchymal signaling, perinatal vitamin D deficiency was associated with altered airway contractility. The findings provide some insight into asthma pathogenesis and the mechanistic effects of vitamin D in perinatally vitamin D-deficient offspring, and further suggest that vitamin D could play a role in the prevention of childhood asthma through perinatal vitamin D supplementation, as has been suggested by Litonjua et al and others (38–40).

Other *in vivo* studies support the role of vitamin D in pulmonary function. Taylor, et al, showed promising and provocative results utilizing a neonatal rat model (41). The investigators randomized one-day-old rat pups to one of three different doses of the active hormone, $1,25(OH)D_2$ and its

physiologic precursor 25(OH)D, or the diluent, via nebulization daily for 14 days. Compared to controls, nebulized vitamin D-treated group had enhanced lung maturation as evidenced by the increased expression of markers of alveolar epithelial (SP-B, leptin receptor), mesenchymal (PPARγ, C/EBPα), and endothelial (VEGF, FLK-1) differentiation, surfactant phospholipid synthesis, and lung morphology without any significant increases in serum 25(OH)D or in serum calcium concentration.

VITAMIN D DEFICIENCY DURING PREGNANCY: HUMAN STUDIES

Observational Studies

For decades, vitamin D was thought to be important to the pregnant woman and her developing fetus only for the maintenance of calcium homeostasis and skeletal integrity. Research during those decades was directed at answering the question of what was necessary to prevent hypocalcemia and prevent bone loss or underdevelopment in the mother and her newborn. Whether it was serendipity or the realization among certain physicians and scientists that vitamin D was involved in other physiological processes, studies began to emerge that linked vitamin D deficiency with long-latency diseases and immune dysregulation.

This new avenue of research extended to the question of whether or not the entity of preeclampsia, which is the leading cause of preterm birth in many parts of the world, was linked in any way to vitamin D deficiency. Reports began to emerge that found an association between the dietary vitamin D_3 intake in pregnant women and preeclampsia (42). Looking to historical studies, it is interesting that now more than half century ago, Olsen and Secher (42), in their studies of pregnant women given halibut liver oil, a rich source of vitamin D_3, found that this supplementation was associated with decreases in preterm birth and preeclampsia, which the authors attributed to marine n-3 fatty acids, with no mention of vitamin D and its potential effect (42). This perspective in the early 1940's was based on the then unknown effects of vitamin D on various systems besides calcium and bone.

More recent studies have delineated vitamin D's extraskeletal and immunological effects, which has brought the question of vitamin D's importance during pregnancy into a new light (38, 43–57). Early observational studies suggested a consistent relationship between maternal circulating concentrations of 25(OH)D and preeclampsia (13, 51–53), altered placental vascular pathology (54), cesarean section rates (55), glucose intolerance (56), adverse birth outcomes due to race and ethnicity (57), brain dysfunction (50) and respiratory dysfunction (38). Since 1980, studies have shown that maternal vitamin D deficiency is a variety of adverse health outcomes, which include abnormal fetal growth patterns (with the likelihood of alteration in growth associated with extreme deficiency) (58), adverse birth outcomes (such as preterm birth) (59, 60), reproductive failure (61–65), and have further strengthened vitamin D's role as a contributing factor in the manifestation and progression of disease leading to preeclampsia (66, 67). A recent meta-analysis of observational studies has found a positive relationship between maternal vitamin D deficiency and the risk of preterm birth (68).

Changing public awareness about vitamin D involves several steps, the first of which has been the publication of observational studies of vitamin D deficiency, alerting scientists and others that vitamin D deficiency has subtle but potentially profound effects. The next step was the conduct of randomized controlled trials, but unlike a pharmaceutical trial where everyone starts off at a baseline concentration of the said drug of 0; in nutrient studies, in this case vitamin D, each study participant has a measurable amount of circulating 25(OH)D, which certainly confounds any such trial. As Dr. Robert Heaney pointed out in his essay on clinical nutrient studies, the importance of a biomarker of a drug, in this case "vitamin" or preprohormone, is underscored (69). Analyses of the effect of vitamin D therapies should use 25(OH)D concentration, and not treatment group, as the better indicator of true effect.

Randomized Clinical Trials

As the gold standards, evidence-based medicine (EBM) and randomized clinical trials are long considered essential in advancing health interventions and practices. This approach has and specific methods has been applied to the evaluation of nutrient effects. As mentioned above, in advancing the cause of nutrient studies, Heaney (69) pointed out that EBM, while appropriate in the evaluation of drugs, is lacking when applied to the study of nutrients. For example, in a clinical trial designed to study a given drug's efficacy, the placebo group would not have been exposed to that drug and would receive none of the drug in question. This is not the case for nutrient studies, including vitamin D studies. To perform a true RCT for vitamin D, the study design would ensure that all subjects were vitamin D-deficient at the study onset, and for the duration of the study, all subjects would have to remain indoors to avoid any sun exposure. In fact, places such as the Middle East where women for cultural and religious reasons have limited sunlight exposure and are not likely to receive vitamin D supplementation, profound vitamin D deficiency exists, and when those women are randomized to vitamin D treatment, there are profound effects noted (70–72). In other regions of the world where vitamin D supplementation is given to pregnant women, a true placebo would be considered unethical, and thus, the dilemma surrounding nutrient study design and interpretation of the data exists.

The five rules of rigor for nutrient studies suggested by Heaney (69) include the following: (1) basal nutrient status must be measured, used as an inclusion criterion for entry into the study, and recorded in the report of the trial; (2) the intervention must be large enough to change nutrient status and must be quantified by suitable analysis; (3) the change in nutrient status produced in those enrolled in the report of the trial must be measured and reported; (4) the hypothesis to be tested must be that a change in nutrient status produces the sought-after-effect; and (5) the status of other nutrients must be optimized to guarantee that the nutrient being studied is the only nutrition-related, limiting factor in the response. We also have added an additional imperative to this list: the nutrient being investigated has to

follow an appropriate dosing schedule matching the physiologic system being investigated, as for example with vitamin D, there is a substantial physiological difference between daily, weekly and monthly dosing (73). Most vitamin D clinical trials conducted thus far would fail based on at least two of these criteria. We thus are forced to look at observational and RCT data that have nutrient study "flaws," but which offer important insights into vitamin D's role in health.

The first studies involving vitamin D supplementation in pregnant women were performed in the early 1980's mainly in Europe (58). These early studies were plagued by small sample sizes, not having meaningful endpoints, or effective treatment/dosing concentrations (58) that could not effectively evaluate the role of vitamin D in the development of certain disease states such as preeclampsia (13, 51–53), asthma (38, 39, 74–76), preterm birth (59, 60, 68, 77), and autoimmune dysfunction (78–82). As a result, the variability in findings led to confusion and limited relevance to the general population, which resulted in stagnation of the field for at least two decades.

As one of the first steps in discerning what is a reasonable, healthy concentration of 25(OH)D, our group in 2001 designed a large RCT to assess the vitamin D requirements during pregnancy. This study represented a radical departure from prior studies in that we proposed a randomized clinical trial of supplementing pregnant women <16 weeks of gestation with up to 4,000 IU/d vitamin D_3 until delivery. The higher dose treatment group was two times the Upper Limit (UL) set forth by the IOM in 1997 (83). Our main goal of the study was to determine the daily dose of vitamin D required to raise circulating maternal 25(OH)D concentrations to at least 80 nmol/L (32 ng/mL) by the third trimester, which, based on mathematical calculations from previous studies (6, 84), was the amount necessary to prevent secondary hyperparathyroidism (85).

Along with our study, several other RCTs have been published (11, 86–95), with the main finding that a daily dose of vitamin D of 4,000 IU/safely elevated circulating 25(OH)D concentrations that, regardless of race, fully and safely normalized vitamin D metabolism and calcium homeostasis in the pregnant women. Using repeated measures, the concentration of 25(OH)D that fully normalized $1,25(OH)_2D$ in our study cohort was determined on each subject and plotted to determine the point at which first order kinetics went to zero order (11), which was 100 nmol/L (40 ng/mL), the beginning point of the plateau at which the production of $1,25(OH)_2D$ became substrate independent (11). Attention to safety in our study as well as other studies showed that serum calcium and urinary calcium/creatinine ratios did not differ between the treatment groups, and thus, 4,000 IU/day vitamin D was deemed to be safe (11, 86–95).

With the emergence from observational vitamin D studies of the favorable efforts of vitamin D on pregnancy outcomes beyond calcium homeostasis, we analyzed health outcomes measures in our pregnancy cohorts. Improved vitamin D status was associated with decreased complications of pregnancy and lower rates of cesarean section (77, 87). Merewood et al previously had shown a similar association between vitamin D and mode of delivery in a cohort of women living in Boston (55). Further, RCT data and analysis by our group and others in various regions of the world have clearly demonstrated that higher doses of vitamin D during pregnancy improve birth outcome data (59, 72, 77, 89, 96).

The list of studies that links vitamin D deficiency to complications of pregnancy continues to grow: vitamin D status has been associated with gestational diabetes (56, 89, 90, 92), aeroallergen sensitization (91), and markers of regulatory immunity (93). Perhaps one of the most far-reaching of these studies was performed by Sablok et al. (89). In their study, vitamin D deficient pregnant women living in Delhi, India, with circulating 25(OH)D concentrations of <25 nmol/L (10 ng/mL), were randomized to receive substantial amounts of vitamin D starting at 20 weeks of gestation or placebo. It is noteworthy that the placebo group remained profoundly vitamin D deficient throughout pregnancy. In the women randomized to vitamin D treatment, there was 100% adherence to protocol, which resulted in a substantial decline in pregnancy complications. In other areas of the world where all women receive at least 400 IU vitamin D/day, there is less deficiency and the differences in clinical outcomes less dramatic. Thus, the effects of vitamin D deficiency appear to be magnified in the cases of severest deficiency, which is the end result of no vitamin D supplementation (placebo).

VITAMIN D-INDUCED GENOMIC ALTERATIONS DURING PREGNANCY

In our original pregnancy study and a subsequent study, from the perspective of an intention-to-treat design, the results are less dramatic (11, 97) than when adherence is taken into account. If instead, circulating 25(OH)D concentrations as the biomarker of vitamin D status is used, the true effect of vitamin D supplementation on preterm birth becomes apparent (59). The same associations from the VDAART trial also hold true for the prevention of preeclampsia (17). Through vitamin D's effect on gene regulation, vitamin D supplementation during pregnancy appears to alter genes related to systemic inflammation and immune responses. The aberration of these genes and processes suggests that there is a specific immune cascade of events associated with vitamin D deficiency that occurs early-on in pregnancy in women destined to develop preeclampsia (16, 65), and likewise, in other comorbidities states of pregnancy such as gestational diabetes (90, 92, 98) and infection (99, 100).

In their recent paper, Al-Garawi et al. (101), in their *post-hoc* analysis of the VDAART RCT study, provide strong evidence of vitamin D's effect on genomic changes during pregnancy, which is one of the first reports of its kind. As part of the parent RCT of vitamin D supplementation in pregnancy at 10–18 weeks of gestation where women were randomized to 400 and 4,400 IU vitamin D_3/day to achieve decreased pediatric asthma risk (86), a subset of blood samples also were collected for RNA analyses for gene expression in the first and third trimesters, for comparison. Using significance of analysis of microarrays (SAM) and clustered weighted gene co-expression network analysis (WGCNA) to identify major biological transcriptional profiles between first and third trimesters of

pregnancy, this team of investigators identified 5,839 significantly differentially expressed genes. Transcripts from these genes clustered into 14 co-expression modules, of which two showed significant correlation with maternal 25(OH)D concentrations. Two modules identified genes enriched in immune defense pathways and extracellular matrix reorganization as well as genes enriched in Notch signaling and transcription factor networks. These important findings suggest that maternal gene expression changes during pregnancy are affected by maternal vitamin D status, which in turn, is a direct reflection of maternal vitamin D supplementation.

The extrapolation of US- and European-based studies to other regions of the world is based on the effects of vitamin D that extend beyond calcium and bone metabolism, and on the findings that vitamin D deficiency impacts immunological function and pregnancy outcomes. Whether a woman attains optimal vitamin D status during pregnancy through sun-exposure or vitamin D supplementation is less of an issue than if she is able to attain optimal status by either method such that her circulating 25(OH)D concentrations are at least 100 nmol/L (40 ng/mL). Women who have profound vitamin D deficiency typically respond to 4,000 IU/day with an exuberant response in the first month since they have upregulated their vitamin D enzymes but by 2 months of supplementation, we have found that their 25(OH)D concentrations will have plateaued and remain so throughout pregnancy. Even these women with initial severe deficiency do not develop hypercalcemia or hypercalciuria in response to treatment (11, 77, 97). If a woman has a higher BMI, she may require additional vitamin D to achieve this goal concentration; or if she is non-compliant with treatment, her vitamin D status will not improve. It is important to follow women with serial 25(OH)D measurements if such risk factors are identified.

There are so many unanswered questions that are the focus of ongoing studies. For example, what effect does maternal 25(OH)D concentration have on fetal development? Are these effects direct or through downstream processes? What about direct effects of maternal gene expression on the fetus? Studies presented at recent vitamin D conferences suggest that there are indeed direct genomic alterations in response to maternal vitamin D status that can alter the health of the mother and birth outcomes (102).

Postnatal Asthma Prevention

Observational studies by Brehm et al suggested that vitamin D supplementation during pregnancy could reduce childhood asthma rates (102). This led to a double-blind multicenter RCT conducted in the US where pregnant women were randomized to 400 or 4,400 IU/d vitamin D_3 across the three major racial/ethnic groups in the US from 10 to 18 weeks of gestation until delivery (the VDAART study) (86). As the authors describe, the primary endpoint—prevention of asthma/wheeze in the infant/child at 1-, 2-, 3-, and 6-years post-birth through vitamin D supplementation in the mother during pregnancy—involved nearly 900 high-risk subjects (86). The results of this study are quite clear: on the basis of intention-to-treat, where there was much non-adherence to protocol there is a strong trend of effect ($p = 0.051$); however, on the basis of maternal circulating 25(OH)D concentrations achieved, vitamin D supplementation during pregnancy will decrease asthma or recurrent wheezing rates in children. This positive finding, however, is dependent upon a circulating 25(OH)D concentration of at least 100 nmol/L (40 ng/mL) at conception (39, 74, 86).

In their RCT study performed in Denmark, Wolsk et al also examined the effect of maternal vitamin D supplementation during pregnancy on later asthma risk in the offspring (75). The Denmark team found results similar to the VDAART study. Combining the VDAART data with the Denmark data (74), the two study teams found that vitamin D_3 given to a pregnant woman reduced the risk of later asthma/wheeze in her child (39, 74).

The wealth of data in the VDAART study led to additional *post-hoc* analyses (39, 74). Those women entering pregnancy with circulating 25(OH)D concentrations $\geq$75 nmol/L (30 ng/mL) who were prescribed 4,000 IU/d vitamin D_3 starting at approximately 10–18 weeks' gestation achieved the maximum protection against asthma development in their infants following birth (39, 74). Further, these data suggest that vitamin D is strongly associated with very early *in utero* lung development in the fetus that cannot be reduced by starting vitamin D supplementation at the end of the first trimester. As discussed earlier, vitamin D-related genes in early lung development are associated with asthma pathogenesis (35).

Are these data from the various studies in the US, Europe, and Iran applicable to other regions of the world? Is there something unique about women in these countries or can the results be extended to women in all regions of the world if they have limited sunlight exposure either through where they live or how they live? Based on vitamin D metabolism and the pharmacokinetics of vitamin D, we believe that the recommendations of 4,000 IU/day during pregnancy should be universal, with the caveat that women with higher BMIs may require higher dosing to attain the optimal 25(OH)D concentration of at least 100 nmol/L (40 ng/mL).

Preeclampsia Prevention

In their *post-hoc* analysis derived from the VDAART RCT, Mirzakhani et al. (17) reported that a key factor in preventing preeclampsia was vitamin D status early in pregnancy: first trimester circulating 25(OH)D concentration of a least 100 nmol/L (40 ng/mL) was associated with a reduction in the risk of developing preeclampsia. Predicted by both observational studies as well as experimental animal models, the association of vitamin D deficiency and its ability to alter placental development and embryo implantation is further supported by the findings of Mirzakhani et al. (15, 17, 103, 104). As mentioned earlier, this effect is likely vitamin D-mediated during a very early point in pregnancy. Beyond this critical period, rescue by further vitamin D supplementation with respect to placentation and the manifestation of preeclampsia, has diminished impact (17). Additional support for vitamin D's role in the development of preeclampsia comes from a recent RCT study where the administration of vitamin D (50,000 IU/week or ~7,142 IU/day)

during the prenatal period reduced the pre-eclamptic rate by half (95).

It is not surprising based on vitamin D's effect on gene regulation that maternal vitamin D supplementation also would be involved in epigenetic regulation. Pathways affected by such regulation include antigen processing and presentation, inflammation, regulation of cell death, cell proliferation, transmission of nerve impulse, neurogenesis, neuron differentiation, sensory organ development (105) and vitamin D metabolism (106). Vitamin D's epigenetic effects on genes involved in metabolism and immune function have been demonstrated in experimental animal models (107, 108) and preliminary findings have been reported in humans (105). The effect of vitamin D status on total genomic changes cannot be ignored and represent one of the most exciting branches of research to be explored.

Neurodevelopment and Autoimmune Consequences

There is emerging data regarding the impact of vitamin D deficiency during pregnancy on neurologic disease and altered development (30, 49, 50). Experimental animal data suggests that there are significant adverse neurological consequences to the offspring if vitamin D is restricted during pregnancy (109–112). A review by Patrick and Ames summarizes cumulative data that support the premise of adverse effects of intrauterine vitamin D deficiency. Vitamin D likely acts through the control of serotonin synthesis in the developing brain: if vitamin D access is restricted, can lead to the later development of autism, attention deficit disorder, bipolar disorder, schizophrenia and impulse behavior (113). A recent study again strongly links vitamin D concentrations during pregnancy with the development of autism spectrum disorder (114).

CURRENT RECOMMENDATION FOR VITAMIN D SUPPLEMENTATION DURING PREGNANCY

Based on our data from our NICHD-sponsored vitamin D supplementation pregnancy trial (11) as well as substantial observational and interventional data from our later studies (16, 60, 97) and that of other investigators around the world (17, 39, 72, 74, 86, 94, 101), we suggest that women considering becoming pregnant (59), and if pregnant, then during the earliest time in pregnancy, maintain a circulating 25(OH)D concentration of a least 100 nmol/L (40 ng/mL). Achieving this goal will reduce the risk of vitamin D-related pregnancy complications, including preeclampsia (17, 115) and preterm birth (60, 97), and later asthma risk in the offspring (17). To achieve this goal, intakes of a least 4,000 IU/d vitamin D_3 will be required because of variable individual abilities to convert vitamin D to 25(OH)D (6). These supplements have proven to be safe in thousands of patients over the past 15 years. Further, this supplementation dose is well within the safe intake level (upper limit, UL) as defined by The Endocrine Society (8). Such supplementation becomes an alternative to direct sunlight exposure, and agrees with data derived from populations living in sun-rich environments (116), whose circulating 25(OH)D concentrations during pregnancy simply from sun exposure are quite similar to those achieved through daily vitamin D supplementation of 4,000 IU (116, 117).

Finally, addressing the original question of this review—does vitamin D qualify as a substance that supports and upholds the Barker Hypothesis? The clear answer is yes! Through its effect on genetic processes, vitamin D deficiency during pregnancy affects both mother and fetus, and, at least in the case of asthma, phenotypic expression in the infant/child.

In summary, the observational and randomized clinical trials present a clear message: that 4,000 IU/d vitamin D_3 supplementation is beneficial to both mother and her developing fetus through optimization of vitamin D metabolism that goes beyond classical calcium and bone homeostasis. While further work is needed to determine what the optimal dose of vitamin D supplementation during pregnancy is based on various genotype differences for the vitamin D binding protein and the vitamin D receptor, body mass index, status at the time of conception, and other factors such as sunlight exposure and latitude, based on our work and that of others, we believe that all individuals, including pregnant women, should achieve a target circulating 25(OH)D concentration of 100 nmol/L (40 ng/mL) as early as possible. Because of individual differences in what is required to attain this target concentration of 25(OH)D, we believe all women should consume at least 4,000 IU/d vitamin D_3 prior to conception and throughout pregnancy.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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25(OH)D Concentration in Neonates, Infants, and Toddlers From Poland—Evaluation of Trends During Years 1981–2011

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Introduction: The numerous evidence showing spectrum of vitamin D effects on human health resulted in both updates of vitamin D supplementation guidelines for general population and concerns on potential risk of hypercalcaemia. The aim of this study was to analyse trends in serum 25-hydroxyvitamin D concentration (25(OH)D) change over the 30 years of operation of a single pediatric diagnostic unit.

Materials and methods: Calcium-phosphate metabolism markers and 25(OH)D concentrations were analyzed in a group that consisted of newborns and infants commissioned for diagnostics due to suspected calcium-phosphate metabolic disturbances (n = 3,163; mean age 8.0 ± 3.0 months).

Results: 25(OH)D <10 ng/ml was noted in 4.5% of patients (n = 163), 10–20 ng/ml in 14.7% (n = 465), 20–30 ng/ml in 23.9% (n = 756) and 30–50 ng/ml in 35.9% (n = 1,136). The mean 25(OH)D concentration in analyzed group was 37.5 ± 24.5 ng/ml. In patients with 25(OH)D concentration <10 ng/ml a normal calcaemia (2.25–2.65 mmol/l) was noted in 83.4% cases (n = 136). Eighty one patients had 25(OH)D concentrations above 100 ng/ml with co-existing calcaemia in range of 2.6–4.38 mmol/l (mean Ca = 2.69 mmol/l). Hypocalcaemia (Ca <2.25 mmol/l) was observed in 0.54%, (n = 17). 13.8% patients revealed calcium levels >2.65 mmol/l (n = 435). In general, the mean calcium-phosphate markers values were within the reference range for age. The highest mean 25(OH)D concentration of 51.8 ng/ml ± 38.8 was noted in years 1981–1999 (n = 305). The lowest mean 25(OH)D value was observed in years 2010–2011 (29.0 ng/ml ± 13.6; n = 412). The trend of decreasing 25(OH)D concentration during analyzed time period was significant ($r = -0.29$, $p < 0.0001$).

Conclusions: Eighty percentage of children aged 0–36 months had 25(OH)D concentration >20 ng/ml, however, during 3 decades a mean 25(OH)D concentrations trended significantly to decrease. A direct relationship between low 25(OH)D concentration and hypocalcaemia was not observed nor between high 25(OH)D concentration and hypercalcemia.

Keywords: vitamin D, 25(OH)D, vitamin D deficiency, calcaemia, infants, toddlers

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INTRODUCTION

Over the last decade, the biologic activity of vitamin D and its metabolites in the human body has become the topic of many publications and intense discussions. The discoveries of the pleiotropic and multi-organ effects extended the knowledge on the basic role of vitamin D, i.e., the regulation of calcium and phosphate homeostasis. It was demonstrated that vitamin D deficiency is associated (or at least coincides to) with many diseases, such as neoplasms (1–5), autoimmune diseases (6–8), type 1 and type 2 diabetes (9–11), cardiovascular disease (12–14), and hypertension (15–17). Therefore, the interest in vitamin D deficiency and vitamin D supplementation as public health problems has increased. In light of increasing life expectancy and high prevalence of chronic diseases as well as negative changes in dietary habits and lifestyle, obtaining and maintaining optimal 25(OH)D concentrations has become an important aspect of health-oriented policies (18, 19).

Vitamin D has been used as an agent for the prevention and treatment of nutritional rickets, however, still majority of epidemiological studies indicate a significant deficiency of this vitamin (20–23). Therefore, it appeared crucial to establish satisfactory recommendations on vitamin D supplementation for the general population. The most commonly quoted position papers are the practice guidelines issued by the Endocrine Society in 2011 (24) and the supplementation recommendations published by the Institutes of Medicine (IOM) in 2010 (25). Vitamin D supplementation guidelines are available also for European countries, including Scandinavian countries (Denmark, Finland, Iceland, Norway and Sweden) (26) or Germany, Austria and Switzerland (27), Poland (28), and Central Europe (29). Despite differences between recommended vitamin D daily doses for general population, international scientific societies recognized 25(OH)D concentrations below 10 ng/ml as extremely low and reflecting severe vitamin D deficiency (20, 24, 25, 28–31). In light of the most recent studies, reference ranges have been changed and the minimum concentration considered as beneficial for health was set at 20 ng/ml or even 30 ng/ml, depending on the reference authority (24, 28, 29, 31). Taking into account the above mentioned criteria, vitamin D deficiency in Poland and in Europe seems to be a common issue (30, 32–34), at least in the age groups not protected by the nutritional rickets prevention programs (20).

However, regular vitamin D supplementation recommended by various scientific societies resulted in significantly increased use of vitamin D supplements by general population, and initiated discussions on vitamin D toxicity and the incidence of hypercalcemia. It has been emphasized that in some cases an excessive doses of cholecalciferol might increase the risk of hypercalcaemia, hypercalciuria, and nephrocalcinosis (35–37). British studies from the 1950s described cases of hypercalcaemia in infants, caused by the use of doses exceeding 4,000 IU a day (38). Some of these patients were of a phenotype later called Williams-Beuren syndrome (39), while others were decades later classified into a new disease entity of a genetic origin—idiopathic infantile hypercalcemia (IIH) (35–37, 40). Similar cases were also observed in Poland in the 1970–1980s (41, 42). Hypercalcaemia manifested itself after the supplementation with doses of 2,500–4,000 IU a day, as well as at loading doses up to 300,000 IU (35, 41, 42). For the above mentioned reasons, supplementation should be carried out carefully, and self-administration of vitamin D without medical supervision seems risky especially in patients with unrecognized hypersensitivity to vitamin D. The risk of vitamin D hypersensitivity is determined genetically (35–37, 43–45). According to the most recent study conducted in Poland the incidence of IIH in Polish population was estimated to be as high as 1:32,465 births (43).

Taking into account the historical aspect of vitamin D use in pediatric population before 2009, the available guidelines in Poland were not nationwide and were limited to nutritional rickets prevention in infants and in small children. Since the mid-1980s, the use of vitamin D loading doses of up to 300,000 IU in the prevention and treatment of nutritional rickets was discontinued in medical practice and the recommended preventive vitamin D intake in neonates and infants was set at a dose of 2,500 IU a day, and since the 1990s it was further lowered to 400–800 IU a day (46). The above approaches did not resulted in an increase of incidence of nutritional rickets in Poland and, most likely, the risk of potential adverse effects and health complications related to overdosing was reduced, at least in individuals with vitamin D hypersensitivity. Unfortunately, there are no extended studies from the 1980s and 1990s that had assessed vitamin D status in the population of neonates, infants and toddlers, especially in the context of vitamin D doses and related outcomes such as 25(OH)D concentrations and calcemia. Further, very few studies from that time had described vitamin D status in newborns from Poland (46) due to the limited availability of the quantitative measurement of 25(OH)D that was carried out only in the reference laboratory facilities. At 1980s and 1990s, the determination of 25(OH)D concentration in Polish children concerned only patients who were suspected of disorders of calcium and phosphate homeostasis and disturbed bone mineralization.

This study data might complement more recent evidence describing vitamin D status in the present populations of neonates, infants and toddlers, however the most of novel data was restricted to regional surveys from small territory and was done at a short period of time and/or was limited by inclusion criteria.

The population of pediatric patients from entire Poland, described below, includes an extensive historical period of the 1980s and 1990s as well as the first decade of the 21st Century. This is a unique group in terms of the period of time, relatively large number of pediatric patients, as well as their clinical and biochemical diversity. Our observations may at least in part uncover different aspects of vitamin D supplementation, vitamin D status and calcemia in the population of the youngest children in the so-far poorly described periods of time. An overview of historical data showing 25(OH)D concentrations and calcemia may add information to discussion on potential trends that occurred over the 30 years as well as give some insights on the relationship between vitamin D status and calcemia. This study was aimed to evaluate 25(OH)D concentration data assayed in newborns, infants and toddlers that were commissioned in the

years 1981–2011 to the single diagnostic unit for the evaluation of vitamin D status and the calcium—phosphate metabolism markers.

PATIENTS AND METHODS

The Study Group

Our study analyzed medical documentation of pediatric patients suspected of calcium and phosphate homeostasis disorders. The analyzed population of patients consisted of neonates, infants and toddlers directed by local general practitioners from entire country for a consultation in the Children's Memorial Health Institute (Warsaw, Poland) in years 1981–2011. Analysis was performed using medical database maintained on an ongoing basis for the scientific and research purposes. The measurements were registered in the database by a physician or nurse after every visit.

The only inclusion criterion for the present study was the availability of 25(OH)D measurement value in a single analysis of a patient's sample. 25(OH)D concentration was analyzed in along with other (measured at the same time) parameters of calcium and phosphate homeostasis, including calcium (Ca), phosphates (PO4), and creatinine levels in serum and urine in a 24 h urine sample or in single sample. Alkaline phosphatase activity (ALP) as well as parathormone (PTH) and $1.25(OH)_2D$ values were also analyzed if data were available.

Every patient's data was analyzed only once and included the first measurement of 25(OH)D concentration, irrespective of the final diagnosis. The study group consisted of children with different calcium and phosphate homeostasis disorders, both congenital and acquired, as well as children who appeared healthy after medical consultation. In total, 3,163 pediatric cases aged 0–3 years (mean age 8 months ± 3) were evaluated.

Methods

Biochemical parameters were measured in each group using the following methods: total calcium (Ca)—colorimetric assay, Dimension system (Dade Behring), since 2008 photometric assay, Cobas system (Roche); phosphates (PO4)—colorimetric assay, Dimension system (Dade Behring), since 2008 photometric assay, Cobas system (Roche); alkaline phosphatase (ALP)—enzymatic method, Dimension system (Dade Behring), since 2008 colorimetric assay, Cobas system (Roche); 25-hydroxyvitamin D (25(OH)D)—manual protein binding method, since 2005 chemiluminescence immunoassay, Liaison system (DiaSorin); parathormone (PTH) – radioimmunoassay, since 2006 immunoradiometric assay (Cisbio Bioassays); 1,25-dihydroxyvitamin D ($1,25(OH)_2D$)—immunoradiometric assay (DiaSource); creatinine—colorimetric assay, Dimension system (Dade Behring), since 2008 enzymatic colorimetric assay, Cobas system (Roche); tubular reabsorption of phosphate (TRP)—calculated indicator. The reference ranges of biochemical parameters used in this study were adapted from the diagnostic laboratory of the Children's Memorial Health Institute as well as from the available reference literature (PO4—in relation to age, TRP, calciuria). For a more detailed analysis of Ca levels in serum, an additional reference range was adopted, which indicated evident hypercalcemia (Ca ≥2.75 mmol/l).

Statistical Analyses

The possible relations between the patient's age, the date of visit, and the 25(OH)D concentration values as well as between the individual biochemical parameters were estimated using statistical correlation models.

The correlation between 25(OH)D concentrations and the year of visit in the clinic was estimated. The studied records were also divided into 9 time periods, each with a comparable number of patients, including the following years: (1981–1999, 2000–2001, 2002–2003, 2004–2005, 2006, 2007, 2008, 2009, 2010–2011). The means ± standard deviation values of 25(OH)D concentrations were calculated for each time period.

The correlation between 25(OH)D concentration values and patients' age was investigated. A studied group was divided into four age-dependent subgroups. Mean values of 25(OH)D and the remaining parameters were calculated. The correlation between 25(OH)D concentration and the patients' age was estimated.

To evaluate possible impact of skin synthesis on vitamin D status, the studied group was divided into four time periods according to the month of the blood sampling in the specific quarter of the year (Q1: January, February, March; Q2: April, May, June; Q3: July, August, September; and Q4: October, November, December). The mean 25(OH)D values were calculated for each quarter. The correlation between 25(OH)D concentration and the date of the visit in each quarter was estimated.

RESULTS

General Characteristic of the Study Group

The total number of patients aged 0–3 years with available 25(OH)D concentration values was 3,163 (mean age 8.0 months ± 3.0) (**Table 1**). The means of the Ca, PO4, ALP, 25(OH)D, PTH, $1,25(OH)_2D$ in serum and Ca, creatinine, and TRP in urine were calculated basing on accessible data. All mean values of investigated biochemical parameters were within the reference ranges for a given age (**Table 1**).

Biochemical Characteristics

As shown in **Table 2**, majority of investigated cases had 25(OH)D concentrations above 20 ng/ml. The mean 25(OH)D concentration was 37.5 ± 24.5 ng/ml. 25(OH)D concentrations of <10 ng/ml were noted in <5% of the patients ($n = 163$), values of 10–20 ng/ml in almost 15% ($n = 465$), values of >20–30 ng/ml in 24% of cases ($n = 756$) and 25(OH)D concentrations >30–50 ng/ml in 36% ($n = 1,136$). The maximum measured 25(OH)D value during operation period of 30 years reached 315 ng/ml and was associated with evident hypercalcaemia expressed as Ca = 3.96 mmol/l.

Among the group with severe vitamin D deficiency, defined as 25(OH)D concentration <10 ng/ml, Ca levels were within the reference range (2.25–2.65 mmol/l) in 83.4% of the patients ($n = 136$), however, two patients were identified with hypocalcaemia (Ca <2.25 mmol/l) and 25 patients (15.3%) revealed Ca

levels indicative of hypercalcaemia (above 2.65 mmol/l). In the subgroup with 25(OH)D values exceeding 100 ng/ml, Ca levels varied between 2.60 and 4.38 mmol/l (on average Ca 2.69 mmol/l; $n = 80$). The percentage of patients with hypocalcaemia (Ca <2.25 mmol/l) was as low as 0.54% ($n = 17$) and elevated (>2.65 mmol/l) Ca levels were noted in 13.8% of the patients ($n = 435$). The minimal Ca level noted in studied group as a whole was 1.55 mmol/l that coincided with 25(OH)D concentration of 27.8 ng/ml. The highest Ca level was as high as 4.38 mmol/l, with coinciding 25(OH)D concentration of 106.7 ng/ml. **Table 3** presents 25(OH)D concentration values in the subgroup of patients with evident hypercalcaemia defined as Ca ≥2.75 mmol/l. The evident hypercalcemia was observed in 3.86% ($n = 122$) cases.

It was determined that the prevalence of evident hypercalcaemia was not significantly related to 25(OH)D concentration range, although the lowest number of cases was noted in range <10 ng/ml. The average Ca level in the group with evident hypercalcaemia was 2.90 mmol/l ± 0.27.

TABLE 1 | General characteristics of assessable biochemical parameters in the group of paediatric patients commissioned for calcium—phosphate a vitamin D status evaluations.

Parameter	Number of participants (*n*)	Mean (SD)	Reference values
Ca mmol/l	3163	2.54 (0.13)	2.25–2.65 mmol/l
PO_4 mmol/l	3163	1.90 (0.22)	1–30 days: 1.25–2.50 mmol/l
			1–12 months: 1.15–2.15 mmol/l
			1–3 year: 1.05–1.80 mmol/l
ALP U/l	3160	319.7 (140.9)	<6 months 120–575 U/l
			6 months-1.5 years 100–550 U/l
25(OH)D ng/ml	3163	37.5 (24.5)	20–50 ng/ml
TRP%	3154	93.8 (6.5)	85–95%
PTH pg/ml	332	23.9 (26.9)	11–62 pg/ml
$1,25(OH)_2D$ pg/ml	46	62.7 (35.4)	0–2 years-25.1–154.0 pg/ml
			>2–4years-21.8–156.0 pg/ml
Mean age (months)	3163	8.0 (3.0)	

TABLE 2 | Presents distribution of the levels of selected biochemical parameters.

Ca mmol/l	%	*N*
<2.25	0.5	17
2.25–2.65	85.7	2,711
2.66–2.75	10.5	331
≥2.76	3.4	104
25(OH)D in serum: min. 0.7 ng/ml, max. 315 ng/ml		
25(OH)D ng/ml	**%**	***N***
<10	4.5	163
10–20	14.7	465
>20–30	23.9	756
>30–50	35.9	1,136
>50–100	17.8	562
>100	3.2	81
Alkaline phophatase in serum: min. 36 U/l, max. 5,010 U/l		
ALP U/l	**%**	***N***
<120	0.5	16
>120–575	96.7	3,056
>575	2.8	88
Tubular reabsorption of phosphate (TRP) in urine: min. 0.66%, max. 100%		
TRP%	**%**	***N***
<85%	8.1	254
>85–95%	38.8	1,225
>95%	53.1	1,674

Analysis of 25(OH)D Concentration Values According to Age

As shown in **Table 4**, the mean 25(OH)D concentrations in subsequent age groups appeared to be higher than 30 ng/ml.

The highest mean 25(OH)D concentration of 38.6 ng/ml ± 26.5 was observed in infants aged 3–6 months ($n = 1112$). The lowest mean 25(OH)D concentration of 27.6 ng/ml ± 33.3 was noted in neonates aged up to 1 month ($n = 27$). Correlation between the age of investigated cases and 25(OH)D concentrations was not significant ($r = 0.0018$; $p = 0.921$), as shown in **Figure 1**.

Analysis of 25(OH)D Levels in the Respective Time Periods

Table 5 presents means of 25(OH)D concentration in the specified time periods that corresponded to the patients' visits in the clinic and 25(OH)D assay done in diagnostic unit.

The highest mean 25(OH)D concentration in analyzed time period was observed in years 1981–1999 ($n = 305$) and reached

TABLE 3 | Distribution of 25(OH)D concentrations among subgroup ($n = 122$) with evident hypercalcaemia (Ca ≥2.75).

25(OH)D, ng/ml	*n* = 122	mean Ca (SD); mmol/l
<10	4	2.80 (0.04)
10–20	20	2.85 (0.15)
>20–30	22	2.84 (0.10)
>30–50	28	2.84 (0.13)
>50–100	32	2.84 (0.14)
>100	16	3.23 (0.57)

TABLE 4 | Mean 25(OH)D concentrations in subsequent age groups.

Age (months)	*n*	25(OH)D ng/ml (SD)
0–1	27	27.6 (33.3)
2	68	29.0 (22.7)
3–6	1,112	38.6 (26.5)
7–12	1,489	32.2 (26.6)
13–18	294	37.7 (24.9)
19–36	173	37.1 (19.4)

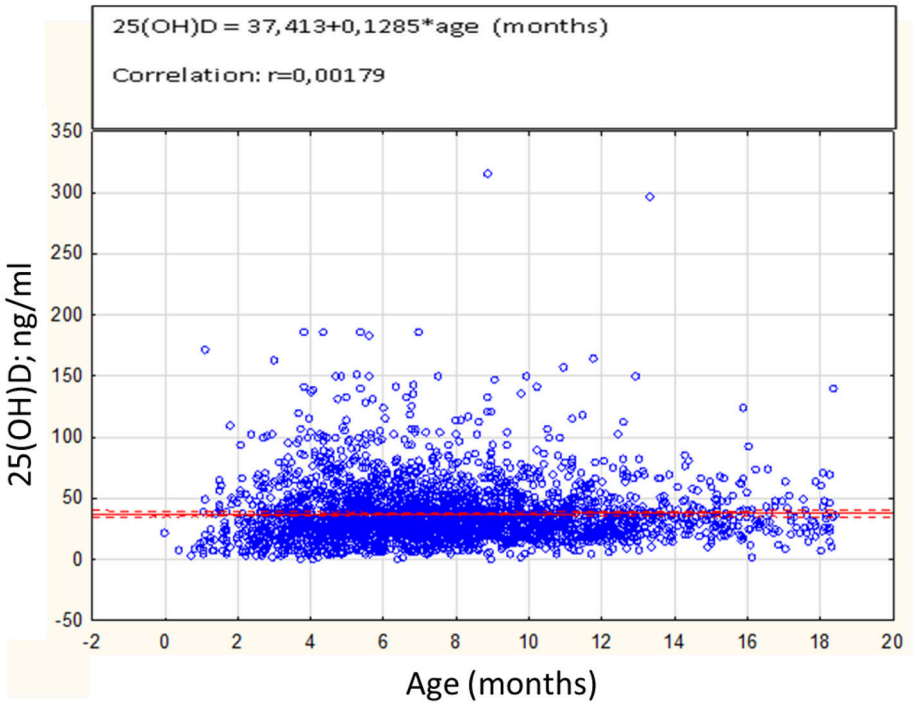

FIGURE 1 | Correlation between 25(OH)D concentrations (ng/ml) and age in months ($n = 3163$).

TABLE 5 | Mean 25(OH)D concentration values calculated in respective time periods during 30 years of operation of single diagnostic unit.

Time period (years)	*n*	Mean age (months)	25(OH)D (SD); ng/ml
1981–99	305	7	51.8 (38.8)
2000–01	212	7	42.9 (27.4)
2002–03	152	7	48.8 (31.7)
2004–05	325	8	34.8 (19.1)
2006	334	8	36.5 (20.0)
2007	485	8	37.2 (23.1)
2008	457	8	32.9 (18.8)
2009	481	8	36.7 (21.7)
2010–11	412	8	29.0 (13.6)

51.8 ng/ml ± 38.8. During the following time periods 25(OH)D concentrations significantly trended to decrease ($r = -0.29$; $p < 0.0001$). The lowest mean 25(OH)D concentration value was observed in years 2010–2011 (29.0 ng/ml ± 13.6; $n = 412$).

25(OH)D Concentrations According to Season of the Year

25(OH)D concentration values in four quarters of the year are shown in **Table 6**. Irrespective of analyzed seasons, 25(OH)D concentrations were not significantly different and appeared above 30 ng/ml.

DISCUSSION

Many reports focused on vitamin D status were published so far. Previously conducted population studies described selected groups, e.g., healthy adults, high-risk groups for vitamin D deficiency—obese or elderly, or were carried out as cross-sectional studies for extensive populations from a single region or country (19, 21–23, 30, 32–34). An increase in interest was concomitant with discussion on vitamin D recommended intakes, reference ranges and potential toxicity risk (24, 25, 31, 47). Most of these reports confirmed that vitamin D deficiency is a serious healthcare problem (22–24, 31, 34).

For many reasons it is not possible to conduct an objective comparison of vitamin D status between modern populations of children and adults, and previous ones from the period of the 1980s or 1990s. Common methods of vitamin D measurement have been used in the laboratory practice since the early 2000s. The methodology was developed simultaneously to discoveries of vitamin D systemic effects. This initiated a dynamic increase in the number of studies evaluating vitamin D status in different groups of patients and/or searching for potential vitamin D—related health benefits. In the last decades of the 20th Century, vitamin D studies were, in general, markedly less often conducted. The 25(OH)D assays were, at that time, based on manual methods that were available only in several reference laboratory facilities, and a scientific surveys included usually a relatively small number of patients.

Our study is based on a representative group of 3,163 pediatric patients admitted to the consultation clinic of calcium and

TABLE 6 | 25(OH)D concentrations in relation to quarter of the year.

Quarter of year	*n*	mean age (months)	25(OH)D (SD); ng/ml
I	820	7	38.9 (28.5)
II	778	7	37.9 (24.6)
III	747	9	37.5 (24.6)
IV	818	12	35.5 (21.5)

phosphate homeostasis disorders at the Children's Memorial Health Institute over the course of more than 30 years. The first characteristic feature of the study group was a previous suspicion of calcium and phosphate homeostasis disorder, and the other was a broad time spectrum (+30 years), in which vitamin D status was evaluated.

We assumed that in the past, as well as currently, preventive supplementation with cholecalciferol was routinely recommended for newborns and infants up to approx. 18 months of age, resulting in relatively high 25(OH)D concentration values noted during the first visit to the consultation clinic.

The evaluation of the mean values of 25(OH)D in our dataset confirmed above mentioned assumptions and indirectly confirmed effective implementation of rickets-preventive cholecalciferol supplementation among children up to 3 years of age. As expected, the lowest 25(OH)D concentrations were observed in neonates and infants up to the end of a 2nd month after birth, but still the calculated mean values were higher than 20 ng/ml.

The observation of changes in vitamin D status in the studied periods of time revealed a higher 25(OH)D concentrations in the last 2 decades of the 20th Century compared to late 2000s. In analyzed period of time 1981–1999, the average 25(OH)D concentrations appeared as high as 52 ng/ml. In this period the optimal and safe preventive dose of vitamin D was set at 2,500 IU a day, and the therapeutic dose at 4,000 IU a day. Interestingly, at that time a mild forms of nutritional rickets were quite often diagnosed in infants supplemented with vitamin D, however, only in cases who also received in their diets the excessive amounts of phosphates (35, 42). In years 2002–2003, 25(OH)D concentration value in 152 newborns and infants appeared still as relatively high and reached on average 49 ng/ml. In the following years the recommended vitamin D dose was decreased to 1,000 IU/day (in the case of breastfed newborns, even to 400 IU), and most likely resulted in a decrease in average 25(OH)D concentrations observed in the next years. The results of our study represent the first data on 25(OH)D concentration and its trends from a perspective of +30 years of an operation of the single diagnostic unit. The similar idea to review historical data have been already utilized, however, both the analyzed time period and the included populations were different. In a similar study conducted by a single laboratory the retrospective analysis of 1957 blood samples collected from 1909 children and adolescents (age 0–17 years) between year 2009 and 2014 revealed median 25(OH)D concentrations for each year from 2009 to 2014 of: 18, 13, 21, 16, 19, and 15 ng/ml, respectively (48). Further, the two time periods 2009–2012 and 2013–2014 that were analyzed before and after increasing recommendations for vitamin D intake in general population (from 200 to 800 IU per day) did not show any trend for change (17 and 17 ng/ml, respectively) (48).

Further, in another study during the period of 10 years (2007–2017), ∼5,000,000 patient samples were tested for 25(OH)D by LC-MS/MS in a single reference laboratory in the USA (49). At the end of summer of 2006, 4.3% of the population being tested had 25(OH)D concentrations <10 ng/ml. This number increased to 8.5% by the end of winter of 2007. Interestingly, after 10 years, a significantly lower percentage of the population had serum 25(OH)D levels <10 ng/ml (0.2 and 3.1% post-summer and post-winter of 2017, respectively). Similarly, the percentage of patients with 25(OH)D concentration range of 10–24 ng/ml decreased steadily between 2007 and 2017. By contrast, the percentage of patients with 25(OH)D concentrations (between 25 and 80 ng/ml) increased from 72.5 to 82.4% post-summer and from 60.6 to 72.9% post-winter during the 10 years period (49). The observation that 25(OH)D concentration values in the general US population have increased during the last decade appeared in opposite to estimated trend noted during 30+ years period by our study, and it has important implications. Population based studies and basic science studies exploring the role of vitamin D metabolism in health and disease pathways have raised public awareness about e?ective modes of vitamin D supplementation that appeared more effective in the USA than in Poland. The very similar approach was also utilized in the study of 74,235 serum 25(OH)D results generated under routine conditions between 2015 and 2016 by Italian and Austrian colleagues who were able to document that females had almost 3 ng/ml higher average 25(OH)D concentration than males, which increased significantly with age (50). 37.9 and 28.3% of males and females, respectively, had vitamin D deficiency defined as 25(OH)D concentration of <20 ng/ml (50).

Our study has some methodological limitations. For example, due to limited number of available data, it was decided to use a possibly controversial method of comparing 20 years-long periods with 1 year-long and 2 years-long periods. Further, the specificity of the analyzed group and the relatively small number of the participants before 1999 are the limitations that should be kept in mind. Nonetheless, our study allowed for estimation of potentially interesting relations, which might be used in further adjustments in the guidelines in order to select the optimal vitamin D supplementation for each age group. The first is the possible relation between skin synthesis (in Poland is restricted to the summer season) and vitamin D status. Taking into account half-life of 25(OH)D (3 weeks), it was assumed that the impact of skin synthesis would be the most evident in Q3 (July-September), and less in Q2 (April-June). However, our study did not reveal any relation between 25(OH)D concentration values and the quarter of the year in which newborns and infants visited the clinic. It was related to a common recommendation to limit the children's skin exposure to sunlight. Despite this, 25(OH)D concentrations higher than 30 ng/ml were observed in every quarter of the year suggesting that cholecalciferol supplementation was maintained throughout the year.

The large variety in individual measurements and relatively high values of standard deviation in the analyzed variables can be explained by the heterogeneity of the group, i.e., presence of patients with disorders of calcium and phosphate homeostasis. The study group included patients that after biochemical and clinical evaluation appeared as healthy (without calcium and phosphate homeostasis disorders) as well as those diagnosed with congenital or acquired disorders, including different types of hyperparathyroidism or hypoparathyroidism, patients suspected for idiopathic infantile hypercalcaemia, hypophosphatemic rickets, etc. The relation between obtained results and the type of disease was not a topic of this survey and was extremely difficult to examine basing on assessable database, what is a limitation of this study.

The analysis of correlation between calcaemia and vitamin D status provided interesting observations. It was revealed that in more than 80% of patients with 25(OH)D <10 ng/ml (severe vitamin D deficiency) Ca levels were within reference range (2.25–2.65 mmol/l). On the other hand, in subgroup with hypocalcaemia (Ca <2.25 mmol/l) severe vitamin D deficiency was not a serious problem. Only 11% of hypocalcaemic patients revealed 25(OH)D concentration values lower than 20 ng/ml, whereas 39% of these patients demonstrated 25(OH)D below 30 ng/ml. In the analyzed subgroup with evident hypercalcaemia (Ca ≥2.75 mmol/l) both very low and high 25(OH)D concentrations were observed, and what seems interesting and potentially meaningful, there was no correlation between the severity of hypercalcaemia and the 25(OH)D concentration values. Moreover, a number of cases with evident hypercalcaemia had 25(OH)D concentrations lower than 10 ng/ml or higher than 50 ng/ml. Using an appropriate set of biochemical and clinical parameters it was possible to identify patients with other characteristic phenotypes of calcium and phosphate homeostasis disorders, e.g., features of hyperparathyroidism.

Another important limitation of our study is related to methodological changes in 25(OH)D measurement over the analyzed time period. In our laboratory, until year 2005, 25(OH)D measurements were carried out using manual protein binding method, and later with the use of automatic Liaison system based on chemiluminescence immunoassay. However, it is worth mentioning that both methods were compared and validated with GLPs, and the high precision of 25(OH)D measurements was confirmed with a DEQAS international quality certificate. Unfortunately, it was not possible to include the more recent 25(OH)D data to indicate possible trends and its changes over the last 15 years, what is limitation of our attempts. Nonetheless, the recent studies conducted in Poland have already uncovered vitamin D status in newborns, infants and toddlers, and confirmed the problem of low 25(OH)D concentrations in the second decade of XXI century. The MAVID RCT study from the Warsaw city area revealed that <10% of mother-newborn pairs had 25(OH)D >30 ng/ml and the median 25(OH)D concentration in newborns was as low as 15 ng/ml (51). In the other study, vitamin D status of newborns and infants was shown to get improved, reaching the 25(OH)D concentration value of 43 ng/ml ± 20 at the 6 months of life (52). Unfortunately, at the 12th month of life, in the same group of infants 25(OH)D concentration values significantly decreased to the mean of 29 ng/ml ($p < 0.0001$), despite that the total vitamin D intake from diet and supplements was close to 1,000 IU/d and was not significantly different at both the 6th and the 12th month of their life. Interestingly, the observed 25(OH)D concentration decrease was related to reduced total vitamin D intake expressed in in international units per kilogram body weight that decreased significantly from 143 IU/kg body weight at the 6th month to 93 IU/kg body weight at the 12th month ($p < 0.0001$) (52).

Further, it was evidenced that in Poland vitamin D deficiency (<20 ng/ml) affects about 17% of children aged 2–3 years (53), in ∼35% of children aged 3–4 years (54) and even in 87% of older children and adolescents, depending on the season of the year and the age group (55).

Nonetheless, more research focused on vitamin D and its metabolites is still required and our study results, due to its limitations, provided only the estimation of unfavorable trends of lowering 25(OH)D concentrations in the youngest kids during the period of 30 years. While a dynamic growth in prospective studies involving children and adults can be predicted, the possibility of the retrospective assessment of data from before 2000 is very limited. The mentioned situation justified analyzing and describing available data from that period, and contributed to the preparation of this study, which also fits in the stream of reports and publications focused on the risk of vitamin D deficiency. What is worth mentioning is that even in that group of children aged 0–36 months, almost 20% revealed vitamin D deficiency expressed as 25(OH)D concentrations below 20 ng/ml. The assessment of the extensive pediatric patient population covering 3 decades can, in our opinion, complement the knowledge of the past and current state and trends in the vitamin D status in very young children residing in Poland.

ETHICS STATEMENT

This study was carried out in accordance to standard diagnostic procedures. The study was conducted basing on medical records of pediatric patients admitted to the consultation clinic of calcium and phosphate homeostasis disorders. Ethics Committee approval was not needed to analyse database.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Vitamin D Insufficiency in Overweight and Obese Children and Adolescents

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Excessive body weight and obesity in childhood and adolescence are becoming more and more important unfavorable factors that entail extremely adverse consequences and require close attention of physicians of any specialty. Along with the high prevalence of obesity and metabolic syndrome in pediatric patients, children and adolescents in the majority of countries are diagnosed with vitamin D deficiency. Among the non-calcaemic effects of vitamin D, a significant role is played by its impact on the hormonal regulation of glucose metabolism and the synthesis of adipokines by fat tissue. The review presents literature data indicative of a close pathogenic relationship between vitamin D insufficiency and impaired tissue insulin sensitivity. It demonstrates the role of vitamin D insufficiency in immune reactions resulting in development of subclinical inflammation in fat tissue infiltrated with macrophages and lymphocytes. It also shows the role of adipokines, immune system cells and pro-inflammatory cytokines produced by them in the pathogenesis of obesity, as well as the function of vitamin D as an endocrine and paracrine regulator of the process of inflammation in adipose tissue. The relationships between the principal adipokines (leptin, adiponectin, resistin) are revealed in the presence of normal vitamin D content and in vitamin D deficiency. The carbohydrate and lipid metabolism parameters in overweight children and adolescents with vitamin D insufficiency are analyzed. A high prevalence of vitamin D insufficiency in overweight and obese children and adolescents (increasing along with the severity of obesity) is demonstrated. The review also presents the current recommendations for the correction of vitamin D insufficiency and underlines the need for higher cholecalciferol doses to achieve serum calcifediol targets in overweight and obese children and adolescents.

Keywords: child obesity, vitamin D, vitamin D and obesity, vitamin D and obesity in children, adipose tissue and autoimmune inflammation

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INTRODUCTION

Prevention of obesity is one of the most important problems of today's medical science, since the rate at which the prevalence of obesity is increasing worldwide indicates a pandemic (1, 2). In 2010, complications related to overweight and obesity resulted in the death of at least 4 million people in the world, in the decrease of the quality of life in 4% of the population every year and

4% of the population become disabled (3). According to WHO data for 2014, 39% of the world's population suffered from excessive weight and 13% from obesity, overweight/obesity afflicted 43 million children under 5 years of age, and this amount is estimated to increase up to 60 million children worldwide by the year 2020 (2). The prevalence of vitamin D deficiency and insufficiency in overweight and obese patients ranges from 5.6% in Canada (4) to 96.0% in Germany (5).

In recent years, there has been a sharp rise in interest in studying the role of vitamin D in the human body. This is due to the fact that there have been accumulated and reappear not only the bone (calcemic) effects of vitamin D, but also completely new effects—non-bone (non-calcemic) (6). According to contemporary views, vitamin D deficiency is associated with an increased risk of diabetes mellitus, arterial hypertension, heart failure, peripheral arterial disease, acute myocardial infarction, various forms of cancer, autoimmune and inflammatory diseases, decreased immune defenses and increased mortality (7). Vitamin D plays an essential role in the regulation of glucose homeostasis, insulin secretion mechanisms, and inflammation associated with obesity (8). Pregnant women, people of color (blacks, Hispanics and anyone with increased skin melanin pigmentation), obese children and adults and children and adults who practice abstinence from direct sun exposure are at especially high risk (9). These studies are the result of understanding that vitamin D is not a vitamin in the classical interpretation. It is a steroidal prehormone with autocrine, paracrine and endocrine action, which through enzymatic processes is consistently transformed into the body into biologically active metabolites that affect various organs and tissues through genomic and non-genomic effects.

PREVALENCE OF OVERWEIGHT AND OBESITY IN CHILDREN AND ADOLESCENTS

The diagnosis and definition of obesity in children is challenging. Obesity is not defined by a standard threshold as it is for adults. Instead, measurements are compared with a reference population. Obesity diagnoses in children are usually determined by calculation of body mass index (BMI). BMI values are then plotted on age-and sex-specific growth charts (10). The Centers for Disease Control overweight is most commonly defined at BMI 85-95 percentile and greater than or equal to 95th percentile for obesity (11). The World Health Organization overweight definition 85–97 percentile and obesity greater than or equal to 97 percentile (12).

Four countries that are leaders in the prevalence of childhood obesity in the world: Greece, USA, Italy and Mexico (13). Most overweight and obese children and adolescents live in economically developed countries, this list is topped by the United States. The prevalence of obesity among American children and adolescents soared dramatically between 1970 and 2000 (from 6.5 to 18.0% in children and from 5.4 to 18.4% in adolescents), and now remains at approximately the same level (4). It is currently estimated that 30% of children in North America are overweight or obese (14).

In economically developed Northern European countries (Denmark, Sweden, Norway), the prevalence of obesity in children remains at approximately the same level among natives and is increasing very significantly among immigrants (15).

A steady rise in obesity prevalence among children is currently seen in countries with medium and low income levels. These countries are following the path trod by economically developed countries 40 years ago, as the prevalence of obesity in their pediatric populations is rapidly growing. The leading country in this list is China where the prevalence rates of obesity among girls and boys increased from 0.45 and 0.16%, respectively, in 1985 to 18.16 and 6.58%, respectively, in 2014 (16). In Eastern European countries (Bulgaria, Croatia, Czech Republic, Hungary, Latvia, Lithuania, etc.), the Russian Federation, and Turkey, the prevalence of obesity (including excessive body weight) is in the range of 14.4–19.2% among boys and 11.8–17.6% among girls (17).

INTERRELATIONSHIP BETWEEN VITAMIN D AND ADIPOSE TISSUE

Vitamin D insufficiency and excessive fat accumulation have mutually negative effects as a result of excessive metabolic processes, enzymatic disorders against a background of decreased activity of alpha-hydroxylase, the key enzyme in the biotransformation of calciferol in a fat-infiltrated liver, resulting in accumulation of inactive forms and decreased bioavailability of vitamin D (8, 18).

In obesity, vitamin D affects insulin secretion, tissue sensitivity to insulin, and systemic inflammation. The direct and paracrine effects of vitamin D lead to VDR activation in pancreatic beta-cells, CYP27B1 expression, and local synthesis of $1,25(OH)_2D$ (18, 19).

Insulin secretion and tissue insulin sensitivity are Ca^{2+}-dependent mechanisms, while vitamin D regulates intracellular concentrations of Ca^{2+} and its passage through the membranes. Additionally, vitamin D positively affects the expression of insulin receptors in peripheral cells and counteracts the systemic immune response by modulating the expression and activity of cytokines (20, 21).

Therefore, the influence of adipose tissue on the metabolism of vitamin D, on the one hand, and its pathogenic role in the obesity development mechanisms, on the other hand, are closely interrelated and represent mutually dependent processes.

Numerous studies have analyzed calcifediol concentrations that may be decreased in obesity. One "superfluous" BMI unit is known to induce a 1.15% reduction in the 25(OH)D concentration (22). In particular, an analysis conducted in 58 obese adolescents demonstrated that a 1% increase in fat weight was associated with a 1.15 ± 0.55 nmol/L reduction in serum calcifediol (23).

There is no consensus as to why calcifediol levels are decreased in obese individuals. The first (and most popular) point of view is that adipose tissue absorbs the fat-soluble vitamin D (24). Some

available data reveal that serum 25(OH)D concentrations show a strong inverse correlation with fat volume and a weaker inverse correlation with BMI (22).

Another hypothesis explains the low 25(OH)D concentrations by the fact that obese people lead a sedentary lifestyle and are less active physically, which entails a decrease in exposure to sunlight and in endogenous synthesis of vitamin D (25).

Other interrelated hypotheses appear to be justified too, specifically that vitamin D metabolism and 25(OH)D synthesis are impaired as a result of hepatic steatosis developing in obesity (26), and that high levels of leptin and IL-6 impair 25(OH)D synthesis by affecting VDR receptors (27).

ADIPOSE TISSUE AND ADIPOSE TISSUE INFLAMMATION

As the body weight grows and the energy balance is positive, the amount of adipose tissue unavoidably increases and its distribution, cell composition, and functions change. An increase in the body's adipose tissue volume results in physiological changes, adipocyte hypertrophy (not hyperplasia), ectopic fat deposition, hypoxia, and chronic stress, which eventually leads to impairment of adipokine secretion. It is adipocyte hypertrophy that plays the key role in the loss of cell insulin sensitivity (28). Hypertrophic adipocytes secrete pro-inflammatory factors (leptin, IL-6, IL-8), as the production of insulin-sensitive adipokines (adiponectin and IL-10) decreases (29).

Adipokines are synthesized by adipocytes and affect carbohydrate and fat metabolism. *In vitro* studies have demonstrated that Ca^{2+} and $1,25(OH)_2D$ regulate the expression of adipokines in visceral adipose tissue, thus leading to an assumption that vitamin D has a modulatory effect on the expression of the genes responsible for secretion of leptin and adiponectin. Protein spectrum studies conducted in obese children, either vitamin D-deficient or with no vitamin D insufficiency, revealed a direct effect of calcitriol that raised adiponectin levels, leading to a conclusion that adiponectin is a key messenger in the mutual influences of vitamin D and progressive obesity in children. According to the majority of authors, adipokines (leptin, adiponectin) are important predictors of impaired sensitivity to insulin, which indirectly decreases gluconeogenesis in the liver, augments glucose transport into the muscles, correlates with the vitamin D reduction, and shows an inverse relationship with insulin resistance (29, 30).

Adipokines include adiponectin, leptin, tumor necrosis factor (TNF-alpha), plasminogen activator inhibitor type I, transforming growth factor (TGF) type I, and resistin (30). Adipokines regulate fat homeostasis by influencing appetite (amount of ingested food), lipid and carbohydrate metabolism, vascular remodeling, and insulin sensitivity (30).

ADIPOSE TISSUE AND ITS EFFECTS ON ADIPOSE TISSUE INFLAMMATION

Adipose tissue is heterogeneous, and contains adipocyte precursors (preadipocytes), nerve endings, blood vessels, and white blood cells. The entire complex is called the "stromal vascular fraction."

In 2003, Xu et al. (31) demonstrated that obesity is associated with a large amount of macrophages in the stromal vascular fraction of adipose tissue. Macrophage migration occurs as a result of impaired functioning of adipose tissue and elevated free fatty acid concentrations (32), production by adipocytes of the proteins chemoattractant-1 and alpha-4 integrin promoting adhesion of macrophages to the endothelial wall, and their subsequent passage through the endothelial barrier (33). Another chemoattractant, LTB4, promotes accumulation of neutrophils in adipose tissue. It is also produced by adipocytes as a result of excessive energy consumption (34). Macrophages accumulate in the visceral pool of adipose tissue. Macrophages migrating into adipose tissue become differentiated in a direction dependent on the volume of the adipose tissue and consequently on the concentration of adipokines generated in adipose tissue. Fat tissue excess is associated with pathological M1-transformation (differentiation) of macrophages. Classical M1 macrophage transformation develops under the influence of T1-helper cells and interferon-gamma or bacterial byproducts. M1-macrophages are pro-inflammatory factors secreting TNF-alpha and IL-1-beta, they have an enormous phagocytic and bactericidal potential (35). On the contrary, Th_2-cells secrete IL-4, IL-10, IL-13 and promote macrophage transformation through the M2 pathway. M2-macrophages have antiparasitic effects, promote tissue repair and remodeling, and secrete the anti-inflammatory mediator IL-10 (36). Accumulation of macrophages in adipose tissue and their inflammatory activity, along with altered balance of pro- and anti-inflammatory cytokines, is a key element in the pathogenesis of diabetes mellitus type 2, cerebrovascular disorders, and non-alcoholic fatty liver disease in patients with obesity (32, 37).

The interactions of immune system cells in healthy adipose tissue and in obesity are shown in **Figure 1**.

Type 2 T-helper cells produce the anti-inflammatory interleukins IL-4, IL-10, and IL-13, which activate M2 macrophage transformation. M2 macrophage transformation is always promoted by T-regulatory cells and eosinophils and mediated by IL-4. M2-macrophages secrete other anti-inflammatory mediators, IL-10, which maintain tissue sensitivity to insulin.

In obesity, Type 1 T-helper cells stimulate M1-macrophage transformation by interferon-gamma; there is also an increased content of other immune cells, B-cells, which synthesize immunoglobulin. As a result, insulin resistance persists. CD8 cells promote macrophage accumulation and augment the expression of pro-inflammatory genes. This results in accumulation of macrophages around dead adipocytes, leading to formation of crown-like structures. M1-transformed pro-inflammatory macrophages secrete TNF-alpha, IL-1-beta, and the marker CD11c.

Obesity-associated insulin resistance is accompanied by elevated levels of pro-inflammatory cytokines, such as TNF-alpha, IL-6, and IL-1-beta (36). Pro-inflammatory cytokines activate intracellular inflammatory pathways, which results in activation of Jan N-terminal kinase−1 (JNK1) and inhibition of kappa-B kinase-beta (IKK-beta). Products of intracellular cytokine activation decrease insulin sensitivity

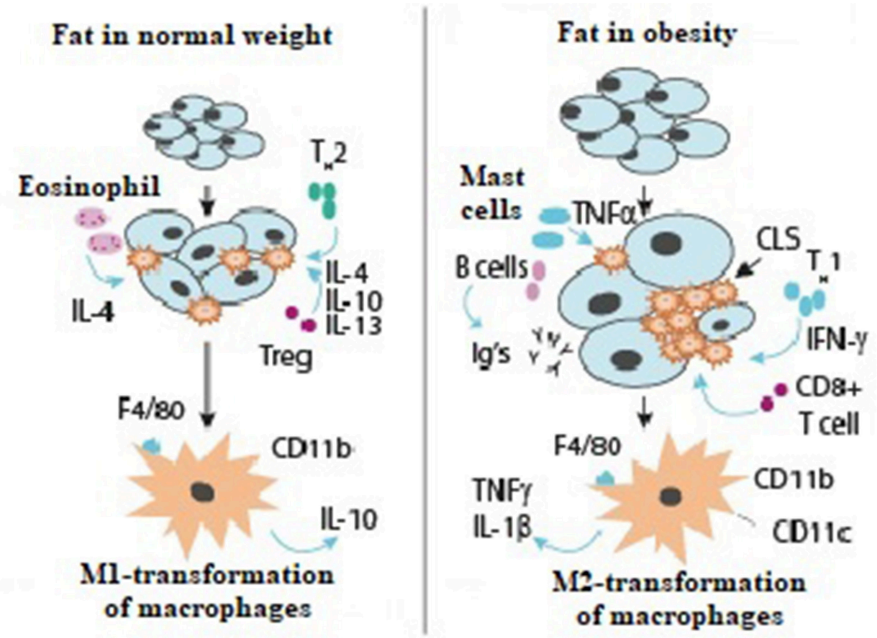

FIGURE 1 | The role of the immune system in healthy adipose tissue and in obesity (37, 38).

of the receptors, thus triggering the development of insulin resistance. The kinase activation in obesity demonstrates how closely interrelated metabolic and immune processes in adipose tissue are. Characteristically, JNK1 and IKK-beta are the kinases activated by inherited immune response mediated by Toll-like receptors (TLR) that can be stimulated by lipopolysaccharides, peptidoglycans, double-stranded RNA, and other microbial components (**Figure 2**) (36).

Jan N-terminal kinase-1 (JNK1) and inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-beta) are inflammatory signaling pathways that promote insulin resistance. Activation of any of the pathways results in phosphorylation of serine, the protein forming a subunit of the insulin 1 receptor, thus counteracting the effects of insulin. IKK-beta also phosphorylates inhibitor of nuclear factor kappa-B (NF-kB), allowing the latter to translocate into the nucleus, bind to the DNA, and activate inflammatory mediators. JNK1 is also able of stimulating the transcription of inflammatory genes in combination with protein transcription activation factor-1 (AP-1). Toll-like receptor-4 (TLR 4), which usually binds lipopolysaccharides (LPS) and saturated fatty acids (FA), promotes activation of JNK1 and IKK-beta. The endoplasmic reticulum (ER) and stress stimulate FA. Excess of nutrients and micro-hypoxia lead to unfolded protein response (UPR). UPR consists of three main pathways: inositol-requiring enzyme (IRE)-1, protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor (ATF) alpha, all of them leading to activation of JNK1 and IKK-beta. Hypoxia also activates a transcription factor, hypoxia-inducible factor-1-alpha (HIF-1α), which induces the expression of different target genes. On the other hand, insulin sensitivity promotes activation of the omega-3 fatty acid receptor (GRP120), which inhibits JNK1 and IKK-beta. PPAR-gamma also augments insulin sensitivity by affecting the NF-kB and AP-1 factors and the subsequent expression of inflammatory genes (36).

The effects of vitamin D on the immune system are multiple and versatile (39). The impact of vitamin D on different elements of immune-mediated inflammation is presented in **Figure 3** (39–42).

MECHANISM OF ACTION OF VITAMIN D IN OBESITY

Vitamin D that was synthesized in the skin or ingested with food enters the systemic circulation and undergoes 2 stages of hydroxylation. The first stage takes place in the liver forming 25(OH)D3, the second stage takes place in the kidneys forming 1,25 (OH) 2D. This active metabolite provides the basic classical (calcemic) effects, phosphorus-calcium metabolism with parathyroid hormone, and when interacting with the VDR (Vitamin D receptor) receptors in the tissues—non-calcemic effects (43). The active metabolite of vitamin D affects the kidneys, regulating the renin–angiotensin–aldosterone system (RAAS), modulates congenital and acquired immunity, exerts effects on adipose tissue and pancreatic beta-cells, alters insulin sensitivity of the cells, and improves the lipid profile. As a result of its influence on the pancreas, and the beta-cells in particular, expression of insulin receptors is increased and insulin sensitivity is augmented.

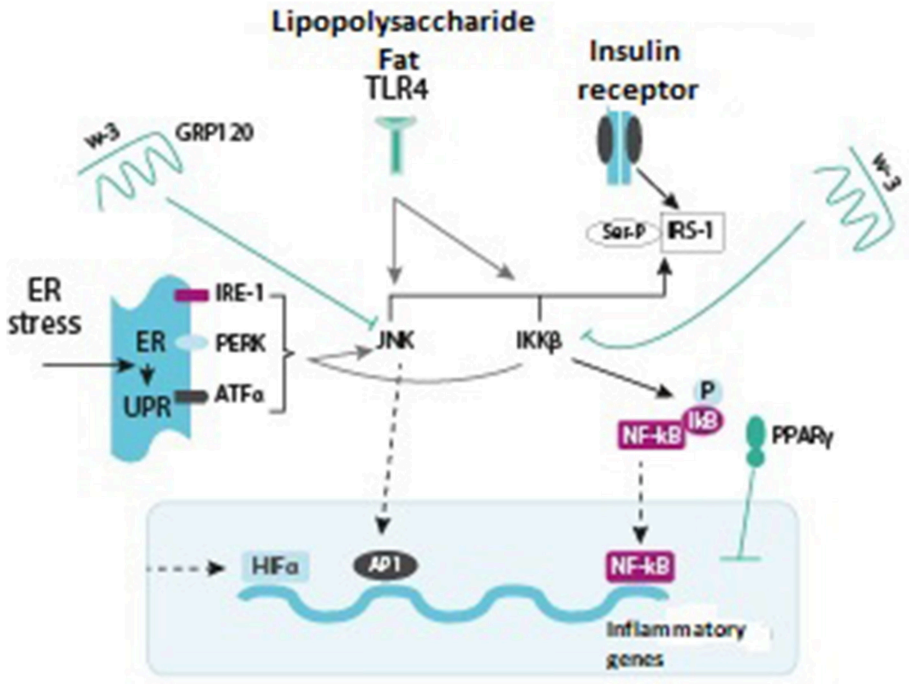

FIGURE 2 | Various signaling pathways stimulating or inhibiting inflammatory signals (green arrows indicate activation, red arrows show inhibition) (36, 37).

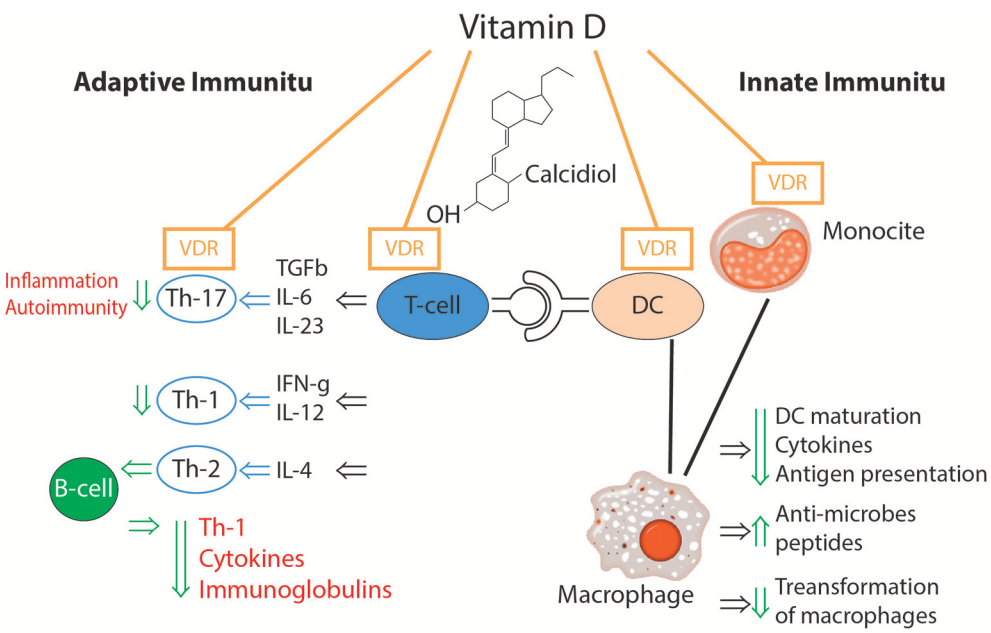

FIGURE 3 | Effects of vitamin D on congenital and acquired immune response (39–42).

In adipose tissue, vitamin D counteracts gluconeogenesis, raises HDL cholesterol concentrations, promotes changes in the adipokine profile, and increases leptin levels. Vitamin D has an important non-calcaemic effect, modifying the risk of diabetes mellitus type 2 and altering the adipokine secretion profile, while not decreasing and not affecting body weight (44). Insulin secretion depends on a number of factors, including calcium (45). Vitamin D affects the function of the

protein calbindin and acts as a modulator of depolarization-stimulated insulin release by re-distributing intracellular calcium (46). Vitamin D has an effect on insulin sensitivity through a number of mechanisms: by stimulating the expression of insulin sensitivity genes (19, 47), by interacting with the VDR-receptor located in the cell nucleus. The result is an increase in the transcriptional activity of the insulin receptor gene increasing the total number of insulin receptors while not changing their affinity (48). $1,25(OH)_2D$ can also augment insulin sensitivity by activating peroxisome proliferator-activated receptor delta (45). Vitamin D insufficiency also leads to elevated parathyroid hormone concentrations, decreased insulin sensitivity, activated lipogenesis, and an increase in fat mass (49). Vitamin D indirectly affects insulin resistance through the RAAS (50).

$1,25(OH)_2D$ after binding the receptor forms a heterodimer with retinoid X receptor (RXR) and translocates to the nucleus, where this complex interacts with specific DNA regions, called vitamin D-responsive elements. By additional interactions with coregulatory proteins, the VDR–RXR complex regulates approximately 3 percent of the human genome (51).

VDR as a member of steroidhormone receptor super family, it has an essential role in modulating immune response and inflammation via binding with its counter ligand vitamin D. The complex of vitamin D and its receptor controls the B-cell insulin secretion (52, 53). VDR is reported to be expressed in human subcutaneous adipose tissue and visceral adipose tissue (54) and human mammary adipocytes (55). VDR are widely distributed along several body tissues, their gene polymorphisms may affect the risk of vitamin D-related metabolic disorders, and could adjust the receptor effectiveness according to vitamin D status (56, 57). Primarily 4 VDR polymorphisms, including the rs10735810 FoKI SNP and 3 additional ones (the rs7975232 ApaI, the rs1544410 BsmI, and the rs731236 TaqI), have been analyzed in relation to genetic predisposition to obesity; however, findings are contradictory (56). There are now studies that confirm both the positive and negative relationship. The Correa-Rodríguez M study in Spain conducted on Caucasian young adults population (58), Hasan et al. (59) study on the Arab adults residing in the United Arab Emirates; Rahmadhani R in Malaysia (60), did not demonstrate a reliable association with obesity, but found association with the metabolic syndrome components, reduced vitamin D levels, insulin resistance (60), and FokI and BsmI—with systolic blood pressure (59). Ruiz-Ojeda in the 1,020 review draws the following conclusions: obesity reported that associations with VDR polymorphisms could be related to either a direct effect of vitamin D in adipocyte differentiation and metabolism, or an indirect effect by modulation of insulin secretion (56).

The effect of vitamin D on inflammation in obesity is made up of the following components: *In vitro*, 1,25(OH)2D inhibits chronic inflammation resulting from obesity, the active metabolite of vitamin D 1,25 (OH) 2D inhibits the pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-12 (39–42, 44, 61), reduces inflammatory activity in adipocytes (62) and reduces inflammation in visceral adipose tissue, while not reducing in subcutaneous fat tissue (62, 63). In obese people a reduced adenosine monophosphate-activated protein kinase and it is closely associated with adipose tissue inflammation. Adenosine monophosphate-activated protein kinase enhances sirtuin 1 by increasing NAD/NADH ratio and decreases adipose tissue macrophage infiltration and inflammation, both have been proposed as key regulators to prevent obesity and obesity-related metabolic dysfunction (64). A 5-year observational study in overweight and obese patients revealed decreased TNF-alpha levels in individuals with normal vitamin D content, as well as a reduction of adipose tissue inflammation (65). In turn, TNF-α regulates the activity of three miRs (miR-146a, miR-150, and miR-155) in adipocytes (66). Vitamin D exerts anti-inflammatory effects mediated by the inhibition of the NF-κB and mitogen activated protein kinase signaling pathways (66), reduced toll-like receptor expression (67). The latter are transmembrane proteins that trigger classical cascade reactions leading to the activation of TNF-alpha (68). The active vitamin D metabolite has an effect on the regulation of NF-kB, the principal transcriptional factor for TNF-alpha; it also blocks the differentiation of dendritic cells and inhibits lymphocyte proliferation (69). *In vitro*, $1,25(OH)_2D$ regulates the differentiation of macrophages, suppresses IL-6, and increases the level of the mRNA factor that affects macrophage transformation (70).

Exerting an immunoregulatory effect, vitamin D helps modulate immune response in adipocytes (54) by changing the concentrations and secretion profiles of adipokines (71), inhibits adiponectin synthesis (72), and increases leptin synthesis (73). The NHANES III trial (74) demonstrated that serum calcidiol levels above 21 ng/mL help reduce the C-reactive protein concentration. Vitamin D counteracts the systemic inflammation effect in patients with type 2 diabetes through a number of mechanisms. $1,25(OH)_2D$ protects pancreatic beta-cells from cytokine-induced apoptosis, affecting the expression and activity of the cytokines (69). The mutual effects of vitamin D insufficiency and obesity are specific in that, apart from abnormal glucose regulation parameters (75, 76), increased HOMA-index values, dyslipidaemia (77, 78), and elevated systolic blood pressure (77), afflicted children are at increased risk for developing atherosclerosis at an early age (79).

A study that was conducted in the Russian Federation by a group of investigators headed by I. L. Nikitina and evaluated the effects of vitamin D in overweight and obese patients yielded data indicating that vitamin D insufficiency in such children aggravates insulin resistance and promotes lipid profile disturbances (80).

One of the latest meta-analyses that examined the association between vitamin D supplementation and systemic inflammation in patients with diabetes mellitus type 2 demonstrated that dietary cholecalciferol supplementation helps achieve a significant reduction in the activity of inflammation, and also confirmed the data showing that sufficient vitamin D levels help decrease C-reactive protein and TNF-alpha concentrations, decrease ESR, leptin concentrations (73).

Figure 4 demonstrates the mechanism of action of vitamin D on different pathogenic elements in obesity (44, 81–83).

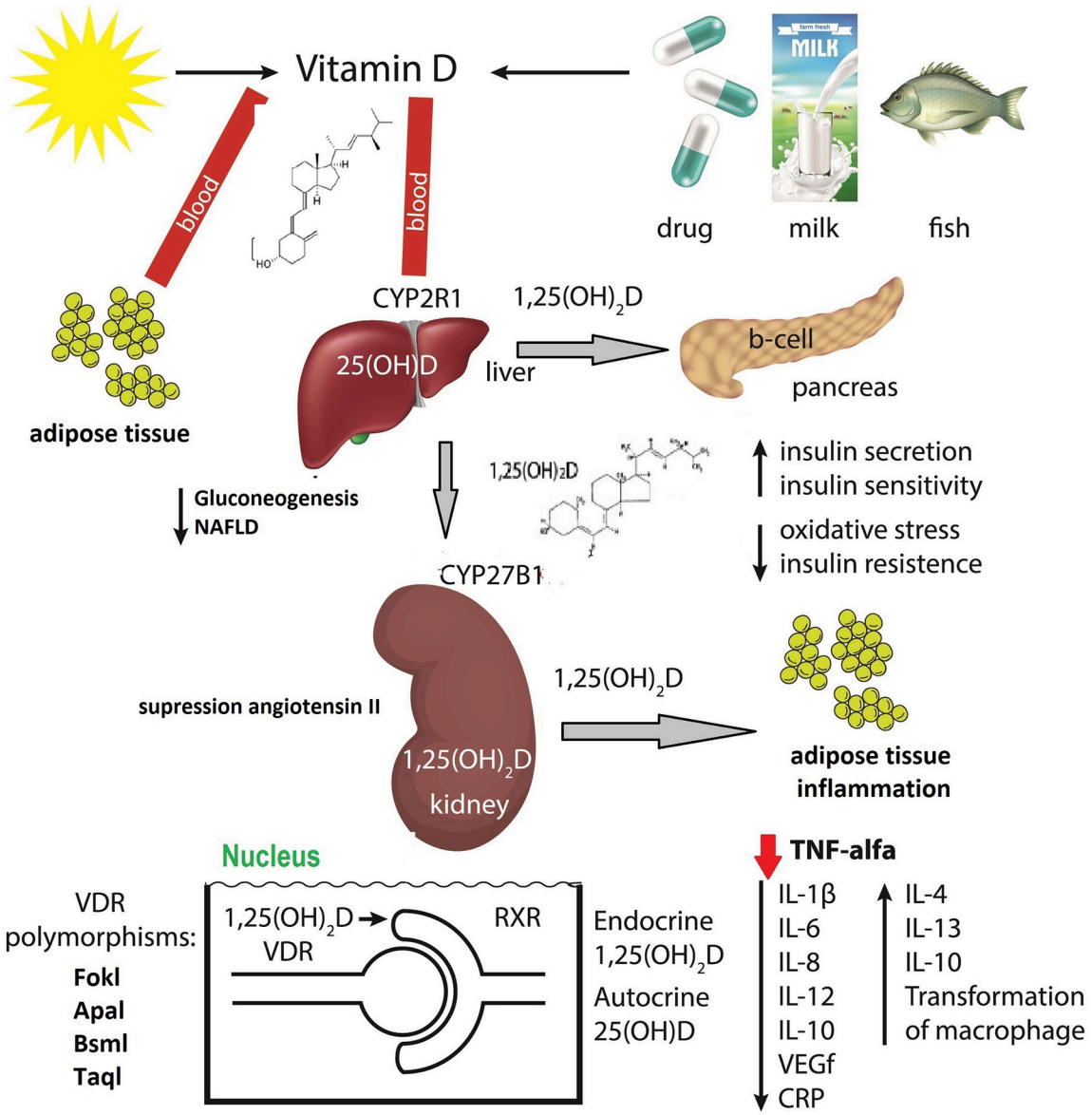

FIGURE 4 | The mechanism of action of vitamin D in obesity (44, 81–83).

PREVALENCE OF VITAMIN D INSUFFICIENCY IN OVERWEIGHT AND OBESE CHILDREN AND ADOLESCENTS

The association between vitamin D insufficiency and obesity has been extensively investigated in adults. The largest meta-analysis performed by Chinese investigators in 2015 demonstrated a high risk of developing vitamin D insufficiency (84).

The meta-analysis, which included 15 studies (3,867 obese individuals and 9,342 healthy subjects), demonstrated a pronounced difference in vitamin D insufficiency prevalence among obese patients, the OR (95%) was 3.70 (2.33–5.06) (**Figure 5**).

The prevalence of vitamin D insufficiency in overweight and obese children and adolescents has been investigated in rather good detail, but no dedicated meta-analyses have been carried out. **Table 1** summarizes the prevalence of vitamin D insufficiency in groups of overweight and obese children by region and vitamin D sufficiency or insufficiency.

Analyzing the data presented in **Table 1**, one can conclude that the prevalence of vitamin D insufficiency in groups of overweight and obese children is very high. The prevalence of vitamin D insufficiency will be even higher if the vitamin D insufficiency threshold is raised to 75 nmol/L (30 ng/mL), as recommended by the United States National Endocrine Society (107).

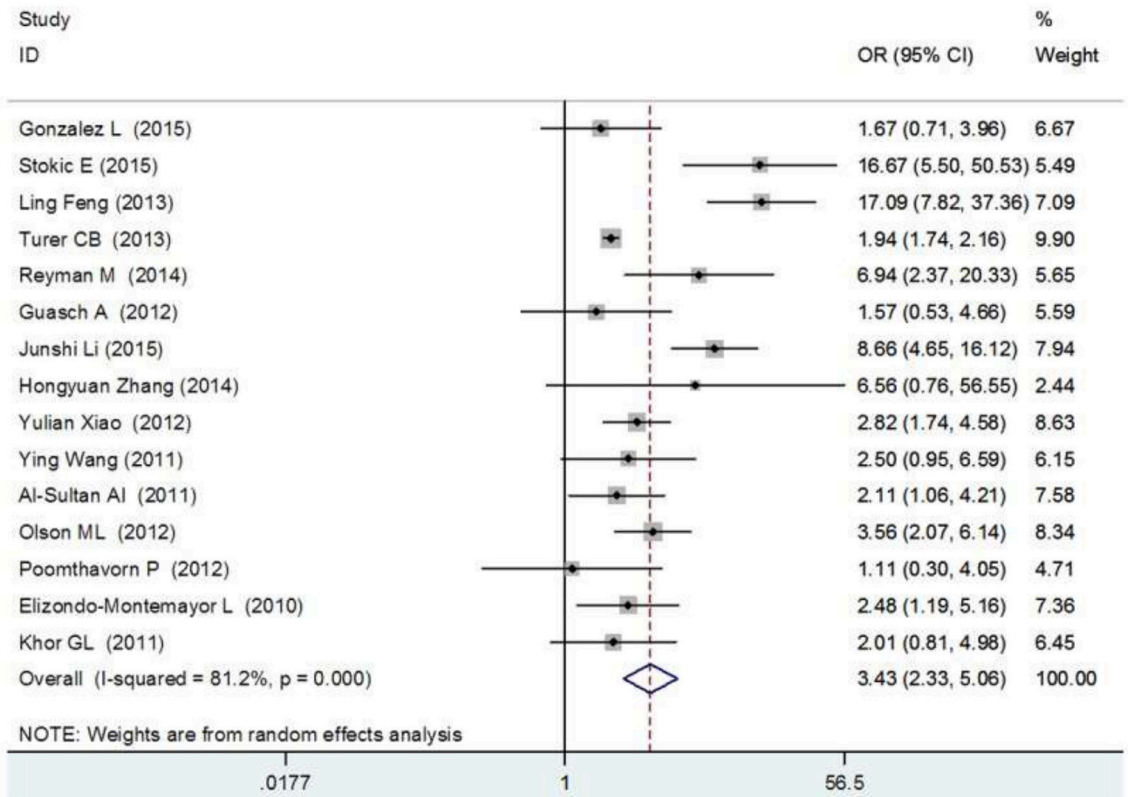

FIGURE 5 | Forest plot between vitamin D deficiency and obesity (84).

This makes northern countries with low sun exposure levels, which are likely to suffer from vitamin D deficiency, fortify foods with vitamin D, as demonstrated in some studies in Canada (85), and recommend prophylactic use of cholecalciferol at high doses and for a long period, as demonstrated in a population of healthy children in Arkhangelsk (The Russian Federation) (108), but overweight children still receive insufficient attention (101).

VITAMIN D DOSES IN OVERWEIGHT AND OBESE CHILDREN

There is no conventional dose universally recommended for the treatment of vitamin D insufficiency in overweight and obese children. A number of individual recommendations are available as part of national consensus documents on the treatment of vitamin D insufficiency or included in a number of prospective studies. In particular, the "National programme for vitamin D insufficiency in children and adolescents in the Russian Federation: the state-of-the-art approaches to treatment" recommends determination of the serum calcifediol concentration in children with excessive body weight or obesity, or administration of the maximum prophylactic doses when such measurement is unfeasible (109).

The Committee on Nutrition of the French Society of Pediatrics recommends administration of vitamin D 80,000 IU single doses and 100,000 IU single doses in the winter months (November and February) for obese children aged 5–10 years or uninterrupted supplementation over the age interval of 1–10 years (110).

The United States Endocrine Society, which published its guidelines on the evaluation, treatment, and prevention of vitamin D deficiency in 2011, recommended a twofold increase in the therapeutic dose of cholecalciferol for overweight and obese patients and setting the calcifediol target at 75 nmol/L (30 ng/mL), with subsequent switching to a maintenance dose (107). The recommended therapeutic dose for *healthy* children aged 1–18 years is 2,000 IU/day for 6 weeks or 50,000 IU once weekly for 6 weeks, and the recommended maintenance dose is in the range of 600–1,000 IU/day.

One of the studies enrolled 18 obese adolescents (median BMI: 32.2 kg/m^2) and 18 non-obese adolescents (median BMI: 20.1 kg/m^2), who received cholecalciferol 2,000 IU/day over a period of 12 weeks and afterwards had their 25(OH)D level determined. Vitamin D insufficiency and deficiency (<75 mmol/L and <30 ng/mL, respectively) was diagnosed in 78.0 and 61.0% of the patients, respectively. After the 12-week therapy, calcifediol concentrations were normalized in 89.0% of healthy subjects and only in 50.0% of obese adolescents. In their conclusion, the investigators recommend a dose increase for adolescents with obesity (111).

TABLE 1 | Prevalence of vitamin D insufficiency in groups of overweight and obese children by region and vitamin D sufficiency or insufficiency.

	Vitamin D insufficiency level	Prevalence of vitamin D insufficiency among overweight/obese children	Reference number
AMERICA			
Canada	<50 nmol/L (<20 ng/mL)	5.6% (77.0%–consumption of vitamin D-fortified milk)	(85)
Canada	<75 nmol/L (<30 ng/mL)	93.0%	(86)
Canada	<75 nmol/L (<30 ng/mL)	76.0%	(87)
Mexico	<75 nmol/L (<30 ng/mL)	36.0%	(88)
USA, *New York*	<50 nmol/L (<20 ng/mL)	55.0%	(89)
USA, *Brooklyn*	<50 nmol/L (<20 ng/mL)	55.2%	(90)
USA, *Alabama*	<50 nmol/L (<20 ng/mL)	78.4%	(91)
USA, Pennsylvania	<75 nmol/L (<30 ng/mL)	27.8% (5–9 years) 35.4% (10–14 years) 50.9% (↑ 15 years)	(92)
USA, *Wisconsin*	<50 nmol/L (<20 ng/mL)	32.3%	(93)
AFRICA			
Ethiopia	<50 nmol/L (<20 ng/mL)	42.0%	(94)
EUROPE			
Denmark	*Deficiency* <30 nmol/L (<12 ng/mL)	16.5%	(95)
Germany	<75 nmol/L (<30 ng/mL)	96.0%	(5)
Greece	<50 nmol/L (<20 ng/mL)	obesity—60.5% overweight—51.6	(96)
Norway	<75 nmol/L (<30 ng/mL)	50.0%	(97)
Spain	<75 nmol/L (<30 ng/mL)	morbid obesity—81.1% obesity—68.2% overweight—55.0%	(98)
Sweden	<50 nmol/L (<20 ng/mL)	33.2%	(99)
The Netherlands	<50 nmol/L (<20 ng/mL)	24.5%	(100)
The Russian Federation, *Arkhangelsk*	<75 nmol/L (<30 ng/mL)	90.0%	(101)
The Russian Federation, *Saint Petersburg*	<75 nmol/L (<30 ng/mL)	92.0%	(102)
ASIA			
Iran	<75 nmol/L (<30 ng/mL)	95.6%	(103)
Malaysia	<50 nmol/L (<20 ng/mL)	obesity—19.2% overweight—17.4%	(104)
Turkey	25–50 nmol/L (<20 ng/mL)	23.0%	(105)
China	<75 nmol/L (<30 ng/mL)	48,6%	(106)

In another trial conducted in 68 obese adolescents (median BMI: 38.0 kg/m^2), administration of vitamin D 50,000 IIU once weekly for a period of 6–8 weeks allowed normalization of 25(OH)D concentrations only in 28.0% of the adolescents, while a repeated course of the same duration and at the same dose level produced no significant changes in the remaining 72.0% (112).

A study that was conducted in the Russian Federation (Saint Petersburg) by I. L. Nikitina, which enrolled children with obesity (median BMI: 29.6 kg/m^2), revealed low vitamin D concentrations (<75 nmol/L, <30 ng/mL) in 92.0% of subjects. Supplementation with cholecalciferol 1,500 IU/day for 3 months, followed by 2,000 IU/day for 3 months, helped normalize calcifediol concentrations in 41.0% of the children. Calcifediol levels returned to normal within the first 3 months, and subsequent supplementation at a higher dose over the same period of time did not change the vitamin D insufficiency rate (80).

SUMMARY AND CONCLUSION

The high rates of excessive body weight and obesity observed worldwide and vitamin D insufficiency are closely interrelated problems of today's medicine that indicate a pandemic and have deleterious health effects in large patient populations.

Excessive body weight results in accumulation of adipose tissue, impaired adipocyte function, development of adipocyte hypertrophy, and an altered adipokine secretion profile. These changes result in migration and transformation of macrophages and in the development of adipose tissue inflammation. As a result of this inflammation, the synthesis of pro-inflammatory cytokines (TNF-alpha, IL-6, and IL-1β) becomes increased and insulin resistance develops.

Vitamin D has a modulatory effect on the expression of the genes responsible for secretion of leptin and adiponectin. *In vitro*, 25(OH)D metabolites inhibit chronic immune-mediated inflammation by suppressing the production of the pro-inflammatory cytokines IL-1β, IL-6, and IL-8.

Long-term monitoring of obese patients receiving vitamin D supplementation revealed an improvement of the adipose tissue inflammation that was a result of inhibited TNF-alpha activity. Vitamin D supplementation in patients with diabetes mellitus type 2 helps decrease C-reactive protein and TNF-alpha concentrations, decrease ESR, and increase leptin concentrations.

The prevalence of vitamin D insufficiency among children and adolescents with obesity is extremely high: 96.0% in Germany, 78.4% in the United States, and up to 92.0% in the Russian Federation.

Despite the consensus achieved with regard to the need to treat vitamin D insufficiency in obese patients, there is no common point of view on the dosage and duration of cholecalciferol administration appropriate for vitamin D supplementation. Currently available data on the treatment of vitamin D insufficiency in obese children and adolescents are contradictory; however, in the overwhelming majority of cases these data allow not only an increase in calcifediol levels but also

a positive effect on carbohydrate and lipid metabolism, as well as on the secretion of adipokines.

AUTHOR CONTRIBUTIONS

IZ: chief of our group; LK: the mechanism of action of vitamin D in obesity; VK: effects of vitamin D on congenital and acquired immune response; IN: prevalence of vitamin D insufficiency in overweight and obese children and adolescents, vitamin D doses in overweight and obese children; SM: prevalence of vitamin D insufficiency in overweight and obese children and adolescents, vitamin D doses in overweight and obese children; SD: prevalence of vitamin D insufficiency in overweight and obese children and adolescents; AK: adipose tissue and its effects on autoimmune inflammation; RA: prevalence of vitamin D insufficiency in overweight and obese children and adolescents; MS: interrelationship between vitamin D and adipose tissue; AT: adipose tissue and autoimmune inflammation; GK: effects of vitamin D on adipokine levels and metabolic specifics in overweight and obese children; AL: prevalence of overweight and obesity in children and adolescents.

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Vitamin D Deficiency and Immune Disorders in Combined Endocrine Pathology

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Introduction: Combined endocrine pathology is a serious healthcare problem in Ukraine. This prospective study assessed the blood levels of 25-hydroxyvitamin D [25(OH)D] and markers of immune function in response to vitamin D intervention in patients with type 1 and type 2 diabetes mellitus (T1DM and T2DM, respectively) and autoimmune thyroiditis (AIT).

Objective: This study evaluated the relationship between the metabolic and immune status of DM + AIT patients with respect to their vitamin D status and changes after vitamin D_3 supplementation.

Material and Methods: Patients with type 1 or type 2 DM in combination with AIT and decreased circulating levels of 25(OH)D were divided into two groups of 30 patients each. All patients with AIT were euthyroid and receiving hormonal replacement therapy. The levels of carbohydrate and fat metabolism markers, Immunologic markers, namely, Th1-type cytokines [interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-6, IL-12], Th2-type cytokines (IL-4, IL-5), IL-10, and IL-17 were measured before and after vitamin D_3 supplementation. The vitamin D status was assessed according to the level of 25(OH)D.

Results: Patients with combined endocrine disorders (DM and AIT) with a decreased vitamin D status had significantly increased background concentrations of Th1-type cytokines and reduced concentrations of Th2-type cytokines (IL-4 and IL-5), IL-10, and IL-17. The results of our study showed that vitamin D_3 supplementation in patients with T1DM and T2DM reduced the concentrations of the inflammatory Th1-type cytokines and increased the levels of Th2-type cytokines.

Conclusion: The presence of two endocrine diseases, aggravated by decreased circulating levels of 25(OH)D, leads to disorders wherein the immune status is markedly changed. These decreased levels of 25(OH)D contribute to an autoimmune inflammatory process and to the progression of complications in addition to the metabolic disorders. A vitamin D intervention resulted in significant changes in the blood levels of 25(OH)D that are related to parameters of autoimmunity and glucose metabolism. Vitamin D_3 supplementation should be considered for the prevention and treatment of combined endocrine pathology.

Keywords: diabetes mellitus, autoimmune thyroiditis, 25-OH D, vitamin D_3 supplementation, immune disorders, cytokine profile, Chernobyl disaster

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INTRODUCTION

The incidence of a combination of endocrine diseases is highly prevalent worldwide. Diabetes mellitus (DM) with coinciding thyroid diseases is a common combination, affecting 17–30% of the cases in the clinical endocrinology practice in Ukraine (1, 2).

Type 1 diabetes (T1DM) is often associated with autoimmune diseases, such as autoimmune thyroid disease (ATD), celiac disease, autoimmune gastritis, pernicious anemia, and vitiligo (3). ATD is the most prevalent endocrinopathy among patients with diabetes (1, 4). The prevalence of ATD during the course of T1DM was as high as 60% of patients, 40% of whom additionally suffered from thyroid disorders including overt hypothyroidism (24%), subclinical hypothyroidism (8%), and hyperthyroidism (8%) (5). According to Krzewska et al. the ATDs Hashimoto's thyroiditis and Graves' disease, are the most prevalent autoimmune diseases in children and adolescents with T1DM. Their incidence is an estimated as 2–4-fold higher than those in the general population, with Hashimoto's thyroiditis the most common clinical form (14–28%) (4). T1DM is frequently associated with autoimmune endocrine and non-endocrine diseases and patients with T1DM are at a higher risk of developing several glandular autoimmune diseases. Familial clustering has been observed, suggesting a genetic predisposition. Various hypotheses of viral- and/or bacterial-induced pancreatic autoimmunity have been proposed; however, a definitive description of the autoimmune pathomechanism is still lacking (5, 6).

Furthermore, there is a deep underlying relationship between diabetes mellitus type 2 (T2DM) and thyroid dysfunction (6). Numerous studies have reported the complex intertwining of biochemical, genetic, and hormonal malfunctions characteristic of this pathophysiological association (4–7). The prevalence of thyroid disorder in a population affected with diabetes was 13.4% and appeared more prevalent (31.4%) in female T2DM patients compared to that in male T2DM patients (6.9%) (1).

Over the past 20 years a steady increase in the incidence of combined endocrine diseases has been observed in Ukraine (8). In addition to this disadvantageous epidemic trend is the substantial healthcare burden for Ukraine. The increasing prevalence of combined endocrine diseases most likely reflects the late health consequences of the Chernobyl disaster (April 26th, 1986), especially radioactive iodine that affected the thyroid gland. In the first decade after the Chernobyl disaster, the combination of diabetes with diffuse toxic goiter predominated; later, the prevalence of diabetes coinciding with thyroiditis increased significantly. The formation of nodules in the thyroid gland in older adults began to increase (9–11). According to Institute of Endocrinology and Metabolism data during the 5 years preceding the Chernobyl nuclear accident, a total of 59 cases of thyroid carcinoma were identified in the birth to 18 years age group (25 cases in those aged 14 years of less and 34 cases in adolescents aged 15–18 years). Between 1986 and 1997, there was a total of 577 of thyroid carcinomas in Ukrainian children and adolescents (358 and 219 cases, respectively). The largest number of cases occurred in patients living in areas of thyroid radiation doses of at least 0.50 Gr. Thyroid cancers developed after a short latent period, were more aggressive at presentation, and expressed regional (57.3%) or distant (14.5%) metastasis. Solid papillary cancers were present in 93.1% and coexisting chronic thyroiditis in 10.2% of cases (10).

Foley et al. focused on the long-term effects of the Chernobyl catastrophe, particularly as an unprecedented event that affected the endocrine system in individuals of all ages and which has provided data on the effects of radiation on humans, including the acceleration of thyroid cancer (12). The first years following the Chernobyl disaster were notable for several striking epidemiologic observations. First, a significant increase of incidence of pediatric thyroid cancer was reported in the two countries that were most contaminated by the release of radioiodine from the damaged nuclear reactor: Ukraine, the site of the Chernobyl plant, and Belarus, located directly to the north. The increased incidence of the thyroid cancer was particularly evidenced in individuals aged 0–4 years at the time of exposure and adults at the time of Chernobyl accident were markedly less affected. From the Ukrainian clinical perspective, the majority of thyroid carcinomas (more than 90%), noted in patients in their growth and maturation periods at the time of the Chernobyl accident, were diagnosed with papillary carcinoma (13).

The autoimmune pathology in clinical practice seems a difficult task for endocrinologists, with many unknowns. Modern laboratory diagnosis with high sensitivity allows the detection of autoantibodies in DM and autoimmune thyroiditis (AIT) and the monitoring of a wide spectrum of markers of the immune status of patients for diagnostic research and treatment. Unfortunately, during the treatment course, it is often very difficult or impossible to effectively reduce auto-aggression (1, 14).

T1DM and T2DM, as well as AIT in combination, have a constant tendency to progress the so-called "Chernobyl footprint" regions. As a result of both therapeutic difficulties and the relatively high prevalence of cases with these combined endocrine diseases in Ukraine, it is important to identify alternative methods to improve the treatment course and clinical outcomes. Among the promising agents and possible methods, proper vitamin D supply offers promise due to its low cost and easy accessibility.

25-hydroxyvitamin D [25(OH)D] is involved in the regulation of many physiological processes in the body and beneficial effects related to treatment with cholecalciferol were noted in immunodeficiency, cardiovascular disorders, anemia, diabetes, various pathologies of the liver, and gastrointestinal tract disorders, as well as for tuberculosis and malignant tumors of the breast and intestine (15, 16). Both genetic predisposition and environmental factors may contribute to the development of autoimmune diseases. Increased levels of immune inflammation markers may affect the course of immuno-endocrine pathology. A gradual development of the autoimmune inflammatory process, when combined with hypothyroidism and DM, may significantly contribute to the development of endothelial dysfunction and the consequent development of vascular complications. Therefore, the aim of our study was to determine the background concentrations of cytokines in patients with combined pathology–T1DM or T2DM and AIT in the context of vitamin D status. An additional objective of the study was to

investigate the protective effect of vitamin D supplementation on the progression of the immune process.

PATIENTS AND METHODS

Study Group

The study group consisted of 60 patients (21 males) aged ≥ 20 years with diabetes and AIT. All patients with AIT included in this study were euthyroid and on levothyroxine hormonal replacement therapy with thyroid-stimulating hormone (TSH) levels within the reference ranges. Among the 60 patients with AIT, 30 each had T1DM and T2DM. The TSH levels and levothyroxine dose did not change significantly during the study period. There were no statistically significant differences in the patients' body mass index (BMI) during the study period.

A separate group of 40 patients with T1DM or T2DM only comprised the control group. Patients who reported taking calcium and vitamin D supplements during the 6 months before the study were excluded. The study group as a whole was evaluated and treated at two in-patient departments of the Kyiv city Center of Endocrinology and Metabolism in Ukraine.

Table 1 describes the general characteristics of the patients included in the study and control groups.

METHODS

Clinical Assessment and Laboratory Investigations

The size and structure of the thyroid gland were evaluated by palpation and by ultrasound investigation. The evaluation of thyroid size by the palpation method was carried out in accordance with the WHO classification (2001) that includes three stages. According to the results of the ultrasound examination, goiter was diagnosed if the thyroid volumes in women and men exceeded 18 and 25 mL, respectively. Ultrasonography of the thyroid gland and volume determinations were performed using an HD 11 XE ultrasound apparatus (No. 453561262961, Philips, Amsterdam, the Netherlands) with an 8-MHz linear sensor. The volume of the thyroid lobes was calculated according to the formula proposed by Brunn (7):

$$V = (\text{length} \times \text{width} \times \text{thickness}) \times 0.479$$

The functional status of the thyroid gland was evaluated based on the levels of thyroid-stimulating hormone (TSH), free thyroxine (fT4), and free triiodothyronine (fT3). The presence of AIT was confirmed by an increased thyroperoxidase (ATPO) titer.

The assay for 25(OH)D measured the total 25(OH)D, including both 25-hydroxyvitamin D_2 and D_3. The 25(OH)D concentration was assessed using a quantitative enzyme immunoassay kit (25-OH D IDS OCTEIA, Immunodiagnostik, Bensheim and Biomedica, Wien, Austria) with an intra-assay precision of <8% and inter-assay precision of <10%. The vitamin D status was assessed using the following criteria: 25(OH)D concentrations <75 nmol/L indicated insufficiency, while concentrations <50 nmol/L indicated vitamin D deficiency.

The glycated hemoglobin (HbA1c) level was determined by immuno-turbidimetry using standard test systems on an Advia 1800 biochemical analyzer, Siemens (Munich, Germany).

Determination of the concentrations of TSH, fT4, fT3 and antibodies to ATPO were performed by immunoassay analysis with standards on an Advia 1800 biochemical analyzer (Siemens, Munich, Germany).

The lipid spectrum of the blood was assessed by colorimetric (for cholesterol) and spectrophotometric (for triglycerides) methods on the Advia 1800 analyzer (Siemens, Munich, Germany).

The total calcium level was determined by colorimetric method on the Advia 1800 and ionized calcium by ion-selective method using the ion-selective electrolyte analyzer on an EasyLyte instrument (Medica Corporation, Bedford, MA, USA).

Determination of insulin concentration and parathyroid hormone (PTH) level was performed by immunoassay using standard sets of test systems on a Centaur biochemical analyzer (Siemens, Munich, Germany).

The levels of Th1-type cytokines (interferon [IFN]-γ, tumor necrosis factor [TNF]-α, interleukin [IL]-2, IL-6, IL-12), Th2-type cytokines (IL-4, IL-5), and IL-10 and IL-17 were measured by flow cytometry.

Study Protocol

Patients underwent traditional treatments for DM and hypothyroidism and received vitamin D_3 supplements at a dose of 2,000–4,000 IU/day, depending on the blood levels of 25(OH)D and the presence or absence of chronic complications of diabetes (in particular, diabetic retinopathy) and obesity (16).

All patients with BMI <29.9 kg/m^2 (24 kg/m^2 for T1DM+AIT patients and 22 kg/m^2 for T2DM+AIT patients) with 25(OH)D deficiency or insufficiency, as well as 14 patients with different grades of obesity, started vitamin D_3 supplementation at a dose 4,000 IU/day. In cases that reached a 25(OH)D concentration of 72.5 nmol/L after the first course of supplementation, the dose was changed to 2,000 IU/day (about 10% of patients). This study used a certified vitamin D preparation with the highest bioavailability, since this preparation is a cholecalciferol protein complex. It was synthesized at the Institute of Biochemistry of Ukrainian National Academy of Medical Sciences.

The vitamin D_3 preparations were prescribed for a period of 2 months (except for the summer period) at intervals of at least 3 months, with the course repeated twice each year.

Patients were examined after 10+/− 1 months with a re-examination of the levels of the metabolic and immune markers. Traditional hypoglycemic therapy in DM patients was not changed significantly during this trial as well as replacement levothyroxine therapy.

The results were analyzed using comparative analysis and variation statistics with calculation of the frequency characteristics of the parameters (P), the mean values, and the variability (standard deviation [SD]). The statistical significance of the differences the between treatment groups for the studied parameters was assessed using Wilcoxon-Mann-Whitney tests. $P < 0.05$ were considered statistically significant.

This study was carried out in accordance with the recommendations of the Ethics Committee of Bogomoletz

TABLE 1 | Characteristics of the patient and control group (mean ± *SD*).

	T1DM + AIT		T2DM + AIT		T1DM		T2DM	
	Male	Female	Male	Female	Male	Female	Male	Female
n	12	18	9	21	9	11	10	10
Age, yrs	36 ± 5	45 ± 7	57 ± 8	61 ± 9	33 ± 7	44 ± 6	57 ± 7	54 ± 7
BMI, kg/m^2	30 ± 4	33 ± 4	36 ± 4	39 ± 4	30 ± 4	32 ± 5	37 ± 4	37 ± 6
AIT duration, yrs	6 ± 2	6 ± 2	4 ± 2	8 ± 2				
DM duration, yrs	9 ± 5	12 ± 5	9 ± 5	16 ± 4	10 ± 4	12 ± 5	10 ± 2	14 ± 3

T1DM, diabetes mellitus type 1; T2DM, diabetes mellitus type 2; AIT, autoimmune thyroiditis; BMI, body mass index; DM, diabetes mellitus.

TABLE 2 | Baseline values of metabolic markers and 25(OH)D concentration evaluated in patients with Diabetes Mellitus type 1, 2 and combined with Autoimmune Thyroiditis, (mean ± *SD*).

Indicators	T1DM	T1DM + AIT	T2DM	T2DM + AIT
Ca++ (mmol/L)	1.1 ± 0.3	1 ± 0.3	1.2 ± 0.3	1.1 ± 0.2
Ca total (mmol/L)	2.1 ± 0.3	2 ± 0.2	2.1 ± 0.4	1.9 ± 0.3
PTH (pmol/L)	6.4 ± 1.5	7.6 ± 1.6*	9.4 ± 1.8	13.5 ± 1.9*
25(OH) D (nmol/L)	49 ± 10	36 ± 9*	47 ± 7	34 ± 6.5*
Insulin (IU/mL)	9.2 ± 2.1	10.4 ± 2.7	16.6 ± 2.7	19.3 ± 2.5*
HOMA-IR	3,6 ± 0.5	4.3 ± 0.8*	6.8 ± 0.9	8.2 ± 1.4*
Triglycerides (mmol/L)	1.7 ± 0.6	2.5 ± 0.5*	2.0 ± 0.5	2.7 ± 0.7*
Fasting glucose (mmol/L)	8.8 ± 0.9	9.3 ± 1.1*	9.2 ± 1.3	9.5 ± 2.4
HbA_1c, %	9.1 ± 1.7	9.7 ± 1.4	9.9 ± 1.4	10.3 ± 1.5
Cholesterol (mmol/L)	5.7 ± 1.3	6.5 ± 1.4*	6.4 ± 1.5	7.2 ± 1.3*

**Significant difference between the T1DM/T2DM and T1DM+AIT/T2DM+AIT groups ($p < 0.05$).*

TABLE 3 | Metabolic markers and 25(OH) D levels dynamic in patients with T1DM + AIT, T2DM + AIT after vitamin D_3 supplementation, (M ± σ).

	T1DM + AIT		T2DM + AIT	
	Before treatment	After treatment	Before treatment	After treatment
Ca++ (mmol/L)	1.0 ± 0.3	1.3 ± 0.25	1.1 ± 0.2	1.28 ± 0.25
Ca total (mmol/L)	2.0 ± 0.2	2.4 ± 0.3*	1.9 ± 0.3	2.5 ± 0.4*
PTH (pmol/l)	7.6 ± 1.6	3.9 ± 1.71*	13.5 ± 1.9	7 ± 1.9*
TSH (mIU/l)	2.5 ± 0.2	2.4 ± 0.3	2 ± 0.2	2.2 ± 0.2
25(OH) D (nmol/L)	36 ± 9	72 ± 8*	34 ± 6.5	69 ± 7*
Insulin (IU/mL)	10.0 ± 3.0	5.3 ± 2.7*	19.0 ± 3.0	6 ± 0.6*
HOMA-IR	4.3 ± 0.8	2 ± 0.2*	8.2 ± 1.4	2 ± 0.6*
Triglycerides (mmol/L)	2.5 ± 0.5	1.4 ± 0.2*	2.7 ± 0.7	1.5 ± 0.8*
Fasting glucose (mmol/L)	9.3 ± 1.1	8.3 ± 1.3	9.5 ± 2.4	8 ± 0.6*
HbA_1c, %	9.7 ± 1.4	8.5 ± 0.9*	10.3 ± 1.5	8.6 ± 1*
Cholesterol (mmol/L)	6.5 ± 1.4	4.9 ± 0.5*	7.2 ± 1.3	6.1 ± 1.3

**Significant difference from the indicator before treatment ($p < 0.05$).*

National Medical University with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Bogomoletz National Medical University.

RESULTS

Baseline Characteristics

The levels of metabolic markers in patients with combined endocrine disorders are shown in **Table 2**.

Follow-up Characteristics

After vitamin D_3 supplementation, the patient's metabolic and immune profile changed as shown in **Table 3**.

Immune Status Before and After Treatment

We evaluated the effects of vitamin D_3 supplementation in patients with both DM and AIT according to the level of Th1-type cytokines (INF-γ, TNF-α, IL-2, IL-6, IL-12) and Th2-type cytokines (IL-4, IL-5, IL-10, IL-17).

Our analyses revealed immunologic shifts in patients with combined endocrine pathology as well as their changes after vitamin D_3 supplementation (**Table 4**).

The concentrations of INF-γ in patients with T1DM and AIT and decreased T2DM and AIT by 3.9-fold and 5.2-fold compared to the concentrations before treatment. The levels of TNF-α after treatment with vitamin D_3 were significantly reduced by 2.3-fold in patients with a combination of T1DM and AIT, and by 3.2-fold in patients with T2DM and AIT. Similar changes were observed for IL-2, with a 3.9-fold decrease in blood concentration after vitamin D treatment in patients with T1DM and AIT and a 5.7-fold decrease with T2DM and AIT. The blood concentration of IL-6 decreased by 4.9-fold and 3.5-fold due to vitamin D_3 supplementation in patients with T1DM and AIT and T2DM and AIT, respectively. In patients with T1DM and AIT, the level of IL-12 after vitamin D_3 supplementation decreased significantly by 2.4-fold and by 4.8-fold in patients with T2 DM and AIT.

In patients with T1DM and AIT, the level of IL-4 in the blood was increased significantly by 10-fold and by 6.7-fold in patients with T2DM and AIT following vitamin D_3 supplementation. As a result of vitamin D_3 supplementation, the level of IL-5 increased by 2.5-fold and 5.9-fold in patients with T1DM and AIT and T2DM and AIT, respectively. In patients with T1DM and AIT, the level of IL-10 increased by 3.5-fold and by 6.6-fold in patients with T2DM and AIT. After vitamin D_3 supplementation, the concentration of IL-17 increased significantly by 3-fold in

TABLE 4 | Comparative evaluation of cytokines, in patients with combined endocrine pathology (DM + AIT) and DM patients before and after vitamin D treatment (mean ± *SD*).

Cytokines, pg/mL	T1DM + AIT		T2DM + AIT	
	Before treatment	After treatment	Before treatment	After treatment
IFN-ɣ	220 ± 50	55 ± 10*	270 ± 60	58 ± 11*
TNF-α	125 ± 16	56 ± 9*	183 ± 25	58 ± 9*
IL-2	113 ± 16	29 ± 7*	124 ± 14	22 ± 8*
IL-6	150 ± 35	30 ± 11*	180 ± 25	52 ± 12*
IL-12	180 ± 50	56 ± 17*	130 ± 20	27 ± 11*
IL-4	16 ± 5	160 ± 22*	25 ± 8	170 ± 30*
IL-5	56 ± 12	140 ± 28*	20 ± 5	120 ± 24*
IL-10	28 ± 16	98 ± 40*	10 ± 3	72 ± 21*
IL-17	45 ± 15	138 ± 33*	40 ± 12	118 ± 22*

**Significant difference with the indicator before treatment ($p = 0.0001$, Wilcoxon-Mann-Whitney tests).*

patients with T1DM and AIT and 2.9-fold in patients with T2DM and AIT.

To detect the presence or absence of relationships between change in HbA1c level and immunological parameters, we conducted a comparative assessment of the dynamics of cytokines on the background of treatment with vitamin D in groups with a small change in the level of HbA1c (under median) compared to that in the group of patients with significant HbA1c improvement dynamics (above median). This analysis was performed separately for patients with T1DM+AIT and T2DM+AIT (**Tables 5**, **6**).

The minimum decrease of HbA1c in the T1DM+AIT group was 0.49% (maximum: −1.9%, median: 1.2%). The patients in this group were divided into two subgroups: HbA1c dynamics <1.2 and >1.2%. The main hypothesis was based on comparing the cytokine dynamics in groups with different levels of HbA1c reduction indicating moderate and significant improvements. There was no statistically significant advantage in the dynamics of cytokines in the group with higher dynamics (ΔHbA1c >1.2%) compared to that in the group with minor changes (ΔHbA1c <1.2%) ($p > 0.05$).

The minimum HbA1c decrease in T2DM+AIT patients was 0.93% (maximum: −2.46%, median: −1.7%). The patients in this group were divided into two subgroups: HbA1c dynamics <1.7 and >1.7%. The main hypothesis was based on comparing the cytokine dynamics in groups with different levels of HbA1c changes, indicating moderate and significant improvement. There was no statistically significant difference in the cytokine dynamics in the group with higher dynamics (ΔHbA1c >1.7%) compared to that in the group with minor changes (ΔHbA1c <1.7%) ($p > 0.05$).

There was a trend toward greater cytokine dynamics in groups with higher dynamics ("c"), but the absence of a statistically significant difference in the cytokine dynamics in groups with minor changes in Δ HbA1c compared to a group of patients with a significant improvement in HbA1c levels does not indicate a priori determinism of cytokine changes due to changes in HbA1c.

Thus, the results of vitamin D_3 supplementation in patients with a combination of DM and AIT showed that vitamin D_3 has a positive effect on the balance of cytokines in the blood of patients with a combined endocrine pathology.

DISCUSSION

25(OH)D deficiency in patients with DM is accompanied by higher levels of glycemia and glycated hemoglobin. 25(OH)D deficiency also causes the early development of complications of DM. As highlighted elsewhere, there is a significant inverse association between serum 25(OH) D and HbA1c. The percentage of 25(OH)D deficiency/insufficiency in the population and the growth of DM prevalence suggest that vitamin D_3 supplementation can improve overall health. There is so much benefit from supplementation with vitamin D or by adding natural vitamin D rich food in the diet, including physical activities with possible sun light exposure. Advising patients with higher HbA1c to get tested for 25(OH)D levels and correct any deficiency/insufficiency if found may result in better blood glucose control and benefit the patient's overall health (7, 17–19). The important result we find from this study is a significant reduction in HbA1c as 25(OH)D levels increased.

DM with coinciding AIT is a serious clinical problem in Ukraine. The combination of these two diseases may affect the severity of the clinical course and therapeutic difficulties may be exacerbated by 25(OH)D deficiency, another well-documented public health challenge in Ukraine (8, 20).

We observed the most severe and resistant decompensation of carbohydrate metabolism on a background of 25(OH) D deficit in patients with T2DM combined with AIT. The homeostatic model assessment of insulin resistance (HOMA-IR) index in patients with T1DM or T2DM combined with AIT was higher than normal, especially in patients with T2DM with AIT. The indicators of fat metabolism, such as triglyceride and cholesterol levels, were higher than normal in patients with combined endocrine disorders compared to those in DM patients. Similar results were noted in other papers (21, 22).

The results of our study revealed a reduction in the total and ionized calcium levels before vitamin D supplementation (at baseline) in all patients, regardless of the type of diabetes. In addition, a compensatory increased PTH level was observed in patients with DM + AIT with coinciding reduced 25(OH)D concentration (less than 75.0 nmol/L). Some authors have postulated the possibility of a relationship with coexisting 25(OH)D concentrations (3, 15, 21).

Vitamin D_3 supplements were prescribed in accordance with blood levels of 25(OH)D at a dose of 2,000–4,000 IU/day according to the Endocrine Society clinical practice guidelines (16).

The experimental data on the hydroxylation of vitamin D in the liver, the involvement of reticulocytes in the deposition of 25(OH)D, and the results of vitamin D supplementation in the clinic demonstrating the possibility of maintaining the required concentration of 25 (OH) D in the blood for 2–3 months; even after discontinuing vitamin D supplementation, we found it

TABLE 5 | Comparison of the cytokine dynamics in groups with different levels of HbA1c reduction in patients with T1DM+AIT.

Cytokines, pg/mL	HbA1c< 1.2%		Δ (95%CI)*	HbA1c> 1.2%		c (95%CI)	P(Δ)**
	Before treatment	After treatment		Before treatment	After treatment		
IFN-γ	220 ± 60	58 ± 7.3	-164.1 (127–201.3)	208 ± 41	51 ± 12	-156.4 (123.7–189)	0.76
TNF-α	124 ± 13	25 ± 9.0	-98.5 (89–108)	127 ± 21	26 ± 9.0	-101.4 (84.2–118.5)	0.94
IL-2	113 ± 13.0	29 ± 8	-83.4 (74.2–92.6)	114 ± 20	27 ± 6	-86.1 (70.4–101.9)	0.25
IL-6	150 ± 30	33 ± 12	-115.1 (95.6–134.5)	150 ± 42	26 ± 7	-124.8 (92.3–157.4)	0.99
IL-12	190 ± 60	61 ± 16	-126.4 (88.4–164.4)	170 ± 37	48 ± 14	-123.7 (93.7–153.6)	0.82
IL-4	17.6 ± 5.3	160 ± 26	142.4 (126.6–158.3)	14 ± 3.3	165 ± 15.3	150.6 (138.8–162.5)	0.11
IL-5	55 ± 13	136 ± 34	81.7 (60–203.5)	58 ± 12	150 ± 15	92.5 (77.9–107)	0.12
IL-10	25 ± 16	100 ± 46	76.1 (46.7–105.3)	33 ± 17	93 ± 30	60.1 (33.9–86.2)	0.7
IL-17	44 ± 15	145 ± 35	100.2 (77.5–122.8)	48 ± 15	130 ± 30	80.2 (54.2–106.2)	0.13

Δ (95%CI): absolute dynamics of indicators before/after treatment, 95% confidence interval.

***P (Δ): evaluation of the statistical significance of the difference between the indicator dynamics in patients with minimal and significant ΔHbA1c changes (<1.2 and >1.2%, respectively) (Wilcoxon-Mann-Whitney test).*

possible to use vitamin D in the exchange rate regimen. We also observed a satisfactory compliance among patients administered vitamin D (29, 30).

The results of vitamin D_3 supplementation in the complex therapy of patients with DM indicated that the normalization of serum calcium level is associated with a decrease in PTH level. Moreover, an increase in 25(OH)D concentration resulted in improvements in carbohydrate metabolism, as assessed by fasting glucose levels in blood and HbA1c level.

25(OH)D deficiency has been associated with autoimmune disturbances, which and may be improved with vitamin D_3 supplementation (23, 24). In patients with T1DM and T2DM and low 25(OH) D concentrations, the background concentration of Th1-profile cytokines was increased and that of the Th2-profile cytokines was reduced, leading to an imbalanced immune status that supported the autoimmune inflammatory process and created conditions for disease progression (25–28).

There is a lack of similar studies of patients with only DM or only AIT. Moreover, the results were contradictory. In some of studies no significant changes in HbA1c or insulin sensitivity in DM patients after Vitamin D repletion were found. (31, 32), in other studies there was an information that Vitamin D_3 supplement improved HbA1C and glycemic parameters in T1DM and T2DM patients. (33, 34). These previous studies focused on the correction of disturbed metabolism in patients with types 1 and 2 DM by vitamin D_3 supplementation.

There is little information on AIT in the literature. Patients with vitamin D deficiency/insufficiency have generally been studied in correlation with serum anti-TPO thyroid antibodies levels. In all studies, vitamin D supplementation led to a decrease in serum anti-TPO levels. "25(OH)D deficiency may be related to pathogenesis of AIT and that its supplementation could contribute to the treatment of patients with AIT," "25(OH)D level is an independent factor affecting the presence of TPOAb in AIT. The causal effect of 25(OH)D deficiency to AIT is to be elucidated" (35, 36).

We found that the combination of AIT and DM significantly disturbed the immune status. The levels of the proinflammatory Th1-type cytokines were increased and the levels of Th2-profile cytokines were decreased. Our comparative analysis showed that the absence of a statistically significant difference in the dynamics of cytokines in groups with minor changes in ΔHbA1c compared to that in patients with a significant improvement in HbA1c levels indicates an absence of a priori determinism of cytokine changes due to changes in HbA1c levels.

The results of our study show that vitamin D_3 supplementation in T1DM+AIT and T2DM+AIT patients reduced the concentration of inflammatory Th1 cytokines (INF-γ, TNF-α, IL-2, IL-6, and IL-12) and increased levels of anti-inflammatory Th2-profile cytokines (IL-4, IL-5) and IL-10 and IL-17. Given the association of proinflammatory cytokines with the development of chronic complications of DM, a decrease in proinflammatory cytokines and an increase in anti-inflammatory cytokines after vitamin D_3 supplementation may retard the development of chronic diabetic complications.

TABLE 6 | Comparison of the cytokine dynamics in groups with different levels of HbA1c reduction in patients with T2DM+AIT.

Cytokines, pg/mL	HbA1c < 1.7%		Δ (95%CI)*	HbA1c > 1.7%		c (95%CI)	P (Δ)**
	Before treatment	After treatment		Before treatment	After treatment		
IFN-ɣ	290 ± 65	62 ± 10.5	-226.9 (187.5–266.2)	240 ± 52	51 ± 9.6	-189.0 (148.6–229.3)	0.76
TNF-α	175 ± 27	33 ± 7.6	-141.7 (124.8–158.6)	195 ± 13.7	36 ± 12	-159.3 (145.3–173.3)	0.11
IL-2	130 ± 12	25 ± 7.3	-105.6 (97.5–113.7)	115 ± 13	17 ± 7.6	-98.3 (87.0–109.4)	0.26
IL-6	177 ± 30	54 ± 14	-123.6 (103.8–143.4)	180 ± 15	48 ± 7.4	-132.9 (119.9–145.9)	0.62
IL-12	130 ± 17	29 ± 11.3	-100.8 (88.6–112.9)	130 ± 14.8	26 ± 12	-104 (89.5–118.4)	0.23
IL-4	22.5 ± 7.1	180 ± 21	156.7 (143.3–169.9)	28.6 ± 7.7	150 ± 35	120.8 (93.8–147.6)	0.09
IL-5	20 ± 6	106 ± 16	85.6 (75.5–95.6)	20 ± 4.7	140 ± 20.8	119 (102.8–135.2)	0.44
IL-10	10.8 ± 3.2	70.5 ± 25	59.7 (44.7–74.6)	11 ± 3.3	74 ± 13	63.2 (73.2–53.1)	0.76
IL-17	120 ± 27	77 ± 7.7	-42.8 (26.0–59.6)	116 ± 12.6	81 ± 13	-34.9 (21.1–48.6)	0.25

Δ (95%CI): absolute dynamics of the indicators before/after treatment, 95% confidence interval.

***P (Δ): evaluation of the statistical significance of the difference between the indicator dynamics in patients with minimal and significant ΔHbA1c changes (<1.7 and >1.7%, respectively) (Wilcoxon-Mann-Whitney test).*

CONCLUSION

Patients with combined endocrine diseases (DM + AIT) accompanied with decreased vitamin D status had significantly increased Th1-type cytokine levels and significantly decreased Th2-type cytokine levels. This finding indicates the presence of immune status disorders in patients, which supports the autoimmune inflammatory process and creates conditions for disease progression. The results of the present study indicate that the combination of endocrine diseases with decreased circulating levels of 25(OH)D may amplify metabolic disorders and generally contribute to early complications in DM. Vitamin D_3 supplementation in DM + AIT patients leads to the normalization of carbohydrate, mineral, and lipid metabolism to reduce the levels of pro-inflammatory cytokines, which may contribute to the effectiveness of the treatment of combined endocrine diseases.

Taking into consideration our results demonstrating the prevalence of 25(OH)D deficiency in these patients, vitamin D_3 supplementation should be recommended in patients with combined endocrine pathology in order to correct metabolic and immunological disorders.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of Bogomoletz National Medical University with written informed consent from all subjects. All subjects provided written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Bogomoletz National Medical University.

AUTHOR CONTRIBUTIONS

YK conceived the idea for the study, contributed to the design of the research, and collected the data. YK and MB analyzed the data and wrote the paper. All authors edited and approved the final version of the manuscript.

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REVIEW
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Rationale and Plan for Vitamin D Food Fortification: A Review and Guidance Paper

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Vitamin D deficiency can lead to musculoskeletal diseases such as rickets and osteomalacia, but vitamin D supplementation may also prevent extraskeletal diseases such as respiratory tract infections, asthma exacerbations, pregnancy complications and premature deaths. Vitamin D has a unique metabolism as it is mainly obtained through synthesis in the skin under the influence of sunlight (i.e., ultraviolet-B radiation) whereas intake by nutrition traditionally plays a relatively minor role. Dietary guidelines for vitamin D are based on a consensus that serum 25-hydroxyvitamin D (25[OH]D)

concentrations are used to assess vitamin D status, with the recommended target concentrations ranging from ≥25 to ≥50 nmol/L (≥10–≥20 ng/mL), corresponding to a daily vitamin D intake of 10 to 20 μg (400–800 international units). Most populations fail to meet these recommended dietary vitamin D requirements. In Europe, 25(OH)D concentrations <30 nmol/L (12 ng/mL) and <50 nmol/L (20 ng/mL) are present in 13.0 and 40.4% of the general population, respectively. This substantial gap between officially recommended dietary reference intakes for vitamin D and the high prevalence of vitamin D deficiency in the general population requires action from health authorities. Promotion of a healthier lifestyle with more outdoor activities and optimal nutrition are definitely warranted but will not erase vitamin D deficiency and must, in the case of sunlight exposure, be well balanced with regard to potential adverse effects such as skin cancer. Intake of vitamin D supplements is limited by relatively poor adherence (in particular in individuals with low-socioeconomic status) and potential for overdosing. Systematic vitamin D food fortification is, however, an effective approach to improve vitamin D status in the general population, and this has already been introduced by countries such as the US, Canada, India, and Finland. Recent advances in our knowledge on the safety of vitamin D treatment, the dose-response relationship of vitamin D intake and 25(OH)D levels, as well as data on the effectiveness of vitamin D fortification in countries such as Finland provide a solid basis to introduce and modify vitamin D food fortification in order to improve public health with this likewise cost-effective approach.

Keywords: vitamin D, public health, food fortification, general population, guidelines, evidence, recommendations, policy

INTRODUCTION

Vitamin D deficiency is common worldwide and potential adverse effects of a poor vitamin D status are of concern for public health (1–4). In this review, we aim to provide an overview on the rationale, current status and implementation plans for vitamin D food fortification as a means to close the gap between widespread inadequate vitamin D intakes and the target vitamin D intakes as recommended by nutritional vitamin D guidelines (4–8). This work should ideally provide a basis for the communication with and guidance for health authorities and regulators that are responsible for food policy and potential food fortification within their respective countries or regions.

This paper is based on a systematic literature search in PubMed until the end of March 2018 using the search terms "vitamin D" and "fortification," but reference lists of retrieved articles and personal references were also used. After an introduction on metabolism and clinical effects of vitamin D, we briefly summarize major nutritional vitamin D guidelines and give an overview on global vitamin D status and vitamin D intakes with a focus on the gap that exists between current estimates for vitamin D requirements and actual vitamin D intakes within populations. Following a section on general approaches on how to prevent and treat vitamin D deficiency, we outline safety issues of vitamin D before we present data on the history and the current status of vitamin D food fortification world-wide. Then, we briefly summarize the approaches and modeling as well as cost-effectiveness studies of vitamin D food fortification. Finally, we present some suggestions and guidance on how to implement vitamin D food fortification.

METABOLISM OF VITAMIN D

Vitamin D has a unique metabolism and is mainly produced in the skin where exposure to ultraviolet-B (UV-B) radiation (in sunlight) induces the conversion of skin produced 7-dehydrocholesterol into vitamin D_3 (cholecalciferol) (9). Dietary intake of vitamin D from natural foods traditionally plays only a minor role with few available natural sources: animal sources such as fatty fish, cod liver oil, or egg yolks contain vitamin D_3, and fungal sources such as mushrooms and yeast exposed to sunlight or UV radiation contain vitamin D_2 (ergocalciferol). Vitamin D_3 and D_2 share, in general, the same metabolism. Therefore, we will not differentiate between these two forms unless otherwise stated and refer to vitamin D (meaning vitamin D_3 and/or vitamin D_2) throughout this manuscript. In terms of sources, vitamin D can also be supplied by supplements and fortified foods but vitamin D and its metabolites may also be stored and released from the body's adipose tissue (9–11). A very rough general estimate is that about 80% of vitamin D supply comes from UV-B induced production in the skin and about 20% from dietary intake, but this varies considerably depending on factors such as season/sun exposure habits, latitude, nutrition/supplement intake or ethnicity (3, 9, 12). Despite a high degree of inheritance of serum 25-hydroxyvitamin D (25[OH]D) in twin studies, data from a

Genome Wide Association Study (GWAS) indicate that serum 25(OH)D concentrations have only a modest overall heritability due to common GWAS single nucleotide polymorphisms (SNPs) of 7.5%, highlighting the great impact of non-genetic factors to the variability in serum 25(OH)D concentrations (13, 14).

Vitamin D itself does not exert significant genomic biological effects and has to be metabolized (9). The common metabolism of vitamin D from any source involves, as a first step, the conversion to 25(OH)D in the liver that is mediated by different 25-hydroxylase enzymes (9). Serum 25(OH)D is the main circulating vitamin D metabolite that is considered to best indicate overall vitamin D status as it reflects vitamin D supply from diverse sources. Serum 25(OH)D has a traced half-life of approximately 2–3 weeks, whereas vitamin D itself has a half-life of only 1 day. In the bloodstream, approximately 85 to 90% of 25(OH)D is bound to vitamin D binding protein (DBP) and 10 to 15% is bound to albumin, so that less than 1% of serum 25(OH)D is unbound or free (15). The classification of vitamin D status is currently based on total serum 25(OH)D concentrations, i.e., the sum of bound and free fractions of both $25(OH)D_2$ and $25(OH)D_3$. It should, however, be acknowledged that there is some discussion regarding whether measuring free 25(OH)D concentrations may also be useful (15, 16). Such considerations are based on the fact that free 25(OH)D may cross the plasma membrane due to its lipophilic properties, whereas only a few organs that are crucial for vitamin D effects such as the kidneys, the parathyroid glands and the placenta are able to take up DBP-bound vitamin D metabolites through endocytosis by the megalin/cubilin complex (15, 16). While this is an active scientific debate, it is well established that 25(OH)D *per se* is hardly biologically active and has to undergo a further hydroxylation step that takes mainly place in the kidneys. In detail, renal 1-alpha-hydroxylase (*CYP27B1)* converts 25(OH)D to 1,25-dihydroxyvitamin D (1,25[OH]2D) that is also called "calcitriol" or the "active vitamin D hormone." Whereas the rate of 25-hydroxylation in the liver is mainly substrate dependent until a plateau is reached at high serum 25(OH)D concentrations, 1-alpha hydroxylation in the kidneys is under tight control by calcium and phosphate metabolism including parathyroid hormone (PTH), which stimulates 1-alpha-hydroxylation and fibroblast growth factor-23 (FGF-23), which inhibits it.

From a physiological perspective, 1,25(OH)2D functions like a classic steroid hormone (similar to sex or thyroid hormones): after binding of 1,25(OH)2D to the vitamin D receptor (VDR), this complex translocates to the cell nucleus and regulates the expression of hundreds of genes by interacting with its vitamin D responsive elements on the DNA. Whereas serum 1,25(OH)2D levels mainly derive from the kidneys and therefore exert classic endocrine functions, there is also a wide expression of extrarenal 1-alpha-hydroxylase that converts 25(OH)D to 1,25(OH)2D on a local/tissue level thereby contributing to autocrine and paracrine functions of 1,25(OH)2D. Importantly, the expression of VDR in almost all human tissues provides a sound scientific basis to postulate that vitamin D is important for overall human health. Further metabolism and degradation of vitamin D metabolites is initiated by 24-hydroxylase (*CYP24A1*), and after additional hydroxylation and oxidation steps, the resulting water soluble metabolites, one of which is calcitroic acid, are finally excreted in the bile and urine. For a more detailed description of vitamin D metabolism, we refer the reader to more detailed reviews on this topic (9, 15, 17).

CLINICAL EFFECTS OF VITAMIN D

Vitamin D is historically known as a substance that can prevent and treat nutritional rickets and osteomalacia (18–20). Rickets is a bone disease that is associated with low serum calcium and low serum phosphate, and is characterized by widening and delay of mineralization of growth plates in bones (18–20). The clinical presentation of rickets includes heterogeneous skeletal and non-skeletal manifestations such as bowing deformities of the bones, development delay or widening of joints (18–20). Severe cases of rickets can lead to hypocalcemic complications including tetany and seizures as well as dilated cardiomyopathy which can be fatal (18–20). Whereas rickets can only occur in open growth plates, osteomalacia constitutes defective mineralization of existing bone (closed growth plates) (18–20). Rickets and osteomalacia can lead to bone deformation (e.g., pelvic deformities in girls with risk of obstructed labor), as well as isolated and global bone pain and muscle weakness (18–20). Apart from rickets and osteomalacia, vitamin D supplementation may prevent falls and fractures in older individuals at risk of vitamin D deficiency, but data from randomized controlled trials (RCTs) on this topic are inconsistent (21–27). This may be explained by different dosing regimens with daily dosing may be beneficial and large intermittent bolus dosing may be detrimental (21–27). Moreover, it is sometimes difficult to disentangle separate effects of vitamin D and calcium, as there exist interactions between them. Several RCTs with a significant benefit used a combined supplementation of calcium plus vitamin D at doses of 17.5–20 μg (700–800 international units, IU) per day (21–27).

Apart from skeletal effects, vitamin D may also have an impact on extra-skeletal health (3, 28–31). Several epidemiological studies have shown that low serum 25(OH)D concentrations are a risk marker for various diseases as well as mortality (3, 28–31) (see **Figure 1** for the association between serum 25(OH)D and mortality). Data from meta-analyses of RCTs suggest that vitamin D supplementation may reduce mortality, respiratory tract infections, asthma exacerbations and pregnancy complications, but more data are required to clearly establish causality and doses-response relationships (32–41). Of particular importance are the RCT data suggesting that vitamin D supplementation during pregnancy may be useful in preventing general complications of pregnancy or infant outcomes such as asthma/wheeze (42, 43).

NUTRITIONAL VITAMIN D GUIDELINES

Recommendations relating to dietary vitamin D requirements in general populations are termed dietary reference intakes (DRI) or dietary reference values (DRV) (5, 6). These are based on the assumptions that total 25(OH)D serum concentrations are a biomarker of vitamin D status and indicate vitamin D intakes

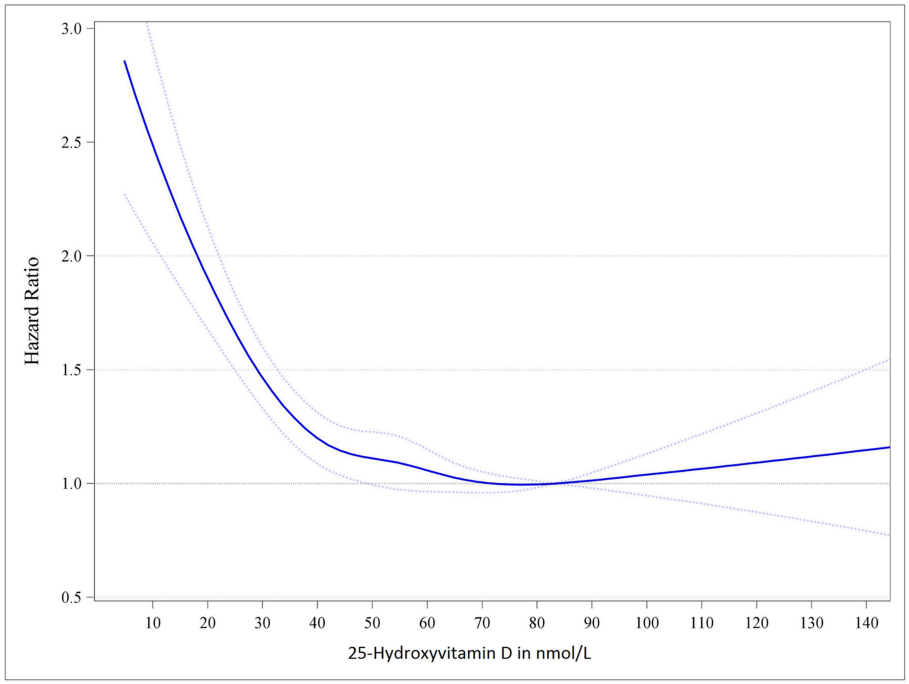

FIGURE 1 | Dose-response trend of hazard ratios of death from all causes by standardized serum 25-hydroxyvitamin D. Dose-response trend of hazard ratios of all-cause mortality by standardized 25-hydroxyvitamin D were adjusted for age, sex, body mass index and season of blood drawing concentrations. Hazard ratios (blue line with 95% confidence intervals as dotted blue lines) are referring to the 25-hydroxyvitamin D concentration of 83.4 nmol/L (i.e., the median 25-hydroxyvitamin D concentration of the group with 25-hydroxyvitamin D concentration from 75 to 99.99 nmol/L). Adopted from Gaksch et al. (28).

in the absence of cutaneous vitamin D production, which is especially the case in winter at northern latitudes (i.e., in regions far away from the equator). The rationale for nutritional vitamin D recommendations is the establishment of a cause and effect relationship between vitamin D intake and specified health outcomes. To date, vitamin D guidelines have generally been based on beneficial effects of vitamin D on musculoskeletal health outcomes (e.g., rickets, osteomalacia, fractures, muscle weakness, falls etc.) and occasionally on extraskeletal health outcomes such as pregnancy-related health outcomes or mortality. The dose-response relationship is then usually characterized by the association between serum 25(OH)D concentrations and these health outcomes.

As part of this process, certain target concentrations for serum 25(OH)D are established that are then used to calculate the vitamin D intakes for the estimated average requirement (EAR), that is the vitamin D intake at the estimated median requirement, and the recommended dietary allowance (RDA), that is the vitamin D intake that meets or exceeds the vitamin D requirements of 97.5% of the population. If the evidence is insufficient to define a RDA, an adequate intake (AI), is defined. The AI is the recommended average daily intake level of a nutrient based on observed or experimentally determined approximations or estimates of intakes that are assumed to be adequate for a group of apparently healthy people. After setting the target serum 25(OH)D concentrations for the EAR/RDA/AI, the vitamin D intakes that are required to achieve these concentrations thresholds, under circumstances of minimal to no UV-B induced cutaneous vitamin D production, are estimated by meta-regression analyses. The DRV/DRI also assume that the requirements for other nutrients such as e.g., calcium are met. In reality, this is usually not always the case, and vitamin D requirements may therefore even be higher in individuals with inadequate calcium intake, and may also vary according to other factors such as body mass index, ethnicity or genetic polymorphisms related to vitamin D metabolism/effects (13, 44).

An excellent overview of nutritional vitamin D guidelines is published elsewhere (5). For the US and Canada, the Institute of Medicine (IOM) report on vitamin D and calcium was released in 2010 and is considered the benchmark for nutritional vitamin D guidelines (45, 46). The IOM DRI report together with the European Food Safety Authority (EFSA) DRV report can be regarded as the main nutritional vitamin D guidelines (45–47). Therefore, we list the DRV/DRI of these two main guidelines together with three of the, in our opinion, most relevant national guidelines [i.e., Scientific Advisory Committee on Nutrition (SACN)report from the UK, the report from the Nutritional Societies in Germany Austria and Switzerland (DACH) and those of the Nordic European countries (NORDEN)] in **Table 1** (45–50). These recommendations are based on conditions of minimal or no endogenous vitamin D synthesis. Apart from these nutritional vitamin D guidelines for the general healthy population, there are also vitamin D guidelines or recommendations published that aim to guide vitamin D

TABLE 1 | Dietary reference values (DRV)/dietary reference intakes (DRI) for vitamin D.

Country (health authority)	USA and Canada (IOM)		Europe (EFSA)	Germany, Austria and Switzerland (DACH)	UK (SACN)	Nordic European countries (NORDEN)
DRV/DRI	**EAR**	**RDA**	**AI**	**AI**	**RNI**	**RI**
Target 25(OH)D in nmol/L	**40**	**50**	**50**	**50**	**25**	**50**
Age group	**Vitamin D intakes in μg per day (1 μg = 40 international units)**					
0–6 months	10			10	8.5–10	
7–12 months	10		10	10	8.5–10	10
1–3 years	10	15	15	20	10	10
4–6 years	10	15	15	20	10	10
7–8 years	10	15	15	20	10	10
9–10 years	10	15	15	20	10	10
11–14 years	10	15	15	20	10	10
15–17 years	10	15	15	20	10	10
18–69 years	10	15	15	20	10	10
70–74 years	10	20	15	20	10	10
75 years and older	10	20	15	20	10	20
Pregnancy	10	15	15	20	10	10
Lactation	10	15	15	20	10	10

IOM, Institute of Medicine; EFSA, European Food Safety Authority; DACH, Germany, Austria and Switzerland; SACN, Scientific Advisory Committee on Nutrition; EAR, Estimated Average Requirement; RDA, Recommended Dietary Allowance; AI, Adequate Intake; RNI, Reference Nutrient Intake; RI, Recommended; Intake; 25(OH)D, 25-hydroxyvitamin D.

diagnostics and supplementation in patients or specific groups, an issue that is beyond the scope of the current article (51–53). A few of these guidelines recommend relatively high target serum 25(OH)D concentrations such as 75 nmol/L (divide by 2.496 to convert nmol/L to ng/mL) because for some musculoskeletal health outcomes and parameters of mineral metabolism such as PTH, these levels may be optimal, whereas target serum 25(OH)D concentrations for effects on the immune system are not clearly established but may even be higher (3, 51, 53).

GLOBAL VITAMIN D STATUS AND VITAMIN D INTAKES

Several studies have investigated the prevalence of low serum 25(OH)D concentrations and of inadequate vitamin D intakes in general populations worldwide (54–63). It is obvious from these various reports that serum 25(OH)D concentrations and vitamin D supply are insufficient to meet the vitamin D requirements in significant sections of the general population worldwide. There exist, of course, regional differences in the burden of vitamin D deficiency, but it can be clearly stated that vitamin D deficiency is a worldwide public health problem. According to recent surveys, serum 25(OH)D concentrations <30 nmol/L and <50 nmol/L are documented in 13.0 and 40.4% of the general population in Europe, and in 6.7 and 26.0% of the general population in the US, respectively (55, 57, 58). Compared to Europe and North America, the prevalence of low serum 25(OH)D concentrations seems to be even higher in many low and lower-middle income countries (56). In India, Tunisia and Mongolia, for example, the prevalence of serum 25(OH)D concentrations below 25/30 nmol/L exceeds 20% in the entire population (56). It should be noted that interpretation of some previous vitamin D status data may be limited due to differences/problems of laboratory assays, whereas many recent surveys were based on well standardized 25(OH)D measurements (55).

Data on dietary vitamin D intakes are less complete compared to data on serum 25(OH)D concentrations, but it can be generally stated that in the majority of the countries worldwide, the median vitamin D intake is below 5 μg (200 IU) per day (62). It should, however, be acknowledged that assessment of vitamin D intakes is not trivial because food composition data are not always up-to-date with regard to actual vitamin D content of food. Furthermore, 25(OH)D content of food has often not been considered although this plays a significant role for vitamin D status in consideration of the fact that 25(OH)D_3 is approximately 5 times as effective as an equivalent intake of vitamin D_3 in terms of increasing serum 25(OH)D concentrations (4).

APPROACHES TO PREVENTION AND TREATMENT OF VITAMIN D DEFICIENCY

Approaches to improve vitamin D status in the population include increasing intake of naturally vitamin D containing food, food fortification, vitamin D supplements, increasing solar UV-B exposure and weight loss (64–76). Promotion of weight loss which may mobilize vitamin D and its metabolites from the adipose tissue as well as increasing intake of naturally vitamin D containing food (e.g., fatty fish) can be considered as general steps toward a healthier lifestyle but such attempts

have usually an insufficient overall impact on vitamin D status. Nevertheless, a meta-analyses of RCTs showed that compared to controls, fish consumption, which is usually the highest food source of vitamin D, raised serum 25(OH)D concentrations on average by 4.4 nmol/L (75). Recommendations regarding more sunlight (UV-B) exposure have the potential to increase serum 25(OH)D concentrations but are limited by adverse effects related to skin damage and skin cancer. Use of vitamin D supplements represents an effective strategy for the prevention and treatment of vitamin D deficiency at the individual level, but adherence within the general population as well as potential overdosing of vitamin D supplements are significant limitations. In the US, 3.2% in the general population take vitamin D supplements at a dose of $\geq$100 μg (4,000 IU) per day (10). It should also be underlined that supplement intake positively correlates with a healthier lifestyle and higher socio-economic status suggesting that recommendations for supplement intake do not adequately reach those people at particular high risk of vitamin D deficiency.

Therefore, vitamin D food fortification seems to be the most appropriate way of improving vitamin D intake and status in the general population in order to meet dietary vitamin D recommendations. In general, food can be enriched with vitamin D by simply adding vitamin D to food (i.e., traditional vitamin D food fortification) or by so called "bioaddition." Bioaddition of vitamin D, which has also been called "biofortification," refers to various ways of increasing vitamin D content of food without direct exogenous addition of vitamin D. Examples of bioaddition include feeding hens with vitamin D (and/or 25[OH]D) to increase the vitamin D (and/or 25[OH]D) content of the eggs, increasing vitamin D content of feed for farmed fish to increase their flesh vitamin D content, likewise with livestock animals in relation to meat, and UV exposure of mushrooms or yeast (that is then used to make bread), which facilitates the conversion of ergosterol to vitamin D_2. These issues are discussed in detail elsewhere (4, 74).

SAFETY ISSUES FOR VITAMIN D

When discussing public health strategies to increase vitamin D intakes in the general population, the potential dual harm of both deficiency and excess of vitamin D must be considered (77–83). Large oral doses of vitamin D increase serum 25(OH)D concentrations while serum 1,25(OH)2D concentrations are usually not materially changed and can even be reduced (79). It has been hypothesized that at very high serum 25(OH)D concentrations the binding capacity of the DBP may be exceeded leading to a release of free and biologically active vitamin D metabolites. Clinically, vitamin D intoxication can lead to hypercalciuria which precedes hypercalcemia. Consequences of hypercalciuria may include the formation of kidney stones, nephrocalcinosis and reduced kidney function. Hypercalcemia can be associated with fatigue, muscle weakness, weight loss, nausea, vomiting, soft tissue calcification or tachycardia. Recent RCTs using relatively high vitamin D doses have significantly increased our knowledge on the safety of vitamin D treatment (84–91).

Guidance on the safety of vitamin D intake is provided by several health agencies that released tolerable upper intake levels (ULs) for vitamin D as shown in **Table 2**. The IOM and EFSA have both set their UL for vitamin D at 100 μg (4,000 IU) per day for adults (45, 46, 78). Given an individual recommendation (e.g., RDA or equivalent) of 10–20 μg (400–800 IU), the safety range is 80–90 μg (3,200–3,600 IU) and the safety factor (UL/RDA) is 5–10. The EFSA report on ULs, after reviewing the literature, concluded that a daily dose of 250 μg (10,000 IU) is considered to reflect a "no observed adverse effect level (NOAEL)" in adults because clinical studies evaluating such doses reported no vitamin D toxicity. Furthermore, this NOAEL seems to be biologically sound because the maximum endogenous vitamin D synthesis by natural sun (UV-B) exposure increases 25(OH)D levels equivalent to oral vitamin D intakes of about 500 μg (20,000 IU) daily (92). In view of some uncertainties around this NOAEL an uncertainty factor of 2.5 was chosen leading to an UL of 100 μg (4,000 IU) for adults. The concept of vitamin D safety also consists of the idea of adequate circulating 25(OH)D concentrations as well as those leading to toxicity. There is, however, uncertainty at which concentrations hypercalcemia occurs although it is frequently quoted that hypercalcemia usually only occurs at serum 25(OH)D concentrations above 375–500 nmol/L. Importantly, the IOM has classified circulating 25(OH)D concentrations of 50–125 nmol/L as adequate and concentrations greater than 125 nmol/L, if sustained, as potentially harmful, although this level is far lower than the serum 25(OH)D concentrations associated with hypercalcemia of approximately greater than 375–500 nmol/L. The considerations regarding the term "potentially harmful" for serum 25(OH)D concentrations above 125 nmol/L until those concentrations leading to hypercalcemia is based on some observational studies indicating increased risk of adverse outcomes such as mortality at high 25(OH)D concentrations. It is important to underline that risk of adverse events at 25(OH)D concentrations above 125 nmol/L has only been inconsistently reported in observational studies and the question of causality is still not answered. However, some RCTs seem to support the cautious approach of the IOM since daily vitamin D supplement doses of 100 μg (4,000 IU) or high bolus doses of vitamin D leading to serum 25(OH)D concentrations >125 nmol/L might in specific population groups adversely impact musculo-skeletal and cardiovascular health (77, 78). On the other hand, several other studies using high doses of vitamin D or studying individuals with very high 25(OH)D concentrations did not report on adverse effects (77, 78, 91). Nevertheless, considering these safety issues and some uncertainty regarding the long term effect of high 25(OH)D concentrations, integrated quantitative risk–benefit assessments according to proposed frameworks are warranted (93–96).

Although the risk of achieving potentially harmful circulating 25(OH)D concentrations by food fortification with vitamin D is likely to be small in the general population, the problem of idiopathic hypercalcemia should not be neglected. A biallelic mutation in the gene encoding for the vitamin D catabolizing enzyme 24-hydroxylase (*CYP24A1*) can cause infantile idiopathic

TABLE 2 | Tolerable upper intake levels for vitamin D.

Country (health authority)	USA and Canada (IOM)	Europe (EFSA)
Age group	**Vitamin D in μg per day (1 μg = 40 international units)**	
0–6 months	25	25
6–12 months	37.5	25
1–3 years	62.5	50
4–8 years	75	50
9–10 years	100	50
11–17 years	100	100
18 years and older	100	100
Pregnancy	100	100
Lactation	100	100

IOM, Institute of Medicine; EFSA, European Food Safety Authority.

hypercalcemia (97, 98). This mutation results in vitamin D hypersensitivity and may have a prevalence of 1:33,000 births in Europe (98). The health consequences of this mutation in the adolescent and adult population are currently not known. When discussing the safety of vitamin D food fortification it must also be noted that improvement of vitamin D status by systematic food fortification may also likewise decrease the prevalence of persons taking vitamin D supplements exceeding the UL. In this context, it should also be noted that intermittent high dose vitamin D supplementation is quite common but may pose risk of adverse events. While daily vitamin D supplements with doses according to the RDA or equivalents are safe, intermittent high dose vitamin D supplementation may even increase the risk of fractures and falls (90). In this context, we believe that systematic vitamin D food fortification with subsequent improvement of vitamin D status in the general population may likewise decrease the potential public health burden (and costs) associated with overuse/overdosing of vitamin D supplements.

HISTORY OF VITAMIN D FOOD FORTIFICATION

Even before vitamin D was discovered, it had been observed that cod liver oil protects against rickets. Interestingly, it has been empirically shown that one teaspoon of cod liver oil, that contains approximately 10 μg (400 IU) of vitamin D per day, is effective in preventing rickets (5). Successful treatment of rickets has also been demonstrated by sunlight or UV exposure of children in the 1920s followed by documentation that irradiation of food such as milk increased its anti-rachitic activity. Vitamin D food fortification has been widely introduced in the 1930s and 1940s in the United States and many other industrialized countries such Great Britain when it became possible to add purified vitamin D itself to food (92). In particular vitamin D fortified milk was produced at that time, but vitamin D has also been added to a variety of foods and beverages including amongst others beer, hot dogs and custard. This food fortification policy was extremely effective in preventing rickets but in the 1950s there was a change in public health policy as food fortification was banned in Great Britain and many other European countries because cases of hypercalcemia were observed that had been suspected to be attributable to vitamin D intoxication. Whether this was really the case is not clear. Beyond the combined effect of vitamin D overdosing due to different sources [heavy vitamin D enrichment of dried milk powder plus vitamin D fortified cereals plus daily supplement with 17.5–20 μg (700–800 IU) of vitamin D] it has also been hypothesized that the hypercalcemic children in Great Britain may have had an inherited disease called Williams syndrome. This syndrome is, apart from other pathologies, associated with hypercalcemia. Unfortunately, methods for measuring circulating 25(OH)D were not available at that time. Some symptoms of hypercalcemia had, however, been observed in infants in the former German Democratic Republic, where infants were supplemented with intermittent doses of 15 mg (600,000 IU) of vitamin D as an effort to prevent rickets (77, 78). In these infants, serum 25(OH)D concentrations increased up to several hundred nmol/L.

CURRENT VITAMIN D FOOD FORTIFICATION POLICIES

Overviews of current food fortification policies have been reviewed elsewhere (62, 73, 74, 99–104). There is a huge variation in availability of vitamin D fortified food or food with vitamin D bioaddition across the countries. In general, there are mandatory and voluntary vitamin D food fortification policies but their differentiation is not always trivial as there can be varying pressure and implementation success of voluntary vitamin D food fortification. In Finland, for example, the Ministry of Trade and Industry recommended vitamin D fortification of fluid milks, margarines/fat spreads in 2003 on a voluntary, and not mandatory, basis, but most companies complied with the option to fortify resulting in a systematic (mass) vitamin D fortification (105–111). Many other countries allow voluntary vitamin D food fortification but with only insufficient effects on vitamin D intakes at population level (104, 112). Legislation is, of course, the basis for vitamin D food fortification and while we cannot discuss this issue in detail, we want to point out that the general regulation of voluntary food fortification is harmonized across the European Union (104, 113, 114). Several countries, however, still refer to national laws restricting addition of vitamins and minerals to food. In Germany, for example, addition of vitamin D to food is limited to margarine, based on a law of 1942.

As the experience with systematic (mass) vitamin D food fortification in the US, Canada and Finland may provide important guidance for health authorities in other regions, we list the main vitamin D fortified foods currently practiced in these countries in **Table 3** (99, 100, 105–111, 115–117).

In particular, the example of Finland can serve as a benchmark for future vitamin D food fortification policies in other countries. In Finland, vitamin D status has recently been assessed in nationally representative samples before and after introduction of systematic vitamin D food fortification (105). These results

TABLE 3 | Vitamin D food fortification in the United States, Canada and Finland.

Food (serving)	United States	Canada	Finland
VITAMIN D PER SERVING IN μg (1 μg = 40 INTERNATIONAL UNITS)			
	Mass fortification (usually mandatory)		
Fluid cow's milk (250 ml or 1 cup)	2.5–5.0†	2.5–5.0††	2.5
Margarine/Fat spread (10 g)		1.5–3.0††	2.0
	Fortification of selected brands		
Yogurt	1.5–5.0 per 170 g	1.0 per 100 g	0.5–1.0 per 100 g
Cheese slice (16 g)	1.5		
Orange juice (125 ml or 1/2 cup)	1.25	1.25	1.25
Plant-based milk such as soy, oat or almond (250 ml or 1 cup)	1.5–3.0	1.5–3.0	1.9–3.75
Margarine 10 g	0.75–5.0		
Bread (100 g)	2.25		1.7
Cereals, ready-to-eat (1/2–3/4 cup)	1–2.5	1.0	3.0 per 100 g

† *FDA in 2016 permitted voluntary "doubling" of mandatory vitamin D in milk.*
†† *Health Canada will require doubling of mandatory amounts by 2020.*

are based on Vitamin D Standardization Program (VDSP)-standardized 25(OH)D data (105), whereas older Finnish reports without VDSP data should only be interpreted with caution (106–110). In 2003, a systematic voluntary food fortification was introduced in Finland with the recommendation to add vitamin D at a dose of 10 μg/100 g to all fat spreads and at a dose of 0.5 μg/100 g to all fluid milk products. In 2010, these fortification recommendations were doubled to 20 μg/100 g in all fat spreads and 1.0 μg/100 g in all fluid milk products. In a nationally representative survey of Finnish adults, changes in serum 25(OH)D concentrations from 2000 to 2011 were investigated (105). Mean serum 25(OH)D concentrations increased from 47.6 nmol/L in the year 2000 to 65.4 nmol/L in 2011. The prevalence of 25(OH)D concentrations below 30, 40, and 50 nmol/L, respectively, was 13.0, 32.0, and 55.7% in 2000, and decreased to 0.6, 3.2, and 9.1%, respectively, in 2011. Importantly, serum 25(OH)D concentrations increased from 2000 to 2011 by about 34 nmol/L in individuals with 25(OH)D concentrations <30 nmol/L in 2000, whereas there was only an increase of about 11 nmol/L in individuals with 25(OH)D concentrations ≥50 nmol/L in 2000. In 2011, only 8 out of 4051 individuals had serum 25(OH)D concentrations ≥125 nmol/L and of these 8 individuals, 7 were vitamin D supplement users. Although food fortification policy in Finland clearly improved vitamin D status over time, it must be mentioned that there was also an increase in vitamin D supplement use from 11% in 2000 to 41% in 2011. Furthermore, a part of the 25(OH)D increase (approximately 10 nmol/L increase) from 2000 to 2011 cannot be explained by vitamin D fortification and increased use of vitamin D supplements. It is also worth mentioning that fat spreads were already a substantial source of vitamin D intake in 2000 as they were recommended to be fortified by 5–10 μg/ 100 g before systematic fortification started in 2003. Nevertheless, contribution to dietary vitamin D intake from fluid milk products, fat spreads, and fish changed from 4, 9, and 57%, respectively, in 2000 to 34, 10, and 38%, respectively, in 2011. When restricting the analyses to individuals with no supplement use, the mean overall increase in serum 25(OH)D from 2000 to 2011 was 6 nmol/L higher in individuals who consumed fluid milks products as compared to those who did not.

Therefore, and to conclude, the Finnish vitamin D nutrition policy, based on appropriate simulations, has considerably improved vitamin D status in the general Finnish population. This implementation of a systematic vitamin D food fortification programme respresents an example of a successful public health action that may inform similar approaches in other countries. Importantly, vitamin D food fortification policies have been re-evaluated and modified if necessary (105, 118). Apart from Western countries, there are also efforts for vitamin D food fortification in countries such as India (with e.g., vitamin D fortified milk), Jordan (with e.g., vitamin D fortified bread) and several others (73, 119–121).

MODELING TO INFORM STRATEGIES FOR VITAMIN D FOOD FORTIFICATION

Identifying the need for systematic vitamin D food fortification requires, of course, the assessment of 25(OH)D status and vitamin D intakes in a respective country or population in order to show that the dietary vitamin D requirements are not met. These data, which should at best be derived from a nationally representative sample of the population, can then serve as the basis for modeling vitamin D food fortification scenarios to meet the vitamin D requirements (122–135). A particular focus on groups at highest risk of profound vitamin D deficiency (e.g., those in high-risk ethnic groups or with restrictive diets) is also important.

Apart from the excellent "real-life" data from Finland on the effect of systematic vitamin D food fortification, there exist of course mathematical models to estimate different scenarios of vitamin D food fortification on vitamin D intakes and 25(OH)D status for a given population or country. In a very simplified view there are three different approaches for modeling effects of

vitamin D food fortification scenarios. First, based on vitamin D intakes and nutrition habits in the population it can be estimated how vitamin D fortification affects nutritional vitamin D intakes by simply adding existing and additional vitamin D intakes by food fortification (112). Second, based on the previous approach and the availability of 25(OH)D concentrations and by use of a dose-response equation of vitamin D intake and 25(OH)D serum concentrations, it can be estimated how vitamin D fortification affects not only dietary vitamin D intakes but also serum 25(OH)D concentrations (127). Third, in addition to the second approach the additional impact of UV exposure with its seasonal variation is considered to model the effect of vitamin D food fortification on 25(OH)D serum concentrations (125, 126, 128). All of these models have their limitations in particular due to some underlying assumptions so that cautious interpretation of the results is warranted. Regarding underlying assumptions it is important to note that when analyzing data from RCTs on vitamin D food fortification, it has been calculated that for every 1 μg (40 IU) ingested vitamin D, the serum 25(OH)D concentration increases by 1.2 nmol/L (95% confidence interval 0.72–1.68 nmol/L) (123). Simple modeling of an equation on the vitamin D intake-serum 25(OH)D relationship does, however, not reflect potentially modifying factors such as body mass index, age, basal serum 25(OH)D concentrations or genetics (136).

COST-EFFECTIVENESS OF VITAMIN D FOOD FORTIFICATION

When considering introduction of systematic vitamin D food fortification, a key question relates to whether or not such a public health intervention is likely to be cost-effective (137–151). In general, micronutrient fortification is considered as being one of the most cost-effective public health interventions (137). With reference to vitamin D food fortification there is, however, only limited evidence available on its cost-effectiveness. Nevertheless, the available studies on this issue point toward the notion that systematic vitamin D fortification (or vitamin D supplementation) may indeed be highly cost-effective (137–153). Regarding the costs for a typical food fortification programme, Fiedler et al. estimated the following distribution of costs: 80% recurrent production costs, 8% marketing and education costs, 7% food control and monitoring costs, and 5% other programme-specific recurrent production costs (137). Using these cost distributions and obtaining annual costs for 20 μg (800 IU) vitamin D per day of 0.11 Euros per person and annual costs for 200 mg calcium per day of 0.22 Euros per person it was estimated by Sandmann et al. that the implementation of a vitamin D plus calcium fortification programme in Germany would cost 41 million Euros per year while saving 365 million Euros per year as a result of reduced fracture costs (139). This would translate into a benefit-cost ratio of 9:1 which is even more conservative than other estimates of the cost-effectiveness of pure vitamin D interventions with even higher benefit-cost ratios (138, 140–149). We are well aware that more data are needed on the cost-effectiveness of systematic vitamin D fortification but we conclude that, despite limited evidence, the available literature suggests that this approach is highly likely to be cost-effective. Despite these promising data, it must be stressed that the overall general health impact of systematic vitamin D food fortification or supplementation can only roughly be estimated (154–156). It should also be mentioned that most studies assessed the cost-effectiveness of vitamin D food fortification in the elderly population and not in the whole population. Beyond cost effectiveness it is, however of course, extremely important that such fortification approaches are also well perceived and accepted by the population itself. This seems to be the case for vitamin D as e.g., shown by a study in Germany (152). The Finnish data also suggest that vitamin D food fortification is well accepted and fortified foods are considered part of the habitual diet (105).

SUGGESTIONS FOR VITAMIN D FOOD FORTIFICATION

There is definitely no clear answer on how to implement systematic vitamin D food fortification in countries where the vitamin D dietary requirements are not met by a significant part of the general population. Nevertheless, we want to provide some guidance for this task (see **Figure 2**).

A first step is, of course, the evaluation of the vitamin D status and intakes from nationally representative nutrition and health surveys. Definition of a precise goal for vitamin D food fortification is not a trivial task, but the general aim is, of course, to improve vitamin D status while avoiding or minimizing risk of potential toxicity related to overdosing of vitamin D. The Guidelines on food fortification with micronutrients by the World Health organization (WHO) define the goal of food fortification as follows: "to provide most (97.5%) of individuals in the population group(s) at greatest risk of deficiency with an adequate intake of specific micronutrients, without causing a risk of excessive intakes in this or other groups" (153). The WHO guideline defines inadequate intakes as intakes below the EAR, which corresponds to a serum 25(OH)D concentration of 40 nmol/L according to the IOM report. Although not clearly outlined in the WHO guideline it appears reasonable to argue that intakes below this goal are a reason for public health actions. Being aware of the heterogeneity of nutritional vitamin D guidelines we are convinced that if significantly more than 2.5% of the population have 25(OH)D concentrations below 25–30 nmol/L there is a justified need for public health interventions including vitamin D food fortification, which becomes imperative if the prevalence increases close to or exceeds 20% in either the entire population or in populations subgroups.

Regarding the goal of vitamin D food fortification we are well aware that in the IOM report, the RDA for vitamin D intakes corresponds to 50 nmol/L of serum 25(OH)D, and one may ask why we should not aim for this level in almost (97.5%) the entire population. Bringing almost everyone to a level of at least 50 nmol/L of serum 25(OH)D is, however, considered unrealistic, costly, ineffective and (in particular) potentially risky because this means that the target median intake

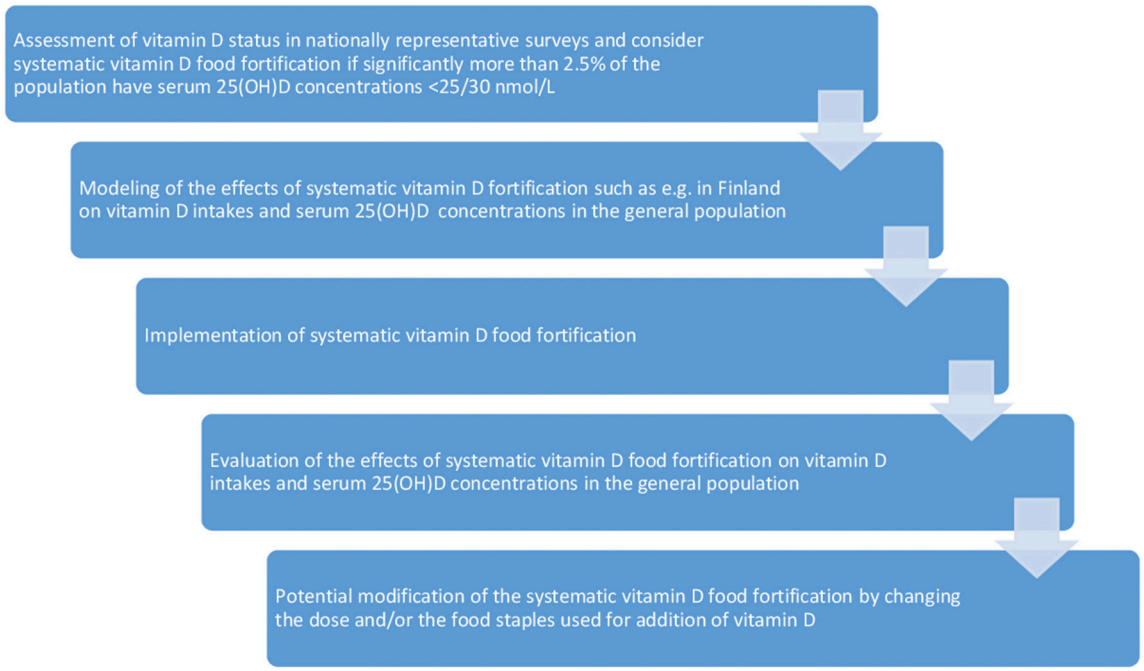

FIGURE 2 | Guidance for vitamin D food fortification.

would need to be set at very high levels (153, 157). It must be considered that even at vitamin D intakes between the EAR and the RDA and respective serum 25(OH)D concentrations of 40–50 nmol/L, the majority of the individuals would meet their dietary vitamin D requirements. The WHO guideline on food fortification suggests the target is to shift the intake distribution upwards so that only 2.5% of the population have an intake below the EAR (154). Thus, nearly everyone in the population should have a daily vitamin D intake of at least about 10 μg (400 IU) per day. This would, for a hypothetical usual intake distribution, result in a target median intake about 1.5 times above the RDA and approximately 20% of the population would have intakes below the RDA (153). This hypothetical example does, however, not fully apply for vitamin D because the distribution of 25(OH)D is different as shown in Finland where achieving a mean serum 25(OH)D concentration of 65 nmol/L by systematic vitamin D food fortification was sufficient to decrease the prevalence of 25(OH)D concentrations <30 nmol/L in the general population below 1% (105). This provides extremely strong arguments for the safety of vitamin D because the Finish data indicate that with vitamin D food fortification there is a much higher increase in 25(OH)D in those with very low 25(OH)D concentrations at baseline when compared to those with high 25(OH)D concentrations at baseline.

In general, we see two broad approaches to implementation of systematic vitamin D food fortification. The first one adheres to previous systematic vitamin D food fortifications in countries with similar population characteristics in terms of vitamin D status and food habits that have been evaluated with regard to safety and efficacy, as it has been done in Finland. It is a reasonable approach to follow the example of the Finnish vitamin D food fortification policy when modeling of the effects of such a vitamin D food fortification results in a significant and safe improvement of vitamin D status and intakes. The second approach is based on an "optimal modeling" of systematic vitamin D food fortification of many different food products to increase vitamin D status. It is clear from hypothetical models of vitamin D intakes and status that fortifying multiple food staples is desirable because such approaches reach broader parts of the population and are theoretically more safe than just fortifying one or a few food staples. However, such approaches are more costly and will likewise have a lower acceptance as there are currently no countries using and evaluating such approaches.

As some of the authors reside in Austria and Germany we wish to briefly outline the conceivable food fortification scenarios in these two countries (158–168). Data from national representative samples on vitamin D status and intakes in Austria and Germany are shown in **Table 4**. It is obvious from the high prevalence of low 25(OH)D concentrations and the low dietary vitamin D intakes that vitamin D food fortification is necessary in these countries to meet the vitamin D requirements. In this context, we wish to underline that there is long experience with vitamin D food fortification of dairy products in different countries covering a wide range of different nutritional habits, lifestyle and latitudes. Therefore, we are of the opinion that systematic vitamin D fortification of milk and margarine/fat spreads (dairy products) according to the approach used in Finland would be a reasonable approach also in Austria and

TABLE 4 | Vitamin D intakes and status in Austria and Germany.

Group	Intakes in μg per day		Serum/plasma 25-hydroxyvitamin D in nmol/L or percentages below a 25-hydroxyvitamin D cut-off concentration						
	Mean (SD)	Median (25th to75th percentile or IQR)	Mean (SD)	Median (25th to75th percentile or IQR)	<25	<30	<40	<50	<75
			nmol/L		Percentages				
AUSTRIA									
Austrian Study on Nutritional Status 2017									
Female adults	2.3 (2.4)	1.7 (1.1–2.8)							
Male adults	2.7 (2.6)	2.0 (1.2–3.4)							
Austrian Study on Nutritional Status 2012									
Girls 7–14 years		1.26 (1.00)		44.9 (32.5)	22.3			62.3	
Boys 7–14 years		1.39 (0.93)		44.7 (36.0)	17.7			55.8	
Women 18–64 years		2.6 (2.2–3.1)		57.4 (47.5)	11.6			39.8	
Women 65–80 years		3.2 (2.5–3.8)		42.3 (28.5)	19.9			42.4	
Men 18–64 years		3.9 (3.1–4.7)		55.9 (51.2)	14.2			43.9	
Men 65–80 years		3.9 (2.9–5.0)		41.8 (28.4)	20.4			44.4	
GERMANY									
German Health Interview and Examination Survey for Children (KiGGS; 2003 until 2006)									
All children			54.0 (19.2)	52.9 (39.4–71.6)	6.0	12.5	25.9	45.6	83.8
Girls 6–11 years		1.3 (0.8–2.1)							
Girls 12–17 years		1.7 (1.2–2.5)							
Boys 6–11 years		1.4 (0.9–2.1)							
Boys 12–17 years		2.2 (1.5–3.3)							
German Health Interview and Examination Survey for Adults (DEGS1; 2008 until 2011)									
All adults			50.1 (18.1)	47.7 (36.1–60.8)	4.2	15.2	34.3	56.0	90.9
German National Health Interview and Examination Survey (GNHIES; 1997 until 1999)									
Women		2.31 (1.53–3.56)							
Men		2.81 (1.89–4.44)							

Germany. Modeling of such a vitamin D fortification scenario on vitamin D intakes and on serum 25(OH)D concentrations is, of course, definitely required. Importantly, in Austria and Germany there is a similar yet slightly lower dairy intake but slightly higher 25(OH)D concentrations compared to Finland before the introduction of systematic vitamin D food fortification, suggesting that the Finnish approach may be a good model for these two countries (169). High prevalences of vitamin D deficiency with a need for improvement of vitamin D status are, however, also observed in many other European countries such as Poland with 16% of the general population having serum 25(OH)D concentrations below 25 nmol/L (170, 171).

In general, following the examples of other countries with vitamin D fortification of milk and margarines/fat spreads may likewise facilitate the implementation and the acceptance of mass fortification in the population. Additionally or alternatively, fortification of other foods such as bread may be considered in particular if dietary vitamin D requirements cannot be adequately met with vitamin D fortification of milk and margarine/fat spreads. Standardized measurements of 25(OH)D status and assessment of overall vitamin D intakes in nationally representative samples before and after implementation of vitamin D food fortification should be, of course, a condition sine qua non. While we hope for and work on the improvement of food fortification approaches in the future, it is now time to take action and work on the improvement of vitamin D status in countries where significant parts of the population fail to meet the dietary requirements. For this aim, the best evaluated vitamin D food fortification strategies with the likewise highest rates of successful implementation should be pursued.

TABLE 5 | Key points.

*Health authorities recommend target serum 25(OH)D concentrations ranging from ≥25 to ≥50 nmol/L (≥10 to ≥20 ng/mL),

corresponding to a daily vitamin D intake of 10–20 μg (400–800 IU)

*Most populations fail to meet these recommended dietary vitamin D requirements

*Systematic vitamin D food fortification is an effective and safe approach to improve vitamin D status in the general population

*Some countries such as the US, Canada, India and Finland have already introduced systematic vitamin D food fortification

*Introduction and/or modification of systematic vitamin D food fortification is required in many countries to improve public health, and should be based on modeling scenarios and efficacy data of vitamin D food fortification from other countries such as Finland

CONCLUSIONS

In this review, we outlined the background, rationale and current status of systematic vitamin D food fortification and also gave some guidance for implementation of such an approach (see **Table 5** for key points). We are of the opinion that the huge gap between the nutritional vitamin D guideline recommendations and the high prevalence of individuals who do not meet their vitamin D requirements calls for public health actions that can be performed by systematic vitamin D food fortification. While there are still many questions surrounding this issue, several countries do have long experience with systematic vitamin D food fortification (172–176). The successful and well evaluated real-life experience with the Finnish food fortification policy may be used as a benchmark for other countries with similar population characteristics. We do hope that our work helps to introduce and modify vitamin D food fortification in those countries where it is needed in order to prevent the significant public health burden of vitamin D deficiency and its adverse consequences.

AUTHOR CONTRIBUTIONS

SP, WM, and AZ drafted an initial version of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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The Association Between Long-Term Acenocoumarol Treatment and Vitamin D Deficiency

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Objective: Both vitamin D and K2 are involved in a number of metabolic processes, including bone metabolism; however, associations between the vitamins are not fully understood. The aim of the study was to evaluate serum concentrations of 25-hydroxyvitamin D [25(OH)D] in adult patients receiving long-term acenocoumarol (AC) treatment.

Participants and methods: In this cross-sectional study, 58 Caucasian patients (31 women, 27 men) with a median age of 65 years receiving long-term AC therapy were evaluated and compared with 35 age- and gender-matched healthy controls. The AC treatment was used due to recurrent venous thromboembolism (34.5%), atrial fibrillation (31%), or mechanical heart valve prostheses (34.5%). Medical records and a questionnaire were used to obtain information about chronic diseases, smoking habits, and the duration of therapy and weekly dose of AC. Anthropometric measurements were performed, and serum concentration of 25(OH)D and total alkaline phosphatase (ALP) activity were measured.

Results: Among the 58 patients receiving long-term AC treatment, a high proportion (46.6%) demonstrated significant vitamin D deficiency with concentrations of 25(OH)D lower than 20 ng/mL. The median concentration of 25(OH)D in subjects receiving AC was significantly lower compared to the control group [20.4 (17.4; 26.1) vs. 28.2 (24; 32.7); $p < 0.001$]. No differences were found between women and men receiving AC therapy. In patients receiving AC, a negative correlation was found between the concentration of 25(OH)D and the weekly dose of AC ($r = -0.337$, $p = 0.01$). Patients with concentrations of 25(OH)D < 20 ng/mL were found to have a significantly higher median dose of AC, compared to those with concentrations of 25(OH)D ≥ 20 ng/mL [21 (17; 31) vs. 17 (12; 28); $p = 0.045$].

Conclusion: In conclusion, treatment with AC is associated with low 25-hydroxyvitamin D levels, although the path leading to this phenomenon is not entirely clear. Long-term administration of AC in adults may increase the risk of chronic vitamin D deficiency, thus, effective supplementation of vitamin D in these individuals needs careful consideration.

Keywords: acenocoumarol, vitamin D deficiency, vitamin K antagonist, chronic disease, long-term treatment

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INTRODUCTION

Vitamin D synthesized in the skin or obtained from the diet is biologically inactive. Enzymatic conversion in the liver and kidney is required for its activation. Cholecalciferol [vitamin D(3)], inherently present in animals, is converted to calcifediol (25-hydroxycholecalciferol) in the liver, whereas ergocalciferol [vitamin D(2)], naturally found in plants, is converted to 25-hydroxyergocalciferol. These two vitamin D metabolites [called 25-hydroxyvitamin D or 25(OH)D] are measured in serum to determine vitamin D status (1). 25(OH)D is further hydroxylated by the kidneys to form calcitriol (1,25-dihydroxycholecalciferol), the biologically active form of vitamin D (2). The presence of 1-α-hydroxylase has been confirmed in bone tissue; hence bone itself is regarded as a source of the active form of vitamin D (3). Vitamin D exerts direct effects on various populations of bone cells, and functions indirectly by regulating both calcium-phosphate homeostasis (4) and the expression of parathormone (5). Vitamin D stimulates proliferation and differentiation of osteoblasts and osteoclasts (6), regulates the expression of numerous genes in the bone cell population (7), plays a role in the synthesis of key proteins secreted by osteoblasts, and inhibits apoptosis of osteoblasts (8). Vitamin D also regulates the expression of proteins involved in intestinal calcium absorption and calcium reabsorption by renal tubules, which ensures adequate mineralization of osteoid. Thus, bone metabolism largely depends on the biological action and adequate levels of vitamin D.

Experimental and observational studies, as well as clinical trials, indicate that insufficient intake of vitamin K2 and/or long-term treatment with vitamin K antagonists (VKAs) are related to bone metabolism disorders and arterial calcification (9–13). The specific mechanism of how vitamin K2 impacts bone metabolism has yet to be fully explained and understood. It is known that dietary calcium is absorbed from the digestive tract with the participation of vitamin D, while both vitamins, D and K2, are responsible for the production of the active form of osteocalcin, an integral protein involved in the synthesis of bone matrix and binding of calcium ions (14). Interestingly enough, an excess of calcium intake, particularly derived from supplementation, when combined with vitamin K2 deficiency, may result in the deposition of calcium in blood vessels (arterial calcification) and may increase the risk of soft tissue calcification (15). VKAs, including acenocoumarol (AC), suppress the synthesis of vitamin K-dependent proteins. Instead, alternative pathways are initiated resulting in the formation of undercarboxylated Gla proteins. In the bone, this results in the synthesis of the functionally inactive form of osteocalcin (16). The information above confirms the existence of links between vitamins D, K2, and bone metabolism, and could explain the potential pathogenic role of VKAs in impaired bone metabolism. For this reason, the possible connection between the use of VKA and vitamin D status may also be worth investigating in the context of chronic diseases and treatment.

According to the current guidelines, hypovitaminosis D is diagnosed by measuring serum concentration of 25-hydroxyvitamin D {[25(OH)D]; calcidiol}, the most commonly accepted indicator of vitamin D status. Levels of 25(OH)D below 20 ng/mL (50 nmol/L) are indicative of a deficiency and levels of 20–30 ng/mL (50–75 nmol/L) indicate an insufficiency, although the definition of deficiency and insufficiency remain a subject of discussion (17, 18). Several negative skeletal effects and bone mineral disorders may be attributed to both vitamin D deficiency and suboptimal vitamin K2 levels. It has been reported that vitamin K2, combined with vitamin D, increases bone mineral density more efficiently than vitamin K2 alone (19). Some study has also shown that vitamin K2 supplementation improves hip bone geometry and bone strength indices among postmenopausal women (20). Given that the effects of vitamin K2 may be generally beneficial for bone health, the suppression of vitamin K2 by VKA may potentially lead to impairment of bone metabolism and, therefore, a deteriorated skeletal status. However, as there are still conflicting reports and inconsistent data, the question arises whether long-term use of VKAs with coexisting vitamin D deficiency may have clinical implications or be a risk for patients.

The objective of the study was to evaluate vitamin D status expressed as serum concentration of 25-hydroxyvitamin D [25(OH)D] in adult patients receiving long-term AC treatment.

MATERIALS AND METHODS

Participants and Study Protocol

The cross-sectional study was conducted among adult patients treated with AC and healthy individuals recruited from the population of 5,834 people of a primary care practice. Participants' data were initially retrieved from an electronic database and medical records of the primary care facility taking part in the study. The obtained data contained information about age, gender, indications for anticoagulant treatment, and type of administered medication. Patients were previously qualified for AC prophylaxis due to recurrent venous thromboembolism, atrial fibrillation, or mechanical heart valve prostheses. The age- and gender-matched control group was recruited from the healthy population of the primary care practice using a random numbers table. The inclusion criteria consisted of written informed consent and duration of AC treatment longer than 3 months. Based on a questionnaire and medical records, participants with chronic diseases (i.e., chronic renal, gastrointestinal, liver, and endocrine diseases), those receiving treatment affecting vitamin D status/metabolism (i.e., anticonvulsants, systemic glucocorticosteroids), and those taking vitamin D and/or K2 supplements, were excluded from the study.

Seventy-two patients treated with AC, and 70 healthy subjects (controls) were invited to the study. Fourteen patients receiving AC, as well as 22 controls, did not meet all eligibility criteria and were excluded. Additionally, 13 healthy subjects refused to participate. Ultimately, 58 Caucasian patients (27 males, 31 females)

Abbreviations: AC, acenocoumarol; ALP, serum total alkaline phosphatase; BMI, body mass index; NS, non significant; VKA, vitamin K antagonist; 25(OH)D, 25-hydroxyvitamin D.

with median age 65 years receiving long-term AC treatment for recurrent venous thromboembolism ($n = 20$; 34.5%), atrial fibrillation ($n = 18$; 31%), and mechanical heart valve prostheses ($n = 20$; 34.5%) were enrolled into the study. Thirty-five healthy subjects (16 males, 19 females) with median age 61 years constituted the control group. Evaluation of all participants taking part in the study was done exclusively on the basis of medical records and a questionnaire. All participants were screened for chronic diseases and therapies known to affect vitamin D status. None of the participants had been supplemented with vitamin D and/or fish oil or vitamin K prior to and during the study. Study subjects were asked about the duration and weekly dose of AC treatment, smoking habits (past and current), time of menopause (if applicable), and the time spent outdoors. The majority of the participants (66 individuals; 71%) were people working in their profession, while others in the sample were not older than 80 years old. Furthermore, neither the patients receiving AC nor any of the controls had conditions that would have made ambulating difficult. Finally, time spent outdoors was comparable among subjects included in the patient and the control group.

The study protocol and procedures were approved by the Ethics Committee of the Medical University of Bialystok (No R-I-002/88/2013).

Measurements

Blood was collected from the study subjects in the winter season (between January and March) to avoid seasonal variations of vitamin D concentration. Blood samples were collected before 9:00 a.m. on the day of the interview after overnight fasting. All samples were centrifuged within 60 min from blood draw and sera were stored at −70°C until measurement.

The Elecsys Vitamin D(3) (25-OH) immunoassay was used for the quantitative determination of 25-hyroxyvitamin D(3) on cobas e immunoassay analyzer (Roche Diagnostics, Indianapolis, IN, USA). Activity of alkaline phosphatase was measured by colorimetric method (Abbott Laboratories, Abbott Park, Illinois, USA) with para-nitrophenyl phosphate as substrate. The reaction was monitored at 404 nm on an Architect ci8200 analyzer (Architect System, Abbott Laboratories, IL, USA).

Standing height and body weight were measured using standard anthropometric methods (wall-mounted stadiometer, electronic scale; Seca, Germany) and body mass index (BMI) was calculated using the standard formula.

Statistical Analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 11.5. The Shapiro–Wilk test was used to examine normal distribution. Since the data were not normally distributed, the results were presented as a median, 25th and 75th percentile. Nonparametric *t*-test and Mann–Whitney *U* test were applied for comparison between groups. Chi^2 test was used for qualitative data. Correlations between parameters were evaluated with Spearman's rank correlation coefficient. For the calculations, $p < 0.05$ was adopted as the level of statistical significance.

RESULTS

No differences in terms of age, gender, BMI, time elapsed, since menopause (females), smoking habits, and ALP activity were found between subjects receiving AC and the control group. All participants had ALP activity within the normal range (normal values: 37–110 IU/L). The median concentration of 25(OH)D in patients treated with AC was significantly lower compared to controls ($p < 0.001$) (**Table 1**). Anthropometric and biochemical parameters did not significantly differ between the groups of patients receiving AC prophylaxis for recurrent venous thromboembolism, atrial fibrillation, and mechanical heart valve prostheses. No differences between women and men receiving AC were found with regards to the studied variables.

Figure 1 presents the proportion of subjects with 25(OH)D level below 20 ng/mL, between 20 and 30 ng/mL, and those above 30 ng/mL. It was found that 27 (46.5%) individuals receiving AC had vitamin D deficiency, i.e., 25(OH)D levels below 20 ng/mL. Such deficiency was observed in only 2 (5.7%) control subjects. Levels of 25(OH)D between 20 and 30 ng/mL were found in 24 (41.4%) subjects treated with AC, and in 20 (57.1%) control subjects. Normal 25(OH)D values, defined as higher than 30 ng/mL, were found only in 7 (12.1%) patients receiving AC treatment and in 13 (37.2%) controls. Patients receiving AC had a significantly larger proportion of decreased 25(OH)D concentration compared to controls ($p < 0.001$).

Among patients receiving AC treatment, a negative correlation was found between the weekly dose of AC and serum concentration of 25(OH)D ($r = -0.337$; $p = 0.01$) (**Figure 2**), and between the weekly dose of AC and age ($r = -0.352$; $p = 0.007$). Also, a positive correlation between ALP activity and age was observed ($r = 0.272$; $p = 0.039$).

In the control group, a negative correlation was found between 25(OH)D level and age, and between 25(OH)D and the time, since menopause ($r = -0.395$; $p = 0.019$ and $r = -0.717$; $p = 0.003$, respectively). ALP activities positively correlated with age ($r = 0.403$; $p = 0.017$).

Among patients receiving AC, the studied parameters were compared between two groups of patients; those with a threshold

TABLE 1 | Basic characteristics of study participants.

Characteristics	Subjects receiving acenocoumarol	Control subjects	*p*
Number, *n*	58	35	
Age, years	65 (59; 73)	61 (59; 72)	NS
Gender (male/female), *n* (%)	27 (46.6)/31 (53.4)	16 (45.7)/19 (54.3)	NS
Body mass index, kg/m^2	28.1 (24.9; 31.3)	27.3 (24.4; 29.4)	NS
Post-menopause, years	15 (8.8; 23.3)	9 (6; 23)	NS
Smokers/non-smokers, *n* (%)	11 (19)/47 (81)	4 (11.4)/31 (88.6)	NS
Duration of AC treatment, years	8 (6.8; 12.3)	0	<0.001
Weekly dose of AC, mg	19 (14; 28)	0	<0.001
Serum 25(OH)D, ng/mL	20.4 (17.4; 26.1)	28.2 (24; 32.7)	<0.001
ALP, IU/L	61 (54.3; 75)	54 (51; 68)	NS

Results are shown as median and quartiles (Q1; Q3).
To convert values for 25-hydroxyvitamin D to nmol/L, multiply by 2.5.

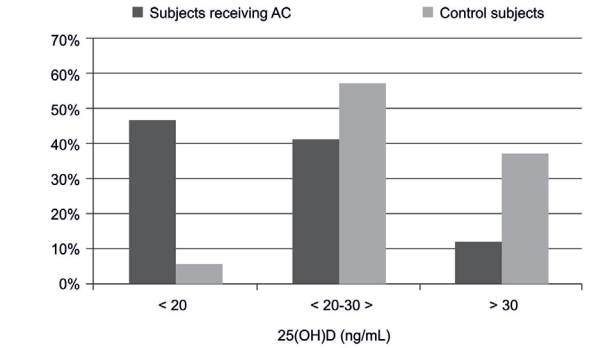

FIGURE 1 | The significant difference in percent distribution of 25(OH)D levels in subjects receiving acenocoumarol treatment and controls (n = 93).

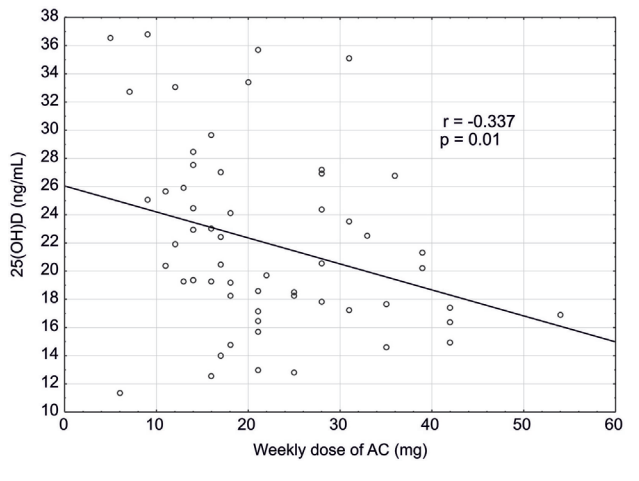

FIGURE 2 | Correlation between concentration of 25(OH)D (ng/mL) and a weekly dose of acenocoumarol (mg) in studied patients (n = 58).

concentration of 25 (OH)D < 20 ng/mL indicating a deficiency, and the group of patients with a concentration of 25 (OH) D ≥ 20 ng/mL. We did not find statistically significant differences in median age, BMI, time from last menstruation, the duration of treatment, and ALP activity between the groups. Both studied groups differed significantly in the median values of weekly dose of AC (p = 0.045), the median of 25(OH)D concentration (p < 0.001), and proportion of female patients (p = 0.015) (**Table 2**).

TABLE 2 | Comparison of studied parameters, depending on 25(OH)D serum concentration in subjects treated with acenocoumarol (AC) (the cut-off value set at 20 ng/mL was used to define the deficiency level).

Characteristics	Subjects receiving AC with 25(OH) D < 20 ng/mL	Subjects receiving AC with 25(OH) D ≥ 20 ng/mL	*p*
Number, *n*	27	31	
Age, years	65 (60; 73)	65 (59; 73)	NS
Gender (males/ females), *n* (%)	8 (29.6)/19 (70.4)	19 (61.3)/12 (38.7)	0.015
Body mass index, kg/m^2	27.9 (24.8; 31.2)	28.4 (24.9; 32)	NS
Post-menopause, years	15 (8.8; 24)	15 (8.5; 19.8)	NS
Smokers/non-smokers, *n* (%)	3 (11.1)/24 (88.9)	8 (25.8)/23 (74.2)	NS
Duration of AC treatment, years	8 (6; 10)	10 (7; 13)	NS
Weekly dose of AC, mg	21 (17; 31)	17 (12; 28)	0.045
Serum 25(OH)D, ng/mL	17.2 (14.8; 18.6)	25.6 (22.6; 29.6)	<0.001
ALP, IU/L	62 (58; 76)	59 (52; 74)	NS

Results are shown as median and quartiles (Q1; Q3).

DISCUSSION

The significantly decreased concentration of 25(OH)D observed in men and women treated with AC may indicate a potential negative effect of this therapy on vitamin D status, and presumably on bone metabolism or possible prospective health outcomes. Our results demonstrated that a large proportion of subjects receiving AC had vitamin D deficiency with the concentrations below 20 ng/mL. Our results are consistent with the results of other cross-sectional studies confirming the low level of vitamin D in patients receiving VKAs. For example, lower 25(OH)D levels in those taking VKAs were also seen in four cross-sectional studies performed among 514 Dutch females (21), 7,553 German males (22), 783 Netherlands geriatric outpatients (23), and 48 Greek children (24). On the contrary, in two other cross-sectional studies (n = 127, n = 116, respectively) and one prospective cohort study (n = 167), no such associations were observed (25–27). Different recruitment methods, designs and methodologies, the influence of still unknown factors, or different duration of treatment may have caused such differences in results across studies presented above.

To the best of our knowledge, our study is the first to demonstrate the inverse association between the dose of AC and vitamin D levels, so there is limited possibility to compare our results with others. The possible relationship between the dose of VKA and the level of vitamin D may be confirmed by the recently published study on the treatment of vitamin D deficiency in patients with venous thromboembolism. The study showed that treatment of vitamin D deficiency in patients with venous thromboembolism, resulted in the control of the international normalized ratio with the lower doses of warfarin. This observation was the first clinical report of an enhancement of the anticoagulant effect of warfarin by vitamin D supplementation (28).

Some common mechanisms or overlapping pathways may be involved in the regulation of the biological effects of these two vitamins. Wang et al. have identified a novel CYP3A4-dependent pathway, of 4-hydroxylation of 25-hydroxyvitamin D(3), the induction of which may contribute to drug-induced vitamin D deficiency. These results suggested that the CYP3A4-dependent metabolism of vitamin D may be important for the regulation of vitamin D(3) levels *in vivo* and in the etiology of drug-induced osteomalacia (29). It is well known, that oxidative metabolism of isomer R-AC is catalyzed by several members of the cytochrome P-450 family in the liver, including CYP3A4. Those data, along with our observations, suggest a mechanism

that leads to decreased vitamin D levels in patients on long-term VKA treatment. The interplay between vitamins K and D is apparent, for instance, in the form of osteocalcin, being essential for the formation of hydroxyapatite crystals in bone tissue (30, 31). Vitamin K2 can act not only through vitamin K-dependent proteins, but is also able to directly impact the gene expression by binding to steroid and xenobiotic receptors (32). Furthermore, vitamin K and D overlap metabolically at the cellular level. The cyclic oxidation and reduction of vitamin K is a source of electron transfer for antioxidant power to protect living cells against oxidative stress (33). Similarly, 1,25(OH)D has anti-oxidative capacity, as demonstrated in animal studies (34). It can, therefore, be concluded that vitamins K and D interact and stimulate each other's metabolism. The activity of vitamin D metabolites can be regarded as a gatekeeper, controlling calcium absorption, while the activity of vitamin K2 can be seen as that of a traffic policeman, directing the calcium ions into the bone (35).

The major limitation of the study was the small sample size of recruited subjects, particularly of the control group (controls were not as numerous) and the observational design of the study, which did not allow us to draw conclusions about causality. Another limitation was the inability to assess concentration of parathormone and osteocalcin among participants. There was also the possibility that some subjects may have had lower vitamin D levels for reasons other than the treatment with AC. By way of example, individuals in poor physical condition are likely to spend more time indoors than healthy ones, thus are prone to inadequate natural ultraviolet B (UVB) exposure which could in turn lead to extremely limited skin synthesis of vitamin D, and decreased 25(OH)D levels. The majority of the participants in this study were people actively working in their profession and none of the participants had a condition that would have made ambulating difficult, thus, in fact, time spent outdoors did not essentially affect the results. Nevertheless, neither the methodological issues nor other factors could detract from the main observation in this report. Our study revealed that AC doses negatively correlated with 25(OH)D concentrations, suggesting a possible association between VKA therapy and decreased vitamin D status. We also found that the median of weekly dose of AC was significantly higher in patients with lower vitamin D levels compared to patients with higher vitamin D levels. Further, patients in poor general physical condition do not generally receive higher doses of AC. Since our study was conducted during the winter months, when sunlight exposure was extremely low (latitude 53–54°N), the potential impact of UVB on vitamin D status among studied participants may be regarded as irrelevant. Nevertheless, explanation of this phenomenon does require further study.

To summarize, the results obtained in this study may indicate that long-term therapy with AC may be related to low levels of vitamin D. Thus far, a large number of data have been published regarding global consequences of vitamin D deficiency in the context of human morbidity, pleiotropic capacity, multifactorial associations between medical therapies, and vitamin D metabolism, in a variety of populations and risk groups (4). The general recommendations for vitamin D supplementation in healthy adult populations, although undergoing a vivid debate, are well known and commonly accepted, however, the list of populations at risk of deficiency have not yet been fully determined (18, 36–39). Future intervention studies warrant explanation and understanding of the underlying mechanisms of our findings, and may presumably allow the determination of precise recommendations for vitamin D-deficient patients treated with VKAs. Our observations, discussed above, suggest that 25-hydroxyvitamin D concentrations should be routinely measured and monitored in all patients receiving specifically long-term treatment with VKAs.

In conclusion, treatment with AC is associated with lower levels of 25-hydroxyvitamin D. Long-term administration of AC in adults may potentiate the risk of chronic vitamin D deficiency, thus, effective well-coordinated supplementation of vitamin D in these individuals needs careful consideration.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Declaration of Helsinki; written informed consent from all participants was obtained. Funded by Medical University of Bialystok. The Medical University of Bialystok in Poland approved the protocol (Approval: No. R-I-002/88/2013).

AUTHOR CONTRIBUTIONS

Design of the study: JS-P and JK. Data collection and analyses: JS-P, EJ, BZ-R, and WJ. Laboratory tests: BZ-R and WJ. Data evaluation: JS-P, JK, and EJ. Writing and preparation of the manuscript: JS-P, JK, EJ, and PA. Proof-reading and editing: JS-P, JK, EJ, and CS.

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Vitamin D Supplementation Guidelines for General Population and Groups at Risk of Vitamin D Deficiency in Poland—Recommendations of the Polish Society of Pediatric Endocrinology and Diabetes and the Expert Panel With Participation of National Specialist Consultants and Representatives of Scientific Societies—2018 Update

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Introduction: Vitamin D deficiency is an important public health problem worldwide. Vitamin D deficiency confers a significant risk for both skeletal and non-skeletal disorders and a number of lifelong negative health outcomes. The objectives of this evidence-based guidelines document are to provide health care professionals in Poland, an updated recommendation for the prevention, diagnosis and treatment of vitamin D deficiency.

Methods: A systematic literature search examining the prevention and treatment strategies for vitamin D deficiency was conducted. Updated recommendations were developed using the Grading of Recommendations, Assessment, Development and Evaluation system describing the strength of the recommendation and the quality of supporting evidence. Twenty-seven contributors representing different areas of expertise and medical specialties, including pediatricians, geriatricians, endocrinologists, epidemiologists, nephrologists, gynecologists and obstetricians evaluated the available published evidence related to vitamin D, formulated the goals of this document and developed a common consolidated position. The consensus group, representing six national specialist consultants and eight Polish and international scientific organizations/societies, participated in the process of grading evidence and drawing up the general and specific recommendations.

Results: The updated recommendations define the diagnostic criteria for the evaluation of vitamin D status and describe the prevention and treatment strategies of vitamin D deficiency in the general population and in groups at increased risk of the deficiency. Age- and weight-specific recommendations for prevention, supplementation and treatment of vitamin D deficiency are presented, and detailed practice guidance is discussed regarding the management in primary and specialized health care.

Conclusion: Vitamin D deficiency remains still highly prevalent in Poland, in all age groups. Currently, there is a great necessity to implement a regular supplementation with recommended doses and to develop an effective strategy to alleviate vitamin D deficiency in the population. These updated recommendations are addressed to health professionals and the authorities pursuing comprehensive health policies and should also be included in public health programs aimed at preventing a broad spectrum of chronic diseases.

Keywords: vitamin D, vitamin D deficiency, recommendations of the experts, supplementation, treatment, vitamin D in Poland

INTRODUCTION

Calcitriol [1,25-dihydroxycholecalciferol ($1,25(OH)_2D$)], an active (hormonal) form of vitamin D, due to its action belongs to a broad group of hormones, being transcription factors of genes for target proteins. In contrast to other hormones of this group (e.g., androgens, estrogens, glucocorticosteroids, mineralocorticosteroids and progesterone), the synthesis of calcitriol is limited by availability of the substrate, 25-hydroxyvitamin D (25(OH)D). 25(OH)D is the most abundant metabolite of vitamin D and its serum concentration defines the status of vitamin D supply. Thus, vitamin D is a prohormone, and the term "vitamin D" should be referred both to ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), as products of conversion of ergosterol and 7-dehydrocholesterol (7-DHC). Biological action of calcitriol is mediated by the intracellular, highly specific vitamin D receptor (VDR)—a transcription factor modulated by a ligand that belongs to the family of genomic receptors for steroids, thyroid hormones and retinoids.

Available data indicate that vitamin D deficiency is a problem affecting general population and patients that is prevalent irrespective of latitude of residence, age, sex and race (1–3). In Poland,

Abbreviations: $1,25(OH)_2D$, 1,25-dihydroxycholecalciferol; UVB, ultraviolet radiation B; DBP, vitamin D-binding protein; MED, minimal erythemal dose; 7-DHC, 7-dehydrocholesterol; D3, vitamin D3, cholecalciferol; D2, vitamin D2, ergocalciferol; DXA, dual-energy X-ray absorptiometry; 25(OH)D, 25-hydroxyvitamin D, calcidiol, calcifediol; FGF-23, fibroblast growth factor 23, phosphatonin-23; RANK, receptor activator of nuclear factor NF-κB; RANKL, RANK ligand; RCT, randomized controlled trials; PTH, parathormone; VDR, vitamin D receptor; ALPL, alkaline phosphatase activity; PO_4, phosphate; Ca, calcium.

vitamin D deficiency of varying severity has been found in 90% of adults, children and adolescents (4–7). Vitamin D deficiency may be associated with its well-known calcemic effect as well as a broad spectrum of pleiotropic effects, the latter having been studied intensely in recent decades. Hence, the problem of vitamin D deficiency and its adequate supply represent an important issue in public health and clinical practice. Guidelines for vitamin D supplementation undergo modifications every few years, in view of new findings resulting in changing the paradigms. The global consensus on prevention and management of nutritional rickets was published in 2016 (8), which due to discrepancies with recommendations for the Central Europe (9) and previous Polish guidelines (10), evoked polemical discussion and dilemmas among doctors of many specialties, particularly among pediatric endocrine and diabetes specialists. Therefore, in 2017 The Board of the Polish Society of Pediatric Endocrinology and Diabetes came up with an initiative on verifying and updating ruling recommendations on prevention and management of vitamin D deficiency, both in the general population and in the risk groups. In cooperation with the European Vitamin D Association (EVIDAS) and other scientific societies and National Consultants, the Expert Panel was constituted to elaborate current guidelines for supplementation and treatment with vitamin D, based on recent literature reviews, personal clinical experience and critical discussion.

METHODS

The Expert Panel with the participation of National Consultants and Representatives of Scientific Societies, basing on the literature review and evaluation of strength and quality of evidence, developed current recommendations for prevention and treatment of vitamin D deficiency in the general population and in the risk groups.

For each point listed below, recommendations and a level of evidence are described, with following modification in the grading evidence: 1 = strong recommendation (application in the general population and in all patients in most circumstances, benefits clearly overweigh the risk) and 2-weak recommendation (consensus opinion of working group or to be considered; the best action may depend on circumstances, benefits and risk closely balanced or uncertain). Quality of evidence was assigned as follows: ⊕⊕⊕ high quality [prospective cohort or randomized controlled trials (RCT) studies, at low risk of bias]; ⊕⊕ moderate quality (observational or clinical trials with methodological flaws, inconsistent or indirect evidence) and ⊕ low quality (case reports, case series or non-systematic clinical observations). The Expert Panel has confidence that vitamin D supplementation or vitamin D deficiency treatment are, on average, safe and beneficial when used according to the strong recommendations. Weak recommendations necessitate more personalized consideration, and, on average, are safe and beneficial as well.

RECOMMENDATIONS—2018 UPDATE

General Recommendations

(1) Prophylactic dosing of vitamin D in the general population should be individualized depending on age, body weight, insolation (season, time of year), sun exposure of an individual, dietary habits and lifestyle (1⊕⊕);
(2) Prophylactic dosing of vitamin D in the risk groups of vitamin D deficiency (**Table 1**) should be implemented according to arrangements for the general population; if no specific practice guidelines are established, the maximal admissible doses for a given age group in the general population are recommended for use in the risk groups of vitamin D deficiency (2⊕⊕);
(3) In the general population, in case of vitamin D deficiency ascertained by laboratory assays, the administration of vitamin D should be based on doses dependent on serum 25(OH)D concentration and chronological (calendar) age, in relation to body weight (2⊕⊕);

TABLE 1 | Indications for assessment of 25(OH)D concentration in serum—groups at risk of vitamin D deficiency.

Disorders	Examples of diagnoses
Disorders of the locomotor system	Rickets, osteomalacia, osteoporosis, bone pains, bone deformations, postural defects, recurrent low energy fractures and aseptic osteonecrosis
Disorders of calcium-phosphorus metabolism	Disorders of calcemia, calciuria, phosphatemia, phosphaturia, hypophosphatasia and hiperphosphatasia
Chronic treatment with some medications	Chronic corticosteridotherapy, treatment with ketoconazole, antiretroviral and antiepileptic therapy
Maldigestion and malabsorption	Maldigestion and malabsorption syndromes, cystic fibrosis and chronic inflammatory bowel disease
Liver diseases	Liver failure, cholestasis, posttrasplant state and non-alcoholic fatty liver disease (NAFLD)
Kidney diseases	Renal failure, posttransplant state and nephrocalcinosis
Endocrine disorders	Hyper- and hypoparathyroidism, hyper- and hypothyroidism, diabetes type 1, growth hormone deficiency, anorexia nervosa and autoimmune polyglandular syndromes
Disorders of somatic development	Short stature, tall stature, obesity and cachexia
Developmental delay	Delay of psychomotor development and intellectual disability
Diseases of the nervous system	Cerebral palsy, chronic immobilization, autism, multiple sclerosis, epilepsy, seizures of unknown etiology, miopathy and muscular dystrophy
Allergy	asthma, atopic dermatitis
Autoimmune diseases	Collagen diseases, rheumathoid arthritis, autoimmune diseases of the skin, diabetes type 1 and Hashimoto disease
Immune disorders	Recurrent infections of the respiratory tract, asthma, recurrent and chronic inflammatory states of other systems
Neoplasms	Blood cancer, malignancy of the lymphatic system and other organs, tumors and states after oncologic treatment
Cardiovascular diseases	Arterial hypertension and ischemic heart disease
Metabolic diseases	Diabetes type 2, lipid disorders, obesity and metabolic syndrome

(4) In the risk groups, the dosing of vitamin D in case of vitamin D deficiency ascertained by laboratory assays, should be based on doses dependent on the 25(OH)D concentration and age, with regard to the nature of the disease, medical therapy and body weight (1⊕⊕);
(5) In the general population, the specific indications for 25(OH)D assay testing are not established and 25(OH)D concentration screening is not recommended (1⊕⊕);
(6) In the risk groups, the evaluation of vitamin D status, based on 25(OH)D concentration assay, is recommended (1⊕⊕);

Recommendations for Vitamin D Supplementation in the General Population

Neonates Born at Term and Infants

i. 0–6 months: 400 IU/day from first days of life, regardless the way of feeding (1⊕⊕⊕);
ii. 6–12 months: 400–600 IU/day, depending on daily amount of vitamin D taken with food (1⊕⊕⊕);

Children (1–10 Years)

i. In healthy children sunbathing with uncovered forearms and legs for at least 15 min between 10.00 and 15.00 h, without sunscreen in the period from May to September, supplementation is not necessary, although still recommended and safe (1⊕⊕⊕);
ii. If above insolation guidelines are not fulfilled, supplementation of 600–1000 IU/day is recommended, based on body weight and the dietary vitamin D intake, throughout a year (1⊕⊕⊕);

Adolescents (11–18 Years)

i. In healthy adolescents sunbathing with uncovered forearms and legs for at least 15 min between 10.00 and 15.00 h, without sunscreen in the period from May to September, supplementation is not necessary, although still recommended and safe (1⊕⊕⊕);
ii. If above insolation guidelines are not fulfilled, supplementation of 800–2000 IU/day is recommended, based on body weight and the dietary vitamin D intake, throughout a year (1⊕⊕⊕);

Adults (19–65 Years)

i. In healthy adults sunbathing with uncovered forearms and legs for at least 15 min between 10.00 and 15.00 h, without sunscreen in the period from May to September, supplementation is not necessary, although still recommended and safe (1⊕⊕⊕);
ii. If above insolation guidelines are not fulfilled, supplementation of 800–2000 IU/day is recommended, based on body weight and the dietary vitamin D intake, throughout a year (1⊕⊕⊕);

Seniors (>65–75 Years) and People With a Dark Complexion

i. Due to decreased efficacy of the skin synthesis, supplementation of vitamin D in the dose of 800–2,000 IU/day, based on body weight and the dietary vitamin D intake is recommended throughout a year (1⊕⊕⊕);

Eldest Seniors (>75 Years)

i. Due to decreased efficacy of the skin synthesis, potential malabsorption and altered metabolism of vitamin D, supplementation of 2,000–4,000 IU/day, based on body weight and the dietary vitamin D intake is recommended throughout a year (2⊕⊕);

Pregnant and Lactating Women

i. Women planning pregnancy should receive adequate vitamin D supply, the same as in the general adult population, if it is possible under the control of 25(OH)D concentration (1⊕⊕⊕);
ii. When pregnancy is confirmed, supplementation should be carried out under the control of 25(OH)D concentration, to maintain optimal concentrations within ranges of >30–50 ng/ml (1⊕⊕⊕);
iii. If the assessment of 25(OH)D concentration is not possible, it is recommended to use vitamin D at a dose of 2,000 IU/day, throughout pregnancy and lactation (1⊕⊕⊕);

Preterm Neonates

Neonates Born at ≤32 Weeks of Gestation

i. It is recommended to start supplementation at a dose of 800 IU/day from the first days of life (if enteral nutrition is possible), regardless the way of feeding (1⊕⊕⊕);
ii. Supplementation should be carried out under the control of 25(OH)D concentration, both during hospitalization (the first control after 4 weeks of supplementation), as well as in the out-patient care (1⊕⊕);
iii. When achieving a total dose of 1,000 IU/day, combining supplements and diet, there is a risk of vitamin D overdose, particularly in neonates with birth weight <1,000 g (1⊕⊕⊕);

Neonates Born at 33–36 Weeks of Gestation

i. 400 IU/day from the first days of life, regardless the way of feeding (1⊕⊕⊕);
ii. There is no need to assay 25(OH)D concentrations routinely (1⊕⊕⊕);
iii. Supplementation under the control of 25(H)D concentration should be considered in children in the risk groups (parenteral nutrition >2 weeks, ketoconazole >2 weeks, anticonvulsant treatment, cholestasis and birth weight <1,500 g) (2⊕⊕);

Supplementation in Groups at Risk of Vitamin D Deficiency

i. A special risk group comprises obese individuals who require double dose of vitamin D in regard to doses recommended for age-matched peers with normal body weight (1⊕⊕⊕); obesity in children and adolescents is defined as BMI > 90th percentile for age and gender; obesity in adults and the elderly is defined as BMI 30+ kg/m^2;
ii. In groups at risk of vitamin D deficiency (**Table 1**), supplementation should be implemented and followed up under the control of 25(OH)D concentrations, in order to maintain the optimal concentration of >30–50 ng/ml (2⊕⊕);

iii. If the assessment of 25(OH)D concentration is not possible, dosing should be carried out according to the guidelines for the general population at the maximal doses for a given age group (2⊕⊕);

Supplementation in Groups at Risk of Vitamin D Hypersensitivity

i. Prior to initiating the supplementation, the probability of vitamin D hypersensitivity should be assessed if feasible (hypercalcemia, hypercalciuria, nephrocalcinosis, nephrolithiasis, *CYP24A1* gene mutation, *SLC34A1* gene mutation or history of other types of vitamin D hypersensitivity in an individual or family members). This recommendation applies to all age groups as well as to groups at the risk of vitamin D deficiency (1⊕⊕⊕);
ii. In groups at the risk of vitamin D hypersensitivity, supplementation should be supervised and carried out carefully and in an individual manner, preferably under the control of calcium-phosphate variables, particularly calcemia, calciuria, parathormone (PTH), 25(OH)D and $1,25(OH)_2D$ (1⊕⊕);

Principles of Supplementation and Treatment With Vitamin D Based on 25(OH)D Concentrations

i. A single loading dose of vitamin D is not recommended in Poland (2⊕⊕);
ii. Vitamin D dosing should be based on 25(OH)D concentrations and antecedent prophylactic management (2⊕⊕);
iii. The diagnostic standards include simultaneous assays of $25(OH)D_2$ and $25(OH)D_3$ [25(OH)D TOTAL], with intraassay variation <5% and interassay variation <10%, being subject to quality assurance by the certifying system DEQAS (2⊕⊕);

Toxic Concentration >100 ng/ml (1⊕⊕⊕)

i. Vitamin D supplementation has to be stopped forthwith; calcemia and calciuria should be assessed, and 25(OH)D concentration should be monitored at 1-month intervals until 25(OH)D concentrations of ≤50 ng/ml are reached (1⊕⊕⊕);
ii. Vitamin D intoxication is defined as the state in which the 25 (OH)D concentration >100 ng/ml is accompanied by hypercalcemia, hypercalciuria and apparent PTH suppression (1⊕⊕⊕);
iii. In case of clinical symptoms of vitamin D intoxication, treatment should be immediately initiated (1⊕⊕⊕);
iv. Verify if previously used supplementation was appropriate, and correct the management accordingly (regularity of intake, dosing, type of preparation and the way of supply) (2⊕⊕);
v. There is a possibility to re-entry vitamin D supplementation at doses recommended for peers from the general population, after reaching normocalcemia, normocalciuria and 25(OH)D concentrations ≤50 ng/ml, followed by excluding vitamin D hypersensitivity (2⊕⊕);

High Concentrations >50–100 ng/ml (1⊕⊕⊕)

i. Verify if previously used supplementation was appropriate, and correct the management accordingly (regularity of intake, dosing, type of preparation and the way of supply) (2⊕⊕);

Concentrations >75–100 ng/ml (2⊕⊕)

i. Vitamin D intake should be suspended for 1–2 months (2⊕⊕);
ii. In neonates, infants and toddlers, calcemia and calciuria should be assessed, vitamin D hypersensitivity should be excluded and the control assay of 25(OH)D concentration should be carried out (2⊕⊕);
iii. There is a possibility to re-entry vitamin D supplementation at minimal doses recommended for peers from the general population, after 1–2 months or, in case of neonates, infants and toddlers after reaching 25(OH)D concentrations ≤50 ng/ml (2⊕⊕);

Concentrations >50–75 ng/ml (2⊕⊕)

i. If vitamin D supplementation was appropriate, it is recommended to reduce the dose by 50%, and to consider assessment of 25(OH)D concentration within the consecutive 3-month period (2⊕⊕);
ii. If vitamin D was supplemented at doses higher than recommended, the vitamin D supply should be ceased for 1 month, and then doses recommended for peers from the general population should be started (2⊕⊕);

Optimal Concentration >30–50 ng/ml (1⊕⊕⊕)

i. Continue previous management (1⊕⊕⊕);

Suboptimal Concentration >20–30 ng/ml (1⊕⊕⊕)

i. Verify if previously used supplementation was appropriate, and correct the management accordingly (regularity of intake, dosing, type of preparation and the way of supply) (2⊕⊕);
ii. If vitamin D supplementation was appropriate, it is recommended to increase the dose by 50% and to consider the assessment of 25(OH)D concentration in 6-month time (2⊕⊕);
iii. If vitamin D was not supplemented previously, it is recommended to start vitamin D intake at doses recommended for peers from the general population (2⊕⊕);

Deficiency >10–20 ng/ml (1⊕⊕⊕)

i. Verify if previously used supplementation was appropriate, and correct the management accordingly (regularity of intake, dosing, type of preparation and the way of supply) (2⊕⊕);
ii. If vitamin D supplementation was appropriate, it is recommended to increase the dose by 100% and to assess 25(OH)D concentration in 3-month time (2⊕⊕);
iii. If vitamin D was not supplemented previously, it is recommended to start vitamin D intake at maximal doses recommended for peers from the general population and to assess 25(OH)D concentration in 3-month time (2⊕⊕);

iv. In patients with skeletal symptoms (bone deformations, bone pain, history of fragility fractures), it is indicated to assess calcium-phosphate metabolism [Ca, PO_4, alkaline phosphatase activity (ALPL), PTH, Ca/creatinine ratio in urine], and, if available—bone mineral density using dual-energy X-ray absorptiometry (DXA) (2⊕⊕);

Severe Deficiency 0–10 ng/ml (1⊕⊕⊕)

i. Verify if previously used supplementation was appropriate, and correct the management accordingly (regularity of intake, dosing, type of preparation and the way of supply) (2⊕⊕);
ii. Therapeutic doses should be implemented, based on age and body weight; the repeated control assay of 25(OH)D concentration should be performed after 1–3 months of therapy (1⊕⊕⊕);
   a. From birth to 12 months of age: 2,000 IU/day (1⊕⊕⊕);
   b. 1–10 years: 3,000–6,000 IU/day (1⊕⊕⊕);
   c. >10 years: 6,000 IU/day (1⊕⊕⊕);
iii. Treatment of severe deficiency should be carried out for 3 months or until the 25(OH)D concentration of >30–50 ng/ml is reached, then it is recommended to use consecutive maintenance dose, i.e., a prophylactic dose recommended for general population, in relation to age and body weight (1⊕⊕⊕);
iv. In patients with skeletal symptoms and bone mineral disorders (bone deformations, bone pain, history of fragility fractures), it is necessary to assess and monitor parameters of calcium-phosphate metabolism (Ca, PO_4, ALPL, PTH and Ca/creatinine ratio in urine), and if available—to examine bone mineral density using DXA (2⊕⊕);

Principles of Calcium Intake During Supplementation and Treatment With Vitamin D

i. During supplementation and treatment with vitamin D, an appropriate dietary calcium intake should be assured (**Table 2**) (2⊕⊕);
ii. If adequate dietary calcium intake is not possible, an additional pharmacological supplementation with calcium salts preparations is recommended, preferably in divided doses, which should be taken with meals (2⊕⊕);

TABLE 2 | Sources of calcium in the diet, equivalent to one glass/one serving of milk (240 mg calcium).

Basic source of calcium	Equivalents
1 average glass of milk = 240 mg of calcium	1 small mug of yogurt (150 g) 1 glass of kefir 1 glass of buttermilk 35 dag of curd cheese 2 small triangles of processed cheese 2 slices of cheese 2 packages of cottage cheese 100 g sardines 100 g almonds 130 g hazelnuts 150 g beans (dry seeds) 260 g spinach 350 g cabbage

EVIDENCE BASE FOR UPDATED POLISH RECOMMENDATIONS

Sources of Vitamin D

Skin Synthesis of Vitamin D

Most of vitamin D in humans is produced in the skin, in the keratinocytes of the epidermal germinative layer from 7-DHC, after exposure to sunlight radiation of wavelength of 280–315 mm [ultraviolet radiation B (UVB)]. Under the influence of the absorbed energy, 7-DHC undergoes transformation to pre-vitamin D_3, and then, due to a thermoconversion, to vitamin D_3. The latter enters the bloodstream, where it is bound to vitamin D-binding protein (DBP). It is estimated that skin synthesis may cover 80–100% of vitamin D daily requirement. In Poland, the skin synthesis of vitamin D may be effective in spring and summer only (from May to September), between 10:00 and 15:00, i.e., at the season and time of a day providing an appropriate angle of sunlight, air temperature favoring sunbath, and predominant cloudless weather (12, 13). In such conditions, an exposure of at least 18% of the body surface (i.e., uncovered forearms and lower limbs) for approximately 15 min should constitute half of minimal erythemal dose (MED; 1 MED results in a light pinkness of the skin) and may lead to natural synthesis of vitamin D in a quantity equivalent to 2,000–4,000 IU/day (12–14). Consequently, the exposure of nearly 100% of the body surface of an adult person may yield 10,000 IU/day. So far no reports showing the risk of obtaining toxic quantities of vitamin D after excessive exposure to sunlight have been published (at least in healthy subjects). This is explained by the fact that possible excess of vitamin D and previtamin D (immediate precursor in the cholesterol biosynthetic pathway) are photodegraded (isomerized) into inactive metabolites—tachysterol, lumisterol, suprasterols and 5,6-trans-vitamin D_3. In the period from October to March, in the regions above 35° north latitude (including Poland; 49°N–54°N), the skin synthesis is considered as not effective (12–15).

Intrinsic and environmental factors such as cloud cover, air pollution, intensive skin pigmentation, advanced age, excessive usage of sun protection cosmetics with sun protection factor above 15, significantly extend the exposure time necessary for achieving sufficient vitamin D supply. The above factors may totally prevent vitamin D skin synthesis even if the appropriate amount of time spent in the sunlight during spring and summer is provided. There are also individuals not adherent to the practice guidelines, or those who fail to follow recommended sensible exposure to sunlight due to specific contraindications. Sun exposure tends to be very limited among children and adolescents because of their indoor extra activities, and also in a large proportion of adult population due to the type of job. In neonates, infants and children younger than 3 years of age, the direct exposure to sunlight without sunscreen is not recommended (http://pediatrics.aappublications.org/content/pediatrics/early/2011/02/28/peds.2010-3501.full.pdf). Other reasons may include chronic diseases preventing from outdoor activities, cancer phobia or fear of skin aging (6, 14, 15).

Diet

The diet is the alternative source of vitamin D for humans; however in natural conditions, at least in Poland, it is a significantly less effective source, compared to skin synthesis. It is estimated that balanced diet covers up to 20% of the required daily vitamin D intake. Dietary vitamin D is present in two forms. In the food products of animal origin the dominant form of vitamin D is cholecalciferol (vitamin D_3), and that of plant and fungal origins—ergocalciferol (vitamin D_2). Natural sources of vitamin D mainly comprise fish, such as eel, wild salmon, herring and to a lesser extent—egg yolk, cheese, milk and some mushrooms (**Table 3**). Evaluation of nutritional habits and food composition in different populations showed that, when an additional source such as skin synthesis is scarce, even varied and balanced diet cannot be regarded to match the complete vitamin D requirement. Therefore, an appropriate vitamin D supplementation plays a crucial role in maintenance of the optimal health outcomes (3, 5, 16).

In terms of prevention of vitamin D deficiency at the population level, a mandatory fortification of selected food products (milk, dairy products, cereals, orange juice, margarine and pasta) is provided in some countries (17). The extent of fortification varies across different world regions, depending on health policy and governmental strategies. So far, the food fortification has not been customarily applied globally or locally in Poland, resulting in pandemic of vitamin D deficiency according to some reports (1–3). Milk formulas for infants and toddlers are an exception, as these products are enriched with vitamin D in a standard way (**Table 3**). The amount of 1 l of commercial formula milk is proven to cover the daily vitamin D requirement sufficiently, at least in context of prevention of vitamin D deficiency and nutritional rickets. It should be underlined that the vitamin D supplementation is necessary in breast-fed infants. The vitamin D content in human milk is fluctuating and small (about 40 IU/l), and seems insufficient for a growing child, even if standard recommended intakes of vitamin D are reassured in a breast-feeding mother (18, 19).

TABLE 3 | Vitamin D content in selected nutritional products in Poland (9, 11).

Product	Vitamin D content (40 IU = 1 μg)
Fresh eel	1,200 IU/100 g
Fresh wild salmon	600–1,000 IU/100 g
Herring in oil	808 IU/100 g
Marinated herring	480 IU/100 g
Salmon (cooked/baked)	540 IU/100 g
Fresh farmed salmon	100–250 IU/100 g
Canned fish (tuna, sardines)	200 IU/100 g
Mackerel (cooked/baked)	152 IU/100 g
Fresh codfish	40 IU/100 g
Shiitake mushrooms	100 IU/100 g
Egg yolk	54 IU/egg yolk
Cheese	7.6–28 IU/100 g
Human milk	1.5–8 IU/100 ml
Human milk during vitamin D supplementation	~20 IU/100 ml
Cow's milk	0.4–1.2 IU/100 ml
First infant formula (0–6 months)	40–60 IU/100 ml
Follow-on formula (7–12 months)	56–76 IU/100 ml
Growing-up formula (2–3 years)	70–80 IU/100 ml

Pharmacological Preparations of Vitamin D

Cholecalciferol (D_3) is the most common preparation used as supplementation and treatment of vitamin D deficiency in Poland and Europe, unlike in the USA, where ergocalciferol (D_2) is largely used. In Poland, vitamin D_3 is available over-the counter at daily doses of 400, 500, 800, 1,000, 2,000 and 4,000 IU. Vitamin D_3 is also available as multivitamin preparations, in composite calcium supplements, cod liver oil and, less frequently, in some food products fortified with vitamin D. The administration of vitamin D as a combination containing calcium or vitamin K2 (MK7) or in conjunction is not recommended presently. Efficacy of simultaneous intake of the vitamins K2 and D, as a factor preventing calcification of vessels and soft tissues, as well as enhancing bone mineralization, has not been proven. Vitamin D should not be administered together with high-fiber cereals (oatmeal and bran), resins binding steroids (colestyramine), laxatives or stool softeners.

Calcifediol, 25(OH)D_3, is an essential preparation improving vitamin D supply, however, this medication is used mainly in patients with impaired hepatic metabolism of vitamin D, coincident chronic liver diseases, cholestasis, long-term therapy with glucocorticosteroids and anticonvulsants (9).

Active metabolites and analogs of vitamin D, such as alfacalcidol (1αOHD_3), calcitriol [1α,25$(OH)_2D_3$] and paricalcitol [19nor1α25$(OH)_2D_2$] should not be considered as an alternative way of vitamin D supplementation, and in consequence, monitoring of the therapy based on active metabolites and analogs, using serial prospective evaluation of 25(OH)D concentration demonstrates a limited value. Alfacalcidol is most often used in cases presenting with impaired renal metabolism of vitamin D and in diseases with a decreased activity of 1α-hydroxylase, such as renal failure, nephrotic syndrome, chronic kidney disease, hypophosphatemic rickets and other vitamin D-resistant rickets, as well as in hypoparathyroidism. Calcitriol has been assigned similar application; however, it is used less frequently and is less available in Poland. Paricalcitol is a newly developed vitamin D analog, used for prevention and treatment of secondary hyperparathyroidism resulting from chronic renal failure (20–22). It should be underlined again, that the use of analogs and active metabolites of vitamin D does not lead to expected changes of 25(OH)D concentrations and is not an alternative mode for supplementation with the use of vitamin D (or with calcifediol in specific cases). The use of analogs does not detract the patient from necessity to a concurrent vitamin D intake, just in the context of pleiotropic health benefits.

Vitamin D Metabolism

Cholecalciferol—produced in the skin, and cholecalciferol (and ergocalciferol)—absorbed in the small intestine (from diet and pharmacological supplements), are transported to the liver and bound to the DBP. In the liver, the first stage of biosynthesis of an active vitamin D is activated. After enzymatic hydroxylation at the carbon-25, 25-hydroxyvitamin D—25(OH)D is formed. This reaction is catalyzed by 25-hydroxylase, which compromises a group of hydroxylases, being part of cytochrome P450 (CYP27A1, CYP3A4 and CYP2R1) (23, 24). The concentration of

25(OH)D in the serum is the best indicator of vitamin D status, mainly due to a higher stability and a longer half-time of 25(OH)D (2–3 weeks), as well as relatively high levels of concentration (expressed in ng/ml or nmol/l) in comparison to the $1,25(OH)_2D$, being under multifactoral regulation and at markedly lower concentrations (half-time 4–6 h, concentration expressed in pg/ml or pmol/l).

The 25(OH)D bound to DBP is subsequently transported from the liver to kidneys (as well as to many other tissues, organs and cells), where an active form of vitamin D—the $1\alpha25(OH)_2D$ is formed via the next key enzyme—1α-hydroxylase (CYP27B1). The activity of 1α-hydroxylase (CYP27B1) is regulated by many factors, including calcium concentration, PTH, fibroblast growth factor 23 (FGF-23) and Klotho, as well as by $1\alpha,25(OH)_2D$ itself, through the mechanism of a negative feedback loop (24, 25). Both active forms of vitamin D [$1\alpha,25(OH)_2D_2$ and $1\alpha,25(OH)_2D_3$] are characterized by similar properties. Due to common occurrence in the nature and the availability of pharmaceutic preparations in Poland, vitamin D_3—cholecalciferol is practically the only one in customary use. Therefore, the prevalent measurable form of an circulating active metabolite is represented by 1α,25-dihydroxycholecalciferol, i.e., calcitriol.

The concentrations of 25(OH)D and $1,25(OH)_2D$ are tightly regulated by enzymatic cleavage in reaction of 24-hydroxylase (CYP24A1), which catalyzes the hydroxylation of calcidiol and calcitriol to metabolites of low biological activity, finally transformed to $24,25(OH)_2D$ and calcitrionic acid, respectively. Calcitriol is a factor inducing 24-hydroxylase expression, present practically in all target cells which are under vitamin D action. This is a feedback system regulating concentration of active metabolites of vitamin and preventing hypervitaminosis D (25). The above metabolic pathway may be disturbed in case of impaired catabolism of 25(OH)D and $1,25(OH)_2D$ (mutations of *CYP24A1* gene coding 24-hydroxylase) (25) or excessive synthesis of $1,25(OH)_2D$ directly resulting from mutation of *SLC34A1* gene coding sodium-phosphate co-transporter (NaPi-IIA) in the kidney (26). In both cases, the risk of hypervitaminosis D is increased even if prophylactic doses of vitamin D are used.

Calcemic Effects of Vitamin D

Classic and well recognized action of vitamin D consists of its key role, besides PTH and calcitonin, in regulation of calcium and phosphorus homeostasis. The main effector organs involved in the regulation of calcium and phosphorus homeostasis by vitamin D action include intestine, bones and kidney. In the intestine, under the influence of $1,25(OH)_2D$, synthesis of a protein binding calcium and calcium absorption are increased, in bones—calcium and phosphates (in case of hypocalcemia) are released, and in kidneys—calcium is reabsorbed with input from PTH activity. The main procalcemic action of calcitriol is the inhibition of PTH secretion in the feedback via calcitriol, resulting in the increase of calcium and phosphates concentrations in serum (27).

Vitamin D action on bone metabolism is mediated by the receptor activator of nuclear factor NF-κB (RANK)/RANK ligand (RANKL) system, responsible for osteoclastogenesis. Calcitriol increases RANKL expression in osteoblasts, which in turn activates RANK receptors in osteoclast precursors leading to formation of mature osteoclasts. The resorption action of osteoclasts releases calcium and phosphates from the skeletal system into circulation (28).

Vitamin D, due to its action regulating calcium-phosphorus metabolism, plays a significant role mainly in tissues and organs rich in these minerals, i.e., the skeletal system and teeth. Vitamin D deficiency in children is a classic risk factor for nutritional rickets, a disease presenting with bone deformations of varying severity as well as impaired mineralization and decreased bone mass. In adults and adolescents, after growth plate fusion, vitamin D deficiency may lead to osteomalacia and osteoporosis. In all age groups, a severe vitamin D deficiency is related to bone pain of various intensity and localization (predominantly in the lower limbs and feet) as well as increased susceptibility to bone fractures. Advanced stages of nutritional rickets and osteomalacia may be even the life-threatening conditions, characterized not only by bone deformations, but also by hypocalcemic seizures (29, 30), tetany (31, 32), severe bone pain and significant muscle weakness (33, 34), hypocalcemic cardiomiopathy, even resulting in circulatory failure (35–38), and by disorders of psychomotor and physical development (39), including short stature (4, 40).

In the scenario of vitamin D deficiency, a three-stage regulatory mechanism was described. Initially, a compensatory increase of PTH secretion to sustain normocalcemia is observed. However, PTH reveals an ability to auto-regulate, and a relative resistance to PTH may develop resulting in decreased calcium concentrations and increased phosphate concentrations (similarly to a PTH resistance in pseudohypoparathyroidism). At this stage, typical symptoms of hypocalcemia, including tetanic convulsions, may occur. Osteopenia is visible on radiographs, without typical rickets lesions. In the next stage, when the severity of vitamin D deficiency continues to progress and PTH secretion is further stimulated, a PTH resistance gets overcome leading to improved calcemia but also to hypophosphatemia and clinical and radiological manifestation of rickets. ALPL increases then, whereas concentration of $1,25(OH)_2D$ is normal or increased. At the final stage, the vitamin D deficiency becomes very severe and $1,25(OH)_2D$ synthesis is markedly inhibited, the calcitriol concentration decreases, and subsequently absorption of both calcium and phosphorus is impaired, along with persistent elevation of PTH and increased ALPL (18).

The mechanism described above highlights that results of biochemical assays may reveal varied values, depending on the severity of vitamin D deficiency. Therefore, isolated assays of $1,25(OH)_2D$ concentrations may be misleading. In moderate vitamin D deficiency, as well as in the course of treatment, concentration of calcitriol may be normal or high, whereas in overdosage of vitamin D it may be normal or decreased (41, 42). These alternations prove a limited clinical utility of $1,25(OH)_2D$ assays, as this metabolite shows low stability and is a subject to multifactorial influences, including hormonal regulation.

Pleiotropic Action of Vitamin D

Development of molecular studies and discovery of VDR in many tissues, which do not take part in calcium-phosphorus metabolism, have initiated an era of intensive research on other non-classic, extra-skeletal functions of vitamin D (43–46).

Furthermore, $1,25(OH)_2D$ concentrations accessible in serum originate primarily from the kidneys and are connected with classic endocrine functions, however, enzymatic activity of extra-renal 1-alpha-hydroxylases that convert 25(OH)D to $1,25(OH)_2D$ in numerous organs, tissues and cells enables important local autocrine and paracrine functions of this "extra-renal" calcitriol. Extra-renal vitamin D metabolic pathways (both anabolic and catabolic mechanisms) are regulated independently of the PTH- and FGF-23-mediated pathways. Importantly, the expression of both VDR and extra-renal 1-alpha-hydroxylases in almost all human tissues provides a sound scientific basis to postulate that vitamin D is important for overall human health.

The activity of vitamin D in effector tissues is exerted through its genomic and non-genomic action. An active form of vitamin D in a number of tissues and cells binds with VDR in the cell nucleus and then it forms a heterodimer with 9-cis retinoic acid receptor (RXR), which shows property of a transcription factor, so the genomic action is initiated. It is estimated that in this way calcitriol takes part in regulation of several hundred genes in the human genome (47, 48). Non-genomic effects are mediated by a receptor localized in a cellular membrane, which is different from the nuclear receptor, and it triggers intracellular metabolic pathways, modulating effects resulting from the gene expression (49, 50). The non-genomic, rapid responses related to calcitriol apply to the regulation of ion channels, phosphatases, phospholipases, kinases and signaling factors that may independently regulate gene expression and its products (49–52).

It has been reported that calcitriol takes part in numerous physiological processes, including enhancement of proliferation and differentiation of immune cells (52, 53). It induces apoptosis of neoplastic cells and inhibits their multiplication (52, 53), increases production of cathelicidin and beta-defensin (51, 54–56), modulates lymphocyte activity (51), Th1 and Th2 lymphocyte ratio (51, 57), decreases concentrations of proinflammatory cytokines (IL-1, TNFα), simultaneously increasing anti-inflammatory cytokines (IL-4, IL-5 and IL-10) (51, 58), decreases renin secretion and in this way reduces activity of the renin–angiotensin–aldosteron system (59, 60), inhibits angiogenesis (61–63), and has favorable effect on calcification processes in blood vessels (64–67), stimulates synthesis of neurotrophic factors (68, 69) and inhibits fibrosis in kidneys (70, 71). Vitamin D deficiency decreases insulin secretion (72). Vitamin D also shows strong immunomodulating action (58, 73). High content of VDR in cells of the immunocompetent system, particularly in macrophages, dendritic cells and lymphocytes T and B supports the concept of the essential role of vitamin D in anti-infectious immunity, in the course of acute and chronic inflammatory processes as well as in autoimmune diseases (45, 46, 58, 73).

The pleiotropic aspect of vitamin D action was tested in numerous observational studies, suggesting association between low serum concentration of 25(OH)D and increased risk of neoplasms (among others cancers of the colon, breast, ovary, prostate, pancreas, skin, kidneys, brain tumors, multiple myeloma and leukemia) (74–78), immunological diseases [multiple sclerosis (79–82), asthma (83, 84), non-specific inflammatory bowel diseases (85, 86) and systemic lupus erythematosus (87, 88)], autoimmune endocrine disorders [diabetes type 1 (89, 90), Addison disease (91, 92), Hashimoto disease (93, 94), Graves-Basedow disease (95, 96) and autoimmune polyendocrine syndromes (97)], immunodeficiencies and recurrent infections (98) (i.e., tuberculosis and influenza), components of metabolic syndrome (including arterial hypertension and cardiovascular diseases, atherosclerosis, ischemic heart disease, diabetes type 2 and obesity) (99–105), as well psychiatric disorders [depression (106), schizophrenia (107, 108)] and neurodegerative diseases [dementia (109–111), Alzheimer disease (112, 113), deterioration of cognitive functions (109, 110, 114)]. Vitamin D deficiency is also associated with increased mortality in the general population (115–117), in patients in intensive care units (118–120), and in patients with neoplasms (121). A better vitamin D supply, reflected by an optimal 25(OH)D concentration, may alleviate invasiveness of a neoplastic process and micrometastases, as well as improve disease prognosis, and also limit risk of recurrence (122). Largely conducted RCT studies, however, have not shown consistent results, likely due to a short time of follow-up, type of regimen and different levels of a supplementary dose (123, 124). Nevertheless, many of these studies showed health benefits related to vitamin D supplementation. Presently, however, it cannot be stated explicitly that vitamin D deficiency is a direct cause of many disorders and their sequelae, and the hypothesis of reverse causality gains significance in the light of systematic reviews of meta-analyses and RCTs (125, 126). Taking into account on-going studies presenting potential health benefits and negligible health risk resulting from supplementation and maintenance of optimal and safe 25(OH)D concentrations, it is indicated to recommend prophylactic administration of vitamin D in the general population, when skin synthesis is insufficient for different reasons.

Vitamin D Safety

Serum concentration of 25(OH)D up to 100 ng/ml is regarded safe in the general population of children and adults, although in preterm neonates, being a specific group, an increased risk of hypercalcemia has been reported at the 25(OH)D values >80 ng/ml (127). No evidence exists until now that these values may be exceeded when appropriate doses of vitamin D are used. In fact, symptoms of vitamin D toxicity are observed very rarely. They are connected with hypercalcemia and hypercalciuria and may occur when vitamin D intake is uncontrolled and excessive, resulting in concentrations of 25(OH)D above 150–200 ng/ml (42, 128). Exceptional conditions comprise individuals with vitamin D hypersensitivity, and also with idiopathic infantile hypercalcemia (IIH) (24, 26), Williams-Beuren syndrome (129), granulomatous diseases (130) and some lymphomas. Vitamin D hypersensitivity may result from impaired catabolism of calcidiol and calcitriol or an excessive, uncontrolled by the feedback, synthesis of calcitriol (local or systemic) (24). The relationship between *CYP24A1* gene mutation and autosomal recessive IIH has been proven (25). It was also shown that subsequently discovered mutations of *CYP24A1* caused loss of function of 24-hydroxylase and clinically manifested as late as in adulthood (131, 132). Hypervitaminosis D may also result from an excessive synthesis of $1,25(OH)_2D$ in the case of mutation of *SLC34A1* gene, coding renal sodium-phosphate co-transporter (NaPi-IIA) (26). It is known that in

an endocrine (renal) pathway, processes of calcitriol synthesis involving CYP27B1, and its degradation involving CYP24A1, are also regulated by FGF-23. Disorders of phosphorus homeostasis as a result of *SLC34A1* mutation lead to the decrease in FGF-23 activity; FGF-23 in physiological conditions limits activity of 1α-hydroxylase (CYP27B1) and stimulates 24-hydroxylase activity (CYP24A1). A decreased activity of FGF-23 due to *SLC34A1* mutation and subsequent hyperphosphaturia and hypophosphatemia, indirectly stimulate synthesis of $1,25(OH)_2D$ that may produce hypercalcemia, hypercalciuria and nephrocalcinosis (133). In case of the diagnosed vitamin D hypersensitivity while supplementing vitamin D deficiency, it is suggested to maintain 25(OH)D concentrations within lower ranges, i.e., 20–25 ng/ml rather than within ranges regarded as optimal, i.e., 30–50 ng/ml (134).

It should be emphasized that at the general population level, vitamin D supplementation with use of daily doses that are recommended for a given age and body mass is safe and reasonable, whereas the incidence of vitamin D hypersensitivity seems to be low or at least it should be precisely investigated. Additionally, upper tolerable limits (UL) have been determined for the general healthy population in order to limit uncontrolled use of vitamin D. Upper tolerable limits should not be confused with recommended doses during well-controlled treatment of vitamin D deficiency. UL are considered safe for the healthy population and are commonly accepted worldwide by international scientific societies [e.g., Institutes of Medicine (IOM, USA) (135), The Endocrine Society (USA) (136) and European Food Safety Authority (European Union) (137)]. In the general population at the neonatal and infantile periods, the UL value equals to 1,000 U/l, in the period of 1–10 years of age—2,000 IU, and from 11 to 18 years of age and in adults—4,000 IU, respectively.

In cases of fully symptomatic vitamin D intoxication, resulting from overdose, the general therapeutic management includes hydration with normal saline followed by loop diuretics and the use of glucocorticoids, bisphosphonates, calcitonin or ketoconazole is often considered as the second-line treatment. Effective therapy is provided also by the anticonvulsant use. Anticonvulsants/antiepileptic drugs are known as potent inductors of cytochrome P450 activity and particularly its isoform CYP3A4. Induction of this enzyme localized in the liver and intestines, contributes to an increase of metabolic clearance of essential vitamin D metabolites, such as 25(OH)D and $1,25(OH)_2D$ (138). Polar products of vitamin D, formed in extra hydroxylation pathways, are then quickly excreted from the body (138, 139). When vitamin D status is normal, abovementioned processes result in vitamin D deficiency and mineral disturbances (138–140). Based on the above phenomenon, there have been two successful published attempts of removal of vitamin D excess in neonates (7 and 1.5 months old), casualties of its erroneous overdose (128, 141). In both cases, apart from other modes of treatment, anticonvulsants were administered (phenytoin 5 mg/kg/day for 17 days, phenobarbital 5 mg/kg/day for 133 days in the first case, and phenobarbital 3 mg/kg/day for 4 months in the other case). In both cases, at the end of treatment period, a decrease of 25(OH)D concentrations from approximately 400 to 40 ng/ml and from 160 ng/ml to normal ranges, respectively, was found. An attempt to withdraw phenobarbital (after 44 for 14 days) caused recurrence of intoxication symptoms (128). Eventually, in both cases monitored parameters of mineral metabolism returned to normal values.

Terminology

The Panel proposed to systematize the terms and nomenclature used in everyday medical practice in Poland. According to panelists' opinion, it is not justified to use the term "hypovitaminosis D" solely on the basis of 25(OH)D concentration value within the range reflecting vitamin D deficiency. It was observed that clinical symptoms might occur or might not, both at the higher (deficiency) as well as at lower (severe deficiency) ranges of 25(OH)D concentration (<10–20 and 0–10 ng/ml, respectively). This phenomenon may be related to individual sensitivity to vitamin D deficiency state, duration of vitamin D deficiency as well as to the status of mineral metabolism, including calcium intake (8). The terms "symptomatic vitamin deficiency" or "hypovitaminosis D" and "non-symptomatic (subclinical) vitamin D deficiency" should be used depending on the presence or the absence of clinical, biochemical or/and radiological signs. The terms should not be limited only to the recent vitamin D status [25(OH)D concentration], although clinical symptoms are usually observed and may develop along with a decreasing 25(OH)D concentration. Therefore, clinically overt and "symptomatic vitamin D deficiency" or "hypovitaminosis D", is a state when clinical symptoms coexist with low 25(OH)D concentration value. "Symptomatic hypervitaminosis D" or "vitamin D intoxication" is recognized by markedly elevated 25(OH)D concentration (usually >150 ng/ml) that coincides with normal or slightly increased $1,25(OH)_2D$, hypercalcemia, hypercalciuria and suppressed PTH. The clinical manifestations of vitamin D intoxication are related to hypercalcemia and include: fatigue, weakness, confusion, difficulty in concentration, drowsiness, apathy, vomiting, constipation, polyuria, polydipsia, abnormalities in electrocardiogram (reduced Q-T interval) and others.

REVIEW OF RECOMMENDATIONS

In available literature, the most commonly quoted and discussed position papers are the guidelines elaborated by IOM in 2010 (135), and the practice guidelines issued by the Endocrine Society in 2011 (136).

Based on the evidence available at the time, the IOM focused on calcium and phosphorus metabolism, including benefits limited to bone tissue. As a result, the target for vitamin D supplementation in the general population, recognized by the IOM, was to obtain 25(OH)D concentration of >20 ng/ml (135). In response to the IOM proposals, the recommendations of the Endocrine Society included general healthy population and populations with chronic conditions; furthermore, both the classic and pleiotropic action of vitamin D were incorporated in the integrated guideline. The Endocrine Society's minimal target value of 25(OH)D concentration was set on 30 ng/ml, and the values of <30 and <20 ng/ml were labeled as insufficient or deficient, respectively (136).

Guidelines concerning the optimal 25(OH)D concentrations and vitamin D supplementation vary across European countries: Scandinavian countries (Denmark, Finland, Iceland, Norway and Sweden) established the target 25(OH)D concentration of ≥20 ng/ml (142), and a similar threshold concept was accepted in Germany, Austria and Switzerland (3).

The recommendations prepared for Central Europe were the closest to the position statement of the Endocrine Society (9). The background in establishing those guidelines were documents by the European Food Safety Authority, published in 2012, and the global discussion of the scientific body over validity and adaptation of the proposals by the IOM and the Endocrine Society. The Guidelines for Central Europe set down the 25(OH)D concentrations of 30–50 ng/ml as optimal in relation to all potential health benefits (9).

Assuming that the aim of vitamin D supplementation is to achieve and to maintain the optimal concentrations of 25(OH)D, as a substrate for renal and extrarenal 1α-hydroxylation (CYP27B1), and in consequence—synthesis of calcitriol, recommendations including endocrine, paracrine and autocrine effects of $1,25(OH)_2D$ seem to reflect a holistic view on vitamin D deficiency and human health. The maintenance of recommended optimal 25(OH)D concentrations (>30–50 ng/ml) is reinforced by results of numerous cross-sectional and epidemiological studies, as well as several prospective trials, showing safety of such concentrations, not causing hypercalcemia or hypercalciuria. Another argument supporting 25(OH)D concentration of >30–50 ng/ml as the optimal, involves kinetics of 25-hydroxylase that showed 50% of its activity at the concentration of 40 ng/ml (143, 144). An important evidence was also reported by Priemel et al., who performed histomorphometric analysis of iliac crest bone biopsies in 675 subjects and revealed the osteomalacia lesions in 26% individuals, including 21% of the examined with 25(OH)D concentrations within a range of 21–29 ng/ml (145). Furthermore, osteomalacia signs were not observed in investigated bone biopsies of cases with 25(OH)D concentrations of >30 ng/ml (145). Moreover, studies carried out in pregnant women showed convincing evidence of health benefits for both woman and child that were associated with vitamin D supplementation and with achieved and maintained 25(OH)D concentrations close to 40 ng/ml (146–148).

Global guidelines published in 2016 considered 25(OH)D concentrations of >20 ng/ml as optimal (8). The supplementation regimen in almost all age groups included significantly lower vitamin D doses as compared to those recommended by the Endocrine Society (136) and for Central Europe (9). As a result, also in Poland, practitioners were forced to choose between the global and local recommendations. It should be emphasized, however, that global recommendations consider supplementation only in the context of prevention and treatment of nutritional rickets, and do not refer to other, widely evidenced health benefits related to vitamin D action, as it was pointed out by the authors of that document (8). Interestingly, the vitamin D doses recommended in the global consensus for the management of vitamin D deficiency confirmed by laboratory assays are very similar to the Central European recommendations. In neonates they are even higher (Central European recommendations—1,000 IU/day and global recommendations—2,000 IU/day; **Table 4**). The global recommendation of using a single loading dose of vitamin D (from 50,000 to 300,000 IU at a time) in treating deficiency in subjects older than 3 months of age, is disputable. A question arises whether it is a return to a historic recommendation of therapy based on a single mega-dose? Absolutely it is not. Loading doses should be justified only in particular situations, when everyday regular supplementation of vitamin D is not possible because of socioeconomic reasons or limitations of the health care system and infrastructure facilitating distribution of vitamin D supplements. In case of loading doses, the risk of hypercalcemia should be carefully taken into account, as it was elsewhere found in 6.5% of children treated with single high doses of vitamin D (8).

In the global consensus, additional attention is paid to relatively low recommended dietary calcium intake, considered as sufficient in preventing nutritional rickets. In children, the following daily calcium intake was recommended: up to 6 months of age—200 mg, 6–12 months—260 mg and after 12 months of age—500 mg. The recommendations for Central Europe (2013) did not arise an issue of calcium intake, however, this was included in Polish recommendations published in 2009 (10). The doses recommended then were definitely higher, particularly in older age groups and increased with age, in the range from 500 to 1,300 mg/day (**Table 4**). The American Institute of Medicine enforces similar recommendations, including calcium intake of 700–1,300 mg/day for the population aged 1–18 years, depending on a child's age (135).

DISCUSSION

Recommendations concerning vitamin D supplementation have been changing over the years and have followed the most recent scientific developments and clinical observations. However, even current doses recommended by scientific societies differ from each other significantly and vary from 200 to 2,000 IU/day (149). This results mainly from discrepancies concerning minimal target 25(OH)D concentration, which was defined by ranges between 10 and 40 ng/ml, depending on how different expert groups perceived vitamin D action (135–137). Most endocrine societies, including the Endocrine Society (USA), and also some dealing with bone health, such as the International Osteoporosis Foundation, reckon the 25(OH)D concentration above 30 ng/ml as that required to achieve health benefits. This value was also determined as a lower range of the optimal 25(OH)D concentration in 2013 Central European recommendations and is now maintained in the present recommendations for Poland (9, 134–137, 150).

The Expert Panel decided on an update of the recommended daily doses for the general population and for the groups at the increased risk of vitamin D deficiency that have been in operation in Poland since 2013. The Panel has decided to add additional target groups for vitamin D supplementation, including adolescents aged 11–18 years, older seniors aged >75 years, and also to modify previous Central European guidelines for the preterm babies.

Indisputably, individuals aged 11–18 years are among the groups of increased risk of vitamin D deficiency, however due

TABLE 4 | Comparison of recommendations of calcium and vitamin D supplementation for Poland (10), for the Central Europe 2013 (9) and global recommendations of prevention and treatment of nutritional rickets 2016 (8).

	Recommendations for Poland 2009	Recommendations for Central Europe 2013	Global recommendations 2016
	Definition of vitamin D supply based on 25(OH)D concentration in the serum (1 ng/ml = 2.5 nmol/l)		
Optimal concentration (sufficiency)	Children and adolescents: 20–60 ng/ml Adults and seniors: 30–80 ng/ml	>30–50 ng/ml	>20 ng/ml
Suboptimal concentration (insufficiency)	Not defined	>20–30 ng/ml	12–20 ng/ml
Deficiency	<10 ng/ml	0–20 ng/ml	<12 ng/ml
Toxic concentration (toxicity)	Not defined	>100 ng/ml	>100 ng/ml
	Recommended doses of vitamin D—supplementation (40 IU = 1 µg)		
0–6 months	400 IU/day	400 IU/day	400 IU/day
6–12 months	400 IU/day	400–600 IU/day	400 IU/day
2–18 years	400 IU/day	600–1,000 IU/day	600 IU/day
>18 years	800–1,000 IU/day	800–2,000 IU/day	600 IU/day
Pregnancy and lactation	800–1,000 IU/day	1,500–2,000 IU/day	600 IU/day
	Recommended doses of vitamin D—treatment of the deficiency (40 IU = 1 µg)		
<1 month	1,000 IU/day	1,000 IU/day	–
<3 months	–	–	2,000 IU/day
1–12 months	1,000–3,000 IU/day	1,000–3,000 IU/day	–
3–12 months	–	–	2,000 IU/day
2–19 years	up to 5,000 IU/day	3,000–5,000 IU/day	–
2–12 years	–	–	3,000–6,000 IU/day
>19 years	up to 7,000 IU/day	7,000–10,000 IU/day	–
>12 years	–	–	6,000 IU/day
	Single loading doses of vitamin D for the management of the deficiency (40 IU = 1 µg)		
<3 months	Not recommended	Not recommended	Not recommended
3–12 months	Not recommended	Not recommended	50,000 IU/3 months
2–12 years	Not recommended	Not recommended	150,000 IU/3 months
>12 years	Not recommended	Not recommended	300,000 IU/3 months
	Recommended calcium (elementary) doses		
0–6 months	300 mg/day	–	200 mg/day
6–12 months	400 mg/day	–	260 mg/day
1–3 years	500 mg/day	–	>500 mg/day
4–6 years	700 mg/day	–	
7–9 years	800 mg/day	–	
10–18 years	1,300 mg/day	–	
19–50 years	1,000 mg/day	–	
>50 years	1,300 mg/day	–	
Pregnancy and lactation			
<19 years	1,300 mg/day	–	
>19 years	1,000 mg/day	–	

to rapid and significant weight gain, an acceleration of skeletal growth, rapid bone turnover and modeling, redistribution of muscle-fat compartments and the other biological and behavioral aspects of pubertal transition, a too low supply of vitamin D during adolescence is of concern. Further, during these critical time frames of development, the risk of vitamin D deficiency and related adverse health outcomes may be exacerbated by sedentary behavior and time spent indoor, dietary habits and even use of restrictive diets. These numerous risk factors for vitamin D deficiency taken together pointed to this group as a target group of special concern and highlighted a need to increase a recommended vitamin D daily dose range to 800–2,000 IU, depending on body weight and season of the year. The British RCT study comprising a group of 110 children and adolescents with normal body weight, aged 14–18 years, evaluated efficacy of vitamin D supplementation at doses of 0, 400 and 800 IU/day, applied in the period between October and March (20 weeks), in order to determine distribution of nutritional requirements to maintain 25(OH)D concentrations ranging from >10 and >20 ng/ml. Data analysis showed that in the examined group of Caucasian children the maintenance of 25(OH)D concentration >10 and >20 ng/ml (in 97.5% of the examined) required a vitamin D supplementation at doses of 400 and 1,200 IU/day, respectively. Interestingly, none of the participants reached the 25(OH)D concentration of 40 ng/ml (151). The RCT of 96 children and adolescents, aged 8–14 years, carried out in the USA (Pittsburg) found that maintenance of 25(OH)D concentrations >20 ng/ml in the period from October to April in 90% of the examined

group required vitamin D supplementation at a dose of 1,543 IU/day, whereas an estimated dose of 2,098 IU/day appeared necessary to provide maintenance of this concentration in 97.5% of studied individuals (152). In another RCT, comparing efficacy of vitamin D supplementation applied for 6 months at doses of 600, 1,000 and 2,000 IU/day in the group of 685 school-aged children, the best effects of the supplementation, as expressed by 25(OH)D concentrations of ≥30 ng/ml, were revealed in the 2,000 IU/day group. In this group 25(OH)D concentration of ≥30 ng/ml was obtained in 60% of children already after the 3 months of trial and the use of 2,000 IU/day resulted in the mean 25(OH)D concentration of 33.1 ng/ml (153). The recent study of 1007 Polish children (6), hospitalized due to symptoms of skeletal disorders, revealed that vitamin D deficiency, including a severe vitamin D deficiency (<10 ng/ml), was noted more frequently at the pubertal period and at adolescence as compared to childhood and the prepubertal children, despite the availability of national guidelines.

In the eldest seniors, aged >75 years, according to the Panel opinion, vitamin D should be supplemented throughout the year at doses of 2,000–4,000 IU/day, depending on body weight. The recommended dosing range for the eldest seniors up to 4,000 IU/day was considered by panelists as effective enough to achieve the target 25(OH)D concentration of >30–50 ng/ml in at least 90% of the elderly in Poland. The group of the eldest seniors is another target group at increased risk of vitamin D deficiency, as well as falls and fragility fractures. Available RCT studies and meta-analyses evidenced that 25(OH)D concentrations ranging >24–50 ng/ml, as a result of vitamin D supplementation of seniors and the eldest seniors, were associated with a significant decrease of risk of falls (by 19%) (154), a significant decrease of risk of proximal femoral fractures (by 37%) (155) and significantly decreased risk of other fractures (by 31%), compared to controls. Although most studies reviewed recommended supplemental doses >800 IU/day, still about half of the seniors and the eldest seniors supplemented with vitamin D did not reach 25(OH)D concentrations considered as optimal. Therefore, after numerous discussions, the Expert Panel recommended a full eradication of vitamin D deficiency, using doses 2,000–4,000 IU/day in order to achieve and maintain the optimal 25(OH)D concentration and also to provide the eldest seniors with potential benefits resulting from pleiotropic vitamin D action. The above recommendation is well-matched to the American Geriatric Society guidelines (156).

The Expert Panel, basing on the review of the literature and RCT studies, has decided to modify the Central European recommendations for preterm babies. RCT studies published during the last 5 years revealed advantages of vitamin D supplementation at doses of 800–1,000 IU/day in neonates born at ≤32 weeks of gestation and in neonates born with very low birth weight (<1,500 g) (157–159). In the study comparing effects of vitamin D supplementation (1,000 vs 800 vs 400 IU/day), the percentage of the preterms with vitamin D deficiency at 36 weeks of the postmenstrual age was 2.5, 9.8 and 22.5%, respectively (159). In a group of more preterm babies (born at ≤28 weeks of gestation), after 4 weeks of vitamin D supplementation (800 vs 200 IU/day vs placebo), the percentage of the preterms with vitamin D deficiency was 0, 16 and 41%, respectively (158). In the subgroup supplemented with vitamin D dose of 800 IU/day, the majority of investigated cases reached 25(OH)D concentrations >60 ng/ml, despite that as high as 67% preterms presented vitamin D deficiency at birth. An observational study of 66 preterm neonates (mean birth weight 970 g, 27 weeks of gestation) showed that vitamin D supplementation at a dose of 800 IU/day was effective to reduce prevalence of severe vitamin D deficiency, evaluated at 36 weeks of the postmenstrual age, from 41 to 0%, as well as to improve prevalence rate of 25(OH)D concentrations >30 ng/ml from 10 to 72% (160). Unfortunately, the problem of vitamin D deficiency in the preterm neonates is also common in Poland (161). The risk of vitamin D deficiency at birth rises along with the shortening of pregnancy duration and the risk of preterm delivery increases with severity of vitamin D deficiency in pregnant women (146–148). The updated vitamin D supplementation doses seem effective for the quick improvement of vitamin D status of preterm neonates, however, after a one month of vitamin D supplementation, according to panelists guidelines for preterms, it is recommended to evaluate 25(OH)D concentration and if necessary modify the dosage. Because of the concern about adverse effects and risk of overdosing, studies with lower vitamin D doses (200 IU/day) were also conducted in the preterms born at ≤32 weeks of gestation. At the 36 week of the postmenstrual age, vitamin D supplementation at a dose of 200 IU/day appeared not fully effective and vitamin D deficiency was noted in up to 40% of cases born at <28 weeks of gestation and 30% of cases born at 28–32 weeks of gestation (162). It seems that in more mature preterm neonates (with relatively lower risk of the severe vitamin D deficiency), vitamin D supplementation at a dose of 400 IU/day, that is also recommended for in-term born neonates, should provide adequate vitamin D supply (163).

The Expert Panel is of the opinion that population-based 25(OH)D concentration screening is not justified, however recognizes strong indications for 25(OH)D concentration assessment in an increasing number of clinical conditions in order to optimize the course and to minimize complications of the underlying disease (**Table 1**). The Panel shares the position statements of the Endocrine Society that the vitamin D supplementation in groups at risk of vitamin D deficiency that need special concern, including women planning pregnancy, pregnant and lactating women and the preterm babies (<32 weeks gestation) should be provided and followed under the control of 25(OH)D concentration and its changes (136). Some available reports pointed on vitamin D supplementation doses of 4,000–6,400 IU/day as safe, effective and beneficial for pregnant and lactating women as well as for offspring (146–148). In contrast, studies performed in Poland and Canada (Calgary, 51°N) evidenced a low effectiveness of vitamin D supplementation at doses 600–800 IU/day for beneficial pregnancy outcomes (16, 164). Considering the safety of vitamin D supplementation during pregnancy (to a lesser extent during lactation) and a high probability of use of multicomponent preparations that usually contain 200 IU vitamin D per serving, the Expert Panel recommends the dose of 2,000 IU/day for the general population of pregnant and lactating women with unknown 25(OH)D concentration. Some pregnant women may require higher doses of vitamin D to achieve optimal 25(OH)D concentration, however, vitamin D supplementation using doses higher than 2,000 IU/day should be carried out based on initial

25(OH)D concentration and its change. The Panel underlines that vitamin D deficiency during pregnancy is associated with a significantly higher risk of preterm delivery and preeclampsia and is considered as a risk factor for low birth weight and bacterial vaginosis. Correction of vitamin D deficiency by regular vitamin D supplementation during pregnancy, starting as early as possible (preferably at the pre-conception stage), may markedly reduce the risk of abovementioned complications and therefore is highly recommended (146–148, 165, 166).

Keeping in mind reports on a group of patients in the Polish population who are genetically predisposed to symptomatic hypercalcemia (carrying *CYP24A1* or *SLC34A1* gene mutations, resulting in decreased catabolism or excessive formation of an active form of vitamin D, respectively) (26), the Expert Panel suggests to consider a directed medical history investigation, anteceding vitamin D supplementation with vitamin D doses higher than recommended for the general population, in order to minimize the risk of adverse events in a individuals with vitamin D hypersensitivity. It was estimated that at least a thousand cases predisposed to symptomatic hypercalcemia live in Poland and the prevalence may be as high as 1:33.000 births (26). If there is a diagnosis of hypercalcemia, hypercalciuria, nephrolithiasis, nephrocalcinosis, *CYP24A1* or *SLC34A1* gene mutations or other form of vitamin D hypersensitivity in a patient or his/her family members, the supplementation should be carried out individually, and controlled by parameters of calcium-phosphate metabolism, particularly calcemia, PTH, calciuria, 25(OH)D and $1,25(OH)_2D$.

In the context of therapeutic dosing of vitamin D, the Expert Panel is of the opinion that single loading doses of vitamin D provided at the 3-month intervals should not be recommended in Poland. An approach for the loading doses use was proposed in the 2016, by global consensus focused on prevention and therapy of nutritional rickets, as an alternative therapeutic procedure for patients suffering from nutritional rickets, exclusively if the regular daily vitamin D supplementation is not possible for various reasons (8). Taking into account a higher risk of hypercalcemia as a result of loading doses (8) and previous Polish and European experience with loading doses (42), as well as a relatively easy, permanent access to vitamin D supplements and health care in Poland, an implementation of very high doses would not be justified.

Recently, a regimen of a single dose of 30,000 IU of vitamin D_3 available on prescription, has been promoted in Poland, with an indication for administration once a month in adults, the elderly and adolescents older than 12 years of age. The standpoint of the Expert Panel is that vitamin D intake at a dose of 30,000 IU, regardless the regimen [once a month according to summary of product characteristics (SPC) or more often according to some positions (167)], is considered neither as appropriate nor as safe management, if a prior assessment of 25(OH)D concentration and the risk factors for vitamin D hypersensitivity were not investigated. In the aspect of prevention of vitamin D deficiency in the general population (considered as healthy) or even in the groups of risk of deficiency, vitamin D supplementation at a cumulative dose equivalent to 15 or 30 daily doses (2,000 or 1,000 IU/day, respectively) rises concerns about safety. In the most extreme regimen of supplementation with use of cumulative dose recently promoted in Poland (30,000 IU), for example in older obese adolescents and obese adults, the recommendation of doses constituting two to three times the dose recommended for peers with normal body weight (i.e., 60,000–90,000 IU, respectively) even twice a month according to some reports (167), should be considered unwarranted and very risky. Panel is of the opinion that vitamin D supplementation at a single dose of 30,000 IU, diverging from the SPC (once a month) may be unfavorable even as an adjunct to osteoporosis treatment. In a RCT of 200 subjects aged >75 years an increased risk of falls as a result of use of 24,000 IU with 300 μg of calcifediol once a month, as well as an increased risk of falls as a result of 60,000 IU once a month were both evidenced (168).

The Expert Panel in the updated Polish recommendations as well as others [the previous Endocrine Society (136) and Central European (9) guidelines] do acknowledge abnormal body weight as a significant variable affecting vitamin D status and recommend obese persons from general population a doubled daily dose of vitamin D. A weak but significant negative correlations were shown between 25(OH)D concentrations and body weight as well as BMI (kg/m^2) in Poland (7). However, vitamin D deficiency is more often noted in obese persons, irrespective of age (169). Our guidelines recommend a two times higher daily dose for obese persons in relation to normal body weight counterparts. Further, age- and body weight related ranges of vitamin D for daily dosing, proposed by experts for use in general population, most likely will help to deal also with underweight cases. Our approach is consistent with the Endocrine Society's recommendations (136) and is supported by results of large surveys that estimated two to three times higher vitamin D daily dose for obese subjects, 1.5 times for overweight, and pointed that underweight persons may need lower vitamin D doses to achieve target 25(OH)D concentration when compared to individuals with normal body weight (170–172).

The Expert Panel recommends that vitamin D supplementation in individuals with an assayed 25(OH)D concentration should be based on the vitamin D status diagnosed according to recommended concentration ranges and should consider previous prophylactic management. Above statement relates to common observation that guidelines for vitamin D supplementation are not implemented or are not carried out properly and the fundamental problem is non-compliance (5, 6). In individuals declaring supplementary vitamin D intake with revealed abnormal 25(OH)D concentration value (low, high, too high, too low, etc.) the first line of management should be based on evaluation of regularity of vitamin D use, the dosage, a choice of preparation and the way of administration (with or without fat-containing products—depending on the preparation). A simple correction of management of vitamin D deficiency usually is sufficient enough. However, if the vitamin D supplementation was compliant to recommended but a response was not satisfying, expressed as 25(OH)D concentration still below optimal value range, it is recommended to increase a daily dose by 50–100% or to introduce therapeutic doses—depending on a severity of vitamin D deficiency. If vitamin D supplementation was not so far implemented, it should be started immediately, including the use of therapeutic doses in individuals showing severe deficiency (25(OH)D <10 ng/ml) (**Figure 1**). The 25(OH)D follow-up and

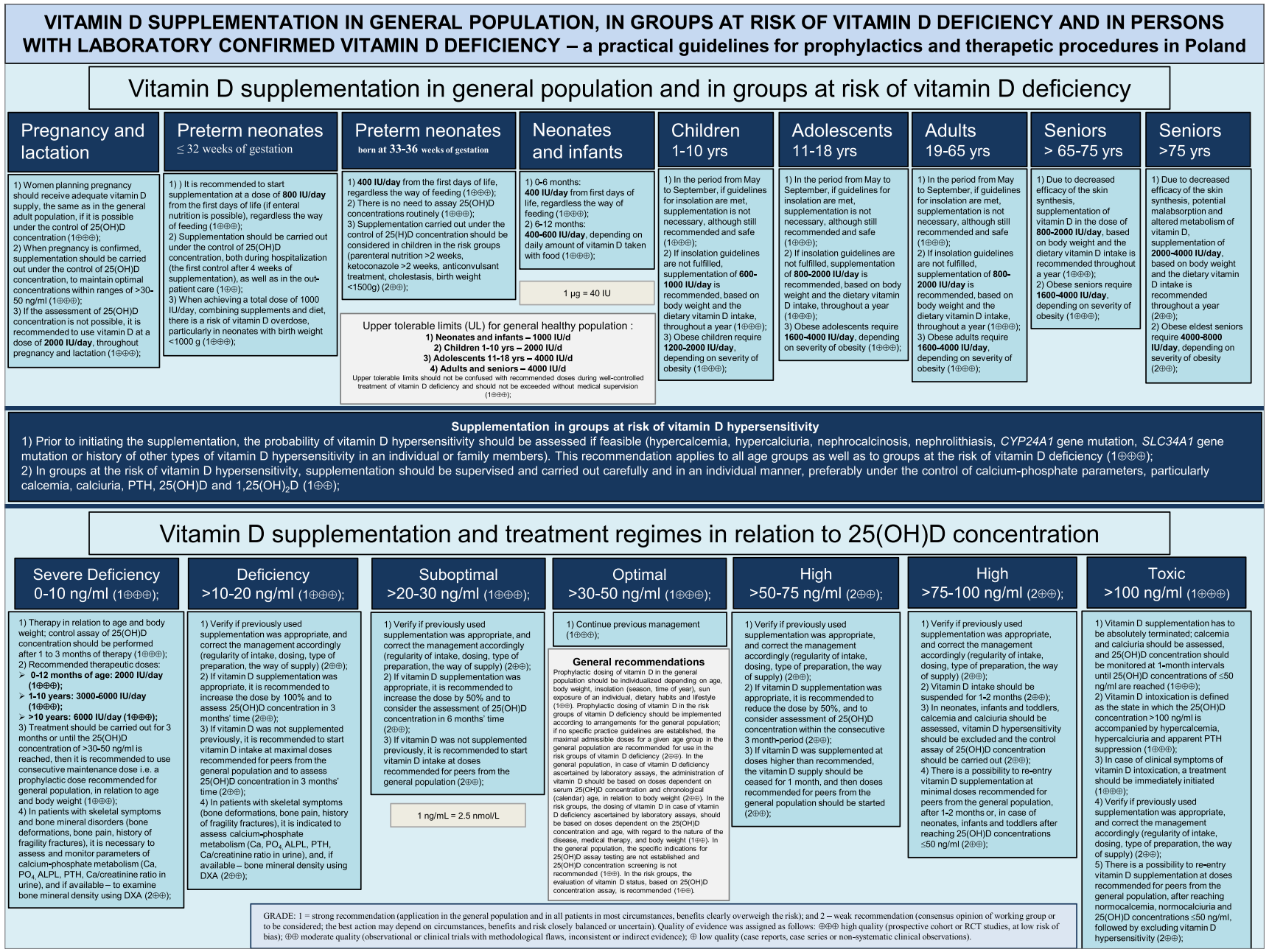

FIGURE 1 | The chart summarizing practical guidelines.

the range of additional investigations should depend on a severity of vitamin D deficiency.

The Expert Panel emphasizes significance of appropriate dietary calcium intake during the course of vitamin D supplementation and treatment of vitamin D deficiency. If dietary sources are considered as not effective, additional pharmacological supplementation with calcium salts preparations is recommended, preferably in a few divided daily doses due to higher absorption rate and the lower risk of periodic hypercalciuria. At the current state of knowledge, it is recommended to maintain existing calcium intake guidelines for Polish population, depending on the age (10).

SUMMARY

Vitamin D deficiency is an important public health problem in Poland that without appropriate preventive actions may escalate as a result of the on-going changes of life style, unfavorable nutritional habits as well as limited vitamin D supply from natural sources (both dietary and UVB) (4–7). It is necessary to introduce and pursue recommendations in its updated form concerning recent perspective on the prevention and treatment of vitamin D deficiency in all age groups. The synopsis of the guidelines is shown in **Figure 1**. This task should be a priority for doctors of all specialties, primarily for general practitioners, as well as people shaping health policies in Poland. It is essential particularly in the context of present knowledge, which provides evidence not only of calcemic action of vitamin D, but also of its pleiotropic effects. The list of classic and non-classic—pleiotropic action of vitamin D and associated health benefits becomes longer and longer. The optimal 25(OH)D concentrations for different endocrine, autocrine and paracrine pathways are indicated as an essential factor in preventing osteoporosis and falls, rickets and osteomalacia, as well as autoimmune diseases, including multiple sclerosis, diabetes type 1, systemic lupus erythematosus, infectious diseases, including tuberculosis and influenza, cardiovascular diseases, neurocognitive disorders, including Alzheimer disease, autism, pregnancy complications, diabetes type 2, as well as decrease of incidence rate and improvement of survival rate and quality of life in malignancy and overall mortality. Despite discussions on causality between vitamin D deficiency and a given disease or a risk of its development, and also in a view of the magnitude of the problem of vitamin D deficiency in the general population and in patients, the Expert Panel recommends implementation of updated guidelines dedicated to prevention and treatment of vitamin D deficiency to a routine everyday practice of physicians and clinical dieticians.

AUTHOR CONTRIBUTIONS

All authors contributed to the preparation of the guidelines, all participated in the data collection, drafting, writing and editing the manuscript. MWa is the National Consultant in Pediatric Endocrinology and Diabetes; President of the Polish Society of Pediatric Endocrinology and Diabetes. MB-K is the President of the Polish Society of Neonatology. DCH-S is the Chairwoman of the Section of Bone Metabolic Diseases in Children and Adolescents at the Polish Pediatric Society. EH is the National Consultant in Neonatology. TJ is the National Consultant in Pediatrics. JKs is the President of the Polish Society for Clinical Nutrition of Children. AL is the National Consultant in Endocrinology. JP-P is the President of the Polish Pediatric Society. MR is the President of the Polish Society of Endocrinology. MWi is the National Consultant in Perinatology; President of the Polish Society of Gynecologists and Obstetricians. DZ is the National Consultant in Pediatric Nephrology. PP is the President of the European Vitamin D Association—EVIDAS.

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Vitamin D Toxicity–A Clinical Perspective

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Confusion, apathy, recurrent vomiting, abdominal pain, polyuria, polydipsia, and dehydration are the most often noted clinical symptoms of vitamin D toxicity (VDT; also called vitamin D intoxication or hypervitaminosis D). VDT and its clinical manifestation, severe hypercalcemia, are related to excessive long-term intake of vitamin D, malfunctions of the vitamin D metabolic pathway, or the existence of coincident disease that produces the active vitamin D metabolite locally. Although VDT is rare, the health effects can be serious if it is not promptly identified. Many forms of exogenous (iatrogenic) and endogenous VDT exist. Exogenous VDT is usually caused by the inadvertent or improper intake of extremely high doses of pharmacological preparations of vitamin D and is associated with hypercalcemia. Serum 25-hydroxyvitamin D [25(OH)D] concentrations higher than 150 ng/ml (375 nmol/l) are the hallmark of VDT due to vitamin D overdosing. Endogenous VDT may develop from excessive production of an active vitamin D metabolite – $1,25(OH)_2D$ in granulomatous disorders and in some lymphomas or from the reduced degradation of that metabolite in idiopathic infantile hypercalcemia. Endogenous VDT may also develop from an excessive production of 25(OH)D and $1,25(OH)_2D$ in congenital disorders, such as Williams–Beuren syndrome. Laboratory testing during routine clinical examinations may reveal asymptomatic hypercalcemia caused by the intake of vitamin D even in doses recommended for the general population and considered safe. That phenomenon, called hypersensitivity to vitamin D, reflects dysregulated vitamin D metabolism. Researchers have proposed many processes to explain VDT. Those processes include elevated activity of 1α-hydroxylase or inhibited activity of 24-hydroxylase, both leading to increased concentration of 1,25(OH)D; increased number of vitamin D receptors; and saturation of the capacity of vitamin D binding protein. Increased public awareness of vitamin D–related health benefits might increase the risk of VDT due to self-administration of vitamin D in doses higher then recommended for age and body weight or even higher than the established upper limit intake values. Consequently, the incidence of hypercalcemia due to hypervitaminosis D might increase.

Keywords: vitamin D, 25(OH)D, toxicity, clinical symptoms, management

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INTRODUCTION

Vitamin D is an important prohormone that plays a vital role in maintaining healthy bones and calcium levels. Vitamin D deficiency leads to hypocalcemia and defects in bone mineralization. Vitamin D deficiency, as suggested in many publications, also is associated with increased risks of extraskeletal complications such as autoimmune diseases, chronic obstructive pulmonary disease, cancer, and metabolic syndrome. Vitamin D deficiency (25-hydroxyvitamin D [25(OH)D] concentration <20 ng/ml; <50 nmol/l) and insufficiency [25(OH)D concentration of 21–29 ng/ml; 52.5–72.5 nmol/l] are both prevalent, being a global problem of public health (1). Because of the growing awareness of vitamin D deficiency and related health problems, vitamin D became a popular supplement, and its use has increased markedly. An increased intake of vitamin D supplements by the general population and a growing number of prescriptions of therapeutic doses (including very high doses) without medical monitoring might result in a greater risk of exogenous hypervitaminosis D, with symptoms of hypercalcemia also known as vitamin D toxicity (VDT) (2). This article presents some problems associated with VDT due to an overdosing and explains some of the problems of hypersensitivity to vitamin D. The existing knowledge related to VDT is based on anecdotal case reports, accidental poisoning, and animal experiments. For ethical reasons, experimentally analyzing VDT in humans is impossible.

DEFINING VDT AND HOW OFTEN IT OCCURS

VDT due to excess of vitamin D (hypervitaminosis D) is a clinical condition characterized by severe hypercalcemia that may persist for a prolonged time, leading to serious health consequences (3).

Hypervitaminosis D with hypercalcemia develops after uncontrolled use of vitamin D mega doses or vitamin D metabolites [25(OH)D, $1,25(OH)_2D$]. In some clinical conditions, hypervitaminosis D may develop as a result of using vitamin D analogs (exogenous VDT). Hypervitaminosis D with hypercalcemia may also be a manifestation of excessive production of $1,25(OH)_2D$ in granulomatous disorders, in lymphomas, and during idiopathic infantile hypercalcemia (IIH) (endogenous VDT) (3).

In healthy individuals, exogenous VDT is usually caused by prolonged use (months) of vitamin D mega doses, but not by the abnormally high exposure of skin to the sun or by eating a diversified diet. The human body can regulate the quantity of previtamin D (tachysterol and lumisterol) produced in the skin by ultraviolet-B radiation. A diversified diet typically does not provide large amounts of vitamin D, and the fortification of food products with vitamin D is modest (4). Exogenous VDT due to vitamin D overdosing is diagnosed by markedly elevated 25(OH)D concentrations (>150 ng/ml) accompanied by severe hypercalcemia and hypercalciuria and by very low or undetectable parathyroid hormone (PTH) activity (4). Hypercalciuria and hypercalcemia are the first measurable manifestations of VDT. The$1,25(OH)_2D$ concentration in patients with VDT may be within the reference range, slightly increased or reduced (less frequently) when an increased level of calcium in serum suppresses PTH activity. $1,25(OH)_2D$ is down regulated both by the inhibition of 1α-hydroxylase activity and by the enhancement of 24-hydroxylase activity (3).

Exogenous VDT may develop in patients taking excessive amounts of $1α,25(OH)_2D$ or other 1α-hydroxylated vitamin D analogs [1α(OH)D], such as paricalcitol and doxercalciferol, used to treat hypocalcemic disorders, including hypoparathyroidism, pseudohypoparathyroidism, osteomalacia, and end-stage renal failure. In those cases, hypercalcemia is an adverse effect of treatment with use of a pharmacological vitamin D agent, not related to 25(OH)D concentration, and the $1,25(OH)_2D$ concentration value is elevated (3, 5).

The increased risk of endogenous VDT is a serious clinical issue in granuloma-forming disorders and in lymphomas as well as in patients with IIH. In those disorders, patients are hypersensitive to vitamin D, and elevated $1,25(OH)_2D$ concentration with hypercalcemia may develop after vitamin D supplementation or from dietary products containing increased amounts of vitamin D or even after uncontrolled sunbathing (3). Patients with Williams–Beuren syndrome also need attention for hypersensitivity to vitamin D; however, both 25(OH)D and $1,25(OH)_2D$ concentration values in that disease may be either normal or elevated, and the pathophysiological explanation is often unclear. In granulomatous diseases such as sarcoidosis, tuberculosis, leprosy, fungal diseases, infantile subcutaneous fat necrosis, giant cell polymyositis, and berylliosis, endogenous VDT is related to the abnormal extrarenal synthesis of $1,25(OH)_2D$ by activated macrophages (3, 6). In lymphomas, the etiology of VDT is multiple, heterogeneous, and still not fully recognized (7). In IIH, a dysfunction of 24-hydroxylase (CYP24A1) activity, an enzyme responsible for degradation of both 25(OH)D and $1,25(OH)_2D$, results in uncontrolled severe hypercalcemia and related consequences (8). IIH may be revealed in early childhood or may persist undiagnosed into adulthood (9). Another recently discovered cause of IIH involves a defect in *SLC34A1*, the gene coding for the sodium-phosphate cotransporter (NaPi-IIA) in the kidney; hypercalcemia is the indirect manifestation of the downregulation of FGF-23 (10). In endogenous VDT, hypercalcemia is related to increased $1,25(OH)_2D$ concentration; in contrast, in VDT due to an overdose of vitamin D (exogenous VDT), hypercalcemia is a consequence of high 25(OH)D concentration (5).

The prevalence of VDT is unknown. As a result of increased intake of vitamin D–containing supplements and the recent information regarding prevalence of the CYP24A1 mutation (8–10) in the general population (estimated to occur in1 of 33,000 births) (11), the incidence of VDT may well increase.

In the past, exogenous VDT was considered a rare adverse effect associated primarily with food fortification. From the1930s through the 1950s, public health officials in the United States and the United Kingdom recommended routine fortification of milk and other foods with vitamin D (4). That policy was implemented initially as an effective public health strategy to

prevent nutritional rickets in children and then as an intervention to improve the general health of the population (4).

In the 1940s, massive doses of vitamin D (200,000–300,000 IU/day) were considered an effective treatment strategy for chronic illnesses as diverse as tuberculosis and rheumatoid arthritis. Because hypercalcemia was observed in some patients thus treated, individual doctors discontinued the massive doses and the symptoms of VDT disappeared after a few months (4, 12). However, those clinical observations alerted physicians to the possibility of VDT, and the practice of administering massive doses of vitamin D was later discontinued nationally. Those observations, however, did not influence fortification of foods and other products with vitamin D, which persisted through the 1950s (4). In the 1950s, several cases of infants with facial abnormalities, supravalvular aortic stenosis, mental retardation, and hypercalcemia were reported mainly in the United Kingdom. That was followed by additional reports of hypercalcemia in some infants in the United Kingdom as well as in other European countries (13).

The Royal College of Physicians and the British Pediatric Association related that unexpected and unexplained increased incidence of hypercalcemia to the excessive intakes of vitamin D from various foods fortified with vitamin D. (At the time, no reliable assessment for measuring vitamin D was available, and no reliable estimates for dietary intake of vitamin D existed). The Royal College of Physicians failed to provide strong evidence for that phenomenon (they based their conclusion predominantly on literature in which pregnant rodents receiving high doses of vitamin D delivered pups with dysmorphic features, aortic stenosis, and hypercalcemia). The British Pediatric Association documented hypercalcemia only in isolated cases of infants who had approximate daily vitamin D intakes of 1,500–1,725 IU. Therefore, the U.K. government strictly regulated vitamin D food fortification and vitamin D supplements to the general public (4, 13). However, in retrospect, hypercalcemia probably resulted from hypersensitivity to vitamin D in infants suffering from Williams–Beuren syndrome and sarcoidosis (4). Nonetheless, in a substantial number of those cases, hypercalcemia was probably due to an excessive daily intake of vitamin D. Later observations of VDT came from the United States, where hypervitaminosis D in eight patients was associated with drinking vitamin D–fortified milk. An analysis of the milk produced at a local dairy revealed excessive vitamin D fortification of up to 232,565 IU per quart instead of the standard 400 IU per quart (14). As a result of that incident, local government agencies around the world prohibited the fortification of milk and alerted physicians to the potential of VDT—a concern that persists to this day (14).

In statements released over the last decade, the Institute of Medicine (IOM) (15) and the Endocrine Society (14) have both concluded that acute VDT is extremely rare in the literature, that serum 25(OH)D concentrations must exceed 150 ng/ml (375 nmol/l), and that other factors, such as calcium intake, may affect the risk of developing hypercalcemia and VDT. Regardless of additional risk factors for VDT, many studies provided evidence that vitamin D is probably one of the least toxic fat-soluble vitamins, much less toxic than vitamin A (4). Dudenkov et al. (2) researched more than 20,000 serum 25(OH)D measurements performed at the Mayo Clinic from 2002 to 2011 to determine the prevalence of VDT, demonstrated by the presence of hypercalcemia. The number of individuals with a serum 25(OH)D concentration >50 ng/ml (>75 nmol/l) had increased by 20 times during that period. However, relatively high 25(OH)D concentrations coincided with a normal serum calcium concentration. Only one patient, with a25(OH)D concentration of 364 ng/ml (910 nmol/l), was diagnosed with hypercalcemia. Pietras et al. (16) reported that healthy adults in a clinical setting, receiving 50,000 IU of vitamin D_2 once every 2 weeks (equivalent to approximately 3,300 IU/day) for up to 6 years, maintained 25(OH)D concentrations of 40–60 ng/ml (100–150 nmol/l) without any evidence of VDT. Those findings were consistent with the observation by Ekwaru et al. (17) that Canadian adults who ingested up to 20,000 IU of vitamin D_3 per day had a significant increase of 25(OH)D concentrations, up to 60 ng/ml (150 nmol/l), but without any evidence of toxicity.

THE PROCESS OF ACUTE VDT

VDT resulting from excessive use of vitamin D is characterized by hypercalciuria, hypercalcemia, elevated 25(OH)D >150 ng/ml (>375 nmol/l), and usually normal or slightly increased $1,25(OH)_2D$ concentration.

Ten years ago, Jones (18) suggested three major hypotheses about the mechanism of VDT. All three involve increased concentrations of a vitamin D metabolite reaching the vitamin D receptor (VDR) in the nucleus of target cells and causing exaggerated gene expression. The three hypotheses to explain VDT are as follows:

1. Toxicity is mediated by increased serum concentrations of the active hormonal form, $1,25(OH)_2D$, which lead to its increased intracellular concentration. That hypothesis is not strongly supported. Only one study, Selby et al. (19) reported elevated $1,25(OH)_2D$ concentration values at VDT. Many other studies revealed that $1,25(OH)_2D$ concentrations were normal or only slightly elevated.
2. $1,25(OH)_2D$ has a low affinity for vitamin D binding protein (VDBP) (20) and a high affinity for VDRs, making it an important ligand with access to the transcriptional signal transduction machinery. In hypervitaminosis D, the concentrations of various vitamin D metabolites, especially 25(OH)D, are markedly increased, saturating the binding capacity of VDBP and in turn enabling other vitamin D metabolites to enter the cell nucleus. Among the various vitamin D metabolites, 25(OH)D in higher concentrations (a dose-dependent effect) has the strongest affinity for VDRs, so that particular metabolite at its high serum concentrations stimulates transcription by itself (20, 21).
3. Vitamin D intake raises the concentration of vitamin D itself and increases concentrations of many other vitamin D metabolites, especially 25(OH)D. In hypervitaminosis D, the concentrations of vitamin D metabolites, such as vitamin D, 25(OH)D, $24,25(OH)_2D$, $25,26(OH)_2D$, and 25(OH)D-26,23-lactone, increase significantly (22). Abnormally increased concentrations of vitamin D metabolites exceed the VDBP

binding capacity and cause a release of free $1,25(OH)_2D$; the latter active metabolite enters the target cells by diffusion and acts through the VDR.

Of those three hypotheses, abnormally high 25(OH)D and free $1,25(OH)_2D$ concentrations are the most credible, although even that concept remains unproven (18, 20).

On the basis of various *in vitro* and *in vivo* studies using animal models, the mechanism of VDT suggested in hypothesis 3 seems unlikely. For example, in one study, a CYP27B1-knockout mouse lacking1α-hydroxylase and unable to synthesize $1,25(OH)_2D$ still suffered from VDT when exposed to doses of vitamin D similar to those given to wild-type controls (23). Thus, the literature favors the concept that VDT involves mechanism 2 and, consequently, that serum 25(OH)D concentration represents an accurate biomarker of the risk of VDT (24).

SIGNS AND SYMPTOMS OF VDT

The clinical manifestations of VDT are varied but are related primarily to hypercalcemia (3, 5).

Symptoms of VDT may be similar to those of other hypercalcemic states and include neuropsychiatric manifestations, such as difficulty in concentration, confusion, apathy, drowsiness, depression, psychosis, and in extreme cases, a stupor and coma. The gastrointestinal symptoms of VDT include recurrent vomiting, abdominal pain, polydipsia, anorexia, constipation, peptic ulcers, and pancreatitis. The cardiovascular manifestations of VDT include hypertension, shortened QT interval, ST segment elevation, and bradyarrhythmias with first-degree heart block on the electrocardiogram. The renal symptoms include hypercalciuria as the earliest sign, polyuria, polydipsia, dehydration, nephrocalcinosis, and renal failure. Other symptoms of VDT caused by hypercalcemia include band keratopathy, hearing loss, and painful periarticular calcinosis (25, 26).

DIAGNOSIS OF VDT

The diagnosis of VDT can be determined clinically. An early diagnosis of VDT requires a detailed clinical and drug history. VDT in most patients is the result of excessive dosages or too-frequent dosing intervals of vitamin D administered for osteoporosis, hypoparathyroidism, hypophosphatemia, osteomalacia, or renal osteodystrophy. Because of vitamin D's current popularity as a treatment agent for many diseases, vitamin D supplementation (including use of therapeutic doses) has become predominant in otherwise healthy individuals. General practitioners should be attentive to the symptoms of VDT in patients who have supplemented with therapeutic vitamin D doses or its metabolites. When hypercalcemia develops, patients with granulomatous diseases or lymphoma have a pervasive active disease. In those cases, the diagnosis of VDT is apparent on examination (3, 5).

Laboratory findings (other than hypercalcemia) inpatients with symptomatic exogenous VDT related to overdosing of vitamin D or 25(OH)D show suppressed PTH (intact), 25(OH)D concentration>150 ng/ml (>375 nmol/l), and normal or increased values of $1,25(OH)_2D$ concentration.

Exogenous VDT, as an adverse result of therapy with use of active vitamin D metabolite [both $1,25(OH)_2D$ and 1α-OHD], is characterized by laboratory findings of suppressed PTH (intact), elevated $1,25(OH)_2D$ concentration, and decreased or normal 25(OH)D concentration values.

Endogenous active metabolite intoxication due to coexisting granulomatous diseases or lymphoma may be characterized by suppressed PTH (intact), decreased or normal 25(OH)D concentration, and elevated $1,25(OH)_2D$.

In a hypercalcemic patient, hyperphosphatemia suggests VDT, whereas hypophosphatemia suggests primary hyperparathyroidism. The latter condition is further characterized by increased PTH activity and increased $1,25(OH)_2D$ concentration but normal 25(OH)D concentration (3, 23).

TREATMENT OF ACUTE VDT

Any one of vitamin D's three forms [vitamin D, 25(OH)D, or $1,25(OH)_2D$] may lead to VDT. Toxicity from vitamin D_2 or D_3 is harder to manage than toxicity due to vitamin D's metabolites [25(OH)D or $1,25(OH)_2D$]. That is partly due to the long half-life in the body because of vitamin D's high lipid solubility in the liver, muscles, and fat tissues and the corresponding large storage capacity (18–22).

Thus, hypercalcemia due to a vitamin D overdose theoretically can last up to 18 months after the administration of vitamin D is discontinued. That is because of the slow release of the stored vitamin D from fat deposits. However, the half-lives of 25(OH)D and $1,25(OH)_2D$ in the body are much shorter, at 15 days and 15 h, respectively. Therefore, an overdose of 25(OH)D may persist for weeks, whereas that related to $1,25(OH)_2D$ lasts only a few days (18, 22).

Treatment of VDT consists of first- and the second-line treatment strategies (3, 25, 27). First-line treatment includes the following:

1. Discontinuation of vitamin D supplementation and the reduction of dietary calcium intake. Patients with granulomatous diseases, lymphoma, and IIH are recommended to avoid exposure to sunlight and other ultraviolet-B light sources.
2. The administration of isotonic sodium chloride solution to correct dehydration and restore kidney function is recommended. Loop diuretics can be added once the volume is restored and maintained. In cases of prolonged sodium chloride and loop diuretic therapy, replacing lost sodium, potassium, and chloride is important.
3. Therapy with glucocorticoids (GS) will decrease plasma calcium levels by reducing intestinal calcium absorption by decreasing transcellular active transport processes and increasing urinary excretion of calcium. Furthermore, GS therapy changes the hepatic vitamin D metabolism to favor synthesizing inactive metabolites. Although that treatment is

efficient (serum calcium levels usually return to normal over several days with GS at doses of 100 mg/day of hydrocortisone or equivalent), the chronic use of systemic (oral or parenteral) GS therapy is unfortunately associated with common adverse events including secondary osteoporosis, osteonecrosis, and muscle weakness.

4. Antiresorptive therapy with use of calcitonin (CT), bisphosphonates (BS), or both can be useful in severe cases in which hypercalcemia is the result of increased osteoclastic bone resorption due to $1,25(OH)_2D$'s direct effect on bone tissue. The response to CT and BS is very different. CT works rapidly, but tachyphylaxis occurs after several days. BS work within a few days, but the effect persists long term. In fact, according to some reports, BS (including oral ones) are the most effective treatment for VDT, at least in children. Clinically, knowing whether increased osteoclastic bone resorption occurs is impossible, although one would assume it to be the case in the presence of significant hypercalcemia. Therefore, use of those compounds cannot be restricted to conditions of increased osteoclastic bone resorption.

Second-line treatments of VDT include the following:

5. Phenobarbital can be a useful treatment for VDT by decreasing 25(OH)D concentrations through induction of the hepatic microsomal enzyme (28).
6. Ketoconazole non-specifically decreases $1,25(OH)_2D$ production by activated mononuclear cells by inhibiting cytochrome P450, CYP27B1, but long-term use is not recommended because it blocks many other important CYPs (29).
7. Aminoquinolines (chloroquine, hydrochloroquine) decrease $1,25(OH)_2D$ production by activated mononuclear cells through an unknown mechanism in granulomatous diseases (30).
8. Specific inhibitors of CYP27B1 (1α-hydroxylase) have been developed that might find utility in specifically blocking the production of $1,25(OH)_2D$ without interfering with other cytochrome P450–containing enzymes (31).
9. The induction of the non-specific liver cytochrome P450 enzymes, including CYP3A4, by drugs such as rifampin results in an alternative catabolic fate from the 24-hydroxylation pathway for vitamin D metabolites and allows for the non-specific breakdown of excess $1,25(OH)_2D$ in IIH patients (32).

POSSIBLE TOXICITY OF MODERATE INTAKES OF VITAMIN D FOR EXTENDED PERIODS

The IOM Report in 2011 not only discussed the upper limits (ULs) for vitamin D intake on the basis of the acute, short-term administration of high-dose vitamin D preparations for limited periods but also emphasized chronic administration of vitamin D over years of supplementation. Acute toxicity would be caused by doses of vitamin D probably in excess of 10,000 IU/day, which result in serum 25(OH)D concentrations >150 ng/ml (>375 nmol/l). That level is clearly more than the IOM-recommended UL of 4,000 IU/day. Potential chronic toxicity would result from administration of doses above 4,000 IU/day for extended periods, possibly for years, that cause serum 25(OH)D concentrations in the 50–150 ng/ml (125–375 nmol/l) range (15).

The IOM cited several association studies that suggest possible deleterious effects of serum 25(OH)D concentrations above 50 ng/ml. Those effects include all-cause mortality, an incidence of certain cancers (breast, pancreatic, and prostate), and falls and fractures. All-cause mortality follows an inverse J curve so that risk of death appears to increase in patients with 25(OH)D concentration values above 30 ng/ml (>75 nmol/l). However, in a recent paper (33), Durazo-Arvizu and colleagues reanalyzed those findings on the basis of standardized 25(OH)D assay results and concluded that the uptick in the reverse J curve is an artifact eliminated at high 25(OH)D values.

In a controversial study, elderly women who received an annual single high dose of vitamin D (500,000 IU) had higher rates of fractures and falls than women in the control group, who received a placebo (34). Though serum 25(OH)D was not measured in the treated group, a sub-study reported that serum 25(OH)D was 48 ng/ml (120 nmol/l) 1 month after dosing. In a more recent study, Bischoff-Ferrari et al. (35) reported a higher risk of falls in men and women older than70 years, who were given 60,000 IU/month, than in control groups given 24,000 IU/month ± 300 μg of $25(OH)D_3$/month over 1 year. Serum 25(OH)D concentrations reached 40 ng/ml (100 nmol/l) in the affected group on doses of 60,000 IU/month and even higher in individuals receiving $25(OH)D_3$.

Consequently, several possible deleterious effects of chronic moderate doses of vitamin D remain unexplained. In contrast with the study of acute VDT, no plausible explanation exists for the mechanism of such deleterious effects on health with chronic VDT. Although no mechanism can yet explain those data, we must continue to question whether chronic moderate vitamin D dosing is potentially harmful.

SUMMARY AND CONCLUSIONS

Although VDT resulting in hypercalcemia is rare, it can be life-threatening if not promptly identified. Many forms of exogenous (iatrogenic) and endogenous VDT exist. The unintentional overdosing due to use of pharmaceutical products is the most frequent cause of exogenous VDT. An overview of VDT cases caused by vitamin D formulation or administration errors that resulted in excessive dosing confirmed that intoxication is extremely rare. However, VDT should always be considered as a differential diagnosis in patients with hypercalcemia (36).

In some clinical conditions, endogenous VDT is also an important clinical issue. Endogenous etiologies may develop from ectopic production of $1,25(OH)_2D$ in granulomatous diseases, such as sarcoidosis and tuberculosis, or in lymphoma. Researchers have proposed many processes to account for VDT, including the inhibited activity of 24-hydroxylase or elevated activity of 1α-hydroxylase, both leading to increased concentration of the active vitamin D metabolite, the increased

number of VDRs, or the saturation of the capacity of VDBP. Despite many controversies related to target 25(OH)D concentration or recommended vitamin D doses for general population, the available guidelines agree that 25(OH)D concentrations >150 ng/ml pose a significant risk of VDT and that vitamin D deficiency treatment regimens with use of high doses (higher than ULs) need regular monitoring (37).

In the general population, the awareness of vitamin D–related health benefits is growing; however, the increased consumption of vitamin D–containing supplements may predispose the general public to an increased incidence of VDT. Therefore, without medical supervision, caution is advised for people who self-administrate vitamin D at doses higher than recommended for age and body weight.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Nordic Walking Training Causes a Decrease in Blood Cholesterol in Elderly Women Supplemented with Vitamin D

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Objective: Different studies have demonstrated that regular exercise can induce changes in the lipid profile, but results remain inconclusive. Available data suggest that correction of vitamin D deficiency can improve the lipid profile. In this study, we have hypothesized that Nordic Walking training will improve lipid profile in elderly women supplemented with vitamin D.

Methods: A total of 109 elderly women (68 ± 5.12 years old) took part in the study. First group [experimental group (EG): 35 women] underwent 12 weeks of Nordic Walking (NW) training combined with vitamin D supplementation (4,000 IU/day), second group [supplementation group (SG): 48 women] was only supplemented with vitamin D (4,000 IU/day), and third group [control group (CG): 31 women] was not subject to any interventions. Blood analysis of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and 25-OH-D_3 was performed at baseline and after the 12 weeks of NW training. Additionally, a battery of field tests specifically developed for older adults was used to assess the components of functional fitness. The same blood analysis was repeated for the EG 6 months after the main experiment.

Results: After 12 weeks of NW training and vitamin D supplementation, in the EG a decrease in TC, LDL-C, and TG was observed. In the SG, no changes in the lipid profile were observed, whereas in the CG an increase in the HDL-C level was noticed. Positive physical fitness changes were only observed in the EG.

Conclusion: Our obtained data confirmed baseline assumption that regular exercise induces positive alternations in lipid profile in elderly women supported by supplementation of vitamin D.

Keywords: physical fitness, exercise, LDL, HDL, health training

INTRODUCTION

Regular exercise has been demonstrated to induce several adaptive changes manifested by an increase in the endurance strength of skeletal muscle. Positive changes in brain structure and function have also been observed in response to physical activity (1). These and other adaptive changes induced by exercise are known to lower the risk of cardiovascular disease, diabetes, cancer, depression, and many others (2), often associated with aging. The pro-healthy effect of physical activity on the risk of these diseases may be partially attributed to beneficial changes in insulin sensitivity, inflammatory markers, and blood lipids post (3, 4). However, the topic continues to raise questions; despite the fact that many studies have reported a positive effect of regular exercise on blood lipids, several studies have shown no effect at all (5).

Vitamin D is an endogenous hormone known to regulate expression of hundreds of genes. Since it is synthetized from 7-dehydrocholesyterol, it is also possible that its status is interrelated with cholesterol.

There is some evidence that apolipoprotein A-I (apo A-I) gene expression can be modified by vitamin D. At the same time, apo A-I is an essential component of high-density lipoprotein (HDL) molecules, positively influencing its quality. The effects of exercise on blood lipids have been shown to depend on applied dietary solutions or some drug compounds (6). In particular, vitamin D combined with exercise has been demonstrated to modify lipid metabolism and blood lipid profile by improving insulin sensitivity (7).

Physical activity itself is associated with better vitamin D status (25-OH-D_3) (8); however, the effect of vitamin D status on blood lipids alone remains unclear. Supplementation of vitamin D at 400 IU for 5 years has been reported to induce no significant changes in blood lipids (9). Conversely, another study has shown that the concentration of 25-OH-D_3 correlated inversely with triglycerides (TG) and total cholesterol (TC) (10). Based on collected data, we have hypothesized that beneficial effects of exercise on the lipid profile may be influenced by the vitamin D status.

Vitamin D deficiency or insufficiency is prevalent in most countries; it is now considered a pandemic (11, 12). Consequently, in this paper, we have studied effects of vitamin D supplementation alone and combined with 12 weeks of Nordic Walking (NW) training on the lipid profile in elderly women. The present study is the first published report to implicate effects of exercise and vitamin D on lipid profile in elderly subjects.

MATERIALS AND METHODS

Three groups of elderly women participated in the study, all aged over 60 years (68.4 ± 5.0 years old). They were randomly assigned to three groups. First group [experimental group (EG)] involved 35 women subjected to 12 weeks of NW training supported with vitamin D supplementation (average 4,000 IU/day). Second group [supplementation group (SG)] involved 48 women, subject only to vitamin D supplementation (average 4,000 IU/day). Vitamin D was supplemented three times per week with appropriate doses to reach 28,000 IU/week which is essentially concordant with current recommendations (13).

Third group [control group (CG)] involved 31 women, who did not receive any supplementation and did not participate in the training. Considering the pleiotropic function of vitamin D on many aspects of human health, we recognized that it would be unethical to include a placebo group in the experiment.

All subjects underwent a medical check-up prior to the experiment. Exclusion criteria from the study included the following: uncontrolled hypertension (systolic blood pressure over 140 mmHg and diastolic over 100 mmHg) a history of cardiac arrhythmia, cardio-respiratory disorders, and orthopedic problems. It was recommended that the volunteers did not change their lifestyle and diet habits throughout the study. Experiment activities were completed at the Gdansk University of Physical Education and Sport.

Ethics Statement

The examination was officially approved by the Bioethical Committee of the Regional Medical Society in Gdansk (KB-26/14) according to the Declaration of Helsinki and was registered as a Clinical trail NCT03417700. Before commencing the training and testing, subjects received verbal description of the experiment. Written informed consent was signed by all participants. The ethics approval was also obtained for referring participants to their family physician upon detection of any abnormal pathology results during the medical check-up.

Blood Analysis

Blood collection was performed following the same timeline for all groups: at baseline and one day directly after the 12-week training program. Additionally, in EG blood collection took place also 6 months after end of NW training program. Blood samples were obtained between 7 and 8 a.m. after an overnight fast. The serum was separated by centrifugation at 1,000× *g* for 15 min and stored at −80°C pending analysis. Red blood cells count (10^6/μL) (RBC), hematocrit (%) (Hct), and blood hemoglobin concentration (g/dL) (Hb), low-density lipoprotein cholesterol level (LDL-C), HDL-C, TC, and triglyceride (TG) were determined from venous blood samples by conventional methods using a BIOSYSTEMS S.A, ANALYZER A25 Costa Brava, Barcelona, Spain.

Vitamin D Assessment

Vitamin D metabolite 25-OH-D_3 was measured by high-performance liquid chromatography mass spectrometry (HPLC-MS). The HPLC system was a Transcend TLX turboflow 2 system attached to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) as described before (14).

Measurements of Physical Fitness

A battery of field tests developed specifically for older adults was used to assess components of functional fitness in the EG. These tests require very little time or equipment and are designed to be conducted in community settings. In accordance with Rikli and Jones (15, 16), we used the following tests at the beginning of

the study and after the 12 weeks of training. The Senior Fitness Test (SFT) consists of the following six items: (1) 30-s chair stand, (2) arm curl, (3) chair sit-and-reach, (4) back scratch, (5) 2-min step, and (6) 8-foot up-and-go. The tests were performed in this order with 1 min of rest in-between. Before each test, the exercises were demonstrated for the participants, who made a trial attempt before completing the actual drill. The walking test was performed only once.

Exercise Protocol

The same group of research assistants and instructors supervised all training sessions. The EG completed 12 weeks of a mesocycle exercise, divided into three microcycles. The training procedure was described in detail in our previous study (17). The participants met three times a week, 1 h after eating a light breakfast and performed the main session of NW training at 60–70% intensity of the maximal HR (10-min warm-up, 45–55-min NW, and 10-min cooldown). Each training unit was recorded with Garmin Forerunner 405 with a built-in GPS.

Statistical Analysis

Statistical analysis was performed using Statistica 12.0 software (Statsoft, Tulsa, OK, USA). All values are expressed as mean ± SD. The Shapiro–Wilk test was applied to assess the homogeneity of dispersion from the normal distribution. The Brown–Forsythe test was used to evaluate the homogeneity of variance. For homogenous results, a paired *t*-test analysis was performed to identify significantly different results. For heterogeneous results, the Wilcoxon signed-rank test was applied. For homogenous results, the analysis of variance (ANOVA) for repeated measurements and the *post hoc* Tukey test for unequal sample sizes were performed to identify significantly different results. For heterogeneous results, the ANOVA Friedman's test and right *post hoc* test was applied. The significance level was set at $p < 0.05$. The relations between variables were evaluated using the Pearson correlation coefficient.

RESULTS

General Outcomes

Baseline descriptive characteristics of participants are summarized in **Table 1**. Values of the body mass index (BMI), percentage, and absolute fat tissue indicate that our groups were within the range of normal to slightly overweight. There were no significant changes in the body composition after the 12 weeks of NW training in the EG nor in the SG or CG.

Level of Physical Fitness

The 12 weeks of NW training improved all measured fitness parameters. Specifically, changes in the level of general shoulder coordination and flexibility were statistically significant (**Table 1**). The applied training program also improved the level of endurance and lowered the heart rate at baseline [from 81 ± 14 to 77 ± 15 bpm, average values during exercise (120 ± 17 to 116 ± 16 bmp) and after the exercise (141 ± 22 to 133 ± 23 bmp)]. These changes, however, were not statistically significant. The level of physical fitness in the SG and CG was lower as compared with the EG; however, the differences did not reach statistical significance (**Table 1**). In addition, some improvement in endurance has been observed in 2,000-m test (1,082 ± 108 vs. 1,059 ± 116 s); however, it did not reach statistical significance ($p = 0.2$, CI − 49 to 11).

TABLE 1 | 12 weeks of Nordic Walking training had no effect on the body composition but had improved physical fitness in elderly women supplemented with vitamin D supplementation.

	Experimental group (exercise and supplement)			Control group		Supplemented group	
	Baseline ($n = 35$)	After 12 weeks ($n = 35$)	After 6 month ($n = 21$)	Baseline ($n = 31$)	After 12 weeks ($n = 31$)	Baseline ($n = 48$)	After 12 weeks ($n = 48$)
Weight (kg)	69.2 ± 10.7	69.0 ± 11.1	66.5 ± 7.9	70.7 ± 11.4	71.2 ± 11.8	69.2 ± 10.1	70.3 ± 10.0
BMI ($kg \bullet m^{-2}$)	26.0 ± 4.0	26.3 ± 4.0	25.6 ± 3.3	27.1 ± 3.7	27.5 ± 4.0	26.4 ± 3.5	26.8 ± 3.7
Fat (kg)	24.8 ± 8.1	24.9 ± 8.6	23.3 ± 6.4	27.0 ± 7.4	27.1 ± 8.0	25.1 ± 7.3	25.9 ± 7.7
Fat (%)	35.3 ± 7.1	35.2 ± 7.7	34.5 ± 6.2	37.3 ± 6.0	37.0 ± 5.7	35.5 ± 6.7	36.1 ± 7.1
FFM (kg)	44.2 ± 5.1	44.1 ± 4.9	43.2 ± 3.6	43.7 ± 5.6	44.1 ± 5.6	44.2 ± 5.0	44.6 ± 5.1
TBW (kg)	32.5 ± 3.8	32.4 ± 3.6	31.7 ± 2.6	32.1 ± 4.1	32.4 ± 4.1	32.5 ± 3.7	32.7 ± 3.8
Chair stand (no. of stands)	21 ± 4	22 ± 3	22 ± 3	19 ± 5	20 ± 5	16 ± 3	17 ± 4
Arm curl (no. of reps)	26 ± 5	28 ± 6[a]	29 ± 3	26 ± 7	26 ± 6	24 ± 5	23 ± 5
Chair sit-&-reach (cm)	7 ± 9	9 ± 9	6 ± 9	4 ± 10	4 ± 10	6 ± 10	4 ± 10
Back scratch (cm)	−1 ± 8	0 ± 8	1 ± 7	−1 ± 7	−1 ± 8	−1 ± 7	−2 ± 6
8-foot up-&-go (s)	4.72 ± 0.5	4.24 ± 0.4[a]	4.56 ± 0.7	4.66 ± 0.7	4.61 ± 0.7	4.49 ± 0.9	4.61 ± 0.7

Values are means (±SD). BMI, body mass index; Fat, fat mass; Fat%, percentage of body fat; FFM, free fat mass; TBW, total body water.
[a]*Significant differences from baseline.*

General Characteristics of Blood Tests

Hematological parameters of the EG were within reference ranges in all subjects at baseline as well as after the training. Nonetheless, a significant drop in Hb, mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH) was observed after training (**Table 2**). Importantly, no iron deficiency (not shown) or anemia was observed in any subject.

Lipid Profile

Vitamin D supplementation combined with the 12 weeks of NW training induced significant changes in the lipid profile in the EG. A significant decrease in TC, LDL-C, and TG was noted. All these changes were accompanied by a significant rise in 25-OH-D_3 concentration owing to the applied supplementation. The training and supplementation caused a decrease in HDL-C; however, the shift was not statistically significant (**Table 3**). A detailed analysis of the ratios between parameters of the lipid profile (LDL-C/HDL-C, TC/HDL-C, TC/LDL-C, TG/HDL-C) showed no additional tendency for change, neither within individual groups nor in the intergroup comparison. Interestingly, 6 months following the experiment, the levels of TC, HDL-C, and LDL-C returned to baseline in the EG (**Table 3**).

In the CG, the concentration of 25-OH-D_3 remained stable over the 12-week period, considerably lower compared with the EG and SG. An increase in HDL concentration was the only change observed in the CG (**Table 4**). At the same time, in the SG, an increase in 25-OH-D_3 was accompanied by a decrease in HDL-C relative to the CG (**Table 4**).

TABLE 2 | Hematological parameters in the experimental group.

Variable	Baseline	After 12 weeks	*p*-Value	CI– 95%	CI+ 95%
Hb (g·dL^{-1})	14.0 ± 0.9	13.7 ± 1.0[a]	0.03	−0.55	−0.02
MCH (pg)	30.1 ± 1.0	29.8 ± 1.1[a]	0.00	−0.52	−0.13
MCHC (g·dL^{-1})	34.0 ± 0.8	33.5 ± 0.8[a]	0.00	−0.64	−0.26
HCT (%)	41.1 ± 2.7	40.8 ± 2.6	0.42	−1.09	0.47
MCV (fL)	88.6 ± 3.1	88.9 ± 3.4	0.14	−0.07	0.56
RBC (min·μL^{-1})	4.6 ± 0.3	4.6 ± 0.3	0.28	−0.14	0.04

Values are means (±SD). CI, confidence interval; Hb, hemoglobin; MCH, mean cell hemoglobin; MCHC, mean corpuscular hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; RBC, erythrocytes. After 12-weeks of comparison between baseline and after the whole period of Nordic Walking training.
[a]*Significantly different from baseline.*

DISCUSSION

Data obtained through this study suggest that regular exercise induced positive changes in the lipid profile in elderly women. We demonstrate that NW training combined with vitamin D supplementation led to a significant decrease in total blood cholesterol in elderly women. As reviewed by Leon and Schantz, many studies have demonstrated that exercise applied alone induced a decrease in LDL and TG, but had no effect on blood TC (18). Conversely, a 12-week program of aerobic exercise did not influence blood lipids despite triggering a decrease in TG and a transient increase in HDL (19). It has also been revealed that the lipid-profile concentrations: serum TG, TC HDL-C, and LDL-C did not differ between athletes and non-athletes (20). In our previous study, we have shown that 4 weeks of regular training in young rowers did not influence the lipid profile unless the subjects were supplemented with vitamin D (21). All of these data indicate that effects of exercise on blood lipids can be modulated by other factors, among many vitamin D. Our preliminary data on 25-OH-D_3 demonstrate that most of the women participating in the study had exhibited vitamin D deficiency or insufficiency. Thus, in our present study, we have investigated the effect of regular training in combination with vitamin D supplementation.

The present study also demonstrates that NW training combined with vitamin D supplementation induced not only changes in total blood cholesterol, but also a significant decrease in LDL-C, yet no shift in HDL-C. Still, a comparison between the SG and the CG shows that vitamin D has a tendency to lower HDL-C. A previously published study supports this observation, as a low dose of vitamin D supplementation (300 IU/day) was shown to lead to a significant drop in HDL-C in postmenopausal women (22). In addition, no differences in the lipid profile between physically active (>3 h exercise/week) and physically inactive (<3 h exercise/week) women were observed. The authors concluded that vitamin D supplementation may have an unfavorable effect on lipids in postmenopausal women undergoing hormone

TABLE 3 | Changes in lipid profile in elderly women when Nordic Walking training is combined with vitamin D supplementation.

	Baseline (*n* = 35)	After 12 weeks of NW training and vit D supplementation (*n* = 35)	*p*	CI (−95%; +95%)	After 6 month without training and vit D supplementation (*n* = 21)
TC (mg/dL)	228.8 ± 36.0	207.7 ± 37.4[a]	0.00	(−34.2; −7.9)	239.0 ± 50.3
HDL-C (mg/dL)	70.8 ± 19.3	67.6 ± 18.5	0.12	(−7.4; 0.9)	73.2 ± 20.5
LDL-C (mg/dL)	134.5 ± 29.6	121.1 ± 32.2[a]	0.02	(−24.5; −2.3)	143.7 ± 47.4
TG (mg/dL)	117.1 ± 55.6	94.6 ± 34.0[a]	0.00	(−36.4; −8.5)	109.8 ± 46.8
LDL-C/HDL-C	2.1 ± 0.6	1.9 ± 0.7	0.49	(−0.2; 0)	2.1 ± 0.8
TC/HDL-C	3.4 ± 0.8	3.2 ± 0.7	0.10	(−0.4; 0)	3.4 ± 0.9
TC/LDL-C	1.7 ± 0.2	1.8 ± 0.2	0.53	(0; 0.2)	1.7 ± 0.3
TG/HDL-C	1.9 ± 1.2	1.6 ± 0.8	0.09	(0; 0.6)	1.7 ± 1.0
Vit D (ng/mL)	20.8 ± 7.7	38.4 ± 14.3[a]	0.00	(12.6; 22.6)	23.6 ± 10.8

Values are means (±SD). TC, total cholesterol; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; TG, triglycerides.
[a]*Significant differences from baseline.*

TABLE 4 | Effect of vitamin D supplementation on lipid profile in elderly women.

	Control group		Supplemented group	
	Baseline (n = 31)	**After 12 weeks (n = 31)**	**Baseline (n = 48)**	**After 12 weeks (n = 48)**
TC (mg/dL)	215.5 ± 41.6	235.0 ± 52.2	218.2 ± 57.3	212.2 ± 56.4
HDL-C (mg/dL)	73.2 ± 16.1	79.8 ± 16.3[a,b]	70.1 ± 14.6	69.1 ± 13.3[b]
LDL-C (mg/dL)	121.5 ± 38.4	133.9 ± 51.6	127.0 ± 54.9	121.0 ± 53.7
TG (mg/dL)	103.7 ± 32.4	106.7 ± 31.7	107.0 ± 37.4	110.5 ± 44.8
LDL-C/ HDL-C	1.8 ± 0.7	1.8 ± 0.8	1.9 ± 1.2	1.8 ± 1.2
TC/HDL-C	3.1 ± 0.8	3.0 ± 0.9	3.2 ± 1.2	3.2 ± 1.3
TC/LDL-C	1.8 ± 0.3	1.9 ± 0.4	1.9 ± 0.4	1.9 ± 0.4
TG/HDL-C	1.5 ± 0.7	1.4 ± 0.6	1.5 ± 0.6	1.6 ± 0.8
Vit D (ng/mL)	24.2 ± 12.1	24.6 ± 10.9	18.1 ± 9.1	40.7 ± 12.1[a,b]

Values are means (±SD). TC, total cholesterol; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; TG, triglycerides.

[a]*Significant differences from baseline.*

[b]*Significant differences between groups.*

replacement therapy (22). It is difficult to agree with this conclusion as a study published in recent years demonstrated that it is the quality rather than quantity of HDL that plays an important role in human health (23–25). The level of HDL in people converted to low-fat high-carbohydrate diet was observed to decrease, while the atheroprotective potential improved (26). Another study has shown the level of HDL-C to decline in bariatric patients after surgical intervention, reaching the preoperative level after 6 months. It has been demonstrated that that during this period a qualitative switch took place as apoE HDL was replaced by apoA-I HDL (25). The effect of vitamin D on the apoA-I gene expression is debatable because both positive and negative shifts have been reported in response to 1,25-OH-D_3 treatment. These data indicate that the effects of vitamin D on blood lipids can be modulated by other factors.

At the same time, in our previously published study we have demonstrated that regular exercise had no effect on the HDL-C level in vitamin-D-deficient young men, but led to its significant reduction when accompanied by vitamin D supplementation (21). It is generally believed that changes are blood lipids result from the adaptive response of skeletal muscle, manifested in an increase in lipid oxidation, and insulin sensitivity (27). We observed some improvement in endurance in the EG, which could have been accompanied by an increase in the mitochondrial oxidative potential. Contrary to our expectation, changes in endurance capacity were accompanied with significant decrease in Hb and MCH; however, all the recorded data were in reference range. More research is needed to understand the nature of these changes. Our data suggest that the shifts in blood lipids induced by NW training were mediated by vitamin D and some factors possibly discharged from exercising muscles (4). Interestingly, in the EG, in the 6-month period after the intervention, during which neither regular training nor supplementation were taking place, all lipids parameters and 25-OH-D_3 returned to baseline values. This happened despite the fact that all participants had attended a talk about benefits of vitamin D and had received recommendations about vitamin D supplementation and exercise. Certainly, however, it is too early to judge the nature of these changes without data about subpopulation of HDL particles. The NW training and vitamin D supplementation had also a positive effect on blood TG. These data are in agreement with a previously published study, where plasma 25-OH-D was inversely associated with TG and TC (10, 28). Certainly, the main limitation of this study is the lack of a placebo group. As mentioned above, we decided not to include a placebo group in our study because of two main reasons. Firstly, most women taking part in our study were vitamin D deficient at the beginning of the experiment. Given that vitamin D deficiency can increase the risk of many morbidities, we decided that maintaining this state would be unethical. Secondly, observations made in this study are supported by our earlier research on young athletes (21). We can, thus, conclude that regular NW exercise induced positive changes in blood lipids in elderly women, in whom vitamin D deficiency was corrected.

ETHICS STATEMENT

The examination was officially approved by the Bioethical Committee of the Regional Medical Society in Gdansk (KB-26/14) according to the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

KrP and JA designed the study and performed the research. JK, JA and EZ performed the research and wrote the paper. KaP, JM, JJ, WS, and ML performed the research.

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Maternal and Cord Blood Vitamin D Status and Anthropometric Measurements in Term Newborns at Birth

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Introduction: Vitamin D deficiency in pregnant women may result in reduced neonatal development due to the fact that systemic vitamin D status during fetal life depends on maternal concentrations. Some authors reported significant differences in neonatal anthropometric measurements depending on maternal vitamin D concentrations.

Objective: The aim of this study is to evaluate the relationship between maternal and cord blood concentrations of vitamin D and neonatal anthropometric measurements at birth.

Materials and methods: This study included 94 pregnant women, at term, who delivered at the Department of Obstetrics, Women's Diseases and Gynecological Oncology, Medical University of Warsaw. Total serum 25(OH)D concentration was measured in mother–child pairs, and newborn anthropometric data were collected. A multiple regression analysis was used for statistical analysis.

Results: No relationship between maternal and neonatal cord blood vitamin D concentrations vs. neonatal weight, length, head, and chest circumference at birth was found ($p > 0.05$). Severe vitamin D deficiency (<10 ng/ml) was detected in 10.6%, deficiency (10–20 ng/ml) in 39.4%, insufficiency (20–30 ng/ml) in 39.4%, and optimal vitamin D concentration (>30 ng/ml) only in 10.6% of the pregnant women. Cord blood vitamin D deficiency (<20 ng/ml) was found in 28.7% of the neonates.

Conclusion: No differences between neonatal anthropometric measurements of infants born to mothers with normal and deficient vitamin D concentrations were found.

Keywords: vitamin D, blood, pregnancy, newborn, health

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INTRODUCTION

Vitamin D is responsible for a number of important functions in the fetus, and vitamin D blood saturation in the neonate is directly dependent on maternal levels. Vitamin D insufficiency in the mother results in neonatal insufficiency (1, 2), which may negatively affect the anthropometric parameters in the neonate, skeletal calcium score, the immune system, and increase the risk for asthma and type 1 diabetes in later life (3–5).

Currently, the recommended intake of vitamin D and its optimal concentration in the body are the source of much heated debate among medical researchers (6, 7). The American Academy of

Pediatrics recommends that serum concentration of 25(OH)D in maternal blood be more than 32 ng/ml (8). In light of various reports, the general consensus is that it should exceed 30 ng/ml (9, 10). However, some experts are of the opinion that maternal levels of at least >20 ng/ml will ensure adequate vitamin D concentration in the neonate (11), while latest publications suggest that 25(OH)D concentration should be at least 40 ng/ml (2). With 30 ng/ml as the threshold value for adequate concentration, vitamin D insufficiency or deficiency is a common occurrence, affecting as many as 99–100% of pregnant women in Turkey (12, 13), 85% in India (14), 69–95% in Central Europe (15–17), 52–85% in Southern Europe (18, 19), 74% in the United States (20), and 63% in China (21). The contemporary lower reference range for cord blood vitamin D level is 20 ng/ml (1, 22). However, as in the case of maternal levels, there is no consensus regarding the optimal concentrations (23–26). The literature reports unanimously agree that cord blood serum concentration of vitamin D is directly correlated with maternal levels. Regardless, cord levels may be higher (24, 27–29), equal to (12, 14, 19), and lower than maternal venous vitamin D concentration (30, 31). Neonates born to mothers with adequate vitamin D concentrations during pregnancy are supplied until 8 weeks of life (2, 32). The contemporary literature questions whether the dose of vitamin D routinely administered to all newborns in the first weeks after birth should depend on the maternal concentrations and infant weight (3, 23).

The aim of this study is to evaluate the relationship between maternal and cord blood vitamin D concentrations and the anthropometric parameters of the newborn (weight, length, and head and chest circumference) as well as the Apgar score.

MATERIALS AND METHODS

Study Design

The cross-sectional study was conducted among 100 pregnant women at the Department of Obstetrics, Gynecology and Oncology, Medical University of Warsaw. The study included women who delivered during two extreme seasons as far as exposure to sun is concerned (winter–summer) to investigate the widest range of vitamin D concentrations in the body. The winter group comprised women who delivered between December 2014 and February 2015, whereas the summer group included women who delivered between July and August 2015. This study included women who presented at the hospital on weekdays in the morning. The exclusion criteria were as follows: non-Polish nationality, multiple gestation, advanced stage of labor, chronic maternal diseases before pregnancy, and threatened course of labor. Informed written consent was obtained. Maternal blood was drawn after admission to the delivery ward (at the same time when blood was drawn for diagnostic purposes), and cord blood was drawn at delivery. Samples were immediately delivered to the hospital laboratory. Only term deliveries (94 patients: 45 from the winter group and 49 from the summer group) were included into the analysis. The Ethics Committee of the Institute of Food and Nutrition approved of the study (Code 10/162/KB/2014). Maternal and neonatal characteristics are presented in **Table 1**.

TABLE 1 | Maternal and neonatal characteristics.

Number of women, *n* (%)	94 (100)
Winter group	45 (48.0)
Summer group	49 (52.0)
Age (years), mean ± SD	29.9 ± 4.3
Education, n (%)	
Higher	63 (67.0)
Other	31 (33.0)
Gravidity, n (%)	
Primiparas	40 (42.5)
Multiparas	54 (57.5)
Prepregnancy maternal body mass index, mean ± SD	22.9 ± 3.7
Weight gain during pregnancy, n (%)	
Low	23 (24.5)
Normal	31 (33.0)
Excessive	40 (42.5)
Gestational diabetes, *n* (%)	9 (9.5)
Smoking during pregnancy, *n* (%)	14 (15.0)
Professionally active during pregnancy, *n* (%)	54 (57.4)
Supplementation with vitamin/mineral preparations, *n* (%)	85 (90.4)
Supplementation with single-component vitamin D preparations, *n* (%)	13 (13.8)
Daily vitamin D consumption	
From diet (μg), median (range)	2.1 (0.2–11.5)
From supplements (μg), median (range)	12.0 (5.0–50.0)
Daily calcium consumption	
From milk and dairy products (mg), median (range)	596 (69–1872)
Dlaily caffeine consumption	
From coffee, tea, and energy drinks (mg), mean ± SD	67 ± 51
Sex of the newborn, n (%)	
Male	48 (51.0)
Female	46 (49.0)
Neonatal weight (g), mean ± SD	3515 ± 500
Neonatal length (cm), mean ± SD	55.4 ± 2.7
Apgar score (points), mean ± SD	9.9 ± 0.1
Neonatal head circumference (cm), mean ± SD	34.8 ± 1.4
Neonatal chest circumference (cm), mean ± SD	34.0 ± 1.9

Laboratory Analysis and Data Collection

Total 25(OH)D [25(OH)D_2 and 25(OH)D_3] concentrations were measured in the blood using immunological tests (LIAISON® 25 OH Vitamin D TOTAL Assay; DiaSorin). The lower detection threshold for vitamin D is 4.0 ng/ml. The intraassay and interassay CV were <8% and <11%, respectively. Neonatal data (sex, weight, length, Apgar score at 5 min, and head and chest circumference) were obtained from the hospital medical records. The anthropometric measurements were taken by the midwives immediately upon delivery. Weight was measured using a physician beam scale. The remaining measurements were taken with the use of a tape measure. The total neonatal length was measured from the vertex of the head to the soles (with the feet kept vertical at 90°). The occipital–frontal head circumference (tape was placed on the maximum protrusion of the occipital and supraorbital ridges) and the chest circumference (tape was placed horizontally on the sternum and lower tip of the shoulder blade) were measured. A Food Frequency Questionnaire, validated at the Institute of Food and Nutrition, was used to assess vitamin D, calcium, and caffeine consumption during the entire course of pregnancy. To precisely evaluate portion size, direct interviewing (face-to-face) and the "Photo Album of Meals and Products" were used for data collection. The questionnaire also included data on supplementation,

patient lifestyle, and weight gain. As advised by the American Institute of Medicine, the recommended weight gain for pregnant women is 12.5–18 kg for underweight, 11.5–16 kg for normal weight, 7.0–11.5 kg for overweight, and 5–9 for obese women (33). These values were also used in our study. Lower or higher weight gain was considered as insufficient or excessive. The nutritional status was estimated using the body mass index (BMI). The content of vitamin D in vitamin/mineral supplements for pregnant women, as well as single-component vitamin D preparations, was estimated based on our earlier analysis (34). As many as 75% of the multicomponent supplements for pregnant women, which are available in Poland, contain a small amount of vitamin D (5–10 μg). Only single-component preparations contain larger amount of vitamin D (mean, 25 μg). The following criteria of maternal serum 25(OH)D concentrations were used: recommended level, >30 ng/ml; insufficiency, 20–30 ng/ml; deficiency, 10–20 ng/ml; and severe deficiency, <10 ng/ml (9, 27). As for cord blood, in accordance with the current recommendations of some experts, the concentration of ≥20 ng/ml was treated as the recommended level, whereas <12 ng/ml signified severe deficiency (1, 22).

Statistical Analysis

A multiple regression analysis was used to investigate a possible relationship between selected baseline characteristics (serum vitamin D concentration, vitamin D and calcium consumption, gravidity, maternal age and education, prepregnancy BMI, weight gain during pregnancy, smoking, caffeine consumption, use of dietary supplements, professional activity during pregnancy, gestational diabetes, and sex of the neonate) and neonatal weight, length, and head and chest circumference. $p < 0.05$ was considered as statistically significant.

RESULTS

Overall, severe vitamin D deficiency (<10 ng/ml) was detected in 10 (10.6%), deficiency (10–20 ng/ml) in 37 (39.4%), insufficiency (>20–30 ng/ml) in 37 (39.4%), and optimal vitamin D concentration (>30 ng/ml) only in 10 (10.6%) pregnant women. The neonates had higher levels of vitamin D than the mothers (mean, 27.0 ± 11.1 ng/ml vs. 19.5 ± 7.8 ng/ml). As a consequence, vitamin D deficiency (<20 ng/ml) and severe deficiency (<12 ng/ml) were found in 27 (28.7%) and 9 (9.6%) cases, respectively.

No relationship between maternal and cord blood vitamin D concentrations and neonatal anthropometric measurements at birth (weight, length, and head and chest circumference) was found ($p > 0.05$). Due to the fact that mean Apgar score was close to the optimal values (9.9 points), that parameter was not included in the statistical analysis. Neontal somatic development was good in all neonates. No statistically significant differences were found between neonatal anthropometric parameters in the winter and summer groups although vitamin D concentrations were higher in the summer compared to the winter group [mean, 22.2 ± 6.5 ng/ml vs. 16.3 ± 8.0 ng/ml ($p = 0.0003$) for the mothers and 31.3 ± 9.4 ng/ml vs. 22.0 ± 11.0 ng/ml ($p = 0.0001$) for the neonates]. Weight gain during pregnancy was the only factor that proved to be associated with neonatal body weight and length. Children born to mothers whose weight gain in pregnancy was lower than the recommendations were 302 g lighter ($p = 0.0405$) and 2.4 cm shorter ($p = 0.0025$), compared to infants born to mothers with normal weight gain (**Tables 2** and **3**).

TABLE 2 | Relationship between selected parameters and neonatal weight.

	Regression beta coefficient (SE)	95% confidence interval	*p*-Value
Maternal serum vitamin D concentration	−5.84 (15.491)	−36.68 to 25.00	0.7070
Cord blood vitamin D concentration	−0.05 (11.230)	−22.41 to 22.31	0.9965
Vitamin D consumption	1.72 (2.951)	−4.15 to 7.59	0.5616
Calcium consumption	5.90 (12.151)	−18.29 to 30.09	0.6284
Supplementation with vitamin/mineral preparations	−232.65 (181.872)	−594.73 to 129.43	0.2046
Pre-pregnancy body mass index:			
underweight	15.69 (199.141)	−380.77 to 412.15	0.9374
overweight/obesity	70.77 (151.218)	−230.28 to 371.82	0.6411
Low weight gain vs. normal gain	−301.77 (144.827)	−590.10 to −13.44	0.0405
Excessive weight gain vs. normal gain	55.03 (137.407)	−218.52 to 328.59	0.6899
Gestational diabetes	120.94 (176.572)	−230.59 to 472.47	0.4954
Smoking	57.71 (159.212)	−259.26 to 374.67	0.7180
Caffeine consumption	−154.11 (138.210)	−429.27 to 121.04	0.2683
Age	10.91 (13.953)	−16.87 to 38.69	0.4365
Education	−144.76 (125.909)	−395.43 to 105.91	0.2538
Gravidity	−57.38 (116.065)	−288.44 to 173.69	0.6225
Professional activity during pregnancy	−60.90 (108.664)	−277.24 to 155.43	0.5768
Neonatal sex	−28.27 (110.419)	−248.10 to 191.56	0.7986

TABLE 3 | Relationship between selected parameters and neonatal length.

	Regression beta coefficient (SE)	95% confidence interval	*p*-Value
Maternal serum vitamin D concentration	−0.00 (0.839)	−0.17 to 0.16	0.9765
Cord blood vitamin D concentration	0.02 (0.061)	−0.10 to 0.14	0.7747
Vitamin D consumption	−0.01 (0.016)	−0.04 to 0.02	0.5247
Calcium consumption	−0.04 (0.066)	−0.17 to 0.10	0.5936
Supplementation with vitamin/mineral preparations	−0.99 (0.985)	−2.95 to 0.97	0.3159
Pre-pregnancy body mass index:			
underweight	−0.24 (1.078)	−2.39 to 1.91	0.8247
overweight/obesity	−0.09 (0.82)	−1.72 to 1.55	0.9176
Low weight gain vs. normal gain	−2.44 (0.784)	−4.01 to −0.88	0.0025
Excessive weight gain vs. normal gain	−0.29 (0.744)	−1.77 to 1.19	0.6970
Gestational diabetes	0.60 (0.956)	−1.30 to 2.51	0.5295
Smoking	−0.14 (0.862)	−1.85 to 1.58	0.8758
Caffeine consumption	−0.93 (0.748)	−2.42 to 0.56	0.2189
Age	−0.03 (0.076)	−0.18 to 0.12	0.6666
Education	−0.68 (0.682)	−2.04 to 0.67	0.3199
Gravidity	0.02 (0.629)	−1.23 to 1.27	0.9740
Professional activity during pregnancy	0.26 (0.588)	−0.91 to 1.44	0.6548
Neonatal sex	−0.07 (0.598)	−1.26 to 1.12	0.9122

DISCUSSION

In our study, we found no association between vitamin D status and the somatic development of the newborns. Despite the fact that maternal vitamin D levels were diverse (minimum, 4 ng/ml; maximum, 37.7 ng/ml), no relationship between any of the investigated anthropometric parameters and maternal values was found. Also, regardless of the fact that predelivery maternal concentrations of vitamin D were too low in approximately 90% of the women, the overall condition of the neonates was good, even in cases classified as "severely deficient in vitamin D" (<10 ng/ml in maternal and <12 ng/ml in cord blood). Lack of a relationship between maternal vitamin D concentrations and neonatal measurements in Poland was reported by Skowrońska-Jóźwiak et al., although their study focused on insufficiently low vitamin D concentrations, which were confirmed in almost 69% of mothers of term infants (16). As for data from other countries, Rodriguez et al. and Eggemoen et al. found no connection between neonatal anthropometric parameters and maternal vitamin D levels, even despite the fact that vitamin D deficiency (<20 ng/ml) was detected in 19.7% and 51% of the women, respectively (18, 35). Loudyi et al. reported no association between maternal vitamin D status and neonatal weight at birth among the investigated women (vitamin D concentration ≤20 ng/ml in 90% of the cases) (36). Shakiba and Iranmanesh and Josefson et al. found no relationship between cord vitamin D concentration and neonatal weight and length (25, 31), while Dalgård et al. reported the same lack of relationship for neonatal weight and head circumference (37).

Nevertheless, some authors have postulated the existence of such a relationship and reported significant differences in neonatal size depending on vitamin D concentrations. An Indian study has demonstrated lower weight (by 480 g), length (by 9.5 cm), and head and chest circumference (by 4.5 and 4.8 cm, respectively) in neonates born to mothers with vitamin D deficiency compared to the recommended levels (14). Nobles et al. found a smaller difference (by 176 g) in the weight of infants born to mothers with vitamin D deficiency in the United States (20). As for cord blood, yet another study reported decreased neonatal length (by 0.5 cm) in a group of children with very low vitamin D concentrations (<4.8 ng/ml) compared to the recommended levels (37). Interesting results were reported by Lykkedegn et al., who demonstrated a U-shaped association between neonatal weight at birth and cord blood vitamin D concentrations. A significant weight gain was observed after the concentration values exceed 24 ng/ml (38).

In light of the conflicting data from individual studies, meta-analyses seem to be the most valuable sources of information. A meta-analysis of observational studies has indicated that lowered maternal vitamin D concentration (<15 ng/ml) results in lower neonatal weight at birth (by 131 g), but has no impact on the length and head circumference (6). Another meta-analysis has concluded that maternal vitamin D concentration of <20 ng/ml increases the risk for small-for-gestational age infant (39).

The conclusions on the effects of vitamin D supplementation during pregnancy are also conflicting. A meta-analysis of randomized clinical trials has found that vitamin D supplementation during pregnancy increases neonatal weight and length but does not affect the course of pregnancy. However, these authors emphasize the need for further studies to obtain more conclusive results (40). Harvey et al., in a systematic review, claimed that the available literature reports are not sufficient to confirm the existence of a relation between vitamin D concentration and neonatal condition at birth, or even to recommend obligatory vitamin D supplementation in pregnant women (7).

The results of our study did not reveal deteriorated neonatal condition at birth among children born to mothers with low vitamin D levels compared to optimal concentrations. Nevertheless, several limitations might have biased the final results, especially the fact that the analysis of vitamin D level was a single test and was conducted on the day of the delivery, which does not signify that the concentration was typical for the entire course of pregnancy or at least its significant part. The second limitation of our study was a relatively small sample size, which was the result of the number of deliveries at the Clinic and the nature of the study, i.e., the winter–summer group, so caution is advised when formulating final conclusions.

CONCLUSION

Our study did not demonstrate a relationship between the anthropometric features of the neonates and maternal and cord blood vitamin D levels. In the absence of differences in neonatal condition between infants born to mothers with vitamin D deficiency and with adequate levels, it seems safe to conclude that the currently recommended vitamin D levels in pregnant women (>30 ng/ml) do not need to be reached to ensure proper fetal growth. Regardless, vitamin D performs various essential functions in the human body, some of which remain to be fully elucidated, so its concentration during pregnancy should not only be perceived through neonatal anthropometric measurements but also be perceived through long-term development and health condition of the children.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of the Institute of Food and Nutrition approved of the study (Code 10/162/KB/2014) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Institute of Food and Nutrition.

AUTHOR CONTRIBUTIONS

RW conceived the idea for the study. RW, MJ, and WS contributed to the design of the research. RW, MK-N, MT, MB, and MS-S collected the data. RW and MK-N analyzed the data and wrote the paper. All authors edited and approved the final version of the manuscript.

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Vitamin D Level and Activities of Daily Living in Octogenarians: Cross-Sectional Study

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Introduction: Despite the growing number of octogenarians, little is known about their vitamin D status and activities of daily living (ADL) relations.

Objective: The aim of this study was to investigate peculiarities of vitamin D and ADL and to assess their relations in octogenarians.

Methods: A cross-sectional study was performed at the National Osteoporosis Centre located in Vilnius, Lithuania. Community-dwelling ambulatory persons aged ≥80 years were included. Current users of vitamin D supplements were excluded. Total 25 hydroxyvitamin D concentration in serum was measured with Cobas E411. Functional status was assessed by Katz ADL and the Lawton Instrumental Activities of Daily Living (IADL) scales. Subjects were divided into three groups according to age and into two groups according to vitamin D level. One-way analysis of variance with *post hoc* test was used to determine between-group comparisons. Associations between vitamin D and ADL score, and IADL score were assessed using Spearman's correlation.

Results: The study was performed on 153 octogenarians: 81 (52.9%) women and 72 (47.1%) men. The average age of subjects was 83.9 ± 3.2 years. Mean total 25 hydroxyvitamin D concentration was 11.2 ± 7.0 ng/ml; 137 (89.5%) persons had vitamin D deficiency, 12 (7.8%) had insufficiency, and only 4 (2.6%) persons were vitamin D sufficient. Positive weak correlation between total 25 hydroxyvitamin D and ADL score ($r = 0.2$, $p = 0.01$) and very weak correlation between total 25 hydroxyvitamin D and IADL score ($r = 0.19$, $p = 0.02$) were found. Total 25 hydroxyvitamin D level was correlated with ADL score in women ($r = 0.23$, $p = 0.04$). In the 80–84 years group ADL score correlated with total 25 hydroxyvitamin D level ($r = 0.23$, $p = 0.02$).

Conclusion: The majority of investigated octogenarians had vitamin D deficiency. The level of vitamin D was associated with the ADL score. There was no association between the vitamin D level and the IADL score, although a weak correlation was found between vitamin D level and category of food preparation.

Keywords: age, octogenarians, total 25 hydroxyvitamin D, activities of daily living, instrumental activities of daily living

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INTRODUCTION

There were 137 million persons aged 80 years or above (octogenarians) living in the world in 2017, and this number is projected to increase more than threefold in 2050, to 425 million (1). In Lithuania, there were 146,319 octogenarians (5.19% of total population) and 33,751 (1.2%) of these were living in the capital city Vilnius living at the end of 2017 (2). It is estimated that 1 billion people have vitamin D

deficiency or insufficiency worldwide, and this is particularly prevalent among elderly people (3). Octogenarians are at risk of total 25 hydroxyvitamin D deficiency for several reasons: a tendency to avoid the sun; decreased skin capacity to produce vitamin D; and diminished intestinal absorption and/or decreased vitamin D dietary intake (4). There is growing evidence that total 25 hydroxyvitamin D deficiency is associated with muscle weakness, increased risk of falling, impaired functional status, lower cognitive performance score, frailty, and poorer quality of life in elderly people (5). The association between total 25 hydroxyvitamin D deficiency and mortality is inconclusive, as some studies report a positive relationship while others do not (6, 7).

Aging is associated with decreased muscular strength and physical function, these changes lead to decreased mobility and independence, such as difficulties in walking and transferring from a bed or chair (8). Activities of daily living (ADL) include the fundamental skills typically needed to manage basic physical needs, comprised of various functional skills in different areas (9).

Despite the growing number of octogenarians, little is known about their vitamin D status and ADL relations. There are few studies performed in the oldest-old group regarding information about the total 25 hydroxyvitamin D status and functional independence relationship (10–14). In three of these studies a positive association between vitamin D level and ADL was found. Houston and colleagues (10) and Nakamura and colleagues (11) revealed that vitamin D deficiency was a predictor of low ADL scores in community-dwelling adults of advanced age. Kotlarczyk and colleagues showed that women in long-term care facilities, who had low levels of vitamin D, had the greatest functional decline (12). In another two studies no association between vitamin D and ADL were found. Navarro-Martínez and colleagues did not find an association between the vitamin D level and ADLs in frail octogenarian women (13). While Formiga and colleagues did not show any association between vitamin D status and IADLs, a positive relationship between vitamin D level and ADL score was found (14).

The aim of this study was to investigate the peculiarities of vitamin D and ADL and to assess their relations in octogenarians.

MATERIALS AND METHODS

This cross-sectional study was conducted at the National Osteoporosis Centre, Vilnius, Lithuania from January 2017 to February 2018. Non-probability convenience sampling method was used for the study population. Subjects were enrolled from outpatient clinics in Vilnius city. Inclusion criteria were: age 80 years and above; community-dwelling ambulatory women and men. The exclusion criteria were: moderate cognitive impairment (mini-mental state examination score < 21); acute illness; chronic diseases in terminal stages; and current use of vitamin D supplements. Subjects were divided into three age groups: aged 80–84 years, 85–89 years, and above 90 years. The height was measured barefoot with a stadiometer to the nearest 0.1 cm. Body weight was measured barefoot and in light indoor clothes with an electronical medical scale to the nearest 0.05 kg. Body mass index (BMI) was calculated as weight in kilograms divided by the height in meter squared (kg/m^2).

Blood samples were collected from 8:00 a.m. till 11:00 a.m., after fasting for at least 12 h. Serum total 25 hydroxyvitamin D (vitamin D) was measured by automated immunoassay (Cobas E411, Roche Diagnostic). Analysis was carried out by fully automated electrochemical luminescence immunoassay method using the original reagents, and in accordance with the manufacturer's instructions, regular calibration, and quality control applied on a daily basis. The total coefficient of variation was 5.9%.

The Endocrine Society Guidelines were used to interpret the total 25 hydroxyvitamin D level in serum: 25(OH)D concentration of 20 ng/ml (50 nmol/l) or below was defined as vitamin D deficiency; vitamin D insufficiency was determined as 25(OH)D concentration of 21–29 ng/ml (52.5–72.5 nmol/l); and concentration of vitamin D equal or above 30 ng/ml (75 nmol/l) was assessed as sufficient (15). In this study, we pooled sufficiency and insufficiency groups. Seasons were defined as winter (December–February), spring (March–May), summer (June–August), and autumn (September–November).

The functional status was evaluated using Katz ADL scale: bathing, dressing, toileting, transferring, continence, and feeding (16). Lawton Instrumental Activities of Daily Living categories were as follows: ability to use telephone; shopping; food preparation; housekeeping; laundry; mode of transportation; responsibility for own medications; and ability to handle finances (17).

The study protocol has been approved by Vilnius Regional Biomedical Research Ethics Committee. All subjects gave their written informed consent prior to enrollment.

Statistical analysis was performed using IBM SPSS Statistics Windows software version 18 (IBM, New York). All data were expressed as mean, SD, or frequencies (number, percentage), as appropriate. Distribution of continuous variables was assessed by the Shapiro–Wilk test. Mean differences of interval variables were compared using Student's *t*-test. The one-way analysis of variance (ANOVA) was used to determine whether there were any significant differences between the means of three or more independent groups. Associations between vitamin D levels and ADL, IADL scores were assessed using Spearman correlation analysis. The level of significance (*p*-value) of <0.05 was considered as statistically significant.

RESULTS

In total, 153 persons participated in this study: 81 women (52.9%) and 72 men (47.1%). Age ranged from 80.0 to 95.6 years: 80.0–92.5 years for women and 80.1–95.6 years for men. Basic descriptive characteristics of study population are shown in **Table 1**. Upon further investigation, none of the IADL categories could be pointed out as cause for difference in IADL scores between women and men.

No association was found between seasons of blood sampling (winter, spring, summer, and autumn) and vitamin D level ($p = 0.65$). In spring, total 25 hydroxyvitamin D was collected from 55 (35.9%) subjects, and their mean total 25 hydroxyvitamin D level was 11.64 ± 8.02 ng/ml. In summer, 24 (15.7%) subjects' blood was sampled and mean total 25 hydroxyvitamin D level was 10.68 ± 6.23 ng/ml. The total 25 hydroxyvitamin D level of 44 (28.8%) subjects' was collected in autumn and mean total

TABLE 1 | Basic descriptive characteristics of study population (mean ± SD).

Characteristic	All subjects (n = 153)	Women (n = 81)	Men (n = 72)	p
Age (years)	83.89 ± 3.18	83.49 ± 2.61	83.94 ± 3.43	0.36
Height (cm)	162.61 ± 9.48	156.29 ± 7.52	169.71 ± 5.65	<0.001
Weight (kg)	72.04 ± 11.81	68.41 ± 12.37	76.13 ± 9.68	<0.001
BMI (kg/m^2)	27.29 ± 4.38	28.07 ± 5.13	26.42 ± 3.17	0.02
Total 25 hydroxyvitamin D (ng/ml)	11.15 ± 7.01	10.61 ± 6.85	11.76 ± 7.19	0.46
– Deficiency	9.41 ± 4.41	9.27 ± 4.5	9.59 ± 4.32	0.36
– Insufficiency	22.19 ± 2.74	21.55 ± 1.39	22.51 ± 3.26	0.23
– Sufficiency	32.42 ± 1.67	33.59 ± 1.52	31.25 ± 0.81	0.29
ADL (score)	4.5 ± 1.07	4.57 ± 1.11	4.43 ± 1.03	0.43
IADL (score)	5.72 ± 2.42	6.12 ± 2.12	5.26 ± 2.67	0.03

p-Value was calculated using Student's t-test when comparing women and men. BMI, body mass index; ADL, activities of daily living; IADL, instrumental activities of daily living.

TABLE 2 | Characteristics of study population in age groups (mean ± SD).

Characteristic	80–84 years (n = 107)	85–89 years (n = 38)	90+ years (n = 8)	Analysis of variance p
Age (years)	82.71 ± 1.38	87.03 ± 1.39	92.21 ± 2.17	<0.001
BMI (kg/m^2)	28.07 ± 4.29	25.63 ± 4.19	24.78 ± 3.72	<0.001
Total 25 hydroxyvitamin D (ng/ml)	11.23 ± 6.65	10.45 ± 8.11	13.37 ± 6.42	0.55
ADL (score)	4.57 ± 0.98	4.47 ± 1.2	3.75 ± 1.48	0.11
IADL (score)	5.86 ± 2.37	5.58 ± 2.43	4.5 ± 3.02	0.29

BMI, body mass index; ADL, activities of daily living; IADL, instrumental activities of daily living.

25 hydroxyvitamin D level was 10.4 ± 6.04 ng/ml. In winter, 30 (19.6%) subjects' blood was collected and their mean total 25 hydroxyvitamin D level was 11.73 ± 7.14 ng/ml. No differences between total 25 hydroxyvitamin D levels were found in women and men. Results of the analysis of the study population in the different age groups are shown in **Table 2**.

Age groups did not differ in total 25 hydroxyvitamin D levels, ADL, and IADL scores. The mean total 25 hydroxyvitamin D level in all age groups was deficient. BMI was higher in the 80–84 years age group compared to the 85–89 years age group ($p = 0.01$). No differences were found between genders.

It was found that in the age group from 80 to 84 years, 94 (87.9%) people were vitamin D deficient, 11 (10.3%) were in the insufficiency group and only 2 (1.87%) octogenarians had sufficient vitamin D levels. In the age group ranging from 85 to 89 years, 36 (94.74%) subjects had deficient levels of vitamin D, none were in the insufficiency category and 2 (5.26%) had normal levels of vitamin D. In the oldest group (90+ years), 7 subjects (87.5%) had vitamin D deficiency, 1 (12.5%) subject had insufficient levels of vitamin D and none of them had sufficient levels of vitamin D.

Of all the subjects, 137 (89.5%) octogenarians had vitamin D deficiency, in 12 persons (7.8%) insufficiency was found, and only 4 (2.6%) subjects were vitamin D sufficient. Characteristics of the study population in vitamin D status groups are shown in **Table 3**.

TABLE 3 | Characteristics of study population in vitamin D status groups (mean ± SD).

Characteristic	Sufficient/insufficient (n = 16)	Deficient (n = 137)	p
Age (years)	83.53 ± 2.78	83.94 ± 3.23	0.59
BMI (kg/m^2)	26.21 ± 3.83	27.42 ± 4.44	0.25
Total 25 hydroxyvitamin D (ng/ml)	24.74 ± 5.2	9.41 ± 4.4	<0.001
ADL (score)	4.69 ± 0.94	4.44 ± 1.1	0.02
IADL (score)	6.88 ± 1.2	5.55 ± 2.45	<0.001

BMI, body mass index; ADL, activities of daily living; IADL, instrumental activities of daily living.

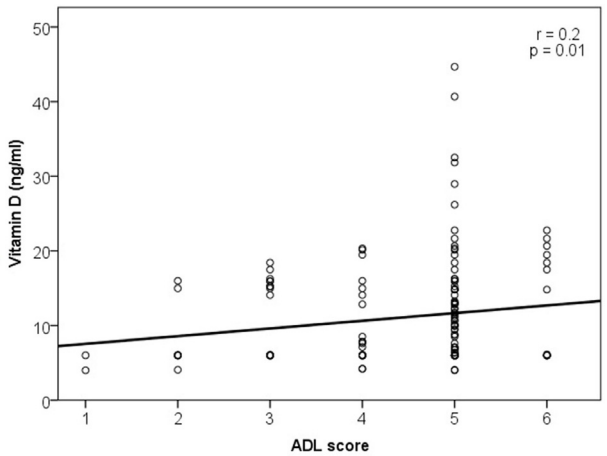

FIGURE 1 | Association of vitamin D level with activities of daily living (ADL).

No differences were found between women and men in the vitamin D status groups. Furthermore, there was no difference between age and BMI. Further analysis showed that subjects with vitamin D deficiency scored lower in ADL than subjects with vitamin D insufficiency ($p = 0.02$) or sufficiency ($p < 0.001$). The difference in the ADL score between insufficiency and sufficiency groups was not significant. The IADL score in the vitamin D deficiency group was significantly lower in the sufficiency group ($p < 0.001$), although there was no difference with the insufficiency group. The vitamin D insufficiency and sufficiency groups did not differ in IADL score.

Vitamin D correlations with ADL and IADL are shown in **Figures 1** and **2**, respectively.

A weak correlation was found between the vitamin D and ADL score ($r = 0.2$, $p = 0.01$) and very weak between the vitamin D and IADL score ($r = 0.19$, $p = 0.02$). There was no association between the serum vitamin D level and any single ADL category (bathing, dressing, toileting, transferring, continence, and feeding). Statistically significant correlations were found in two of eight IADL categories: weak correlation of vitamin D with food preparation ($r = 0.2$, $p = 0.02$) and very weak with the ability to use telephone ($r = 0.19$, $p = 0.02$).

Further analysis revealed a weak correlation between the vitamin D level and ADL score in women ($r = 0.23$, $p = 0.04$), and no correlations were found in men. When vitamin D level associations with ADL and IADL scores were analyzed in different

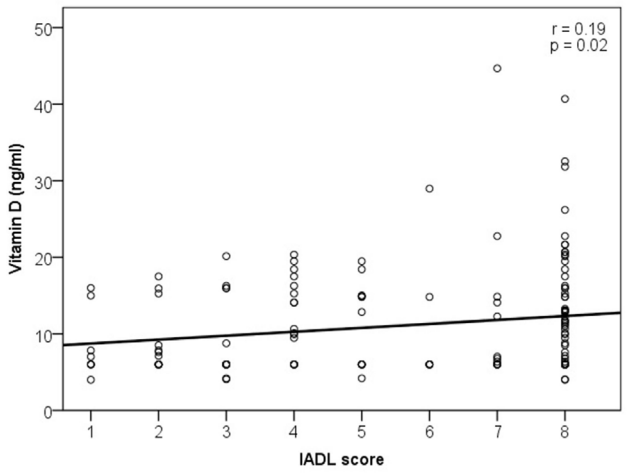

FIGURE 2 | Association of vitamin D level and instrumental activities of daily living (IADL).

age groups, correlation was found only for the ADL score in the 80–84 years group ($r = 0.23$, $p = 0.02$). No correlation between vitamin D level and ADL or IADL scores was found in different vitamin D status groups.

DISCUSSION

The results of our study show a high prevalence of vitamin D deficiency in community-dwelling ambulatory subjects aged 80 years and older—it was found in more than 89% of study subjects.

Data on the vitamin D status of oldest-old adults are scarce: only a few studies were aimed to investigate the vitamin D status in octogenarians (10, 13, 17–20). Some other investigators had also found quite a high amount of vitamin D deficiency in 52.5–80.9% of oldest-old subjects investigated, depending on region (4, 6). Usually vitamin D deficiency is investigated in elderly with specific conditions.

Bruyère and colleagues (19) have reported a high prevalence of vitamin D inadequacy in 1984 European women aged over 80 years with osteoporosis and osteopenia. Results of this study showed that the average level of total 25 hydroxyvitamin D was 21.4 ng/ml, and different levels have been shown for different European countries. Our data show that the mean vitamin D level in women (10.61 ng/ml) was lower than the lowest level found in Belgian women (18.3 ng/ml). The difference of results could be explained by different study populations: only women with osteopenia and osteoporosis were investigated in the study conducted by Bruyère and colleagues (19), while we have included subjects regardless of their illness. Moreover, we did not include persons taking vitamin D supplementation.

Navarro-Martínez and colleagues (13) reported the significantly reduced vitamin D concentration in frail institutionalized women. Even in frail women, authors have found higher levels of vitamin D (28 ng/ml) as compared to our results. Bruyère and colleagues (19) have also found higher concentrations in Spain women—32.7 ng/ml. These differences might be explained by different geographical area: study was performed in Spain which is located at lower latitude than Lithuania.

In oldest-old Americans vitamin D deficiency was lower compared to their European counterparts and was found in 21.5% of subjects (18). A higher prevalence of vitamin D deficiency is seen in women rather than in men (75.6 vs. 24.4%) (6). Also, some studies found that an age-associated fall in serum total 25 hydroxyvitamin D starts earlier in women than in men (10, 21). In our study no difference in vitamin D levels were found between octogenarian men and women.

Results of this study show a weak correlation between total 25 hydroxyvitamin D levels and ADL in women. According to the results of Navarro-Martínez and colleagues (13), no correlation between vitamin D levels and frailty syndrome was found in institutionalized octogenarian women. Nakamura and colleagues (11) reported that low serum vitamin D levels were associated with low ADL levels, but ADL was assessed in a younger population and using a different scale—the Barthel index. Kotlarczyk and colleagues (12) investigated the association of vitamin D deficiency with functional changes assessed by ADLs and IADLs. The authors concluded that women with vitamin D deficiency had a greater decline in physical function. However, the study population was very different from our subjects in number and social status (residing in long-term care facilities). Cardiovascular Health Study All Stars (10) revealed that older adults with deficient total 25 hydroxyvitamin D levels were approximately 50% more likely to report ADL disability. However, most of the studies were cross-sectional in design and it is difficult to assess whether low vitamin D levels preceded the onset of reported functional skills or whether individuals had low total 25 hydroxyvitamin D levels because their functional status was poorer and, because of limited time outdoors, had less exposure to the sun and less endogenous vitamin D synthesis. No association between serum vitamin D and any single ADL or IADL categories was found, and further research with a large study population is needed.

The strength of our study is that we have investigated female and male octogenarians without vitamin D supplementation.

Our study also has some limitations. The results of this study could not represent the whole Lithuanian population. We have not assessed the physical activity and muscle strength of study subjects, or nutritional status. This would help to make a more comprehensive assessment of octogenarians.

In conclusion, this study reports a high prevalence of vitamin D deficiency in the studied octogenarians. Our findings suggest a weak correlation between vitamin D and activity of daily living. Weak correlation was found between vitamin D levels and instrumental activities of daily living category of food preparation. Further studies are needed to address the issue.

ETHICS STATEMENT

Study protocol has been approved by Vilnius Regional Biomedical Research Ethics Committee. All participants gave their written informed consent prior to enrolment.

AUTHOR CONTRIBUTIONS

VA, AM, and MT were involved in the design of the study. VA, JK, and AM were involved in the management of the

study. JK and AM performed the statistical analysis. VA, JK, AM, and MT drafted the manuscript and contributed in the final manuscript. All authors read and approved the final manuscript.

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Vitamin D Genomics: From *In Vitro* to *In Vivo*

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The vitamin D_3 metabolite 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is the exclusive high-affinity ligand of the vitamin D receptor (VDR), a transcription factor with direct effects on gene expression. Transcriptome- and epigenome-wide data obtained in THP-1 human monocytes are the basis of the chromatin model of vitamin D signaling. The model describes, how VDR's spatio-temporal binding profile provides key insight into the pleiotropic action of vitamin D. The transcription of some 300 primary target genes is significantly modulated through the action of genomic VDR binding sites in concert with the pioneer transcription factor PU.1 and the chromatin organizer CTCF. In parallel, the short-term vitamin D intervention study VitDbol (NCT02063334) was designed, in order to extrapolate insight into vitamin D signaling from *in vitro* to *in vivo*. Before and 24 h after a vitamin D_3 bolus chromatin and RNA were prepared from peripheral blood mononuclear cells for epigenome- and transcriptome-wide analysis. The study subjects showed a personalized response to vitamin D and could be distinguished into high, mid, and low responders. Comparable principles of vitamin D signaling were identified *in vivo* and *in vitro* concerning target gene responses as well as changes in chromatin accessibility. In conclusion, short-term vitamin D supplementation studies represent a new type of safe *in vivo* investigations demonstrating that vitamin D and its metabolites have direct effects on the human epigenome and modulate the response of the transcriptome in a personalized fashion.

Keywords: vitamin D, vitamin D receptor, vitamin D target genes, vitamin D intervention trial, chromatin, epigenome, immune system

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INTRODUCTION

The energy of sunlight-derived UV-B (290–315 nm) is used in human skin to convert the ubiquitous cholesterol precursor 7-dehydrocholesterol into pre-vitamin D_3 that isomerizes in a non-enzymatic reaction to the secosteroid vitamin D_3 (1). The hydroxylation of vitamin D_3 to 25-hydroxyvitamin D_3 [25$(OH)D_3$] and then to 1,25$(OH)_2D_3$ is necessary to generate the biologically most active metabolite (2). Lifestyle choices, such as preferential indoor activities and coverage by textile outdoors, in combination with climatic and seasonal changes are the main reasons for insufficient UV-B exposure of the majority of today's human populations (3). In order to avoid deficiency due to this low endogenous production, vitamin D_3 needs to be taken up by diet or supplementation with pills.

Abbreviations: 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 25$(OH)D_3$, 25-hydroxyvitamin D_3; AGPAT1, 1-acylglycerol-3-phosphate O-acyltransferase 1; ChIP-seq, chromatin immunoprecipitation sequencing; FAIRE-seq, formaldehyde-assisted isolation of regulatory elements sequencing; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; TAD, topologically associated domain; TSS, transcription start site; VDR, vitamin D receptor.

Well-known physiological roles of vitamin D are (i) control of intestinal absorption of calcium and phosphorus from diet, (ii) renal reabsorption of calcium, and (iii) remodeling of bones (4). However, vitamin D and its receptor, the transcription factor vitamin D receptor (VDR), are involved in far more functions than maintaining calcium homeostasis and bone integrity (5). Highest *VDR* gene expression is found in metabolic tissues, such as intestine, kidneys, and bone, but low to moderate *VDR* levels can be observed in more than half of the some 400 tissues and cell types forming the human body (www.proteinatlas.org/ENSG00000111424-VDR/tissue). For example, vitamin D modulates the responsive of both the innate and the adaptive immune system, i.e., it supports the human body in its fight against infections and in parallel prevents autoimmune disorders (6). Accordingly, vitamin D deficiency results not only in problems with bones, which are rickets in children or increased fracture risk for adults, but also weakens vitamin D's protective role of against diseases like tuberculosis, multiple sclerosis, and type 1 diabetes (7).

The lipophilic structure of vitamin D_3 and its metabolites allows the molecules passing through biological membranes. Thus, gene regulation by vitamin D is more direct and less complex than that of peptide hormones, growth factors, cytokines, and other hydrophilic signaling molecules. Since VDR is the only protein binding $1,25(OH)_2D_3$ with high-affinity (8), the physiological effects of vitamin D are largely identical to those of its receptor. Thus, comprehensive insight into vitamin D signaling requires the understanding of VDR's molecular actions.

Vitamin D receptor belongs the nuclear receptor superfamily, most members of which are activated by small lipophilic molecules (9). Within VDR's ligand-binding domain some 40 amino acids, which are mostly non-polar, form a ligand-binding pocket that fixes $1,25(OH)_2D_3$ with high specificity and affinity (10). The binding of ligand induces a change in the conformation of the ligand-binding domain, so that VDR's protein–protein interaction profile alters from that of a repressor to that of an activator (11, 12). Thus, VDR functions as a vitamin D-sensitive switch that attracts a set of nuclear proteins, like co-factors and chromatin modifying enzymes, to its thousands genomic binding sites (**Figure 1**). This leads to local changes in chromatin accessibility at many genomic loci, i.e., the epigenome responds to vitamin D.

The expression of a primary vitamin D target gene is modulated, i.e., in most cases increased, when it co-locates with a prominent VDR binding site within the same higher order chromatin structure, referred to as topologically associated domain (TAD) (13). An additional condition is that the transcription start site (TSS) of the vitamin D target gene and a VDR-binding enhancer region are within accessible chromatin (12). Thus, changes in the epigenome are the first events after stimulation of a cell with vitamin D before the transcriptome gets modulated.

This review describes a transition in the understanding of vitamin D signaling. The latter was on *in vitro* cell culture models and now gets new insights from *in vivo* investigations in the context of short-term vitamin D intervention trials.

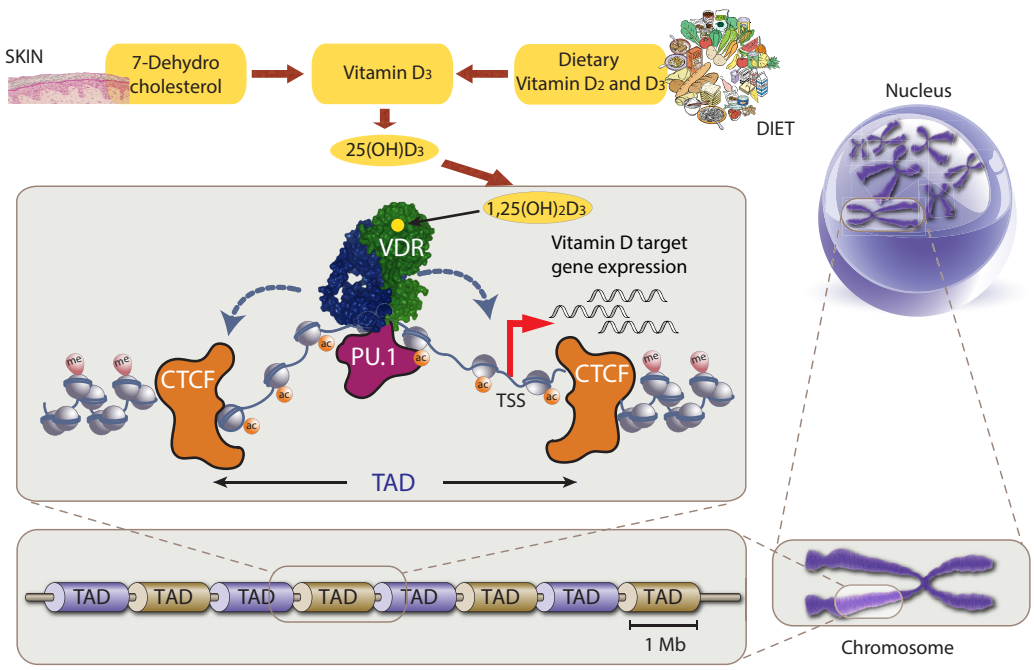

FIGURE 1 | Chromatin model of vitamin D signaling. Top: production of vitamin D_3 and its metabolites $25(OH)D_3$ and $1,25(OH)_2D_3$. Center: vitamin D receptor (VDR) (green) binds accessible genomic DNA in complex with a partner protein (RXR or others, blue). VDR's DNA binding is supported by the pioneer factor PU.1 (purple). The genomic region that can be influenced by $1,25(OH)_2D_3$ (*via* binding to VDR) is restricted by CTCF proteins (orange) defining left and right topologically associated domain (TAD) borders, i.e., only vitamin D target genes within the TAD will be stimulated to produce more mRNA copies. Bottom and right: schematic illustration of TAD size on relation to chromosomes and the nucleus.

GENOME-WIDE VDR BINDING PATTERNS *IN VITRO*

During the past years, the method chromatin immunoprecipitation sequencing (ChIP-seq) was widely used for the description of the genome-wide VDR binding pattern, the so-called "VDR cistrome" (14). VDR ChIP-seq data have been obtained in a number of human *in vitro* cell culture models, such as GM10855 and GM10861 human B lymphocytes (15), LS180 colorectal cancer cells (16), LX2 hepatic stellate cells (17), and lipopolysaccharide-polarized THP-1 macrophage-like cells (18). In parallel, in mouse cells VDR ChIP-seq had been performed with 3T3-L1 pre-adipocytes (19), IDG-SW3 osteocytic cells (20), pre-osteoblastic and differentiated MC3T3-E1 osteoblastic cells (21), as well as with bone marrow-derived mesenchymal stem cells differentiating into bone and fat cells (22). However, the presently most comprehensive analysis of the spatio-temporal VDR binding pattern has been performed in undifferentiated THP-1 human monocyte-like cells (13, 23).

Cell culture models have the advantage of rather homogenous cell populations that mostly display an unlimited growth potential. This allows performing biological repeats without the risk of major variations. Moreover, growth media can be depleted from lipophilic molecules, such as vitamin D_3 and its metabolites, so that a stimulation with pharmacologic doses of $1,25(OH)_2D_3$ (10–100 nM) results in maximal induction in reference to untreated cells. Accordingly, VDR ChIP-seq datasets obtained from *in vitro* cell models unanimously demonstrate that stimulation with $1,25(OH)_2D_3$ significantly increases the number of genomic VDR binding events 2- to 10-fold (18).

The cistrome of ligand-stimulated VDR comprises some 2,000–10,000 sites per cell type. The VDR binding pattern is rather cell-specific and only the small subset of some 50 sites is found in all investigated cell types (18). Therefore, most *VDR* expressing tissues and cell types have a rather different set of vitamin D target genes (14, 24).

In agreement with findings of the ENCODE project (25) VDR binds equally likely both up- and downstream of genes, i.e., VDR binding sites are distributed in a Gaussian fashion in relation to the TSSs of primary vitamin D target genes. Accordingly, the more distant VDR binding sites are from a TSS, the less likely they are functional for the respective gene. In addition, the VDR binding site within an enhancer region and the TSS of a primary vitamin D target gene under the control of the receptor have to be located within the same TAD. Interestingly, out of 11,600 VDR binding sites identified in THP-1 cells, the small subgroup of only 339 highly conserved persistent VDR loci is well suited for describing most vitamin D gene regulatory scenarios (13). In THP-1 cells almost all primary vitamin D target genes are located within $1,25(OH)_2D_3$-modulated TADs (more details below). Conserved persistent VDR sites control 168 of the 311 primary vitamin D target genes, whereas 120 genes are close to transiently occupied VDR sites. The equal distribution of persistent VDR binding sites over the human genome suggests that they may be strategically positioned, in order to provide the whole genome with sensitivity to vitamin D (13). The similarly equal genomic distribution of primary vitamin D target genes (26) supports this concept. Thus, the time-dependent binding profile of a few 100 VDR loci is sufficient for regulating most primary vitamin D target genes.

CHROMATIN RESPONSES TO $1,25(OH)_2D_3$

Genomic DNA is not "naked" but always wrapped around nucleosomes forming a protein–DNA complex that is referred to as chromatin (**Figure 1**). Nucleosomes are composed of eight histone proteins that are rich in the basic amino acids lysine and arginine. In particular at the histone's protruding amino-termini these amino acids are often post-translationally modified by methyl or acetyl groups. Such histone modifications alter the structure of chromatin by affecting the non-covalent interactions within and between nucleosomes.

The epigenome is the genome-wide representation of (i) some 100 different histone marks, (ii) the level of DNA methylation at CpG islands, and (iii) higher order chromatin organization (27). The epigenome dynamically responds to extra- and intracellular signals, such as ligand activation of the VDR (28). However, most chromatin regions are intrinsically repressed, so that the binding of transcription factors and other nuclear proteins to genomic DNA is prevented, i.e., the epigenome controls the access to the genome (29, 30). In consequence, in a differentiated cell the so-called "epigenetic landscape" is restricted to some 100–200,000 chromatin loci (**Figure 2** shows an example genomic region) that are accessible to transcription factors and RNA polymerases (25). This represents less than 10% of the whole chromatin and primarily refers to regions carrying TSSs and enhancers.

Chromatin modifying and remodeling enzymes read, write, or erase chromatin marks and reposition nucleosomes, respectively (31). These enzymes are modulated in their activity by signal transduction cascades originating from intra- and extracellular signaling molecules and/or form complexes with transcription factors specifically binding to respective genomic regions (32). VDR communicates with chromatin modifying enzymes *via* direct and indirect interaction, such as up- and down-regulating their genes (33) or being part of the same large protein complex in the nucleus (34).

On the genome-wide level, vitamin D-induced alterations in the chromatin accessibility profile can be measured *via* the method formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq, **Figure 2**). In THP-1 cells, FAIRE-seq identified 62,000 accessible chromatin loci, nearly 9,000 of which are significantly modulated by $1,25(OH)_2D_3$ (28). A 2 h stimulation with $1,25(OH)_2D_3$ resulted at more than 3,300 genomic loci in significant changes in chromatin accessibility, after 24 h even more than 4,500 sites responded, while after 48 h only some 2,400 regions were targets of vitamin D. This suggests that maximal epigenome-wide effects occur after 24 h. In parallel, this indicates that the process of chromatin opening by vitamin D includes multiple steps. Although the exact molecular mechanisms of these vitamin D-triggered epigenome changes are not fully understood, it is obvious that they are secondary consequences of genome-wide VDR binding.

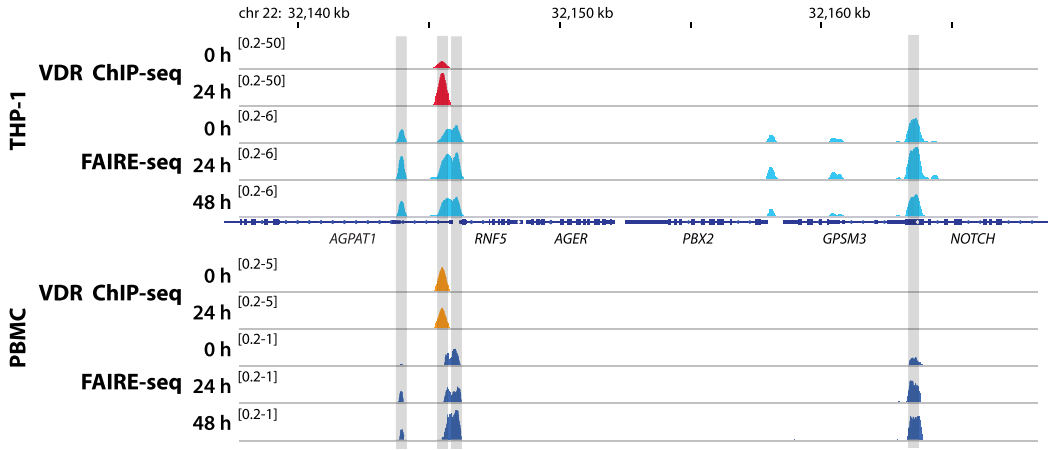

FIGURE 2 | Vitamin D receptor (VDR) binding and chromatin opening of the 1-acylglycerol-3-phosphate O-acyltransferase 1 (*AGPAT1*) locus *in vitro* and *in vivo*. Top: THP-1 cells were stimulated for 0, 24, and 48 h with 1,25(OH)$_2$D$_3$ and VDR chromatin immunoprecipitation sequencing (ChIP-seq) and formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq) were performed. Bottom: in an analogous *in vivo* experiment (phase II context of the VitDbol study) one individual was challenged with a vitamin D$_3$ bolus (2,000 µg). The average raise in 25(OH)D$_3$ serum concentrations at days 1 and 2 after the vitamin D$_3$ bolus was 11.9 and 19.4 nM, respectively. Peripheral blood mononuclear cells (PBMCs) were isolated before (day 0) and at days 1 (24 h) and 2 (48 h) and VDR ChIP-seq and FAIRE-seq were performed. The integrative genomics viewer browser was used to visualize the *AGPAT1* gene locus. The peak tracks represent mergers of each three biological repeats. Gene structures are shown in blue.

THE CHROMATIN MODEL OF VITAMIN D SIGNALING

The human genome is subdivided into at least 2,000 chromatin loops (35), which segregate each chromosome into TADs. The latter are functionally independent chromatin subdomains in the size of hundreds of kilobases to a few megabases. Insulator regions separate TADs from each other (36) and contain binding sites for the transcription factor CTCF. This makes CTCF a key protein in organizing chromatin into active and inactive regions. However, from the 20,000 genome-wide CTCF loci, only some 15% are involved in forming TAD anchor regions. Interestingly, in THP-1 cells the binding of CTCF to some 1,300 sites is affected significantly by a stimulation with 1,25(OH)$_2$D$_3$ (37). More than half of the vitamin D-modulated CTCF sites mark one or both anchors of some 600 TADs, each of which comprises at least one VDR binding site and one vitamin D target gene. Interestingly, in the same cellular system, 587 genes are regulated significantly by 1,25(OH)$_2$D$_3$ (13).

In addition to the chromatin organizer CTCF, VDR also functionally associates with pioneer factors, such as PU.1 (38) or GABPA (39). A pioneer factor is a transcription factor that (i) displays many genomic binding sites, (ii) shows some promiscuity in DNA binding, and (iii) has a high diversity in protein–protein interactions (40). Accordingly, after a cellular perturbation pioneer factors are the first protein binding enhancers interacting with chromatin modifying enzymes. This makes chromatin more accessible for regular transcription factors like VDR. Interestingly, in THP-1 cells a 24 h stimulation with 1,25(OH)$_2$D$_3$ significantly modulated PU.1 binding at more than 5,600 sites (38).

In summary, in the THP-1 model system the epigenome-wide outcomes of a 24 h stimulation with 1,25(OH)$_2$D$_3$ are (i) VDR binding at more than 10,000 sites, (ii) chromatin opening at some 4,500 loci, (iii) changes in CTCF-based TAD anchors affecting some 600 chromatin loops, and (iv) increased PU.1 pioneer factor binding at more than 5,000 regions. This led to the chromatin model of vitamin D signaling (**Figure 1**). In this model, VDR already binds, in the absence of ligand, to a limited number of loci within accessible chromatin, while 1,25(OH)$_2$D$_3$ stimulation increases, *via* support of pioneer factors like as PU.1, the number of DNA-bound VDR molecules. This VDR binding leads to changes in chromatin accessibility, which increases the binding strength of TAD anchor forming CTCF sites upstream and downstream of prominent VDR binding loci (41).

Some 300 conserved persistent VDR sites act as key nodes, at which not only primary contacts of VDR ligands with the genome are established, but also functional consequences of vitamin D induction are coordinated throughout the whole stimulation period (13). For more than half of all primary vitamin D target genes a regulatory scenario applies, where each gene is controlled by one or more conserved persistent VDR sites being located within the same TAD. In addition, a few 100 transient VDR sites mediate more tissue-specific primary functions of vitamin D, such as immune system regulation (13). In total, five TAD classes are distinguished that differ in the number of persistent and transient VDR sites and contain sets of genes that represent different physiological functions of vitamin D. Most of the remaining VDR sites are involved in mediating secondary effects of vitamin D.

IN VIVO INVESTIGATIONS OF VITAMIN D SIGNALING

In vitro cell culture models, such as THP-1 cells, use experimental setups that are designed for obtaining maximal effects of 1,25(OH)$_2$D$_3$ in short time periods, such as 24 h, but may

not reflect the reality of the endocrinology of vitamin D *in vivo* (2, 42). In fact, the genetic origin of today's populations from East Africa and respective minor changes in physiology in the limited time, since the exodus some 50,000 years ago, suggest that humans are still primarily adapted to a constant vitamin D levels rather to changes in 25(OH)D_3 serum concentration between winter and summer (43). This raises the question of how far results from *in vitro* experiments represent vitamin D's actions *in vivo*.

The VitDbol vitamin D intervention trial (NCT02063334, ClinicalTrials.gov) studied under *in vivo* conditions vitamin D-dependent gene regulation in humans. From healthy young adults, peripheral blood mononuclear cells (PBMCs) were isolated at days 0, 1, and 2 after supplementation with a vitamin D_3 bolus. In phase I of VitDbol, changes in chromatin accessibility were measured at selected genomic regions (44) and alterations in gene expression were determined (45). The serum 25(OH)D_3 concentrations of the subjects raised in average by some 20 nM, i.e., a 20–40% increase in the vitamin D status is sufficient to open chromatin and to activate genes.

VitDbol participants differed significantly both on the level of changes in chromatin accessibility as well as on vitamin D target gene expression (44, 45). Accordingly, they could be segregated into low, mid, and high responders to vitamin D. Together with comparable results from the long-term vitamin D intervention study VitDmet (46), the VitDbol results served as the basis for the concept of the personalized vitamin D index (47). Some of the differences between individuals may be based on variations in their genome, such as SNPs, affecting the vitamin D status (48). However, in analogy to common aging-related disorders, more likely differences in the epigenome of the study participants are the main molecular explanation for alterations in the underlying traits. Accordingly, throughout their entire life a significant proportion of the human population may have a vitamin D status that is significantly lower than the needs of the respective individual for optimal function of vitamin D endocrinology.

In contrast to suggestions from classical pharmacogenetics, an individual's health or disease status cannot be deduced reliably from a single genotyping experiment (49). Therefore, persons need to be profiled on the level of their epigenome and transcriptome in time series experiments. In phase II of VitDbol, one individual received once a month a vitamin D_3 bolus three times in a row (50). **Figure 2** illustrates changes in chromatin accessibility of PBMCs within 2 days. FAIRE-seq was used to detect accessible chromatin at 5,205 genomic loci, the 853 most prominent of which were categorized into early, delayed, and non-responding genomic regions. Already after 1 day 70 loci showed significant chromatin opening or closing and after 2 days 361 additional genomic sites were affected. Although in PBMCs, the number of chromatin sites with significantly changed accessibility is far lower than THP-1 cells (28), some 85% of the most prominent genomic loci are found both *in vitro* and *in vivo* (50).

The main cellular components of PBMCs, lymphocytes, and monocytes, belong to the adaptive and innate immune system, respectively. This fits well with the observation that the human leukocyte antigen (HLA) region in chromosome 6 is an epigenome "hotspot" in PBMCs (50). Interestingly, the epigenome at the *HLA* cluster is very responsive to vitamin D. This provides a first molecular explanation that how vitamin D may modulate actions of the immune system (51).

CONCLUSION

Vitamin D is known as a molecule that controls calcium homeostasis and bone formation, but in humans VDR's genome-wide actions are investigated primarily in the hematopoietic system. This emphasizes the impact of vitamin D in innate and adaptive immunity. The VitDbol study demonstrated that the human epigenome responds already within 1–2 days to vitamin D. Importantly, the design of VitDbol allows safe human *in vivo* experiments. Nevertheless, such *in vivo* investigations cannot provide the same level of reproducibility than *in vitro* cell culture experiments, in which conditions, such as nutrient availability and temperature, are far more constant.

AUTHOR CONTRIBUTIONS

This is a single author mini-review. CC has written all text and created both figures.

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Vitamin D Receptor *TaqI* Polymorphism Is Associated With Reduced Follicle Number in Women Utilizing Assisted Reproductive Technologies

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Purpose: Calcitriol, or 1,25-hydroxycholecalciferol, is the active form of vitamin D. It binds and activates vitamin D receptor (VDR). Infertility and defective folliculogenesis have been observed in female *vdr*-knockout mice; however, whether *VDR* polymorphisms affect human ovarian responses to controlled ovarian stimulation (COS) remains unclear. We hypothesized that *VDR* polymorphisms are associated with infertility and COS responses. Thus, we evaluated the association between the *TaqI*, *BsmI*, and *FokI* *VDR* polymorphisms and ovarian responses in women undergoing COS.

Methods: In this study, we recruited a control group (n = 121) comprising volunteers with a history of natural conception and a second group of women undergoing COS (n = 70). *TaqI*, *BsmI*, and *FokI* genotyping was performed *via* restriction fragment length polymorphism analysis or TaqMan qPCR and Sanger sequencing. Intrafollicular 25(OH)D contents were measured in follicular fluid collected from COS patients during oocyte retrieval. Ovarian response parameters were obtained from patient medical records.

Results: There were no significant differences in the genotype frequencies of *VDR* polymorphisms (*TaqI*, *BsmI* and *FokI)* between the control and COS groups. However, the allele frequency of *TaqI* (C allele) was significantly lower in the COS group than in the control group (p = 0.02). Follicle number but not oocyte number was lower in patients with *TaqI* polymorphic (TC/CC) genotypes (p = 0.03). Importantly, the ratio between

Abbreviations: BMI, body mass index; COS, controlled ovarian stimulation; FF, follicular fluid; E2, estrogen; P4, progesterone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; 1,25(OH)2D, 1,25-Hydroxycholecalciferol; 25(OH)D, 25-Hydroxyvitamin D; 25(OH)D3, 25-Hydroxycholecalciferol; 25(OH)D2, 25-Hydroxyergocalciferol; HWE, Hardy–Weinberg equilibrium; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; LH, luteinizing hormone; OR, odds ratio; RFLP, restriction fragment length polymorphism; SNP(s), single-nucleotide polymorphism (s); VDR, vitamin D receptor.

the number of follicles retrieved and intrafollicular estradiol concentrations was higher in patients with the TC/CC *TaqI* genotypes ($p < 0.02$).

Conclusion: We identified an association between the *VDR TaqI* polymorphism and reduced follicle number in women undergoing COS, suggesting that *VDR* signaling affects the ovarian response to stimulation *via* unknown mechanisms.

Keywords: calcitriol, *VDR* polymorphisms, 25(OH)D, *TaqI*, folliculogenesis, infertility

INTRODUCTION

Calcitriol, or 1,25-hydroxycholecalciferol (1,25(OH)2D), is the active form of vitamin D, a steroid hormone that exerts classical functions in calcium and phosphorus homeostasis and bone mineralization (1). Calcitriol binds its nuclear receptor, vitamin D receptor (VDR) (2), and has an array of actions in the immunological, cardiovascular (3), and reproductive systems of both genders (4). In particular, a number of studies have demonstrated an association between 25-hydroxyvitamin D (25(OH)D) concentrations and different causes of infertility in animals (3, 5–8) and humans (9–14).

VDR expression has been reported in different central (hypothalamus and hypophysis) and peripheral reproductive organs (ovary, uterus, placenta, and oviduct) (13, 15, 16). Evidence linking calcitriol and reproductive function has been demonstrated in 7-week-old female *vdr*-knockout mice. These animals exhibited uterine hypoplasia, defective folliculogenesis (the absence of mature follicles), and associated infertility (6, 7). Moreover, female *vdr*-knockout mice exhibited decreased aromatase expression and activity in the ovary, and these effects were associated with elevated serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations, indicating a peripheral rather than a central defect (8) and suggesting a role for calcitriol in regulating folliculogenesis.

Genetic alterations in the *VDR* gene may lead to important defects in gene activation. Alterations were reported to affect calcium metabolism, cell proliferation, and immune function (17). Furthermore, some *VDR* single-nucleotide polymorphisms (SNPs) may contribute to a genetic predisposition to certain diseases. SNPs present in the *VDR* gene alter receptor length and decrease its activation in target cells (18). Among these polymorphisms, the best described are *TaqI*, *BsmI*, and *FokI*. *TaqI* (rs731236, changes T/C, exon 9) and *BsmI* (rs1544410, changes G/A, intron 8) are present in the 3′ untranslated region (3′ UTR) of the *VDR* gene and are related to modulation of gene and protein expression of the receptor. The *FokI* polymorphism (rs2228570, changes T/C, exon 9), in turn, is present in a translated region and effects functional activity by generating a longer VDR protein with reduced transcriptional activity (19).

These polymorphisms have been previously associated with increased risk of developing diabetes (20), tuberculosis (*FokI* polymorphism) (21), specific cancers (22, 23), and multiple sclerosis (24). Conversely, they were also associated with protection against breast cancer (*TaqI* polymorphism) (25), osteoporosis (26), and asthma (*FokI* polymorphism) (27). However, no associations between *VDR* polymorphisms have been reported in conditions such as osteoporosis (28), colorectal cancer (29), and metabolic syndrome (30). In the context of reproductive medicine, *VDR* polymorphisms have been associated with polycystic ovarian syndrome and endometriosis (17, 31–38), although these results are inconclusive and require further investigation.

25(OH)D deficiency is now recognized as a pandemic condition (39). In Brazil, 25(OH)D deficiency is largely detected in women of different ages, including elderly and postmenopausal women (40) and women of reproductive age (41). Controlled ovarian stimulation (COS), which aims to increase the success rate of *in vitro* fertilization (IVF) through stimulation of folliculogenesis, revealed a decrease in the pregnancy (42) and fertilization rates (43) in women with lower 25(OH)D concentrations.

Moreover, other studies have demonstrated that women with replete serum concentrations of 25(OH)D (42) or at least sufficient 25(OH)D in the follicular fluid (FF) had lower pregnancy and fertilization rates (44). A recent study from our group demonstrated that women with lower follicular 25(OH)D concentrations exhibited better outcomes when treated with the COS protocol in that they produced more larger follicles and had higher serum estradiol concentrations (45). Despite these controversial data, *in vitro* and animal model studies strongly support a significant role of calcitriol in orchestrating reproductive processes and IVF outcomes (46). However, further studies are warranted to demonstrate a causal relationship between 25(OH)D status and infertility.

In the present study, we hypothesized that *VDR* polymorphisms are associated with infertility and response to COS. The identification of specific *VDR* polymorphisms that can be shown to be related to infertility and response to COS may help clarify the causes underlying female infertility and poor ovarian response.

MATERIALS AND METHODS

Patients

Two groups of patients were enrolled for each polymorphism analysis. The control group comprised volunteer women with no history of reproductive disorders. To be included in the control group, volunteers had to declare that they had become pregnant through natural conception at least once and had never experienced any difficulties in conceiving. The COS group consisted of women who underwent COS for intracytoplasmic sperm injection (ICSI) at the Fertipraxis Center for Human Reproduction, a clinic certified by the Brazilian Health Surveillance Authority (ANVISA) and the Latin American Network of Assisted Reproduction (REDLARA). We enrolled 62 controls and 47

COS-treated women in the *TaqI* polymorphism analysis, 57 controls and 49 COS-treated women in the *FokI* analysis, and 86 controls and 54 patients in the *BsmI* analysis.

This study was approved by the local Ethics Committee and was registered on the Brazilian platform of research under the number 02213812.4.0000.5275. All the enrolled subjects (volunteers and patients) provided written informed consent before joining the study. In the COS group, clinical data, including hormone concentrations [serum and follicular estrogen (E2), progesterone (P4), LH, and FSH] and indicators of ovarian response (number of follicles and oocytes retrieved), were obtained from patient medical records. Clinical data for the control group were obtained during patient enrollment and interviews. All patients underwent blood collection for further *VDR* polymorphism genotyping.

COS Protocol

Controlled ovarian stimulation protocols were performed according to the specific clinical requirements of the patients. Briefly, the gonadotropin-releasing hormone antagonist analog cetrorelix acetate (Cetrotide® 0.25 mg, Merck-Serono, Italy) was administered to induce hypophysis suppression, and on the second day of menstruation, ovarian stimulation was initiated with synthetic FSH alone (Gonal-F®, Merck-Serono, Italy; or Bravelle®, Ferring Pharmaceutical, Germany) or FSH and LH (Pergoveris®, Merck-Serono, Italy; or Menopur®, Ferring Pharmaceutical, Germany) treatments. FSH dosage varied from 150 to 300 IU/day, and LH dosage ranged from 75 to 300 IU/day.

When at least one follicle had reached 18 mm or at least two follicles had reached 16 mm (assessed by ultrasound), human chorionic gonadotropin (hCG) (Ovidrel® 250 µg, Merck-Serono, Italy) was administered to mimic LH. Thirty-five hours post-Ovidrel® administration, the oocytes were retrieved, and FF was obtained during the follicular aspiration procedure. In addition, blood samples were collected for *VDR* genotyping following FF isolation.

DNA Extraction

Blood (1 ml) was submitted for genomic DNA extraction from peripheral leukocytes *via* the salting-out technique (47) using a commercial Wizard® Genomic DNA purification kit according to the manufacturer's instructions (A1120, Promega, Madison, WI, USA). After the extraction, DNA quantity and quality were examined using a NanoPhotometer (Implen, Munchen, Germany).

Genotyping

The genotyping of *TaqI* (rs731236) and *FokI* (rs2228570) polymorphisms was performed using the restriction fragment length polymorphism (RFLP) technique. **Table 1** shows the primer sequences used for the *VDR* polymorphism analysis, which were validated through the Primer Blast program to ensure PCR quality; intron-spanning primers were used to avoid contamination with external genomic DNA. To perform the PCR reactions, a commercial kit (GoTaq, Promega, USA) was used and the conditions were as follows: 95°C for 4 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and 72°C for 7 min. DNA samples were digested by *TaqI* and *BseGI* (*Btscl* isoschizomers that recognize the same sequence recognized by the *FokI*) endonucleases (Thermo Scientific, EUA). The mixtures were incubated at 65°C and 55°C, respectively, to promote cleavage. The samples were then subjected to electrophoresis on 2–4% agarose gels to determine the lengths of the fragments and genotyping results (Figures S1 and S2 in Supplementary Material).

BsmI (rs1544410) polymorphism genotyping was performed using TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA, 4371355) and a TaqMan® SNP Genotyping Assay (Applied Biosystems, PN4351379) in a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Allele discrimination was analyzed using the ViiA™ 7 software, and genotyping was performed with Genotyping version 3.1 from Thermo Fisher Cloud. Furthermore, Sanger sequencing (Big Dye® Terminator v 3.1 Cycle Sequencing Kit) was performed on the four amplified products for which real-time PCR did not achieve accurate results to confirm the genotyping assay results (Figure S3 in Supplementary Material). The same primers used for amplification were used for genotyping assessment (5′CAACCAAGACTACAAGTACCGCGTCAGTG3′ and 3′AACCAGCGGGAAGAGGTCAAGGG5′) with 1 cycle at 96°C for 1 min, 25 cycles at 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min. Products of the sequencing reactions were assessed in a Genetic Analyzer ABI3500. Sequence analysis was performed using MacVector, version 14.

FF Collection for 25(OH)D Measurement

Follicular fluid collection was performed during oocyte capture, as previously described (45, 49). Briefly, follicle aspiration was undertaken with a transvaginal ultrasound probe as a guide (Medison X8®) and a 17G oocyte aspiration needle (Wallace®) connected to a closed vacuum system under 90 mmHg of negative pressure, which was used to empty the follicles. The follicle exhibiting the largest diameter, greater than 16 mm, was selected, captured and placed in a sterile container. FF was extracted after oocyte detection and subsequently frozen in liquid nitrogen. This technique allowed the collection of fluid from a single follicle and decreased the chance of blood contamination.

TABLE 1 | Sequences of primers used to amplify each polymorphism and their respective fragments with or without endonucleases.

Polymorphism	Sense	Antisense	Length	Reference
TaqI (rs731236)	CAGAGCATGGACAGGGAGCAA	GCAACTCCTCATGGCTGAGGTCTC	495 bp uncut 290, 245 bp cleaved	(48)
FokI (rs2228570)	AGCTGGCCCTGGCACTGACTCTGCTCT	ATGGAAACACCTTGCTTCTTCTCCCTC	265 bp uncut 196, 69 bp cleaved	

Pb, pair of bases.

Quantification of 25(OH)D

FF 25(OH)D concentrations were assessed using an electrochemiluminescence fixation assay (ElecsysTotal Vitamin D total assay, Roche Diagnostics, Brazil). The range of measurements was 3–70 ng/ml. Inter- and intra-assay variations were 5.9 and 5.2%, respectively. This technique is based on competition, and a vitamin D-binding protein binds 25-hydroxycholecalciferol (25(OH)D3) and 25-hyroxyergocalciferol (25(OH)D2).

Statistical Analysis

Genotype and allele frequencies were calculated based on the observed genotypes. Departure from Hardy–Weinberg equilibrium (HWE) in the distribution of the genotypes was estimated with the χ^2 test. If the χ^2 test resulted in a p value greater than 0.05, the population was considered to be in HWE. The influence of each *VDR* polymorphism on COS variation was assessed by an odds ratio (OR) analysis. We performed χ^2 tests to analyze heterogeneity, and a value of $p < 0.05$ was considered to indicate statistical significance. The dominant model, in which heterozygous and homozygous minor alleles were grouped, was analyzed.

The Mann–Whitney test was used to investigate possible associations between polymorphisms and ovarian response variables and to test associations between polymorphisms and FF concentrations of 25(OH)D. A p value <0.05 was considered statistically significant. All comparisons were performed using SPSS (version 22) software. Graphics were generated using Prism (version 6) software.

RESULTS

Clinical Data

To determine whether the presence of polymorphisms in the *VDR* gene affected the ovarian response of women undergoing COS, we extracted clinical data from control volunteers who had declared a history of natural conception and from women who underwent the COS protocol and ICSI treatment. The demographic parameters of the control and COS groups are depicted in **Table 2**.

The COS infertility diagnoses in our group were as follows: unexplained (37%), tubal factors (18%), ovarian failure (13%), endometriosis (13%), female anatomical causes (6%), and other causes of infertility (13%), including hypogonadism, colonic surgery, ovarian failure and tubal factors, female endocrine factors, breast cancer, tubal factors and endometriosis, or polycystic ovarian syndrome and endometriosis.

TABLE 2 | Demographic parameters of control and COS groups.

	Control	COS	*p*-Value
Age (years)	44 ± 0.9	35 ± 0.5	<0.0001
Height (cm)	161 ± 0.7	164 ± 0.7	<0.01
Weight (kg)	66 ± 1.0	61 ± 1.2	<0.002
BMI (kg/m^2)	25.5 ± 4.1	22.5 ± 3.9	<0.0001

BMI, body mass index; COS, controlled ovarian stimulation.

Analysis of *TaqI*, *BsmI*, and *FokI VDR* Polymorphisms

Table 3 shows the genotype frequencies of the *VDR* polymorphisms studied in the control versus COS groups. No differences were found, and the *FokI* polymorphism was in HWE (control: $p = 0.6$, COS: $p = 0.23$). **Table 4** shows the allele frequencies of the *VDR* polymorphisms studied in the control versus COS groups. No differences were observed in the *BsmI* and *FokI* allele frequencies between the control and COS groups. However, the *TaqI* polymorphism exhibited a higher frequency of the C allele and a lower frequency of the T allele in the COS group [$p = 0.02$; OR: 1.95 (1.097–3.5)]. We then applied the dominant model and identified a considerable trend in the genotype distribution for the *TaqI* polymorphism [$p = 0.056$, OR: 2.106 (0.979–4.53)].

TaqI Polymorphism and COS-Related Variables/25(OH)D Associations

Because an association was detected between infertility and the frequency of *TaqI* alleles, we next examined whether the *TaqI*

TABLE 3 | Genotype frequencies of *VDR* polymorphisms.

Polymorphism	Genotype frequencies (%)		χ^2 (*p*)
	Control (*n*)	COS (*n*)	
***TaqI* (rs731236)**	*n* = 62	*n* = 47	
TT	62.9 (39)	42.6 (20)	4.47 (0.10)
TC	22.6 (14)	34.0 (16)	
CC	14.5 (9)	23.4 (11)	
p (HWE)	<0.01	0.13	
***FokI* (rs2228570)**	*n* = 57	*n* = 49	
TT	50.9 (29)	47.0 (23)	0.76 (0.68)
TC	36.8 (21)	35.0 (17)	
CC	12.3 (7)	18.0 (9)	
p (HWE)	0.60	0.23	
***BsmI* (rs1544410)**	*n* = 86	*n* = 54	
GG	54.7 (47)	42.6 (23)	2.22 (0.33)
GA	9.30 (8)	14.8 (8)	
AA	36.0 (31)	42.6 (23)	
p (HWE)	<0.01	<0.01	

COS, controlled ovarian stimulation; HWE, Hardy–Weinberg equilibrium (p value < 0.05 is not considered in HWE; p value > 0.05 is considered in HWE). χ^2—chi-squared test (values above 3.84; p < 0.05).

TABLE 4 | Allele frequencies of *VDR* polymorphisms.

Polymorphism	Allele frequencies (%)		OR (95% CI)	χ^2 (*p*)
	Control (*n*)	COS (*n*)		
***TaqI* (rs731236)**	*n* = 124	*n* = 94		
T	74.0 (92)	60.0 (56)	1.95 (1.097–3.5)	5.24 (0.02)*
C	26.0 (32)	40.0 (38)		
***FokI* (rs2228570)**	*n* = 114	*n* = 98		
T	69.0 (79)	64.0 (63)	1.25 (0.71–2.21)	0.60 (0.44)
C	31.0 (35)	36.0 (35)		
***BsmI* (rs1544410)**	*n* = 172	*n* = 108		
G	59.3 (102)	50.0 (54)	1.45 (0.90–2.35)	2.32 (0.12)
A	40.7 (70)	50.0 (54)		

COS, controlled ovarian stimulation. OR, odds ratio. IC, confidence interval. χ^2, chi-squared test (values above 3.84; p < 0.05).
**Significant p value.*

polymorphism is associated with variables related to the COS protocol. We, therefore, sorted the COS group according to genotype based on the dominant model (**Table 5**). A comparison of the *TaqI* genotypes did not reveal any differences in LH, FSH, E2, or P4 concentrations on day 1 of the COS protocol. However, there was a trend for women possessing the TC/CC genotypes to have a lower number of antral follicles than were found in women with the TT genotype ($p = 0.08$). The duration of COS and the FSH dose administered did not differ according to the *TaqI* genotype (**Table 5**). Similarly, a comparison of *TaqI* genotypes at baseline (**Table 6**) before COS revealed that there were no differences in 25(OH)D, E2, and P4 concentrations on the day of oocyte retrieval.

We further analyzed the number of follicles and retrieved oocytes according to the *TaqI* genotypes (**Figure 1**). A lower number of mature follicles was found in women with the TC/CC genotypes than in women possessing the TT genotype ($p = 0.03$). However, we found no significant differences in the number of oocytes retrieved. We also analyzed the serum concentrations of E2 and P4 on the day of hCG administration as well as the ratio of intrafollicular E2 to follicles retrieved according to *TaqI* genotype (**Figure 2**). There were no significant associations between *TaqI* genotypes and serum concentrations of E2 or P4 on the day of hCG administration (**Figure 2**). However, the ratio of intrafollicular E2 to retrieved follicles was higher in women with TC/CC genotypes than in women with the TT genotype ($p < 0.02$) (**Figure 2**). Our analysis of comorbidities between the two groups (TT and TC/CC genotypes) revealed no differences. There were no smokers in either group. Hypertension and diabetes were not found in any of these patients. The only comorbidity found was thyroid dysfunction. In all, 10% of the women with the TT genotype and 14% of the women with the TT/CC genotypes had thyroid dysfunction.

TABLE 5 | Ovarian stimulation-related variables according to *TaqI* genotype.

		TT	TC/CC	*p*
Day 1 of COS	Antral follicles	13.1 ± 1.03	10.9 ± 0.74	0.08
	FSH (mU/ml)	6.5 ± 0.74	5.9 ± 0.56	0.54
	LH (mU/ml)	4.9 ± 0.59	6.5 ± 0.59	0.08
	E2 (pg/ml)	53.5 ± 7.2	83.1 ± 10.8	0.11
	P4 (pg/ml)	361 ± 43	340 ± 32.4	0.69
COS duration (days)		9.5 ± 0.45	10.0 ± 0.34	0.39
FSH (UI)		1835 ± 152	1941 ± 168	0.64

COS, controlled ovarian stimulation; E2, estradiol; P4, progesterone; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone.
The data are expressed as the medians ± SEM.

TABLE 6 | Intrafollicular 25(OH)D, E2 and P4 concentrations according to *TaqI* genotype.

		TT	TT/CC	*p*
Intrafollicular (capitation day)	25(OH)D	22.5 ± 3.1	25.6 ± 2.4	0.4
	E2 (ng/ml)	423 ± 91	488 ± 75	0.58
	P4 (pg/ml)	18.34 ± 2.7	23.87 ± 41.3	0.69

E2, estradiol; P4, progesterone; 25(OH)D, 25-hydroxycholecalciferol.
The data are expressed as the medians ± SEM.

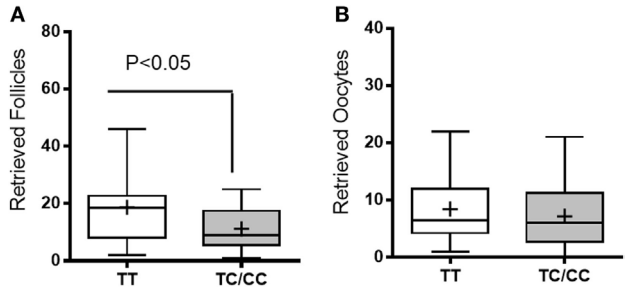

FIGURE 1 | Follicles **(A)** and retrieved oocytes **(B)**. White bars represent women carrying the TT genotype, whereas gray bars represent women carrying the TC/CC genotypes. Unpaired *t*-test. The data are presented as the mean ± min and max.

DISCUSSION

This work provides the first demonstration of an association between the *VDR TaqI* polymorphic C allele frequency and decreased follicle production by women exhibiting different causes of infertility. There were no significant differences in the genotype and allele frequencies of the *FokI* and *BsmI* polymorphisms between the COS and control groups. Instead, the higher frequencies observed in the group with polymorphic *TaqI* alleles in the COS group indicates that this *VDR* polymorphism is a potentially important SNP candidate that may be involved in female fertility.

There is some disagreement in the literature regarding the relationship between *VDR* polymorphisms and infertility disorders. Several studies have found that there is a negative association between the presence of *TaqI, FokI*, and *BsmI VDR* polymorphisms and the risk of developing reproductive disorders, such as polycystic ovarian syndrome and endometriosis (31–35). Conversely, other studies have found positive associations (36–38, 50) or no association at all (17) for these variables, suggesting that there is a need for further studies to clarify this important question.

We did not find an association between follicular 25(OH)D concentrations and any specific *TaqI* polymorphism allele (C or T), suggesting that these polymorphisms do not alter FF 25(OH)D concentrations. Importantly, while serum concentration of 25(OH)D were not evaluated in this study, recent findings reported by our (49) and other groups (13, 14, 43, 44, 51) have demonstrated that FF accurately reflects plasma 25(OH)D concentrations (14, 44) in both fertile and infertile patients.

The above results indicate a lack of a direct relationship between FF concentrations of 25(OH)D and infertility and suggest that the *TaqI* polymorphism does have a role in this context. In contrast, some studies have demonstrated an association between the *TaqI* C allele and decreased serum 25(OH)D concentrations in women with colorectal cancer (52), whereas another study performed in a healthy cohort in India (53) demonstrated that the *TaqI* C allele was directly associated with higher serum concentrations of 25(OH)D.

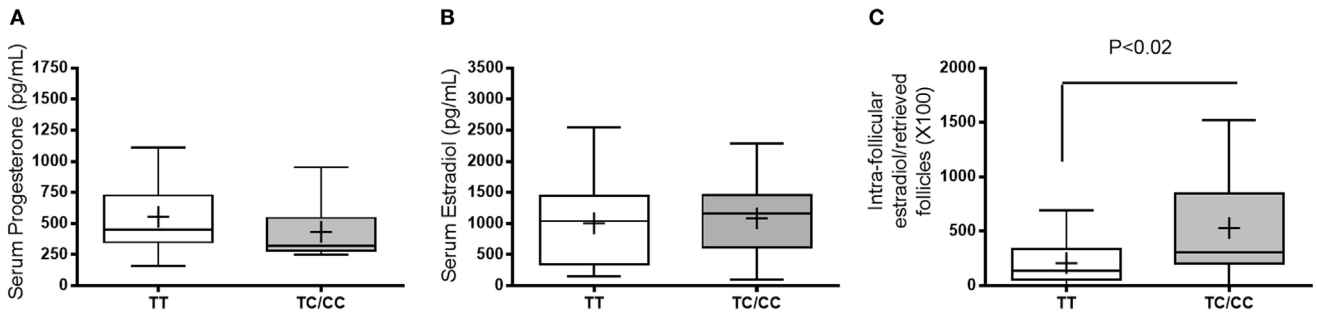

FIGURE 2 | Serum concentrations of progesterone **(A)** and estradiol **(B)** on the day of human chorionic gonadotropin administration and the ratio of serum estradiol/to retrieved follicles **(C)**. White bars represent women carrying the TT genotype, whereas the gray bars represent women carrying the TC/CC genotypes. Unpaired *t*-test. The data are presented as the mean ± min and max.

However, a study of polycystic ovarian syndrome in Caucasian women (32) found no association between *TaqI* polymorphic genotypes (TT, TC, CC) and 25(OH)D deficiency. This finding is in line with our results, given that we did not found any associations between *TaqI* polymorphism genotypes and intrafollicular 25(OH)D concentrations. Altogether, these data highlight the relevance of the *TaqI* polymorphism under different conditions and suggest the need for further studies investigating the relationship between *VDR* polymorphisms and 25(OH)D serum concentrations in different pathologies, including infertility disorders.

Our study has some limitations, including the relatively low number of included patients and the fact that we did not genotype all three *VDR* polymorphisms in all samples we evaluated. We also observed that there was a lack of HWE in the control population due to the exclusion criteria. This decreased the size of the study population and may have contributed to the observed imbalance in genotype and allele frequencies, resulting in a lack of HWE in the study populations. However, the COS population was under HWE and exhibited an association between the *TaqI* TC/CC polymorphic genotypes and the production of fewer ovarian follicles. These results suggest a possible role of the C allele in determining the number of pre-ovulatory follicles.

The above observation is supported by our data showing a higher ratio of retrieved follicles to intrafollicular E2 in women with TC/CC genotypes than in women carrying the TT genotype, i.e., women who have the TC/TT genotypes exhibited lower E2 availability in pre-ovulatory follicles. This finding demonstrates an important impact of the *TaqI* polymorphism on follicular development and hormone secretory function.

Recent studies have shown that 25(OH)D is present in FF (15, 19). While there is some controversy regarding the importance of FF 25(OH)D concentrations in positive IVF outcomes [for example, in patients with chemical pregnancies, embryonic implantation problems, chemical pregnancy (β-hCG level higher than 25 mIU/ml), in fertilization rates and in the numbers of embryos transferred and oocytes retrieved] (13, 14, 44), *VDR* mRNA and 1-α hydroxylase enzyme are expressed in the ovary (i.e., in ovarian cells and granulosa cell cultures) (12, 38), indicating that calcitriol activity affects local synthesis and autocrine and/or paracrine actions in the ovaries.

In conclusion, this study revealed an association between the presence of the C *VDR TaqI* polymorphism allele and infertility. This association is likely mediated by impaired calcitriol signaling, which may impact the number of follicles in women undergoing COS *via* mechanisms that are yet to be described.

ETHICS STATEMENT

This study was approved by the local Ethics Committee of the Maternidade Escola of the Federal University of Rio de Janeiro, which was registered on the Brazilian platform of research under the number 02213812.4.0000.5275. All of the enrolled subjects (volunteers and patients) provided written informed consent before joining the study.

AUTHOR CONTRIBUTIONS

Conceptualization of the experiments. Formal analysis. Performed experiments. Writing review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00252/full#supplementary-material.

FIGURE S1 | Agarose gel electrophoresis of DNA against a 100 bp DNA ladder (Promega) following PCR amplification and restriction fragment length polymorphism with the *TaqI* restriction enzyme. **(A)** Lane 1, uncut band with a length of 495 bp. Lanes 2, 3, 5, and 6 contain uncut 495 bp fragments, indicating that these samples have the TT (homozygous) genotype. Lane 4 contains two bands at 290 and 205 bp, indicating the CC (homozygous) genotype. **(B)** Lanes 1, 3, 4, 5, and 6 contain 495, 290, and 205 bp fragments, indicating the TC (heterozygous) genotype. Lane 2 contains an uncut band with a length of 495 bp. Lane 7 contains two bands at 290 and 205 bp, indicating the CC (homozygous) genotype.

FIGURE S2 | Agarose gel electrophoresis of DNA against a 100-bp DNA ladder (Promega) following PCR amplification and restriction fragment length polymorphism with the *BseGI* restriction enzyme. **(A)** Lane 1 contains an uncut band at 265 bp. Lane 2 contains fragments at 265 bp, indicating the TT (homozygous) genotype. Lane 3 contains two bands at 196 and 69 bp, indicating the CC (homozygous) genotype. **(B)** Lane 1 contains an uncut band with a length of 265 bp. Lanes 2 and 3 contains fragments at 265 bp, indicating an uncut fragment and, therefore, the TT (homozygous) genotype.

FIGURE S3 | DNA fragment sequences in affected and unaffected individuals. The BamH1 restriction site sequence is underlined, and the arrows indicate the polymorphic site. **(A)** DNA sequence electropherogram of the wild-type G/G genotype (arrow). **(B)** DNA sequence electropherogram of the homozygous A/A polymorphism (arrow). **(C)** DNA sequence electropherogram of the heterozygous G/A polymorphism (arrow).

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Association of Vitamin D Receptor Gene Variation With Osteoporosis Risk in Belarusian and Lithuanian Postmenopausal Women

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Vitamin D receptor (VDR) is one of the main mediators of vitamin D biological activity. VDR dysfunction might substantially contribute to development of postmenopausal osteoporosis (PMO). Numerous studies have revealed the effects of several *VDR* gene variants on osteoporosis risk, although significant variation in different ethnicities have been suggested. The main purpose of this work was to assess the frequency of distribution of *VDR* genetic variants with established effect and evaluate their haplotype association with the risk of PMO in a cohort of Belarusian and Lithuanian women. Case group included women with PMO (n = 149), the control group comprised women with normal bone mineral density (BMD) and without previous fragility fractures (n = 172). Both groups were matched for age, height, sex, and BMI—no statistically significant differences observed. *VDR* gene polymorphic variants (ApaI rs7975232, BsmI rs1544410, TaqI rs731236, and Cdx2 rs11568820) were determined using polymerase chain reaction and restriction fragment length polymorphism. The lumbar spine (L1-L4) and femoral neck BMD was measured using dual-energy X-ray absorptiometry. Association between each *VDR* variant and PMO risk was assessed using multiple logistic regression. The genotyping revealed statistically significant difference in the rs7975232 genotype frequencies between the patients and the controls (homozygous C/C genotype was overrepresented in patients, p = 0.008). Patients with osteoporosis were also three times more likely to carry the rs1544410 G/G genotype, when compared to controls. We found that rs7975232, rs1544410, and rs731236 variants were in a strong direct linkage disequilibrium ($p < 0.0001$), suggesting that risk alleles of these markers are preferably inherited jointly. For the bearers of C-G-C haplotype (consisting of rs7975232, rs1544410, and rs731236 unfavorable alleles), the risk of PMO was significantly higher (OR = 4.7, 95% CI 2.8–8.1, $p < 0.0001$) compared to controls. This haplotype was significantly over-represented in PMO group compared to all other haplotypes. Our findings highlight the importance of identified haplotypes of *VDR* gene variants. Complex screening of these genetic markers can be used to implement personalized clinical approach for prevention, treatment, and rehabilitation programs.

Keywords: vitamin D receptor, genetic variants, polymorphism, haplotype, postmenopausal osteoporosis

INTRODUCTION

Vitamin D and its active metabolites are important components of the immune and hormonal systems that not only control phosphorus and calcium homeostasis but also play important role in providing numerous biological effects, involved in the regulation of processes of cell differentiation and proliferation in many target organs and tissues. So-called classical effects of $1.25(OH)_2D$ and other vitamin D metabolites are participated in the processes of mineralization of bone tissue, maintaining calcium homeostasis and, finally, direct effect on bone remodeling mediated through the vitamin D receptor (VDR), encoded by *VDR* gene. Research published during the past two decades has established that pleiotropic effects of vitamin D and their genetic revelations are associated with a wide variety of diseases (1). This study is focused on association of *VDR* gene variants with susceptibility to postmenopausal osteoporosis (PMO).

Osteoporosis is characterized by reduced bone mineral density (BMD) and increased bone fragility. Homeostasis of bone tissue during lifetime is mainly maintained by balanced processes of bone resorption and formation, resulting from the combined action of multiple genes and environmental factors. Identification of gene variants, responsible for low BMD, will help to reveal individuals with increased risk of osteoporosis and suggest a personalized clinical approach to prevent or at least to delay the development of this pathology. The prevalence of osteoporosis is different in various ethnicities (2). Evaluation of genetic predisposition to osteoporosis is especially important, because this disease is asymptomatic; the first clinical manifestation in the majority of cases is low energy fractures, and the number of older people, having elevated risk of fragility fractures, is increasing. Postmenopausal women lose important bone protectors (estrogens), and their bone resorption rate increases dramatically (3).

The human *VDR* gene is located on the short arm of chromosome 12. It consists of 9 exons and encodes a 427 amino acid protein (4). In the *VDR* gene, several polymorphic sequence variations have been reported, which can occur in coding or noncoding parts of the gene and lead to changes in the protein sequence or affect the degree of gene expression (5). These include single nucleotide polymorphisms (SNP) that can be identified with the appropriate restriction endonuclease enzymes, such as ApaI (rs7975232), BsmI (rs1544410), FokI (rs2228570), TaqI (rs731236), and Cdx2 (rs11568820).

VDR ApaI (rs7975232) gene polymorphism is located in the 3′-regulatory region of *VDR* gene (in intron 8). There is no functional effect of this variant described, although some authors suggested its effect on mRNA stability (5). It was found that BsmI (rs1544410) variant is significantly associated with the increased risk of developing PMO (6) and antiresorbtive treatment responses (7). *VDR* TaqI (rs731236) gene polymorphism is located in exon 9 and it has been proved to affect mRNA stability, influencing biological function of vitamin D (8). These three SNPs are located at the 3′-terminus of *VDR* gene and frequently reported to be in linkage disequilibrium (LD). The FokI (rs2228570) polymorphism is located in the second exon of the *VDR* gene, plays an important role in post transcriptional processes and causes the production of two different VDR protein variants: long and short variants (5). Compared with the long VDR form, the short form has greater transcriptional activation capability. In meta-analysis, *VDR* FokI polymorphism was significantly associated with higher risk of developing PMO in Asian, but not in Caucasian populations (6). Cdx2 variant is located in the 5′-promoter region of the *VDR* gene. The *VDR* Cdx2 G-variant reduces transcriptional activity of the gene to 70% of the A allele (9).

In recent years, multiple studies have been performed to investigate correlation between *VDR* gene variants and osteoporosis risk, suggesting the presence of ethnic differences in the genetic association with osteoporosis. But there is still no clear evidence about their effects in performed recent meta-analyses (6, 10–12). Therefore, to improve the significance of associations, it is necessary to perform evaluation of combinations of genetic variants with established effects on independent cohort.

The aim of this study was to compare the associations of selected polymorphic variants within *VDR* gene with the risk of osteoporosis in Belarusian and Lithuanian postmenopausal women.

MATERIALS AND METHODS

Subjects and Clinical Assessment

Based on a case–control design, 149 patients with postmenopausal osteoporosis (PMO group) and 172 asymptomatic controls (CON group) participated in the study. One hundred twenty-one Belarusian patients and 127 controls were recruited from Minsk City Center for osteoporosis and bone-muscular diseases prevention (Minsk, Belarus), while 28 Lithuanian patients and 45 controls were recruited from National Osteoporosis Center (Vilnius, Lithuania). All subjects signed written informed consent after being fully informed about the nature of the study according to Helsinki Declaration of 1975, as revised in 2000. Local Research Ethics Committee at the Belarusian Medical Academy of Postgraduate Education and Lithuanian Regional Biomedical Research Ethics Committee approved the study protocol. The exclusion criteria for both groups were chronic diseases, oncology, hypercalcemia, and use of glucocorticosteroids. The women with the clinical diagnosis of osteoporosis (the BMD *T*-score of −2.5 or lower at the femoral neck or the lumbar spine) and at least 2 years postmenopausal were defined as the patients with PMO. The control group comprised postmenopausal women with BMD *T*-score of >−2.5 and without previous fragility fractures. The data of the medical history and the fracture history were obtained by a clinical expert.

BMD Measurement

Bone mineral density was measured at the lumbar spine and both proximal femurs using dual-energy X-ray absorptiometry (Prodigy, GE Lunar, Madisson, WI, USA). The lowest value from right or left femur and lumbar spine L_1–L_4 BMD was taken and used in further comparative analysis.

Genotyping

For genetic analyses, venous blood samples were taken from the cubital vein using the Vacutainer system with EDTA

(Beckton-Dickinson, Franklin Lakes, NJ, USA). DNA was isolated from bloodspots dried on special NucleoSafe cards (Macherey-Nagel, Germany) using the standard proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation. The DNA solution was extracted with a phenol–chloroform–isoamyl alcohol mixture to remove protein contaminants and then was precipitated with 100% ethanol. The DNA was pelleted after the precipitation step, washed with 70% ethanol to remove salts and small organic molecules, and resuspended in a buffer at a concentration suitable for further investigation (20–120 ng/μL). The quality and purity of DNA samples were checked using Qubit 2 Fluorimeter (Thermo Fisher Scientific, USA).

Selected polymorphic variants (ApaI rs7975232, BsmI rs1544410, TaqI rs731236, and Cdx2 rs11568820) in *VDR* gene were determined using the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis as described earlier (13). Briefly, the PCR reaction system consisted of 10-μL 10 × PCR buffer (1 × buffer = 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.25 mM $MgCl_2$), 1.0 μL of 10 × dNTPs (0.2 mM), 1.0 μL of each primer, 0.5 μL of polymerase, 3.5 μL of mQ water, and 10 ng of genomic DNA. The PCR was performed with an initial denaturation at 95°C for 15 min, followed by 28 cycles of denaturation at 99°C for 1 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. The PCR amplification was carried out in an automated thermal cycler (C1000, Bio-Rad, USA). The final extension was performed at 72°C for 1 min. The PCR products were size-separated by electrophoresis on the 10% polyacrylamide gel at 125 V for 1 h. The 100-bp DNA ladder (Thermo Fisher Scientific, Lithuania) was used to determine the fragments size.

Statistical Analysis

Kolmogorov–Smirnov test was used to assess the normality of data distribution. Normally distributed data are presented as mean and compared using Student's *t*-test. The data which are not normally distributed are presented as median (25, 75% interquartile range) and compared using Mann–Whitney *U*-test.

Based on the determined frequencies of genotypes and by using the Pearson chi-square (χ^2) test, the Hardy–Weinberg equilibrium was assessed. Crude odds ratios (OR) were reported with 95% confidence intervals (95% CI) and calculated in comparison to reference (wild-type) genotype. Logistic regression models were used to assess difference between the characteristics of PMO and CON groups for categorical data and for comparison of allele, genotype, and haplotype frequencies between these groups. The statistical analysis was performed using the freely available programming language R (http://r-project.org) with package "SNPassoc" (version 1.9-2). LD between the genetic variants was determined using "haplo.stats" R-package. The differences between the groups were considered statistically significant at $p < 0.05$.

RESULTS

Participant Characteristics

Each subject was supplied with questionnaire, containing personal information and variables, necessary for the study. The participants within the PMO and CON groups were matched for age, height, sex, and BMI—no statistically significant differences were found (**Table 1**). Both groups included Belarusian and Lithuanian individuals—postmenopausal women of the same age. The comparison of Belarusian and Lithuanian groups has not revealed any significant differences in alleles and genotypes distribution. The CON group had significantly higher spine and femur neck BMD level compared to PMO group ($p < 0.01$).

Genotype and Allele Frequencies Distribution of Single *VDR* Gene Variants

Four *VDR* gene restriction fragment length polymorphisms (ApaI, BsmI, TaqI, and Cdx2) were selected following the literature review for consideration in Caucasian population (5, 6, 14, 15). Search of risk alleles and assessment of their combined action in present study was performed on the independent cohort. The genotype and allele frequencies of the analyzed *VDR* gene single SNPs are presented in **Table 2**. The distribution of all four analyzed gene polymorphisms in controls and in patients with postmenopausal osteoporosis was in correspondence with the one expected from the Hardy–Weinberg equilibrium ($p > 0.05$ in all cases).

There was a statistically significant difference in genotype and allele distribution for *VDR* ApaI, BsmI, and TaqI gene variants revealed between PMO and CON groups (**Table 2**). The *CC* genotype of *VDR* ApaI was significantly over-represented in PMO (36.9%) group when compared to the CON (22.1%) group (OR = 2.1, 95% CI 1.3–3.4, $p = 0.008$). The CON group individuals were more likely to carry *VDR* ApaI A allele (56.4%), compared to the PMO group (40.6%, OR = 0.5, 95% CI 0.4–0.7, $p = 0.0007$). The differences in the genotype ($p = 0.002$) and allele ($p = 0.0001$) frequency distributions of the *VDR* BsmI gene variant between the PMO and CON groups were also significant. More specifically, the risk of PMO was significantly higher for the bearers of GG-genotype compared to reference wild-type AA-genotype (OR = 3.0, 95% CI 1.6–5.4, $p = 0.002$).

There was, however, no statistically significant difference in *VDR* Taq genotype distribution ($p = 0.1$), though the T allele was over-represented in the asymptomatic CON (55.2%) group compared to the PMO (46.3%) group (OR = 0.7, 95% CI 0.5–0.95) and the C-allele was over-represented in the PMO (53.7%) group

TABLE 1 | Clinical characteristics of analyzed patients with postmenopausal osteoporosis (PMO) and control (CON) groups.

	PMO ($n = 149$)	CON ($n = 172$)	*p*-Value
Age, years	61.4 (6.5)[a]	57.5 (7.3)[a]	>0.05
Height, cm	159.2 (8.3)[a]	165.1 (5.8)[a]	>0.05
Weight, kg	64.5 (7.2)[a]	73.3 (5.1)[a]	>0.05
Body mass index, kg/m^2	26.7 (3.2)[a]	27.3 (4.7)[a]	>0.05
Years after menopause	11.3 (4.5)[a]	8.2 (2.7)[a]	>0.05
Spine bone mineral density (BMD), g/cm^2	0.944 (0.831; 1.090)[b]	1.152 (1.024; 1.240)[b]	<0.01
Femoral neck BMD, g/cm^2	0.803 (0.707; 0.914)[b]	0.983 (0.913; 1.13)[b]	<0.01

[a]*SD.*
[b]*IQR, 25–75% interquartile range.*

TABLE 2 | The genotype and allele frequencies (in %) of *VDR* gene variants.

Gene variant	Genotype, major allele	PMO, $n = 149$	Control, $n = 172$	OR (95% CI)	p-Value
VDR ApaI rs7975232	AA	18.1	34.9	1.0	
	AC	45.0	43.0	1.1 (0.7–1.7)	**0.008**
	CC	36.9	22.1	**2.1 (1.3–3.4)**	
	A allele	40.6	56.4	**0.5 (0.4–0.7)**	**0.0007**
HWE p-value		0.41	0.12		
VDR BsmI rs1544410	AA	21.8	37.2	1.0	
	AG	42.9	42.4	1.7 (1.0–3.0)	**0.002**
	GG	35.4	20.4	**3.0 (1.6–5.4)**	
	A allele	43.2	58.4	**0.5 (0.4–0.7)**	**0.0001**
HWE p-value		0.13	0.12		
VDR TaqI rs731236	TT	25.5	33.7	1.0	
	TC	41.6	43.0	1.3 (0.8–2.2)	0.1
	CC	32.9	23.3	1.9 (1.0–3.0)	
	T allele	46.3	55.2	**0.7 (0.5–0.95)**	**0.02**
HWE p-value		0.05	0.09		
VDR Cdx2 rs11568820	GG	68.4	71.6	1.0	
	GA	30.1	27.6	1.3 (0.7–2.2)	0.15
	AA	1.5	0.8	–	
	G allele	83.5	85.4	0.9 (0.5–1.4)	0.52
HWE p-value		0.28	0.2		

compared to the CON (44.8%) group (OR = 1.4, 95% CI 1.1–2.0, $p = 0.02$ in both cases). No significant differences in the *VDR* Cdx2 genotype distribution ($p = 0.15$) and allele frequencies ($p = 0.52$) between the PMO and CON groups were observed.

LD Analysis of *VDR* Gene

The results of LD analysis are presented in the **Figure 1**. LD plot was constructed using combined genotype data from PMO and CON groups. The three SNPs, ApaI (A/C), BsmI (A/G), and TaqI (T/C) of *VDR* gene are in a very strong LD (the measure *D*' is very close to 1, $p = 0.01$). The positive coefficient of correlation *r* suggests that major alleles of *VDR* ApaI, BsmI, and TaqI gene variants are likely to be inherited together, as well as minor alleles.

No significant LD was found between *VDR* Cdx2 and other three analyzed gene variants. Thus, two haplotypes A-A-T and C-G-C are inferred with high probability from revealed *VDR* polymorphisms with high *D*' measure, allowing further complex analysis of allelic combinations.

Haplotype Analysis

The haplotype analysis was performed for three *VDR* gene polymorphisms: ApaI, BsmI, and TaqI (**Figure 2**). Haplotypes were constructed from all possible allelic combinations of three *VDR* polymorphisms and compared between the PMO and CON groups.

Five haplotypes (A-A-T, C-G-C, C-A-C, A-G-C, and C-G-T) of the possible eight combinations were inferred at a frequency of greater than 5%. The A-A-T haplotype was the most frequent haplotype (total frequency 36.8%), constructed from three wild-type allele variants. This haplotype was significantly over-represented in CON group (46.0%) compared to the PMO group (26.7%, $p < 0.01$). The total frequency of C-G-C haplotype was

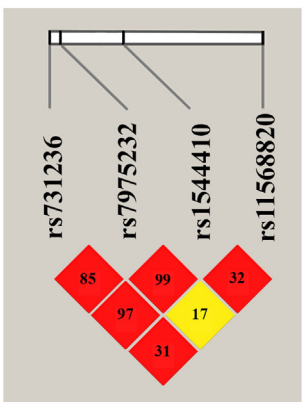

FIGURE 1 | Linkage disequilibrium plot for *VDR* ApaI, BsmI, TaqI, and Cdx2 variants. The LD plot was build using combined genotype data from postmenopausal osteoporosis and control groups (constructed with "haplo.stats" package for R). LD is displayed as pairwise *D*' values multiplied by 100 and given for each single nucleotide polymorphism combination within each cell. Red cells correspond to $p < 0.0001$, yellow—$p > 0.1$.

32.4%, it was significantly under-represented in CON group (23.7%) compared to PMO group (43.2%, $p < 0.01$). For the bearers of C-G-C haplotype, the risk of PMO was significantly higher compared to the reference (wild-type) haplotype (OR = 4.7, 95% CI 2.8–8.1, $p < 0.0001$). None significant association was found for other revealed haplotypes.

DISCUSSION

This case–control study was performed to investigate the role of *VDR* gene in PMO in combined cohorts of Caucasian

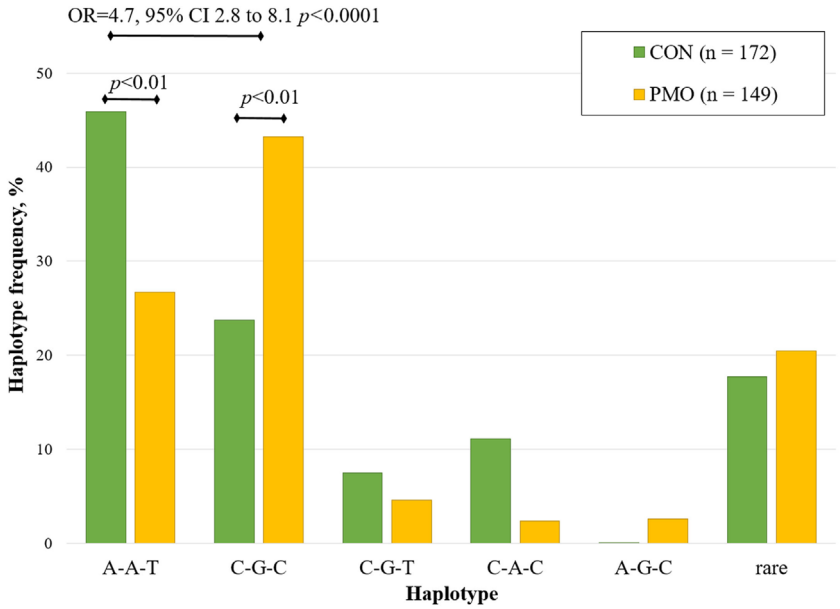

FIGURE 2 | Estimated haplotype frequency distribution of *VDR* ApaI, BsmI, and TaqI variants in the postmenopausal osteoporosis (PMO) and control (CON) groups.

(Belarusian and Lithuanian) women. Because PMO is a multifactorial disorder, genetic variants within the *VDR* gene, which is an important regulator of bone remodeling, may be a contributing risk factor. For the best of our knowledge, this is the first study demonstrating an association of four *VDR* variants with PMO risk in cohort of Belarusian and Lithuanian patients.

VDR gene variants were selected from key publications with established associations with PMO (5, 6, 14, 15) and analyzed in independent population for assessing their combined action. A total of four polymorphic variants of *VDR* gene were selected and evaluated for this study. These variants are established as PMO risk factors, so we are mainly interested to know if their effect is similar to other populations or may be different and what will be their combined action.

The cases and controls involved in the analysis were well defined with similar inclusion criteria. The descriptive analysis of our random selection of subjects showed that PMO and CON groups were matched for age, height, weight, sex, and BMI. Spine and femoral BMD levels were significantly different between PMO and CON groups. The observed genotype frequencies in CON group did not deviate from Hardy–Weinberg equilibrium ($p > 0.05$ in all cases), while in PMO group it deviated (*VDR* Taq, $p = 0.05$). We also revealed significant differences in *VDR* polymorphic alleles (ApaI, BsmI, and TaqI) and genotypes (ApaI and BsmI) frequency distribution between PMO and CON groups.

Obtained genotype and allele frequencies in CON group (**Table 2**) for *VDR* ApaI were close to those of Caucasian subjects reported in HapMap (16), for *VDR* BsmI—in British (17), Spanish (18), and Slovenian (19) populations, *VDR* TaqI—in Czech population (20), *VDR* Cdx2—in Slovenian population (19). The contrast with the data reported in other Caucasian studies (6) is likely related to both ethnic differences and differences in inclusion criteria.

The main finding of single *VDR* gene variants association analysis is that the presence of *VDR* ApaI CC and BsmI GG homozygous genotypes is significantly associated with increased PMO risk, being over-represented in PMO group. By comparison of *VDR* TaqI allele frequencies in CON and PMO groups, we found that allele C also represented a risk factor (OR = 1.4, $p = 0.02$). This data are in accordance with the data reported in meta-analysis (6), where ApaI and BsmI were the most frequent markers, associated with PMO risk. Currently it is accepted that *VDR* BsmI polymorphism is related to BMD level, but its effect is relatively small and strongly influenced by external factors like diet (1). Unlike *VDR* BsmI, ApaI, and TaqI polymorphisms affect mRNA stability, which results in change of biological functions of vitamin D (6, 21).

The absence of significant association for *VDR* Cdx2 may be explained by very low number of individuals with AA-genotypes (less than five individuals in each subgroup).

As shown in **Figure 1**, three of four investigated variants were in strong LD. This finding is in agreement with previous studies that have found strong linkage between ApaI, BsmI, and TaqI variants of *VDR* gene located within the chromosomal region 12q12. The greatest degree of LD was found between ApaI and BsmI, followed by BsmI and TaqI, and then ApaI and TaqI. The same trend was reported in the meta-study for Caucasian populations (22), as well as for British population (23), opposite trend observed in Italian population (24). Most likely, the strong LD coefficient may be explained by the location of all three markers in the ninth exon of *VDR* gene, as well as by the adaptive advantage of a particular allelic combination. The absence of LD between Cdx2 (located in the promoter region of *VDR* gene) and other three variants is in good accordance with the literature (23).

Positive correlation between *VDR* ApaI, BsmI, and TaqI variants suggests that their major alleles are associated together,

making them likely to be inherited jointly. Using logistic regression, we have analyzed the global distribution of all revealed haplotypes in PMO and CON groups. Inferred haplotypes of ApaI, BsmI, and TaqI variants were significantly associated with PMO risk (global haplotype association $p < 0.0001$).

The most frequent haplotype was wild-type A-A-T (36.8%) followed by C-G-C (32.4%). These haplotypes were more common than other haplotypes in CON and PMO groups. Very close haplotype frequency distribution was reported for Caucasian (22), Dutch (25), and Italian (24) women. By excluding too rare haplotypes, most common were compared with reference haplotype A-A-T. The data show that for the bearers of non-favorable haplotype C-G-C, the risk of PMO is significantly higher. No protective variants were revealed in this study.

It is necessary to mention that, in contrast to ApaI and BsmI, *VDR* TaqI variant is constituted by the silent replacement of thymine (T) by cytosine (C) and does not change the amino acid sequence of VDR protein (5). This evidence partly contradicts with the result of this study, where significant association of PMO risk with single *VDR* TaqI allele variants or *VDR* ApaI-BsmI-TaqI haplotypes was found. These three SNPs are located at the 3′ end of the *VDR* gene and are at very high degree of LD. Therefore, with high probability the risk allele of TaqI variant is inherited together with risk alleles of ApaI and BsmI variants, and *vice versa*. For this reason, the effect of *VDR* TaqI variant can be explained by strong LD, when they are found in specific haplotypes, and due to the effects of ApaI and BsmI polymorphisms on PMO risk. Another possible explanation of *VDR* TaqI significant association with PMO risk are epigenetic (methylation) processes (26).

Considering the contrasting results in different studies of the genetic predisposition to osteoporosis, it seems that the best interpretations are possible if a complex of genetic variants and haplotypes is evaluated. In addition, it is very important to analyze the association of established risk variants on independent cohort, which will help to escape risk score summarization paradox. The present study is the first such research, performed on independent cohorts for evaluation of combined effect of different genetic variants with known associations, as well as to assess if the overall pattern of association is similar to other populations. Moreover, basic studies will help to reveal a deeper knowledge of the molecular mechanisms of bone disorders regulated by vitamin D through its receptors.

The limitations of current study include a low number of Lithuanian participants, which is not sufficient for comparison of *VDR* variants genotype distribution between Lithuanian and Belarusian populations. Also, the sample size was not enough big to perform the adjustments for additional covariates (such as BMI, height, weight, vitamin D level, fractures incidence). Further, well-designed studies with larger sample sizes of both ethnic populations will clarify data, obtained in present studies.

Thus, the most important result of this study is that for the women-bearers of C-G-C haplotype, there is a 4.7-fold risk of developing PMO. Our study suggests that variants of *VDR* gene are associated with the risk of PMO, although different polymorphisms might have different influences. The overall pattern of known *VDR* markers of PMO risk in combined Belarusian and Lithuanian population is close to other Caucasian populations. Further work will also include analysis of interaction between *VDR* variants and other genetic markers, playing important roles in genetic predisposition to osteoporosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

PM collected the data, performed statistical analysis, and drafted the research paper. ER, IM, MT, and VA supervised and guided the design and analysis of the research project. AR and VS collected patient sample and performed clinical survey of Belarusian patients. MT collected patient sample and performed clinical survey of Lithuanian patients. KK performed DNA extraction and genotyping. All authors were involved in drafting and revising the manuscript.

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ORIGINAL RESEARCH
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Relationship Between Vitamin D Status and Vitamin D Receptor Gene Polymorphisms With Markers of Metabolic Syndrome Among Adults

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Introduction: Recent studies have demonstrated that vitamin D deficiency contributes to the development of metabolic disorders, including obesity and type 2 diabetes mellitus (T2DM). Several vitamin D receptor (VDR) gene polymorphisms had been described to play a role in these conditions since vitamin D receptors were found in many tissues. The aim of this study was to assess the relationship between vitamin D status and VDR gene polymorphisms with metabolic syndrome (MS) parameters in Russian middle-aged women.

Materials and Methods: A total of 697 women aged between 30 to 55 years were included in this cross-sectional study. Serum 25-hydroxyvitamin D (25(OH)D) level and four VDR gene polymorphisms rs1544410 (*BsmI*), rs7975232 (*ApaI*), rs731236 (*TaqI*), and rs2228570 (*FokI*) were measured. We applied the International Diabetes Federation (IDF) criteria to identify subjects with MS.

Results: 9.3% of subjects had normal vitamin D level, while 90.7% were insufficient or deficient. Abdominal obesity (AO) was seen in 75.5%, impaired glucose tolerance (IGT) or T2DM was observed in 33.3%, reduced high-density lipoprotein cholesterol (HDL-C) level in 32.2% and hypertriglyceridemia in 23.4%. Serum 25(OH)D level in women with or without MS did not differ (48.6 ± 1.8 and 51.1 ± 1.5 nmol/l, $p > 0.05$). Subjects with vitamin D deficiency showed an increased risk of AO [CI 95% 2.23; 1.15–4.30] and low HDL-C [CI95% 2.60; 1.04–6.49] compared to subjects with normal 25(OH)D level. IGT and T2DM risk was increased only when 25(OH)D concentration was less than 39.0 nmol/l [CI 95% 7.17; 2.99–17.7], but risk of MS did not differ in normal vitamin D status subjects and insufficient/deficient ones ($p > 0.05$). T allele carriers (*A*) of rs7975232 had higher total cholesterol and low-density lipoprotein cholesterol levels compared with the GG (*aa*) genotypes. Similarly, GG (*BB*) genotype carriers of rs1544410

had higher triglyceride levels than subjects with A (*b)* allele carriers. However VDR gene polymorphisms did not seem to be associated with an increased risk of MS.

Conclusions: Vitamin D deficiency, rs7975232, and rs1544410 VDR gene variants are associated with MS parameters in Russian middle-aged women.

Keywords: metabolic syndrome, obesity, diabetes, dyslipidemia, VDR gene polymorphisms

INTRODUCTION

It is well known that obesity, impaired glucose tolerance (IGT) or diabetes mellitus type 2 (T2DM), hypertension, high low-density lipoprotein cholesterol (LDL-C), hypertriglycedemia and decreased high-density lipoprotein cholesterol (HDL-C) are associated with high risk of cardiovascular disease and mortality (1–5). Different combinations of these factors have been defined as "Metabolic Syndrome" (MS). The International Diabetes Federation (IDF) defines MS as abdominal obesity (waist circumference ≥94 cm for males and ≥80 cm for females) plus two of the others cardiovascular risk factors: hyperglycemia, T2DM, hypertension, hypertriglyceridemia, and low HDL-C level (6). However, there are other definitions of MS, for example the World Health Organization includes insulin resistance as an essential component to define MS (7).

Interestingly, recent studies showed a possible negative effect of vitamin D deficiency in the development of metabolic disorders, including obesity, IGT, T2DM, dyslipidemia and hypertension (8–11). These relationships are not clear. Some studies demonstrated inverse associations between serum 25-hydroxyvitamin D (25(OH)D) level and MS (12–14), but others have failed to do so (15–17). Analysis of data from the National Health and Nutritional Examination Survey (NHANES) demonstrated that concentrations of 25(OH)D were significantly associated with MS prevalence among Americans (18). In addition, dose-response meta-analysis showed a relationship between serum 25(OH)D level and MS in cross-sectional but not in longitudinal studies (19).

It is not surprising that vitamin D could be associated with cardiovascular risk factors. Vitamin D is not only involved in calcium and bone metabolism, but also in the regulation of proliferation and differentiation of many cells, in addition to its immunoregulatory, antiangiogenetic, and antioxidant properties. Pleiotropic extraskeletal effects of vitamin D are mediated by the activation of vitamin D receptors (VDR) which are widely expressed in different cells (20, 21).

Several polymorphisms have been reported for the VDR gene such as rs7975232 (*ApaI*), rs1544410 (*BsmI*), rs2228570 (*FokI*), and rs731236 (*TaqI*). Recent studies demonstrated that some of these polymorphisms are associated with T2DM and insulin secretion (22) as well as with metabolic disturbances in obese people (23).

Some studies in the Russian Federation confirmed high prevalence of MS and its components in the general population (24). Studies have also demonstrated high prevalence of vitamin D deficiency and insufficiency in Russia (25). However the relationship between MS components and vitamin D status in this population has not been assessed.

The aim of this study was to find out whether there is an association between vitamin D status and VDR gene polymorphisms with MS parameters.

MATERIALS AND METHODS

Study Population

We conducted a cross-sectional study of 697 Caucasian women aged between 30–55 years and residing in Saint-Petersburg, Russia. The subjects were recruited during outpatient clinic visits for minor medical problems. Exclusion criteria included clinically significant kidney and gastrointestinal diseases, history of diabetes mellitus, regular insolation (every week) and use of vitamin D supplements. The study was conducted in compliance with the principles of the Declaration of Helsinki, and each participant gave written informed consent before enrollment. The study was approved by the local ethics committee.

Data Collection

Anthropometric measurements included body weight (kg), height (cm), waist circumference (WC; cm), and body mass index (BMI) that was determined using the following formula: BMI = weight (kg)/height (m2). All measurements were performed in the morning with the participants dressed in light clothing, without shoes. Waist circumference was measured with the subjects standing and at the midpoint between the lower rib margin and the iliac crest parallel to the floor. Abdominal obesity was defined as WC ≥ 80 cm. Blood pressure was measured in the right arm in a sitting position after 10-min rest.

Blood samples were taken after an overnight fast and stored at −70°C. Serum 25(OH)D concentrations were measured by a chemiluminescent immunoassay (Abbott Architect 8000, Deerfield, IL, USA; intra-assay CV of 1.60–5.92%, inter-assay CV ranged from 2.15 to 2.63%).

Fasting plasma glucose (FPG) was determined enzymatically using commercially available kits and auto analyzer (UniCel DxC 800, Brea, CA, USA). Serum lipids: total cholesterol (TC), triglycerides (TG), HDL-C, and LDL-C were measured by enzymatic colorimetric assays with the analyzer COBAS INTEGRA 400/700/800 and standard kits (Roche Diagnostics, Mannheim, Germany).

Genetic Analysis

Genomic DNA was extracted from peripheral white blood cells using phenol extraction method. VDR gene polymorphisms

rs7975232 (*ApaI*), rs1544410 (*BsmI*), rs2228570 (*FokI*), and rs731236 (*TaqI*) were detected by polymerase chain reaction-restriction fragment length polymorphism method. VDR alleles and genotypes were determined by the presence or absence of *BsmI, ApaI, TaqI*, and *FokI* restriction enzyme sites. Digestion pattern assessment indicated the following VDR genotypes: *BsmI (BB, Bb, bb), ApaI (AA, Aa, aa), TaqI (TT, Tt, tt), and FokI (FF, Ff, ff)*. VDR genotyping was performed in 454 study subjects, while 236 healthy subjects represented the control group for genetic analysis.

Data Analysis

Metabolic syndrome was diagnosed using IDF criteria: systolic blood pressure (SBP) ≥130 mmHg or diastolic blood pressure (DBP) ≥85 mmHg or treatment for hypertension; TG ≥1.7 mmol/L and HDL-C <1.29 mmol/l or treatment for dyslipidemia; plasma glucose level more than 5.6 mmol/l or IGT/T2DM during oral glucose tolerance test (6).

Vitamin D status was classified according to the Endocrine Society criteria (26). Vitamin D deficiency was defined as serum 25(OH)D level <50 nmol/l, insufficiency 50-75 nmol/l and sufficiency ≥75 nmol/l.

Statistical analyses were performed using SPSS software 17.0 for Windows (SPSS Inc, Chicago, Ill). Data were presented as a percentage or mean ± SD. Frequencies of qualitative indicators were compared by nonparametric techniques using chi square (χ^2). Comparison of quantity indicators was performed using ANOVA. Statistical significance was defined as $p < 0.05$.

RESULTS

The study group included 697 Caucasian women aged 30–55 years, mean age was 43.5 ± 0.3 years. One hundred and seventy one participants (24.5%) had WC less than 80 cm whereas abdominal obesity was revealed in 526 subjects (75.5%). Thirty one patients (4.5%) were newly diagnosed with T2DM and IGT was noted in 201 participants (28.8%). One hundred and seventy five patients (25.1%) had arterial hypertension. Increased TC was observed in 342 women (49.0%), hypertriglyceridemia in 163 (23.4%), elevated LDL-C in 443 (63.6%), and reduced HDL-C in 224 (32.2%) participants.

The results demonstrated significantly higher BMI, BP, levels of fasting plasma glucose, TC, LDL-C, TG, and lower HDL-C in subjects with abdominal obesity compared to those with WC < 80 cm.

Serum 25(OH)D assessment revealed vitamin D deficiency and insufficiency in 632 women (90.7%), while only 65 participants (9.3%) had 25(OH)D concentrations ≥75 nmol/l. Vitamin D deficiency was more frequently observed in overweight and obese women ($\chi^2 = 4.32$, $p < 0.05$ and $\chi^2 = 6.29$, $p < 0.05$, respectively). Prevalence of vitamin D deficiency and insufficiency did not differ between subjects with and without abdominal obesity. The basic characteristics of study participants are presented in **Table 1**.

Statistical analyses revealed an association between vitamin D deficiency and presence of abdominal obesity [CI (95%) 2.23; (1.15–4.30)] as well as low HDL-C [CI (95%) 2.60; (1.04–6.49)].

TABLE 1 | Clinical and laboratory characteristics of women with and without abdominal obesity.

Characteristics	Abdominal obesity (+)	Abdominal obesity (–)	*P*-value
Age, years	45.0 ± 0.3	40.4 ± 0.6	< 0.05
WC, cm	95.7 ± 12.1	72.4 ± 4.7	< 0.001
BMI, kg/m^2	30.5 ± 5.9	22.3 ± 2.5	< 0.001
BP, mmHg			
Systolic	127.8 ± 17.9	118,86 ± 18.7	NS
Diastolic	80.1 ± 12.7	75,1 ± 13.1	NS
FPG, mmol/l	5.65 ± 1.53	5.06 ± 0.65	< 0.001
Total cholesterol, mmol/l	5.45 ± 0.93	5.11 ± 1.12	< 0.01
LDL-C, mmol/l	3.55 ± 1.11	3.10 ± 1.62	< 0.01
HDL-C, mmol/l	1.42 ± 0.37	1.56 ± 0.37	< 0.05
Triglycerides, mmol/l	1.32 ± 0.74	0.95 ± 0.50	< 0.001
25(OH)D, nmol/l	46.62 ± 19.48	52.23 ± 19.93	< 0.05
VITAMIN D STATUS			
Deficiency, *n* (%)	342(65)	88(51)	NS
Insufficiency, *n* (%)	147(28)	61(36)	NS
Sufficiency, *n* (%)	37(7)	22(13)	NS

Results are presented as means ± SDs and percentages. WC, waist circumference; BMI, body mass index; FPG, fasting plasma glucose; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; BP, blood pressure; 25(OH)D, 25-hydroxyvitamin D.

Serum 25(OH)D <39.0 nmol/l was associated with increased risk of IGT and T2DM [CI (95%) 7.17; (2.99–17.7)].

Metabolic syndrome components were evaluated in 397 women with abdominal obesity and genetic data. Among this group 187 subjects (47.1%) met IDF criteria of metabolic syndrome. Apart from abdominal obesity 187 subjects (47.1%) had increased plasma glucose or T2DM, 180 (45.3%) had reduced HDL-C, 152 (38.3%) were diagnosed with arterial hypertension, hypertriglyceridemia was revealed in 111 subjects (28.0%). The study showed that participants with MS had significantly higher systolic and diastolic blood pressure, fasting plasma glucose, LDL-C, TG, and lower levels of HDL-C compared to those with abdominal obesity but without MS. No significant differences were found for serum 25(OH)D concentration between the groups with and without MS. Vitamin D status did not influence risk of developing MS ($p > 0.05$). Clinical and laboratory characteristics of the subjects with and without MS are presented in **Table 2**.

Genotyping for rs1544410 (*BsmI*), rs7975232 (*ApaI*), rs731236 (*TaqI*), and rs2228570 (*FokI*) polymorphisms in VDR gene was performed in study subjects and in controls. Genotype and allele distribution did not differ between the groups. The observed genotype frequencies were consistent with Hardy-Weinberg equilibrium. Genotype distribution of VDR gene polymorphisms is shown in **Table 3**.

There was no difference in serum 25(OH)D concentration and anthropometric characteristics between rs1544410 (*BsmI*), rs7975232 (*ApaI*), rs731236 (*TaqI*), and rs2228570 (*FokI*) genotypes. Laboratory results demonstrated significantly higher TG levels in subjects with GG (*BB)* genotype of rs1544410

TABLE 2 | Characteristics of patients with and without metabolic syndrome.

Characteristics	MS (+)	MS (–)	*P*-value
Age, years	45.4 ± 9.9	43.3 ± 5.1	<0.05
WC, cm	98.9 ± 11.1	87.6 ± 11.6	<0.01
BMI, kg/m^2	31.5 ± 5.7	27.9 ± 5.1	<0.001
BP, mmHg			
Systolic	133.8 ± 18.7	121.17 ± 11.0	<0.001
Diastolic	85.9 ± 11.9	77.80 ± 11.7	<0.001
FPG, mmol/l	6.20 ± 1.78	5.30 ± 0.72	<0.01
Total cholesterol, mmol/l	5.60 ± 1.23	5.28 ± 0.87	<0.01
LDL-C, mmol/l	3.66 ± 1.23	3.34 ± 1.45	<0.05
HDL-C, mmol/l	1.21 ± 0.41	1.58 ± 0.43	<0.001
Triglycerides, mmol/l	1.70 ± 0.96	1.01 ± 0.29	<0.001
25(OH)D, nmol/l	48.62 ± 1.81	51.14 ± 1.56	NS

Results are presented as means ± SDs and percentages. WC, waist circumference; BMI, body mass index; FPG, fasting plasma glucose, LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; BP, blood pressure; 25(OH)D, 25-hydroxyvitamin D.

TABLE 3 | Distribution of VDR genotypes in study subjects and controls.

VDR genotypes (%)	Study population	Controls	*P*-value
rs1544410 (*BsmI*)			
GG *(BB)*	24.3	24.1	>0.05
GA *(Bb)*	57.7	54.2	
AA *(bb)*	18.0	21.7	
N	449	212	
rs7975232 (*ApaI*)			
TT *(AA)*	25.1	25.9	>0.05
TG *(Aa)*	54.9	51.5	
GG *(aa)*	20.0	22.6	
N	454	212	
rs731236 (*TaqI*)			
TT *(TT)*	45.1	45.3	>0.05
TC *(Tt)*	42.7	45.3	
CC *(tt)*	12.2	9.4	
N	454	212	
rs2228570 (*FokI*)			
CC *(FF)*	28.4	34.3	>0.05
CT *(Ff)*	50.5	51.3	
TT *(ff)*	21.1	14.4	
N	402	236	

(*BsmI*) polymorphism compared to A (*b*) allele carriers GA, AA (*Bb, bb*). Subjects with genotypes TT (*AA*) and TG (*Aa*) of rs7975232 (*ApaI*) polymorphism had significantly higher levels of TC and LDL-C compared to genotype GG (*aa)* carriers. No other differences between studied genotypes were observed (**Table 4**). Multiple regression analysis adjusted to age, smoking status, WC, BMI, and 25(OH)D level demonstrated no association of VDR gene polymorphisms with MS risk.

DISCUSSION

High prevalence of vitamin D deficiency is currently a global health problem (27). Low vitamin D status corresponding to either deficiency or insufficiency has been observed in all age groups from different countries and ethnicities (26, 28). According to our previous data, prevalence of vitamin D deficiency and insufficiency in northwestern region of Russia was observed in over 80% of the population studied (25), and was reconfirmed in this study. Such a high prevalence of low vitamin D status in middle-aged women is probably related to inadequate natural sunlight exposure, which is known to be a major cause of vitamin D deficiency (28). But what might be the impact of low vitamin D levels? The main physiologic function of vitamin D is maintenance of calcium and phosphate homeostasis and bone metabolism control but vitamin D effects are not just limited to bone tissue regulation as witnessed by the fact that most cells in the body have vitamin D receptors as well as para/autocrine vitamin D metabolic machinery (27, 29, 30). There have been an increasing number of investigations on the extraskeletal actions of vitamin D including regulation of cellular proliferation and differentiation, as well as effects on cardiovascular and immune systems (29, 30). Apart from musculoskeletal problems vitamin D deficiency has been found to affect a number of acute and chronic diseases (31). Recent studies have demonstrated an association between vitamin D deficiency and some metabolic disorders such as obesity, MS, and T2DM (32). As we know, MS is a combination of abdominal obesity and cardiovascular risk factors such as hyperglycemia, hypertension, and dyslipidemia associated with increased risk of cardiovascular disease (CVD). According to the IDF consensus abdominal obesity is defined as waist circumference ≥94 cm in males and ≥80 cm in females and is the main component of MS (6). Prevalence of abdominal obesity in our study was 75.5%. Patients with abdominal fat distribution had significantly higher levels of some metabolic parameters such as BP, FPG, TC, LDL-C, and TG and lower HDL-C related to CVD. Our findings appeared to be consistent with the previously reported data concerning association between central obesity and higher incidence of cardiovascular risk factors (33). Some studies have demonstrated an association between obesity and vitamin D deficiency but the causal relationship between these two conditions remains to be determined (34). In our study vitamin D deficiency was associated with increased risk of abdominal obesity and was noted more frequently in women who were overweight or obese. These results support previously demonstrated evidence of inverse association between 25(OH)D concentration and body mass index (35, 36).

Observational and prospective studies have shown the possible relationship between 25(OH)D concentrations and glucose levels (37). However, a recent study reported no association between 25(OH)D and risk of diabetes (38). According to data from systematic review and updated meta-analysis, 25(OH)D concentration was inversely associated with the incidence of T2DM (39). Our results did not show a relationship between glucose and 25(OH)D values in middle-aged women, however subjects with 25(OH)D levels less than 39 nmol/l had IGT or T2DM more frequently. We could not confirm

TABLE 4 | Lipid profile, plasma glucose level and serum 25(OH)D concentration in relation to VDR genotypes.

Genotypes	TC, mmol/l	LDL-C, mmol/l	HDL-C, mmol/l	TG, mmol/l	FPG, mmol/l	25(OH)D, nmol/l
rs1544410(*BsmI*)						
GG *(BB)*	5.44 ± 0.14	3.44 ± 0.12	1.37 ± 0.04	1.54 ± 0.09*	5.66 ± 0.21	49.72 ± 2.57
GA *(Bb)*	5.37 ± 0.07	3.44 ± 0.06	1.39 ± 0.02	1.28 ± 0.04	5.59 ± 0.08	48.68 ± 1.96
AA *(bb)*	5.56 ± 0.13	3.60 ± 0.14	1.36 ± 0.10	1.40 ± 0.08*	5.72 ± 0.10	48.31 ± 3.18
b allele carries	5.40 ± 0.06	3.45 ± 0.06	1.38 ± 0.02	1.32 ± 0.04**	5.66 ± 0.07	50.05 ± 1.95
	*$p < 0.05$ compared to *Bb* genotype, **$p < 0.05$ compared to *BB* genotype					
rs7975232 (*ApaI*)						
TT *(AA)*	5.49 ± 0.15#	3.47 ± 0.13#	1.40 ± 0.05	1.46 ± 0.10	5.68 ± 0.13	54.39 ± 3.31
TG *(Aa)*	5.53 ± 0.08#	3.37 ± 0.07	1.37 ± 0.03	1.32 ± 0.05	5.80 ± 0.13	48.88 ± 2.42
GG *(aa)*	5.14 ± 0.15	3.25 ± 0.12	1.36 ± 0.05	1.40 ± 0.05	5.74 ± 0.14	49.86 ± 4.27
A allele carries	5.52 ± 0.07#	3.54 ± 0.06#	1.38 ± 0.02	1.36 ± 0.05	5.76 ± 0.10	51.11 ± 1.96
	#$p < 0.05$ compared to *aa* genotype					
rs731236 (*TaqI*)						
TT *(TT)*	5.48 ± 0.08	3.53 ± 0.08	1.38 ± 0.03	1.35 ± 0.05	5.66 ± 0.09	48.29 ± 2.27
TC *(Tt)*	5.35 ± 0.07	3.39 ± 0.07	1.37 ± 0.03	1.31 ± 0.06	5.78 ± 0.13	49.88 ± 1.98
CC *(tt)*	5.61 ± 0.22	3.58 ± 0.19	1.36 ± 0.47	1.48 ± 0.13	5.59 ± 0.13	50.33 ± 3.60
rs2228570 (*FokI*)						
CC *(FF)*	5.42 ± 0.12	3.40 ± 0.06	1.40 ± 0.04	1.33 ± 0.07	5.60 ± 0.08	51.45 ± 4.21
CT *(Ff)*	5.47 ± 0.09	3.59 ± 0.08	1.34 ± 0.03	1.38 ± 0.05	5.71 ± 0.11	47.53 ± 3.56
TT *(ff)*	5.33 ± 0.11	3.41 ± 0.13	1.35 ± 0.21	1.33 ± 0.08	5.51 ± 0.7	45.90 ± 2.85

Results are presented as means ± SDs. VDR, vitamin D receptor gene; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; FPG, fasting plasma glucose; 25(OH)D, 25-hydroxyvitamin D.
**compared to Bb genotype, **compared to BB genotype, #compared to aa genotype.*

that vitamin D deficiency played a role in increasing the risk of developing T2DM and hence a prospective study is required.

Furthermore, other studies have reported a link between vitamin D status and dyslipidemias and showed that low 25(OH)D concentrations were associated with increased TG (40, 41) and decreased HDL-C or apolipoprotein A1 levels (42). We found a positive association between 25(OH)D values and HDL-C, but no associations between 25(OH)D and total cholesterol, TG and LDL-C values. It is known that vitamin D and cholesterol share a common 7-dehydrocholesterol pathway and hence, it is possible that the relationship between 25(OH)D and dyslipidemias could be related to the synthesis of vitamin D precursor and lipoproteins in the liver.

Moreover, the effects of vitamin D are mediated by VDR that belongs to the steroid receptor family and modulates expression of target genes. VDR is a protein which consists of two functional domains (N-terminal dual zinc finger DNA binding domain and C-terminal ligand-binding activity domain) and linking region (43, 44). VDRs are widely expressed in different tissues. The gene encoding VDR is located on chromosome 12 (12q12-14) (45).

Several single nucleotide polymorphisms have been described in the VDR gene which are supposed to affect metabolic disorders related to vitamin D deficiency (46). Single nucleotide polymorphisms (SNPs), including rs1544410 (*BsmI*), rs7975232 (*ApaI*), and rs731236 (*TaqI*), located at the 3′ untranslated region of VDR gene have been shown to influence mRNA stability and VDR expression (22, 46) whereas rs2228570 (*FokI*) SNP located near the promoter region results in altered VDR activity due to change in amino acid sequence of this protein (47). Genetic variants in VDR gene have been reported to be associated with MS and its components including anthropometric parameters related to obesity, insulin resistance, T2DM, and atherogenic lipid abnormalities in different populations (21, 23, 48). In contrast to these data, some studies have demonstrated no association between VDR gene polymorphisms and the risk for MS development (49–51). Our study found no significant relationship between VDR SNPs and serum 25(OH)D concentration, anthropometric characteristics, FPG, and MS risk, however an associations of rs1544410 (*BsmI*) and rs7975232 (*ApaI*) variants with atherogenic lipid profile were revealed. Thus, GG (*BB)* carriers of rs1544410 (*BsmI)* showed significantly higher TG levels, whereas, women carrying TT (*AA)* and TG (*Aa)* genotypes of rs7975232 (*ApaI)* had significant increase in TC and LDL-C. Consistent with our results, some previously reported studies showed no association between VDR gene polymorphisms and MS risk but observed the relationship of VDR SNPs with dyslipidemia (49, 51).

CONCLUSION

Our study demonstrated very high prevalence of low vitamin D status in middle-aged women from North-West Russia. We found an association between vitamin D deficiency and increased risk of MS components such as abdominal obesity, reduced HDL-C, IGT, and T2DM. VDR gene polymorphisms rs1544410 (*BsmI*), rs7975232 (*ApaI*), rs731236 (*TaqI*), and rs2228570 (*FokI*) were

not associated with MS risk in our study but the relationship between rs1544410 (*BsmI*) variants with TG levels and rs7975232 (*ApaI)* variants with TC and LDL-C was identified. Our findings suggest that vitamin D deficiency and VDR gene polymorphisms may contribute to the development of some MS components in adult female population. Future studies are required to clarify the causal relationship between VDR gene polymorphism, low vitamin D status and metabolic disorders including developing T2DM.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethics committee of Pavlov First Saint Petersburg State Medical University. The protocol was approved by the ethics committee of Pavlov First Saint Petersburg State Medical University. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

TK designed the study, performed the research, wrote the paper. EG and OB designed the study, performed the research. AB, AA, and AK performed the research, wrote the paper. EJ and PP wrote the paper.

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Vitamin D_3 Modulates Impaired Crosstalk Between RANK and Glucocorticoid Receptor Signaling in Bone Marrow Cells After Chronic Prednisolone Administration

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The effectiveness of vitamin D_3 (cholecalciferol) in counteracting the side effects of glucocorticoid (GC) therapy has been demonstrated previously. Abnormalities in systemic hormonal and local (cytokine) regulation of bone marrow (BM) cells may underlie GC-induced imbalance between osteosynthesis and bone resorption. The cytokine system receptor activator of nuclear factor kappa-B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) is considered as an integrating link in the NF-κB-mediated interaction of various cells involved in maintaining osteoblastic-osteoclastic balance, which makes it a pharmacological target for regulation and correction of the bone remodeling process. We studied GC-induced impairments of the RANKL/RANK/OPG axis in BM cells depending on vitamin D bioavailability and whether these changes were mediated by glucocorticoid (GR) and/or vitamin D (VDR) receptors. Female Wistar rats administered with prednisolone (5 mg/kg b.w., 30 days) showed a decrease in the GR protein level and the number of GR-positive BM cells. GC caused a marked elevation of RANKL and RANK levels in BM, while OPG decreased. Flow cytometry data indicated GC-elicited increase in the number of circulating RANK-positive osteoclast precursors (OCPs) in BM, peripheral blood, and spleen. In full accordance with the data that the interaction of RANKL-RANK leads to transcriptional activation of NF-κB and subsequent differentiation of osteoclasts, we found an increase in the level of phosphorylated p65 subunit of NF-κB with a simultaneous decrease in the NF-κB inhibitor (IκB) level. These changes were accompanied by vitamin D insufficiency and downregulated expression of CYP27B1 and VDR, which are responsible for synthesis and hormonal signaling of $1,25(OH)_2D$. Notably, we observed VDR and RANK co-localization in OCPs. Cholecalciferol co-administration (1,000 IU/kg b.w., 30 days) with prednisolone resulted in elevated GR synthesis in BM. Cholecalciferol prevented prednisolone-elicited disturbances of the RANKL/RANK/OPG, which correlated with improved bioavailability and vitamin D signaling through VDR. This caused the lowering of phosphoNF-κB p65 level and inhibiting NF-κB translocation to the nucleus that could reduce the circulating OCPs pool in

BM, peripheral blood, and spleen. Our findings suggest that prednisolone-induced abnormalities in GR and RANKL/RANK/OPG signaling pathways are associated with the impairments of vitamin D auto/paracrine system in BM cells and can be ameliorated by cholecalciferol supplementation.

Keywords: prednisolone, osteoporosis, vitamin D, vitamin D receptor, glucocorticoid receptor, RANKL/RANK/osteoprotegerin axis, nuclear factor kappa-B, osteoclastogenesis

INTRODUCTION

Glucocorticoids (GCs) have been extensively used in clinical applications as an effective therapy for a variety of severe inflammatory and autoimmune disorders (1, 2). However, long-term or high dose administration of GCs causes osteoporosis, which is characterized by a rapid and severe bone loss and microarchitectural changes in bone tissue, resulting in easy fracturing, and even disability (3). Deleterious side effects of GCs on multi-system pathways linked to osteoblast and osteoclast differentiation and apoptosis, marrow adipogenesis, mineral, and lipid metabolism are the most prevalent pathological features of glucocorticoid-induced skeletal disorders (4).

Several studies suggested that pathogenic mechanisms of GC-associated osteoporosis involve an initial acceleration of osteoclast-induced bone resorption followed by a decrease in osteoblast-mediated bone formation and attenuation of osteoclasts activity. Nevertheless, the exact mechanisms are still controversial and not conclusive. Although GCs are known to affect osteoblast differentiation and function, there have been conflicting reports about the effect of GCs on osteoclast formation and activity, leading to the assumption that the character of their influence depends largely on which synthetic GC is applied, its dosage and treatment duration. It was demonstrated that the differentiation deficiencies of osteoclast precursors (OCPs) may contribute to corticosteroid osteoporosis; however, the stimulating effect of GCs on these cells have also been reported. The latter was limited to the early phase of osteoclast differentiation and the enhanced priming of osteoclast progenitors [bone marrow (BM)-derived monocytes/macrophages] toward differentiation into mature osteoclasts (5).

Bone disruption associated with GC action may be attributed to abnormal regulation of bone remodeling at systemic (hormonal) and local (cytokine) levels. In the complex system of bone remodeling, the receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) pathway is the coupling factor between bone formation and bone resorption. RANKL, one of the tumor necrosis factor (TNF) superfamily members, is a potent stimulator of osteoclast formation and bone resorption, which acts through the RANK (receptor activator of nuclear factor κB—NF-κB) and often contributes to the pathologies of bone metabolism. RANKL belongs to the group of regulatory glycoproteins that is produced by BM stromal cells, osteoblasts, activated dendritic cells, and T-lymphocytes (6). This emphasizes the importance of RANKL-dependent and NF-κB-mediated osteoimmune interaction in the process of bone tissue remodeling. OPG, synthesized by various cells, acts as a decoy receptor for the RANKL and prevents its osteoclastogenic activity. The proper balance between RANKL and OPG determines the degree of proliferation and osteoclastic activity (7) and impaired OPG to RANKL ratio can be considered as an essential pathogenetic factor in the development of osteoporosis.

Available scientific data concerning the effects of GCs on osteoprogenitors or mature bone tissue cells are mainly obtained in studies on cell cultures or related to changes in bone tissue associated predominantly with short-term administration of high doses of synthetic hormones. Some studies have shown that GCs inhibit the expression of the OPG mRNA and further protein synthesis and secretion, thus leading to the RANKL mRNA overexpression in the culture of osteoblastic cells regardless of the differentiation stage (8). Because glucocorticoid response elements were found within the RANKL gene, the expression of this cytokine may in all probability be regulated by enhanced transcriptional activity of the corticosteroid (9). GCs were also shown to promote osteoclastogenesis by transrepressing the OPG gene through the AP-1 site (10). Although the components of RANKL/RANK signaling pathway are synthesized by the precursor cells of osteoblasts and osteoclasts in the BM; however, it remains an open question whether changes in this cytokine system may lead to the defective osteosynthesis in GC-induced osteoporosis.

Vitamin D_3 (cholecalciferol) is a unique compound, which is effectively used in the treatment of bone diseases. As a prohormone, it fulfills its biological effects through the mechanism of hormonal action, and as such it cannot be considered a classic vitamin. The hormonally active form of vitamin D_3 is 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which through its specific receptor (VDR) provides transcriptional regulation of the expression of about 500 genes in human cells (11). Less recognized is that vitamin D_3, like other nuclear steroids, can also exert non-genomic effects in various tissues (12).

VDR is a zinc-finger containing protein of the nuclear hormone receptor superfamily. Mechanistically, ligand binding induces conformational changes in VDR that promote receptor heterodimerization with the retinoid X receptor (RXR). After ligand activation, it binds directly to the vitamin D response elements and recruits a variety of co-regulatory complexes that perform the additional functions to modify transcriptional activity (13). Importantly, VDR-mediated gene regulation requires the involvement of multiple modular enhancers at a range of locations many kilobases upstream, downstream, or within the transcription units (14).

One of the main biological functions of vitamin D_3 is to ensure the normal growth and development of bones as well as the prevention of rickets and osteoporosis. Cholecalciferol regulates *in vivo* mineral metabolism and promotes the deposition of calcium in bone tissue. This action of vitamin D_3 is provided by

its hormonal effect on calcium homeostasis and VDR-mediated regulation of proliferation, differentiation, and apoptosis of various cell types involved in osteogenesis (osteoblasts, osteoclasts, osteocytes, immunocompetent cells). Nevertheless, the molecular mechanisms by which $1,25(OH)_2D_3$ stimulates bone resorption were also discovered. It has been demonstrate that regulation of *Rankl* gene expression by $1,25(OH)_2D_3$ is mediated by at least five distal regions in osteoblastic cells that, in addition to the GC receptor, contain binding sites for VDR and RXR (15). *In vitro* exposure of osteoblastic cells to $1,25(OH)_2D_3$ stimulates RANKL expression, which in turn induces osteoclastogenesis (16). Other results suggest that $1,25(OH)_2D_3$ can increase bone resorption by directly enhancing the formation and maturation of osteoclasts (17). Thus, recent advances in bone cells and vitamin D_3 biology have led to a more detailed understanding of bone tissue formation/resorption pathways and clear difference between *in vitro* (osteoclastogenic) and *in vivo* (antiresorptive) effects of active vitamin D_3 metabolites have been demonstrated.

The urgent scientific problem is to elucidate the role of VDR-mediated signaling in the impairment of osteoblastic–osteoclastic interaction, which provides the realization of bone tissue remodeling and maintenance of bone homeostasis in various pathologies of bone tissue, including GC-induced osteoporosis. Despite the decisive role of vitamin D_3 and its receptor in the process of bone remodeling, it remains controversial whether the interaction of vitamin D_3 with the signaling pathways of glucocorticoid receptor (GR) and RANKL/RANK/OPG has any effect on the differentiation of the OCPs after the concurrent administration of cholecalciferol and GCs. In this study, we examined the role of vitamin D_3 in the regulation of RANKL/RANK/OPG axis in primary BM cells and its possible relationship with abnormal interaction between GR and VDR signaling pathways in the BM after chronic administration of synthetic GC prednisolone.

MATERIALS AND METHODS

Experimental Design

A total of 45 four-week-old female Wistar rats (100 ± 5 g) were randomly divided into the following groups: (1) the control group; (2) the prednisolone group that received orally synthetic GC prednisolone at dose 5 mg/kg of b.w. for 30 days; and (3) the group that received concurrently prednisolone (5 mg/kg of b.w.) and vitamin D_3 (1,000 IU/kg of b.w. for 30 days, orally). All experiments were conducted in accordance with the international recommendations of the European Convention for the Protection of Vertebrate Animals used for Research and Scientific Purposes (Strasbourg, 1986) and are ethically acceptable. The protocol of animal experiments was approved by the ethics committee on controlling the rules of research work with experimental animals of the Palladin Institute of Biochemistry, Kyiv, Ukraine.

Total, Nuclear, and Cytoplasmic Protein Extract Preparation and Western Blot Analysis

Total protein extracts were prepared from frozen BM samples using standard protocol with RIPA buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 1 mM EGTA; 0.1% SDS, 1% sodium deoxycholate, 10 mM sodium pyrophosphate). Briefly, BM samples (100 mg) were lysed for 20 min in RIPA buffer in the presence of protease inhibitor cocktails (PIC, Sigma, USA), then centrifuged for 20 min (14,000 *g*) at +4°C. Nuclear and cytoplasmic protein fractions were isolated by the method as described (18). Frozen BM samples (100 mg) were ground in a porcelain mortar with liquid nitrogen and lysed by incubation on ice for 20 min in 0.9 ml of 0.5% NP-40-phosphate-buffered saline (PBS) containing PIC. The cell lysates were centrifuged at 1,500 *g* for 5 min, and cytoplasmic proteins were separated from nuclei and further centrifuged at 14,000 *g* for 20 min at +4°C. The nuclei pellets were washed with 0.5% NP-40-PBS containing the PIC and centrifuged at 1,500 *g* for 5 min twice, then mixed with RIPA buffer and PIC by vortexing at +4°C, followed by centrifugation at 14,000 *g* for 20 min. Protein concentration of all supernatants were determined by Lowry's method. Total, cytoplasmic, and nuclear lysates were stored at −80°C. Equal amounts of protein (for total lysates—50 μg, nuclear fraction—40 μg, cytoplasmic fraction—50 μg) were loaded and separated by 10–15% SDS polyacrylamide gels (depending on the molecular weight of target proteins), followed by transfer of proteins onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in PBS plus 0.05% Tween-20 (PBST) followed by incubation overnight with primary antibodies against RANK (1:400; Santa Cruz Biotechnology, USA), RANKL (1:250; Santa Cruz Biotechnology, USA), OPG (1:250; Santa Cruz Biotechnology, USA), CYP27B1 (1:200; Santa Cruz Biotechnology, USA), NF-κB p65 (1:250; Thermo Fisher Scientific Inc., USA), NF-κB p65 phosphorylated at Ser 311 (1:200; Santa Cruz Biotechnology, USA), IκB (1:500; Santa Cruz Biotechnology, USA), GR (1:250; Santa Cruz Biotechnology, USA), β-actin and lamin B1 (1:20,000 and 1:1,000, respectively; Sigma, USA) in PBS supplemented with 0.1% (vol/vol) Tween-20 and 5% (wt/vol) non-fat milk. Primary-antibody-bound membranes were then incubated with peroxidase-conjugated secondary antibodies: anti-mouse IgG (Fab Specific)-Peroxidase (1:2,500; Sigma, USA), anti-rabbit IgG (H + L)-HRP conjugate (1:4,000; Bio-Rad Laboratories, Inc., USA) or anti-goat IgG (H + L) (1:2,500; Invitrogen, USA). Thereafter the membranes were developed with chemiluminescent agents: p-coumaric acid (Sigma, USA) and luminol (AppliChem GmbH, Germany). Tissue levels of target proteins in total and cytoplasmic lysates were normalized to β-actin; the levels of target proteins in nuclear lysates were normalized to lamin B1. The immunoreactive bands were quantified with Gel-Pro Analyzer32, v3.1.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNAs from BM were obtained using the innuPREP RNA Mini Kit (Analytik Jena AG, Germany). mRNA concentrations were determined by DS-11 Spectrophotometer/Fluorometer (DeNovix, USA). Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA) was used to synthesize cDNAs in a standard reverse transcriptase reaction. The cDNA samples were then used as templates for real-time PCR

analysis, which was performed on an qTOWER 2.0 Standard real-time PCR Thermal Cycler (Analytik Jena AG, Germany). Specific primer sequences for the *Rankl*, *Vdr*, and *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase), that was used as a reference gene, were designed by Primer BLAST software: *Rankl*—forward 5′-CCAGCATCAAAATCCCAAGT-3′; reverse 5′-TGAAAGCCCCAAAGTACGTC-3′; product length—201 bp; *Vdr*—forward 5′-TCATCCCTACTGTGTCCCGT-3′; reverse 5′-TGAGTGCTCCTTGGTTCGTG-3′; product length—161 bp; *Gapdh*—forward 5′-TGAACGGGAAGCTCACTGG-3′; reverse 5′-TCCACCACCCTGTTGCTGTA-3′; product length—307 bp. Target genes were amplified for 50 cycles using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Inc., USA). Data were normalized to an internal housekeeping gene *Gapdh* and then calculated as the fold change relative to control using the ΔΔCt method.

Isolation of Splenocytes, Peripheral Blood, and BM Mononuclear Cells

Spleens from rats were removed aseptically, and placed in 3 ml of media (RPMI 1640 with 10% fetal bovine serum) in a small petri dish. Using sterile tweezers, the spleen was placed on a sterile wire mesh screen and then carefully pushed through it with the plunger of a 10 ml syringe into the petri dish without transferring the capsule of the spleen in the media. Screen was rinsed with the 3 ml of media and the spleen mixture was gently pipetted, transferred into the tubes, and centrifuged at 500 *g* for 10 min at +4°C. Final pellets of splenocytes were diluted to 1×10^6 cells/ml and stored on ice until fixation (19). Peripheral blood mononuclear cells were isolated using Histopaque-1083 (Sigma, USA) as described in the standard manufacturer's protocol. Briefly, 5 ml of PBS without calcium and magnesium were added to 3 ml of whole blood, collected in heparin, and mixed. 3 ml of Histopaque-1083 were added to a centrifuge tube, then 8 ml of the blood–saline mixture were carefully layered on top. The mixtures were centrifuged at 400 *g* for 30 min, the upper layer was aspirated to within 0.5 cm of the opaque interface containing the mononuclear cells. The opaque interface was carefully transferred to a clean centrifuge tube and 10 ml of PBS were added, centrifuged at 250 *g* for 10 min. Supernatant was discarded. Lymphocyte pellets were resuspended with 5 ml of PBS and gently mixed, then centrifuged at 250 *g* for 10 min. After repeating the last steps twice the resulting pellets were diluted to 1×10^6 cells/ml and stored on ice until fixation. BM mononuclear cells were obtained as follows: rat femurs were excised, dissected, and BM cells were harvested by repeated flushing of the femoral cavities with 1 ml syringe using PBS 10 times. Total marrow isolates were collected by centrifugation at 400 *g* for 1 min at +4°C and resuspended in PBS. The resultant cell suspension was filtered through successive 70- and 40-μm nylon cell strainers followed by 3 washes at 400 *g* for 5 min at +4°C. Subsequently, red blood cells (RBC) were lysed with RBC Lysis Buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA, pH = 7.4) and the BM mononuclear cells were washed twice, centrifuged, and final pellet was resuspended in PBS (1×10^6 cells/ml) and stored on ice until fixation.

Cell Fixation and Permeabilization

Cell fixation was performed using 4% paraformaldehyde (Sigma, USA) for 10 min, then cells were rinsed with PBS for three times. If permeabilization was required (GR, pNF-κB p65 and VDR), 0.1% Triton X-100 was used for 10 min, followed by repeated rinse steps with PBS for three times. Nonspecific binding was blocked by incubation with PBS/1% bovine serum albumin for 45 min at room temperature.

Flow Cytometry and Confocal Microscopy

RANK-, VDR-, and GR-positive BM cells were quantified using flow cytometry, RANK- and pNF-κB p65-positive cells were visualized by confocal microscopy. To detect the RANK, VDR, pNF-κB p65 and GR-positive cells (surface expression of RANK or cytoplasmic/nuclear localization of VDR pNF-κB p65 and GR), fixed splenocytes or peripheral blood/BM mononuclear cells were incubated with anti-RANK (1:150; Santa Cruz Biotechnology, USA), anti-VDR (1:100; Santa Cruz Biotechnology, USA), anti-pNF-κB p65 (1:250; Santa Cruz Biotechnology, USA) anti-GR (1:100; Santa Cruz Biotechnology, USA) for 60 min, then washed three times and incubated with specific DyLight 488-conjugated goat anti-rabbit IgG antibody or Alexa Fluor 568-conjugated goat anti-mouse IgG (H + L) antibody for 45 min in the dark box. The percentage of positive cells as well as the level of fluorescence was measured by an EPICS XLTM flow cytometer (Beckman Coulter, USA) using the excitation/emission wavelengths of 495/515 nm. The level of fluorescence was calculated as the mean fluorescent intensity of positive cells and data were expressed as folds of control. Background fluorescence was assessed by staining with control isotype-matched antibodies. All data were analyzed using FCS Express software. Immunoflourescence cell staining for confocal microscopy was performed as described above. Additionally, cell nuclei were visualized by Hoechst. Diode 405-30 laser (for Hoechst), Tunable Argon 458/477/488/514 nm at 30 mW laser (for DyLight 488) and He-Ne 543 nm at 1 mW (for Alexa Fluor 568) were used. Fluorescence was detected using the following channels: 420–480 nm, 505–530 nm, and >560 nm, respectively. Images were acquired using Carl Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Germany) at 40× or 100× magnification and processed using Zeiss LSM Image Browser software. Laser power and the detector settings were kept constant to maintain consistency in the data collection system. For visualization studies, 10 slides were examined in random fields in at least three experiments. Based on the series of pictures obtained by scanning (with a step of 0.32 μm) of single RANK- or pNF-κB p65-positive cells (at least five in each group) the 3D models of RANKL/RANK interaction and pNF-κB p65 translocation into the nucleus were build using Zeiss LSM Image Browser software.

ELISA Blood Serum 25-Hydroxyvitamin D Level Assay

Commercial ELISA kit "25-OH-Vitamin D_3" (Immunodiagnostics, Germany) was used in accordance with the manufacturer's instructions to assess the vitamin D_3 status by determining of 25-hydroxyvitamin D (25OHD) concentration in rat serum.

Statistical Analysis

Data distribution was analyzed using the Kolmogorov–Smirnov normality test. Normally distributed data are expressed as the mean ± SEM. Statistical differences between the groups were analyzed by the one-way ANOVA test. Differences were considered to be statistically significant when a p-value was less than 0.05. All statistical analysis was performed using Origin Pro 8.5 (OriginLab Corporation, Northampton, MA, USA).

RESULTS

Assessment of the RANKL/RANK/OPG Axis and Quantifiation of Preosteoclasts in the BM

The results of the study presented in **Figures 1A,B** show that a long-term prednisolone administration caused an increase in the *Rankl* mRNA and RANKL protein content in the BM by 4.14- and 1.27-fold ($p = 0.0003$ and $p = 0.0007$), respectively, compared with the control. At the same time, western blot analysis data revealed a 1.38-fold ($p = 0.0042$) lower level of OPG in the BM of prednisolone-administered rats than in control animals (**Figures 1A,E**). Physiological effects of OPG and RANKL on the skeletal system cells are known to be largely determined by the ratio of their synthesis (20). It was established a significant (2.0-fold) decrease in the OPG to RANKL proteins ratio induced by the prolonged action of prednisolone compared with the control. A decreased ratio of OPG/RANKL may contribute to the differentiation and activation of osteoclasts responsible for the enhancement of bone resorption. Vitamin D_3 treatment caused a 1.6- and 2.4-fold decrease in the *Rankl* mRNA and RANKL protein levels ($p = 0.0003$ and $p = 0.0007$), respectively, as compared with prednisolone-administered rats. Although it has been found that cholecalciferol had a slight lowering effect (by 1.22-fold, $p = 0.0042$) on the level of OPG in the BM compared with the action of prednisolone, its ultimate impact on OPG/RANKL was, nevertheless, changed toward normalization.

Given that RANK is constitutively expressed in mature osteoclasts of the bone tissue, and its level in the BM also reflects the amount of immature precursors of osteoclasts, we examined the RANK protein expression, as well as the number of RANK-positive cells in the BM. It was shown that prednisolone upregulated (by 1.53-fold, $p = 0.01$) the relative RANK protein content in the BM, as compared with the control (**Figure 1C**). Consistent with the data obtained by flow cytometry, this effect was due to an increase in the number of osteoclasts precursors expressing RANK on their surface. **Figure 1D** illustrates that prednisolone induced a 1.7-fold ($p = 0.0007$) increase in the quantity of RANK-positive cells in the BM compared with the control. In addition, RANK-positive precursors of osteoclasts were visualized by confocal microscopy using indirect immunofluorescence labeling and the changes similar to those established by flow cytometry were confirmed (**Figure 1F**).

Since the effect of prolonged administration of GCs on the circulating pool of OCPs is not yet clarified, it was essential, apart from the BM, to estimate the number of RANK-positive cells among the monocytes/macrophages of the peripheral blood and spleen. Quantitative cytometric analysis of RANK-positive cells in the monocytes fraction isolated from rat peripheral blood and spleen showed an increase in their number by 4.2- and 1.26-fold ($p = 0.049$ and $p = 0.012$), respectively, as compared with the control (**Table 1**). Consequently, prednisolone induced more pronounced changes in the quantity of RANK-positive cells in the peripheral blood than in the spleen and BM.

When vitamin D_3 was co-administered with prednisolone, the relative content of RANK protein in the BM cell lysates was completely normalized, presumably due to the established decrease in the number of RANK-positive cells. Notably, the quantity of preosteoclasts in the BM reached the level that was found in control rats. The pool of circulating RANK-positive preosteoclasts after the administration of vitamin D_3 almost returned to the control level in the peripheral blood, whereas in the spleen it reached values below the control.

Based on available scientific evidence that stromal cells are considered as one of the sources of RANKL, and RANK-positive cells are targets for regulatory control of this cytokine, we have used double (RANK plus RANKL) immunofluorescence labeling of the BM cells. Images obtained by confocal microscopy clearly demonstrate the co-localization of RANKL with the RANK-positive cells (**Figure 1F**). In addition, by scanning single cells with a step of 0.32 μm, a 3D model was constructed using the resulting series of images. One can notice the presence of RANKL bound to its receptor in RANK-positive cells isolated from animals that were given prednisolone (**Figures 1G,H**). It is also noteworthy that cells other than RANK-positive showed negligible immune-specific signal from RANKL.

Thus, prednisolone increased the number of circulating OCPs that was accompanied by the activation of the RANKL/RANK/OPG axis. Vitamin D_3 significantly reduced the preosteoclastic pools and partially normalized the RANKL/RANK/OPG levels.

NF-κB-Mediated Signaling in the BM

To address whether GC-induced excessive RANKL levels in the BM are implicated in upstream transcriptional activation through RANK signaling pathway, we explored the involvement of NF-κB in the mechanism of GC action and assessed potential therapeutic efficacy of vitamin D_3.

It is known that after the phosphorylation of main isoform of inhibitory nuclear factor κB (IκB-α) by the IκB kinase (IKK) complex, IκB-α is degraded and releases NF-κB, which translocates to the nucleus (21). Therefore, we first analyzed the effect of prednisolone on the IκB-α level in BM lysates and demonstrated its reduction by almost 1.2-fold ($p = 0.0009$) in GC-administered rats compared with control animals (**Figures 2A,B**). IκB-α level paradoxically further significantly decreased following the treatment with vitamin D_3. The levels of total NF-κB p65 and pNF-κB p65 estimated in whole lysates of the BM cells were also found to be elevated by 1.58- and 1.48-fold ($p = 0.047$ and $p = 0.0007$), respectively, after prednisolone administration (**Figures 2C,D**). To determine the activation and nuclear translocation of NF-κB, the protein content of Ser311 phosphorylated large subunit of NF-κB (pNF-κB p65) was next quantified by western blot analysis in cytosol and nuclear fractions of the BM cells. Prednisolone elevated the levels pNF-κB p65 in cytoplasm and nuclei

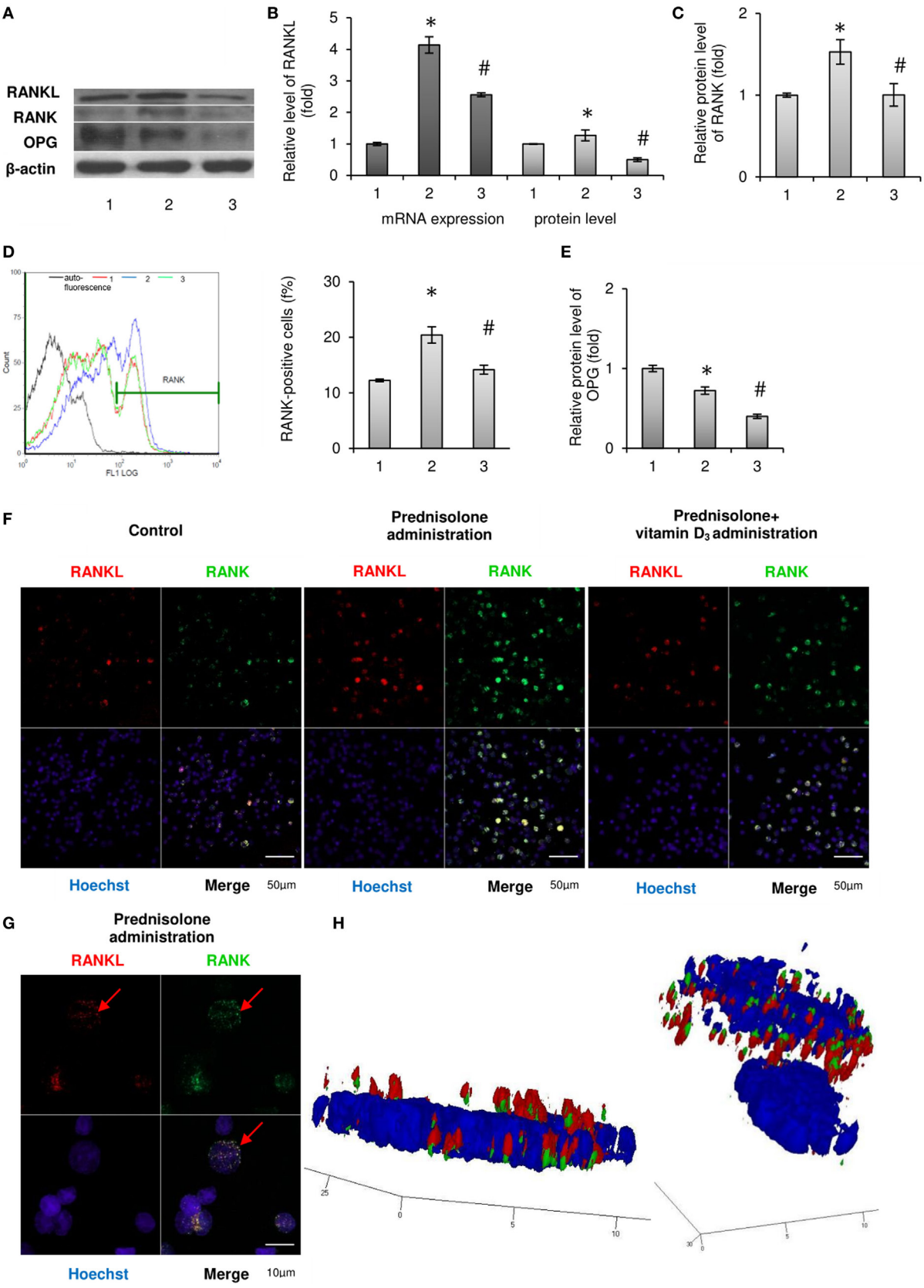

FIGURE 1 | Continued

FIGURE 1 | Effects of prednisolone and vitamin D_3 administration on the RANKL/RANK/osteoprotegerin (OPG) signaling pathway: 1—control; 2—prednisolone administration (5 mg/kg of b.w.); and 3—prednisolone and vitamin D_3 (1,000 IU/kg of b.w.) administration. Immunoblotting analysis of RANKL, RANK, and OPG in rat bone marrow (BM): representative immunoblots are shown **(A)** and quantified using β-actin as a loading control for total BM lysates. The bar graphs of RANKL **(B)**, RANK **(C)**, and OPG **(E)** are presented as means ± SEM (n = 6/group). Quantitative polymerase chain reaction of *Rankl* in rat BM **(B):** data were normalized to *Gapdh* and pooled from two independent experiments (n = 6 rats/group). RANK-positive BM cells **(D):** representative histograms (count—the number of events; FL1 LOG—fluorescence intensity) and quantification of RANK positive cells documented by flow cytometry analysis. All data are shown as means ± SEM; $^*p < 0.05$ vs. control, $^\#p < 0.05$ vs. prednisolone administration. Immunocytochemical analysis of RANKL-positive (red fluorescence) and RANK-positive (green fluorescence) BM cells **(F)**. Hoechst (blue fluorescence) was used for nuclear staining. Scale bars indicate 50 μm (magnification 40×). Red arrows indicate co-localization of RANK and RANKL in BM osteoclast precursors in prednisolone group, suggesting RANKL–RANK direct interaction. Acquiring 3D model of RANKL–RANK interaction on the surface of BM cell in prednisolone-administered rats: based on the series of pictures obtained by scanning (with a step of 0.32 μm) of single RANK-positive cells (at least 5, scale bars indicate 10 μm, magnification 100×) **(G)** the 3D model of RANKL–RANK interaction **(H)** were build using Zeiss LSM Image Browser software.

TABLE 1 | RANK-positive mononuclear cells from spleen, blood and bone marrow (BM).

Groups	Control	Prednisolone administration	Prednisolone ± vitamin D_3 administration
RANK-positive cells isolated from spleen (%)	3.60 ± 0.12	4.53 ± 0.50*	2.36 ± 0.19#
RANK-positive mononuclear cells isolated from whole blood (%)	1.14 ± 0.05	4.08 ± 0.92*	1.87 ± 0.09#
RANK-positive mononuclear cells isolated from BM (%)	12.23 ± 0.24	20.42 ± 1.46*	14.17 ± 0.81#

*Results represent the percentages of RANK-positive cells determined by flow cytometry. Values represent the means ± SEM for three experiments (M ± m, n = 7) done in triplicate. *p < 0.05 vs. control; #p < 0.05 vs. prednisolone administration.*

(**Figures 2E,F**) of the BM cells by 1.30- and 1.77-fold ($p = 0.00001$ and $p = 0.00015$), respectively, indicating a significant GC-elicited shift of pNF-κB p65 localization from the cytosol to the nuclear fraction of the cells. Nuclear translocation of pNF-κB is consistent with the probable transcriptional activation.

Vitamin D_3 treatment diminished the levels of both phosphorylated and non-phosphorylated large subunits of NF-κB in whole lysates of the BM cells and partially blocked pNF-κB p65 translocation to the nucleus. The level of phosphorylated NF-κB p65 in the nuclear fraction was decreased to a greater extent than in cytosolic fraction, indicating a successful prevention of pNF-κB translocation and probable inhibition of its transcription activity.

In order to further confirm the translocation of pNF-κB p65 to the nucleus associated with prednisolone action, not using exclusively western blot analysis, the pNF-κB-positive BM cells were visualized by confocal microscopy after their indirect immunofluorescence labeling (**Figure 2G**). Corresponding to the results obtained in immunoblotting studies of pNF-κB p65, GC therapy significantly increased total immunofluorescence of pNF-κB p65 in the BM cells. Based on the scans of single pNF-κB-positive cells and the resulting series of images, a 3D model of pNF-κB translocation to the nucleus was constructed (**Figure 2H**). Our data confirmed the stimulatory effect of prednisolone on the translocation of pNF-κB to the nucleus, while the transcription factor remained more diffused in the cytoplasm from rats of the control and vitamin D_3-administered groups.

In summary, we found GC-induced increase in the levels of both NF-κB phosphorylated and non-phosphorylated p65 subunits in BM cells and demonstrated that vitamin D_3 administration attenuated transcriptional activation of the NF-κB p65.

The BM Levels of GC Receptors

Considering that cell-specific effects of GCs are usually mediated by the GC receptor (22), it was advisable to determine the relative content of GRs in the BM. GC receptor protein levels evaluated in isolated BM cells showed a slight difference between GC-administered and control animals. Western blot analysis revealed a 1.26-fold ($p = 0.0033$) lowering effect of prednisolone on protein synthesis of GC receptors as compared with the control (**Figure 3A**). Consistent with the GC-induced downregulation of GR protein expression, there was a threefold ($p = 0.00012$) decrease in the number of GR-positive cells in the BM, which were quantified by immunofluorescence staining and flow cytometry and further visualized using confocal microscopy (**Figures 3B,C**). Low expression of GC receptors may indicate the involvement of the negative feedback mechanism of their regulation, or even desensitization, induced by excessive prednisolone load. Vitamin D_3 supplementation had profound effect on the abundance of GRs and GR-positive cells. It increased GR protein and the amount of GR-positive cells to the levels 2.45- and 10.50 times ($p = 0.0033$ and $p = 0.00012$) as much as those in control rats. These results indicate that GR signaling in progenitor cells, playing a crucial role in coupling bone formation and resorption, could be effectively targeted by vitamin D_3 co-administered with prednisolone.

Characteristics of the Vitamin D Auto/Paracrine System in the BM

Since most of the BM disturbances associated with the chronic effect of prednisolone have been significantly attenuated by the administration of vitamin D_3, we carried out an assessment of 25-hydroxyvitamin D (25OHD) level in the blood serum as a reliable marker of vitamin D bioavailability. A significant reduction of 25OHD was found in the blood serum of GC-administered animals (by 2.46-fold, to 38.78 ± 1.03 nmol/l) compared with the control (95,42 ± 1,36 nmol/l, $p = 0.00015$), indicative of a severe GC-induced vitamin D deficiency (**Figure 4A**). Vitamin D_3 supplementation to GC-administered animals resulted in almost full normalization of 25OHD level.

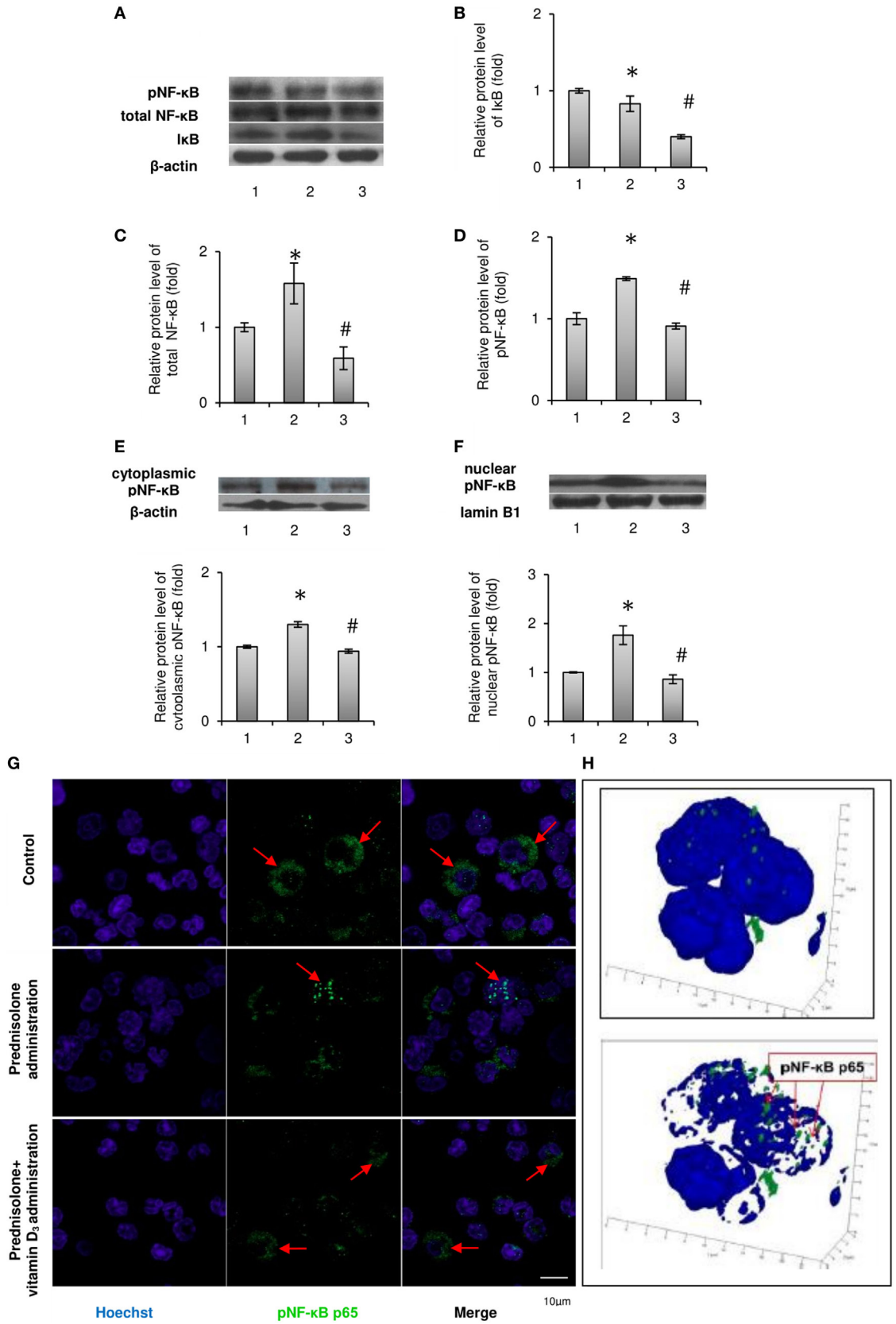

FIGURE 2 | Continued

FIGURE 2 | Levels of total, phosphorylated NF-κB p65 (Ser311) and IκB-α in rat bone marrow (BM): 1—control; 2—prednisolone administration (5 mg/kg of b.w.); and 3—prednisolone and vitamin D_3 (1,000 IU/kg of b.w.) administration. Immunoblotting analysis of phosphorylated at Ser311, total NF-κB p65, and IκB-α in whole BM lysates: representative immunoblots are shown next to the bar charts **(A)** and quantified using β-actin as an internal control. The bar graphs of IκB-α **(B)**, total NF-κB p65 **(C)**, and phosphorylated at Ser311 **(D)** are presented as means ± SEM (n = 6/group). Immunoblotting analysis of NF-κB p65 subunit phosphorylated at Ser311 in cytoplasmic **(E)** and nuclear **(F)** lysates: representative immunoblots are shown above the bar charts and quantified using β-actin and lamin B1 as the loading controls for the cytoplasmic and nuclear fractions, respectively. The bar graphs of phosphoNF-κB p65 in cytoplasmic and nuclear lysates are presented as means ± SEM (n = 6/group),*$p < 0.05$ vs. control, #$p < 0.05$ vs. prednisolone administration. Immunocytochemical analysis of phosphoNF-κB p65-positive (green fluorescence) BM cells **(G)**. Hoechst 33342 (blue fluorescence) was used for nuclear staining. Scale bars indicate 10 μm (magnification 100×). Red arrows show diffuse cytoplasmic distribution of phosphoNF-κB in the control and prednisolone + vitamin D_3 administration groups and the nuclear localization of phosphoNF-κB in the prednisolone group. Acquiring 3D model of phosphoNF-κB nuclear translocation in BM cells in prednisolone-administered rats: based on the series of pictures obtained by scanning (with a step of 0.32 μm) of single phosphoNF-κB-positive cells (at least 5, magnification 100×) the 3D model of phosphoNF-κB nuclear translocation **(H)** were build using Zeiss LSM Image Browser software.

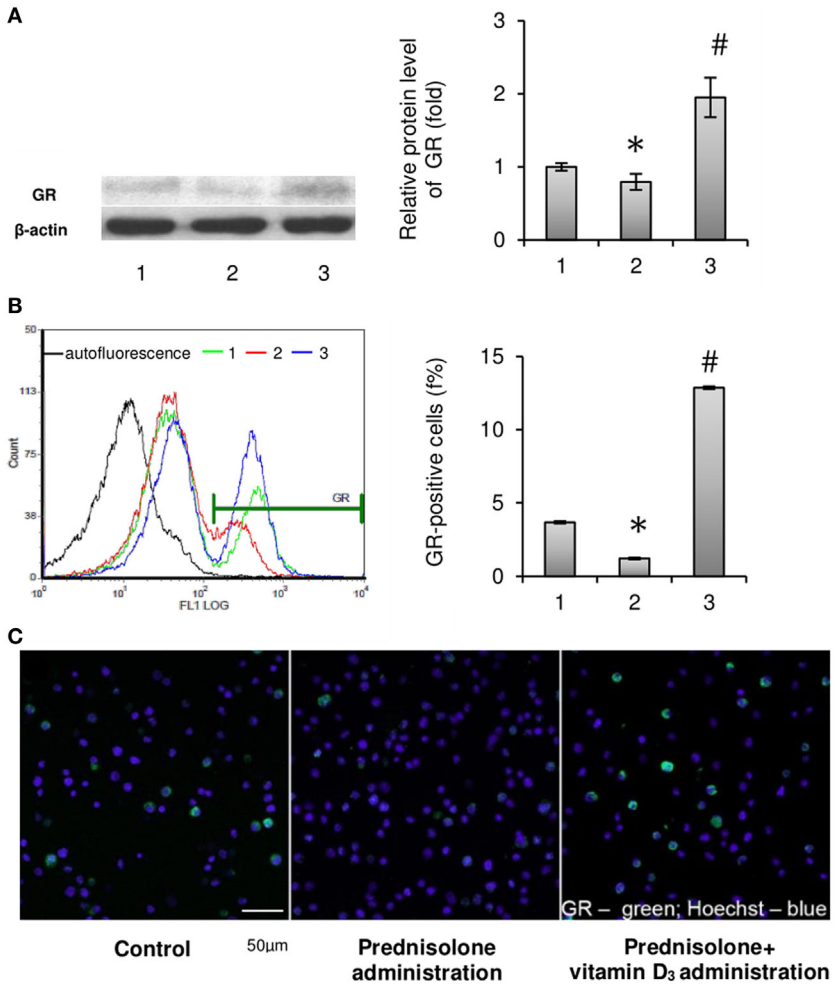

FIGURE 3 | Effects of prednisolone and vitamin D_3 administration on the level of glucocorticoid receptors in rat bone marrow (BM): 1—control; 2—prednisolone administration (5 mg/kg of b.w.); and 3—prednisolone and vitamin D_3 (1,000 IU/kg of b.w.) administration. Proteins from total BM lysates were separated by SDS-PAGE and western blot analysis was performed using the antibodies against GR. Representative immunoblot and quantification of three experiments **(A)** are shown. Protein levels were normalized to β-actin. The bar graphs of GR are presented as means ± SEM (n = 6/group). GR-positive BM cells: representative histograms (count—the number of events; FL1 LOG—fluorescence intensity) and quantification of GR-positive cells documented by flow cytometry analysis **(B)**. All data are shown as means ± SEM; *$p < 0.05$ vs. control, #$p < 0.05$ vs. prednisolone administration. Confocal microscopy of GR-positive (green fluorescence) BM cells **(C)** shows a significant decrease in the number of GR-positive cells after prednisolone administration. Hoechst 33342 (blue fluorescence) was used for nuclear staining. Scale bars indicate 50 μm (magnification 40×).

Consistent with the evidence that 1α-hydroxylase (CYP27B1), which catalyzes $1,25(OH)_2D$ synthesis in the kidney, is also expressed in other tissues, we assumed that 25OHD may be converted to $1,25(OH)_2D$ locally in the BM, where it could exert autocrine/paracrine regulatory effects. As shown in **Figure 4B** prednisolone caused significant (2.14-fold, $p = 0.0037$) decrease

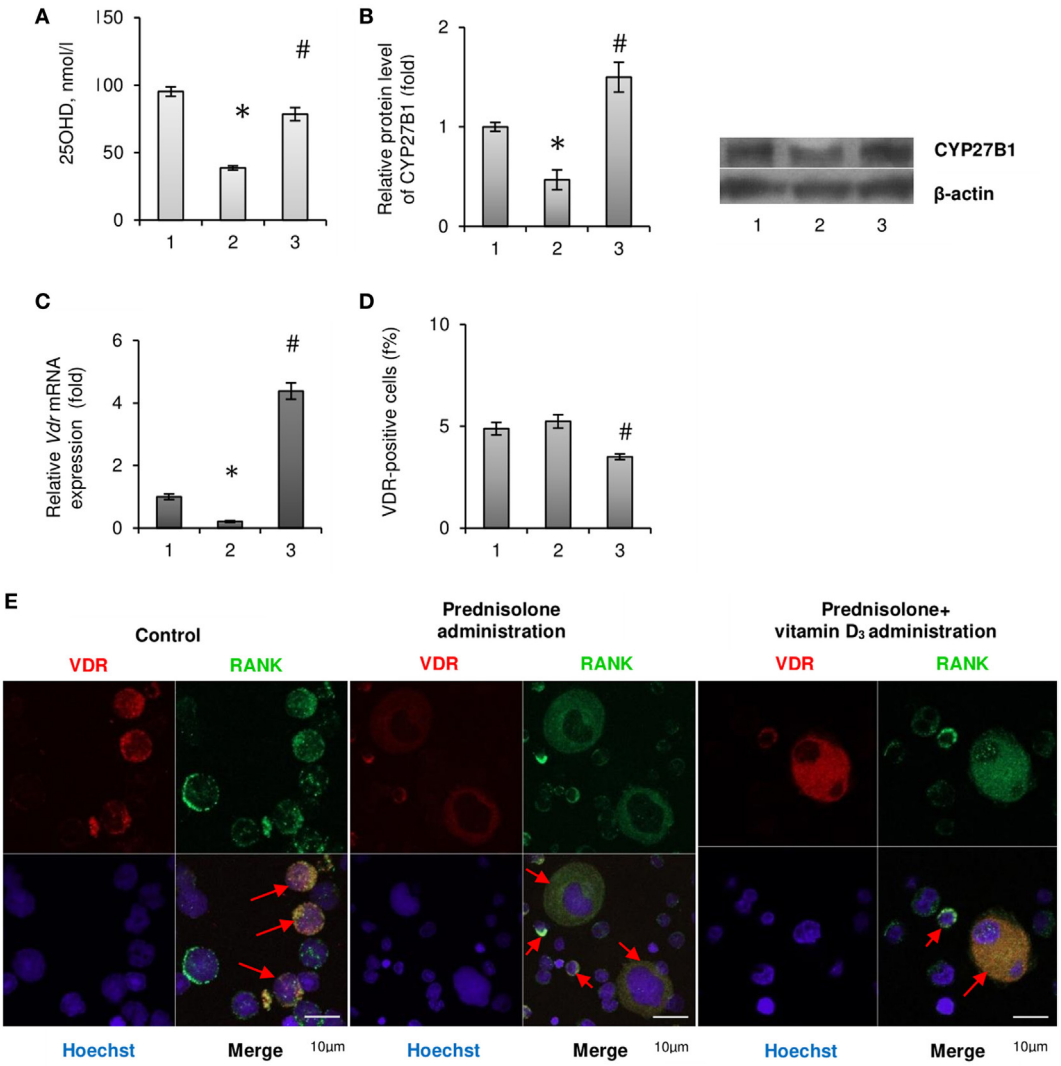

FIGURE 4 | Vitamin D auto/paracrine system in bone marrow (BM) and vitamin D bioavailability after prednisolone and vitamin D_3 administration: 1— control; 2—prednisolone administration (5 mg/kg of b.w.); and 3—prednisolone and vitamin D_3 (1,000 IU/kg of b.w.) administration. 25OHD concentration was measured by ELISA **(A)**. Immunoblotting analysis of CYP27B1 in rat BM: the bar graphs of CYP27B1 **(B)** are presented as means ± SEM (n = 6/group) and representative immunoblots are shown next to the bar chart and quantified using β-actin as a loading control. Quantitative polymerase chain reaction of *Vdr* in rat BM **(C)**; data were normalized to *Gapdh* and pooled from two independent experiments (n = 6 rats/group). Quantification of VDR-positive BM cells using flow cytometry analysis **(D)**. Data are shown as means ± SEM; $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. prednisolone administration. Immunocytochemical analysis of double RANK-positive (green fluorescence) and VDR-positive (red fluorescence) BM cells **(E)**. Hoechst 33342 (blue fluorescence) was used for nuclear staining. Scale bars indicate 10 μm (magnification 100×). Red arrows show co-localisation of RANK and VDR in BM mononuclear osteoclast precursors (OCPs) in control group as well as in multinuclear OCPs in prednisolone and prednisolone + vitamin D_3 group.

in the protein expression of CYP27B1 in the BM compared with control animals. Vitamin D_3 treatment restored CYP27B1 to the level that was even 1.5-fold higher than in the control ($p = 0.0037$).

As the biological effects of hormonally active form of vitamin D_3 in different cell types are mediated through specific receptors to $1,25(OH)_2D$—VDR, we also examined the expression of *Vdr* gene in the BM cells. Following prednisolone administration, the level of *Vdr* mRNA in BM was shown to be diminished by 5.0-fold ($p = 0.0027$), indicating a possible decrease in cell responsiveness to vitamin D_3 action (**Figure 4C**). It was found a strong increasing effect of vitamin D_3 on *Vdr* mRNA expression that reached the value 4.38-fold higher than in the control.

In addition, we used immunofluorescence staining of the BM cells for flow cytometric analysis and confocal microscopy to detect the quantity of cells that express vitamin D receptor (VDR-positive cells). GC administration showed the number of VDR-positive cells similar to that observed in the control, whereas their amount decreased below the control level after vitamin D_3 treatment (**Figure 4D**). Finally, confocal microscopy was used to study the precursors of osteoclasts with the double immunofluorescence staining for RANK and VDR (**Figure 4E**).

What is the most interesting is that among the BM cells isolated from rats of prednisolone group, in addition to mononuclear cells, a large number of multinucleated and fused RANK-positive cells (preosteoclasts) were identified. Such cells were not seen in the control animals and were rarely found in the group of rats which received both prednisolone and vitamin D_3 (**Figure 4E**). These data demonstrate vitamin D-mediated modulation of GC's effects on rat bone progenitor cells and identify a role for VDR and RANK as mediators of this process.

Collectively, we established that prednisolone induced the vitamin D deficiency and lowered the levels of key components of the vitamin D auto/paracrine system in the BM, while cholecalciferol supplementation normalized, at least partially, these parameters.

DISCUSSION

A large body of evidence has demonstrated increased risk of secondary osteoporosis associated with a chronic GC treatment. The findings from clinical and experimental studies suggest that GC-induced loss of skeletal mass arises from changes in the numbers of bone cells, altering a balance between osteoblast-dependent bone formation and osteoclast-mediated bone resorption (23). Disturbances in the bone tissue caused by GCs may also be closely related to deleterious changes in the vitamin D endocrine system. Here, we found that molecular mechanisms of GC-induced bone loss involve aberrant interaction of GR and VDR signaling pathway and associated impairments of RANK/NF-κB axis in the BM that most likely results in abnormal osteoclastogenesis/osteosynthesis coupling. In addition to the well-recognized role of vitamin D and its receptor in mineral metabolism and bone formation, we confirmed the positive effects of cholecalciferol on GC-induced dysfunctions of the BM osteoprogenitors. In this investigation, we followed the prevention paradigm to counter pathogenic alterations elicited by chronic prednisolone therapy using concurrent vitamin D_3 administration.

Glucocorticoids exert their numerous effects in cells largely through GR, which is a ligand-regulated transcription factor and the member of nuclear hormone receptor superfamily (22). Most of our findings can, at least in part, be explained by the alterations of GRs expression and impairment of the GR signaling. Prednisolone evoked a decrease in protein synthesis of GRs in the BM and a significant drop in the quantity of cells capable of expressing detectable amounts of GRs. It has been recently proposed that transrepression mediates most of the beneficial effects of GCs, whereas transactivation promotes most of their adverse effects (24). We can hypothesize that negative impact of chronic prednisolone therapy on cellular function may be mediated through insufficient interaction of GC receptors with prooxidant and/or inflammatory transcription factors to repress transcriptional activity, i.e., transrepression. Additionally, the underlying mechanism for prednisolone-associated abnormalities may also be attributable to the inhibitory action of the hormone on the expression of factors that limit the manifestation of side effects.

Osteogenesis is known to be a highly regulated process, in which subpopulations of BM cells differentiate into mature bone cells of skeletal tissues (25). Precursors of main bone-forming cells (osteocytes and osteoblasts) are mesenchymal stem cells residing in the BM. Osteoclasts originate from precursors of the myeloid/monocyte lineage and circulate in the monocyte fraction of the peripheral blood until they become resident cells of the bone tissue (26). GCs modify osteoclastic cell differentiation, number, and function either directly or through influencing the recruitment of their precursors from the BM, but the precise mechanisms are contradictory and not fully defined (27). RANK is known to be a key component of the osteokine system RANKL/RANK/OPG, localized on the cell surface of preosteoclasts, which facilitates their differentiation into osteoclasts and activation of mature osteoclasts responsible for bone resorption (20). Therefore, the RANK protein content strongly correlates with the number of osteoclastic cells and their activity in the bone tissue, and is also considered a reliable marker of preosteoclasts. With an augmented level of RANK protein currently established in the BM, we detected a significant increase in the number of RANK-positive cells (preosteoclasts) among the isolated BM cells. Moreover, prednisolone-induced migration of OCPs from the BM and the appearance of their significant amounts in the peripheral blood and spleen were also shown in our study. These results are in accordance with the suggestion that spleen can act as the reservoir of hematopoietic OCPs under pathological conditions (28). Although the mechanism underlying the massive recruitment of preosteoclasts into the peripheral blood and the redistribution of their pool between the BM, bloodstream, and spleen remains unclear, the findings indicate abnormal RANK-mediated regulation of the maturation of osteoclast BM progenitors following GC administration. In all probability, elevated number of RANK-positive cells in the BM indicates prednisolone ability to stimulate proliferation of osteoprogenitor cells, which is very likely associated with GR desensitization and decreased cellular signaling *via* GR.

We established the corrective effect of vitamin D_3 on the pool of circulating RANK-positive precursors of osteoclasts, which is generally consistent with the antiproliferative activity inherent to this molecule (29). The effects of cholecalciferol may be due to its ability to relieve the arrest of the cell cycle of RANK-positive OCPs, the so-called "cell cycle-arrested quiescent osteoclast precursors" that can switch the differentiation of these cells from osteoclasts to dendritic cells (30).

The receptor activator of the NF-κB ligand is the central player in the regulation of osteoclastogenesis, and the quantity of RANKL presented to OCPs is essential for determining the intensity of osteoclast formation. The proper balance between the bone formation and resorption is reliably maintained by an adequate OPG to RANKL ratio. In the bone tissue OPG is synthesized mainly by osteoblasts and osteocytes and acts as an endogenous soluble decoy receptor for the RANKL (31). OPG, by binding RANKL, prevents RANK activation on the cell surface of the preosteoclasts and reduces both osteoclastogenesis and resorptive activity of mature osteoclasts. The harmonized effect of these cytokines was substantially disturbed by the administration of prednisolone, which exhibited a stimulatory effect on the formation of RANKL with a significant reduction in the content of OPG. Consequently, the detected reduction in the ratio of

OPG/RANKL most likely contributes to the elevation of RANKL-mediated osteoclastogenesis.

Within the context of GC-induced deregulation of the RANKL/RANK/OPG axis, the possible relationship between BM progenitor cells and bone metabolism can be discussed. A number of research efforts unambiguously indicate that osteoblasts play a crucial role in the occurrence of secondary osteoporosis associated with chronic GCs treatment primarily due to hypofunction and increased apoptosis of osteoblasts and their progenitors (32). As for osteoclastogenesis, available scientific data indicate that both the decrease and increase in this process can account for the bone loss, provided that there is a concomitant decline in the formation and functional ability of osteoblasts (33, 34). Several recent studies which largely correspond to experimental design of the present investigation has demonstrated that chronic exposure of high GC doses had inhibiting effects both on bone formation and bone resorption (35, 36). The discrepancy between prednisolone-induced osteoclastogenic profiles of cytokines, the increase in the number of circulating RANK-positive cells, and the low bone turnover can be explained by suppressed recruitment of OCPs from the BM to the bones. In particular, GCs were reported to inhibit osteoclastogenesis through the downregulation of beta3 integrin, which plays an important role in the formation of multinucleated osteoclasts (37).

Vitamin D_3 treatment was found to partially normalize altered RANKL level as well as the OPG/RANKL ratio that is consistent with the genomic or non-genomic VDR-modulated effects of the prohormone on cytokines production and cell-to-cell communication. The corrective effect of the compound points to vitamin D_3 as a potential modulator of the pool of circulating OCPs both in the BM, from which they originate, and in the peripheral blood and spleen.

Because preosteoclastic cells are repoted to be a major target for RANKL, we also studied the regulatory mechanisms of RANKL subcellular signaling in isolated BM cells. The process of osteoclast formation requires the involvement of signaling *via* NF-κB activated in response to a key osteoclastogenic cytokine, RANKL, which controls the activation and maturation of osteoclasts through binding to RANK. RANKL and some proinflammatory cytokines, including TNFα, activate the NF-κB-associated signaling pathways, thereby positively regulating osteoclast formation and function (38).

Normally, the association of GR with the proinflammatory transcription factor NF-κB antagonizes its activity and is reported to be a primary mechanism by which GCs suppress inflammation (39). Several studies suggest that the GR and the p65/p50/inhibitory κB-α complex directly interact and GC/GR binding leads to the inhibiting of p65 transactivation function (40). However, the beneficial or deleterious role of GC/GR signaling in bone homeostasis seems to depend on the dose as well as treatment duration. Our findings convincingly indicate that chronic prednisolone administration induced transcriptional activation of the NF-κB signaling cascade in BM cells by the "classical" pathway, as is evidenced from increased total NF-κB p65 level and enhanced nuclear translocation of phosphorylated at serine 311(active) NF-κB p65 form. Also as expected, prednisolone destroyed NF-κB p65 sequestration complex with IκB-α, that promoted downstream NF-κB signaling. We can suggest that reduced protein level and possible resistance of GRs due to prolonged GC treatment may cause impaired interaction between the GR and p65/p50/IκB complex leading to the elimination of the inhibitory control of GR necessary to block NF-κB p65 transactivation.

The mechanism of osteoclasogenesis in prednisolone-induced osteoporosis can additionally be discussed considering NF-κB of BM cells as a target for regulation by the protein kinase Cξ (PKCξ) signaling. It has been recently reported that TNF-α-dependent NF-κB transcriptional activation may result from the PKCξ-mediated Ser311 phosphorylation of NF-κB p65 (41). As both RANKL and TNF-α belong to the same superfamily of cytokines, it is not excluded that RANKL may have similar effect on NF-κB Ser311 phosphorylation. It is possible that increased Ser311 phosphorylation of NF-κB p65 following prednisolone administration in our study, may occur in response to TNF-α stimulation and excessive reactive oxygen species formation.

We have shown that vitamin D_3 can effectively inhibit prednisone-induced overactivation of the NF-κB signaling cascade in rat BM cells by modulating the NF-κB-associated signaling pathways and thereby promoting the normalization of osteoclastogenesis and restoring the balance between bone formation and resorption. Previously, Cohen-Lahav et al. (42) revealed that vitamin D_3 can reduce NF-κB nuclear translocation by upregulating IκB-α level *via* increasing mRNA stability and decreasing IκB-α phosphorylation. In contrast to this report, our findings indicate that normalization of NF-κB signaling pathway was accompanied by a significant decrease in the protein level of IκB-α after vitamin D_3 supplementation. Consistent with a marked vitamin D_3-induced elevation of the GR level in the BM cells, we can speculate that cholecalciferol inhibits NF-κB activity by direct protein–protein GR/NF-κB interaction, preventing translocation p65 subunit to the nucleus (43). It restors the function of GR, which is recognized as a potent repressor of this transcriptional factor.

Vitamin D_3 is one of the main regulators of bone tissue formation in the process of its development and remodeling throughout the life cycle. Through direct action on bone cells, the hormonally active form of vitamin D_3 controls the proliferation of mesenchymal stem cells, their differentiation to osteoblasts and can directly or indirectly regulate the differentiation and activity of osteoclasts (17). Considering the presence of severe vitamin D_3 deficiency following prolonged GC therapy and the effectiveness of cholecalciferol administration, the established changes in the BM may, at least in part, may be due to impairment of cholecalciferol biological effects in regulation of osteoclastogenesis and indicate direct or indirect VDR-mediated involvement of vitamin D hormone in these processes. According to our data, the negative effect of prednisolone includes abnormal vitamin D metabolism and impaired functioning of the vitamin D auto/paracrine system in the BM that can lead to deregulation of the VDR-mediated interaction of various cell types with the involvement of the RANKL/RANK/OPG cytokine system.

Importantly, synergistic anti-inflammatory and antiproliferative action of combined GC and cholecalciferol treatment has recently gained increased attention (44, 45). GCs have been previously shown to modulate effects of $1,25(OH)_2D$ through regulation of VDR expression. Furthermore, numerous putative

GC response elements were found in the *Vdr* gene (46). In whole, these facts contradict to adverse effects of prednisolone that have been successfully prevented by cholecalciferol in the present study. While GCs and 1,25(OH)$_2$D may have similar effects on the cells, the specific mechanisms and pathways are probably involved, allowing vitamin D$_3$ to counteract a harmful influence of prednisolone on rat BM. Vitamin D$_3$ could act through separate mechanisms from those utilized by GCs, most likely through differences in transcriptional targets and through different effects on shared targets, providing benefits in treating prednisolone-induced BM disorders. Determining whether these shared and non-shared pathways, suggestive of potential mechanisms, mediate a combined effect of prednisolone and vitamin D$_3$ will require further investigation.

CONCLUSION

Prednisolone administration caused impairments of the VDR and GR signaling that contributed to the RANK/NF-κB axis activation in bone marrow cells and increased pool of circulating preosteoclasts. Sufficient vitamin D bioavailability and proper VDR expression are important for restoration of crosstalk between osteoclastogenic signaling pathways. Although clinical use of the study is restricted, novel development that have risen from our research in vitamin D biology and discoveries in the bone remodeling process can be expected to result in further treatment options based on vitamin D for various bone disorders.

DATA AVAILABILITY STATEMENT

Datasets are available on request: the raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All experiments for the research work "Vitamin D$_3$ modulates impaired crosstalk between RANK and glucocorticoid receptor signaling in bone marrow cells after chronic prednisolone administration" were conducted in accordance with international recommendations of the European Convention for the Protection of Vertebrate Animals used for Research and Scientific Purposes (Strasbourg, 1986), General Ethical Principles of Animal Experimentation, approved by the First National Congress on Bioethics (Kyiv, 2001) and are ethically acceptable.

AUTHOR CONTRIBUTIONS

OL and IS contributed to the study design. OL, IS, AM, and DL collected and analyzed data. OL, IS, and MV interpreted the results and wrote and revised the final draft of the manuscript.

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Three Therapeutic Strategies: Cinacalcet, Paricalcitol or Both in Secondary Hyperparathyroidism Treatment in Hemodialysed Patients During 1-Year Observational Study—A Comparison

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Introduction: Secondary hyperparathyroidism (sHPT) is a common hormonal complication of chronic kidney disease. There are several therapeutic options for sHPT management aiming at calcium-phosphorus balance normalization and decrease of parathormone secretion.

Objectives: The aim of this retrospective, observational study was the outcome assessement of three most common therapeutic strategies of secondary hyperparathyroidism treatment with vitamin D receptor activator-paricalcitol, calcimimetic-cinacalcet or both agents administered together during in 12-months period.

Methods: One hundred and thirty-one haemodialysed patients with uncontrolled parathyroid hormone secretion have been treated with paricalcitol administered intravenously (group PAR—60 patients) or cinacalcet per os (group CIN—50 patients). The last group (group PAR+CIN—21 patients) received paricalcitol i.v. and oral cinacalcet administered simultaneously.

Results: In all groups, the iPTH level decreased significantly, however in group 1 treated with paricalcitol administered intravenously iPTH level decrease was greater than in group 2 treated with cinacalcet and in group 3 treated with paricalcitol and cinacalcet in parallel. The most substantial change of iPTH level was noticed after 3-months of observation. After this period the iPTH level was stabilized and maintained till the end of observation. Safety level of all strategies was comparable. No severe hypercalcemia or hypocalcemia was observed during the whole period of observation.

Conclusions: The results of observation show significant advantage of intravenous paricalcitol treatment. Complementing cinacalcet therapy with paricalcitol does not improve treatment outcomes. In case of unsatisfactory results after 3-months treatment,

potential continuation should be considered carefully. Among three available therapeutic options, the treatment with paricalcitol i.v. should be considered in all haemodialysed patients with inadequate control of serum PTH level. The second option—with cinacalced administered orally should be considered in PD patients and when severe hypercalcemia occurs.

Keywords: cinacalcet, hemodialysis, outcome, paricalcitol, secondary hyperparathyroidism, vitamin D

INTRODUCTION

The number of patients with chronic kidney disease (CKD) requiring renal replacement therapy is growing every year. It makes secondary hyperparathyroidism (sHPT) a rising medical issue. SHPT is one of the most frequent hormonal complications connected with CKD, especially stage 4 and stage 5. Uncontrolled hyperparathyroidism contributes to the development and progression of mineral and bone disorder in chronic kidney disease (CKD-MBD)–serious complication leading to vascular and soft tissues calcification, abnormalities of bone turnover and mineralization, and decreased quality of life. There are several therapeutic strategies for sHPT management—from dietary restrictions which leads to limitation of phosphorus intake, through pharmacological intervention with phosphate binders, vitamin D or its analogs supplementation, calcimimetics administration to partial parathyroidectomy. According to the latest KDIGO (Kidney Disease: Improving Global Outcomes) guidelines limitation of "dietary phosphate intake in hyperphosphatemia treatment alone or in combination with other treatments" (2D level of evidence means that we suggest and the grade is very low) in CKD stages 3A−5D (dialysis) i.e., phosphate-binders are suggested (1). Moreover, restriction of calcium-based phosphate binders dose (2B level of evidence means that we suggest and the grade is moderate) is suggested as well. The KDIGO guidelines suggest "calcimimetics, calcitriol, or vitamin D analogs, or a combination of calcimimetics with calcitriol or vitamin D analogs" (with level of evidence 2B) to lower PTH in dialysis (1). In case of weak or lack of effects of pharmacological treatment partial or total parathyroidectomy should be considered. Data on the comparison of different SHPT treatment are very scarce and generally limited to studies on the effectiveness of different vitamin D analogs and vitamin D receptor analogs (VDRA).

The most popular intervention is supplementation with active vitamin D or the VDRA or calcimimetics administration. Administration of both agents (VDRA and calcimimetics) in parallel seems to be very promising due to opposite effects on serum calcium and different mechanism of action of drugs. However, in the literature the data on combination therapy are very limited. In the FARO-2 study this option was not analyzed due to low number of patients (2).

Taking all these data into consideration, we tried to analyze the real world data and compare efficacy of these therapeutic strategies, including combination treatment.

PATIENTS AND METHODS

This is an observational, retrospective study on 131 haemodialysed patients with inadequate control of PTH level treated with either intravenous paricalcitol (Paricalcitol Fresenius/Fresenius Medical Care Nephrologica)−60 patients aged 66 (SD = 11, Me = 66)–group PAR or cinacalcet (Mimpara/Amgen)−50 patients aged 60 (SD = 14, Me = 63)–group CIN. In the third group (PAR+CIN) of 21 patients aged 54 (SD = 13, Me = 51) treated with oral cinacalcet, intravenous paricalcitol has been added due to inability to reach target iPTH levels (more than 9 x higher than laboratory values limit), and then both agents were administered simultaneously.

The evaluation of the data, in particular, in the combination group was possible owing to introduction of therapeutic program for sHPT allowing to use either cinacalcet, paricalcitol or both agents. Every month of treatment iPTH, P, Ca, and ALP levels has been checked in all patients.

The treatment procedures were provided according to the therapeutic program approved by Polish Ministry of Health. Patients provided their informed consent for the therapeutic program. However, the authors also turned to the Ethical Committee of Regional Physicians Chamber in Poznan (Wielkopolska Izba Lekarska) and received their approval (Opinion no 89/2018).

Paricalcito doses were calculated according to Summary of Product Characteristics Paricalcitol Fresenius (3). Paricalcitol is a vitamin D3 analog. The chemical classification of paricalcitol is cholecalciferol. Chemical structure is (1R,3R)-5-[(2E)-2-[(1R,3aS,7aR)-1-[(E,2R,5S)-6-hydroxy-5,6-dimethylhept-3-en-2-yl]-7a-methyl-2,3,3a,5,6,7-hexahydro-1H-inden-4-ylidene]ethylidene]cyclohexane-1,3-diol.

The initial dose was established on the basis of serum iPTH concentration in pg/ml divided by 80. Subsequent doses were modified on the last iPTH serum concentration level checked monthly. The average dose of paricalcitol during whole observation period (12-months) was 6.76 mcg/dialysis session (Me = 5.00, Q1 = 3.84, Q4 = 35.00). Paricalcitol was administered intravenously to the bloodline during dialysis session. Cinacalcet is a naphthalene derivative and a calcium -sensing receptor agonist. Chemical structure is N-[(1R)-1-naphthalen-1-ylethyl]-3-[3-(trifluoromethyl)phenyl]propan-1-amine.The dosage of cinacalcet was based on Summary of Product Characteristics Mimpara Amgen (4) and the average dose during whole observational period was 0.6 mg/kg b.w. (SD = 0.3). Cinacalcet was taken by patient individually 2 times daily. Hyperphosphatemia was controlled by administration

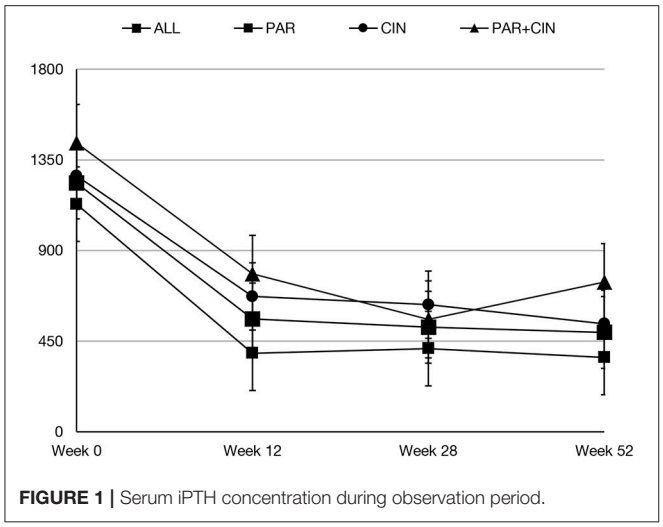

FIGURE 1 | Serum iPTH concentration during observation period.

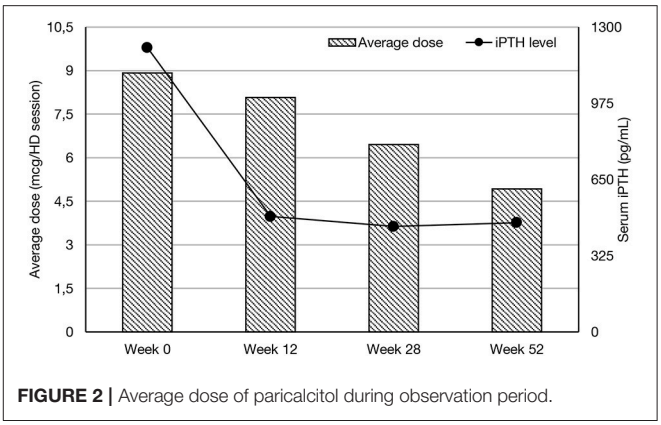

FIGURE 2 | Average dose of paricalcitol during observation period.

of calcium—based phosphate binders or non-calcium based phosphate binders as shown in **Table 1**. The dialysis was provided on Fresenius 5008S HD machines with FX Cordiax dialysers. The average dose of HD was 4 h three times a week.

The results are presented as percentage for categorical values, mean with one standard deviation in case of variables normally distributed. For non-normally distributed variables median and range were presented (tested by Lillefors test). The statistical significance was considered when P-value was <0.05. For statistical significance assessment T-Test, One-Way Anova, Chi-Square, Wilcoxon, and Mann-Whitney tests were used accordingly.

RESULTS

In all groups iPTH serum level significantly decreased. The most substantial changes were observed in first period of observation (week 0–12). In the next period iPTH serum concentration stabilized and remained on the similar level till the end of observation, as shown on **Figure 1**. Changing the average paricalcitol dose based on the current iPTH level was easy and allowed to keep this parameter at the target level (see **Figure 2**). Comparison between all groups showed that the highest control of iPTH level is achievable with paricalcitol intravenous treatment. Statistically significant difference was observed between groups PAR and CIN in this parameter. Calcium and phosphate serum concentration changed in all groups during the period of observation, however no severe hypercalcemia or hypocalcemia was observed. Biochemical parameters during treatment are shown in **Table 2**.

DISCUSSION

Secondary hyperparathyroidism and its consequences referred to as mineral and bone disorder in chronic kidney disease, despite new drugs and diagnostic methods development, remains an important factor of chronic kidney disease morbidity and mortality (5, 6).

Effectiveness and safety profile of sHPT treatment with calcimimetics as well as paricalcitol are widely described in literature (7–22).

However, there are no clear answers to key questions—whether sHPT management with active vitamin D or calcimimetics reduce mortality in HD population and which therapeutic approaches are most appropriate—vitamin D supplementation, and if so which of the available analogs is the most appropriate, the use of calcimimetics alone, or the administration of both preparations together. It is important to choose the form of administration—intravenously, during hemodialysis session or oral. Evidence for the reduction of mortality in hemodialysis patients receiving vitamin D supplementation origin mainly from observational studies. Several retrospective studies have identified the relationship between vitamin D administration in hemodialysis patients with chronic kidney disease in stage 5 and reduced mortality. A meta-analysis of the effects of active vitamin D on reducing mortality in patients with CKD in 2013 showed a significantly lower risk of death for any cause (23%) and a significantly lower risk of cardiovascular mortality (37% reduction) (23). Similarly, prospective FARO study showed statistically significant reductions in mortality from any cause and cardiovascular mortality in patients taking vitamin D analogs compared to untreated patients (24).

Therefore, when choosing the most beneficial treatment for a patient, the safety profile of the individual preparations and the mechanism of action must be taken into account.

Our study showed that in real life all employed strategies—oral cinacalcet, intravenous paricalcitol, and both agents administered simultaneously—are safe and efficient. The combination of cinacalcet and paricalcitol did not improve the efficacy, however cinacalcet might reduce vascular calcification as it was reported by others. There was no effect on serum calcium and phosphorus levels using the both agents. However, the combination of two drugs may be considered is some selected cases—e.g., hypocalcemia during treatment with cinacalcet, but in this particular case, the cost-effectiveness of such therapy should be carefully evaluated.

In several studies the comparison between paricalcitol and cinacalcet has been provided (25–29). In randomized IMPACT

TABLE 1 | Clinical and demographic characteristics.

Parameters	All ($n = 131$)	PAR ($n = 60$)	CIN ($n = 50$)	PAR+CIN ($n = 21$)	*P*-value
AGE, YEARS					
Mean (*SD*)	n/a	66 (11)	n/a	54 (13)	$p = 0.01$
Median	63	n/a	63	n/a	
Quartile 1	53	n/a	51.5	n/a	
Quartile 4	90	n/a	82	n/a	
SEX, *n*					
Female	51	21	19	11	$p = 0.3$
Male	80	39	31	10	$p = 0.3$
Use of phosphate binders, %	42	45	40	40	$p = 0.7$
Calcium containing only	34	40	25	40	n/a
Sevelamer only	0	0	0	0	n/a
Combination of calcium containing and sevelamer	8	5	15	0	n/a
Other binders	0	0	0	0	n/a
PARICALCITOL DOSE, mcg/HD SESSION					
Initial dose (week 0)					
Mean	n/a	8.89	n/a	8.88	$p = 0.06$
Median		8.84		5.76	
Quartile 1		6.05		5.00	
Quartile 4		25.00		21.53	
Week 12					
Mean	n/a	7.19	n/a	10.64	$p = 0.1$
Median		5.00		7.69	
Quartile 1		5.00		4.46	
Quartile 4		30.00		35	
Week 28					
Mean (*SD*)	n/a	5.89	n/a	8.24	$p = 0.2$
Median		5.00		5.00	
Quartile 1		4.51		4.61	
Quartile 4		20.00		22.69	
Week 52					
Mean	n/a	4.74	n/a	4.94	$p = 0.4$
Median		4.23		4.61	
Quartile 1		3.08		3.07	
Quartile 4		27.69		18.84	
CINACALCET DOSE, mg/kg B.W./DAY					
Initial dose (week 0) Mean (*SD*)	n/a	n/a	0.6 (0.3)	0.6 (0.3)	$p = 0.5$
Week 12 Mean (*SD*)	n/a	n/a	0.6 (0.3)	0.6 (0.3)	$p = 0.5$
Week 28 Mean (*SD*)	n/a	n/a	0.6 (0.3)	0.6 (0.3)	$p = 0.5$
Week 52 Mean (*SD*)	n/a	n/a	0.6 (0.3)	0.6 (0.3)	$p = 0.5$

sHPT study cinacalcet was compared with paricalcitol in respect of iPTH reduction, serum calcium and phosphorus concentration changes, alkaline phosphatase activity, and FGF23 concentration. In 28 week observation, the iPTH level reduction was significantly higher in the group treated with paricalcitol. Additionally, the percentage of patients who reached the iPTH level in the range of 150–300 pg/ml was higher in this group (25–27). Incidence of adverse events during the 28 weeks of follow-up were comparable in both groups, however, paricalcitol use was associated with higher prevalence of hypercalcemia, whereas nausea and hypocalcemia were more frequent in the cinacalcet group (26). In addition, Sharma et al. (28, 29),

TABLE 2 | The effect of treatment on serum PTH, calcium, phosphate, and alkaline phosphatase.

		iPTH (pg/mL)		*P*-value	Ca (mg/dL)		*P*-value	P (mg/dL)		*P*-value	ALP (U/L)		*P*-value
Group		**Week 0**	**Week 52**		**Week 0**	**Week 52**		**Week 0**	**Week 52**		**Week 0**	**Week 52**	
All	Average	1235	493	$p < 0.0001$	8.07	9.12	$p < 0.0001$	5.40	5,61	$p = 0.13$	199	120	$p < 0.0001$
	SD	–	–		–	–		1.49	1.60		–	–	
	Median	1072	339		8.56	9.20		–	–		125	84	
	Q1	863	228		7.84	8.47		–	–		87	63	
	Q4	2833	2490		16.00	11.52		–	–		1679	1211	
PAR (*SD*)	Average	1130	369	$p < 0.0001$	8.64	9.54	$p < 0.0001$	5.10	5.87	$p = 0.002$	151	88	$p < 0.0001$
	SD	–	–		0.90	0.75		1.36	1.47		–	–	
	Median	972	268		–	–		–	–		119	67	
	Q1	872	212		–	–		–	–		87	58	
	Q4	2472	1800		–	–		–	–		813	466	
CIN (*SD*)	Average	1271	536	$p < 0.0001$	7.13	8.49	$p = 0.02$	5.68	5.03	$p = 0.02$	627	380	$p = 0.2$
	SD	–	–		3.16	0.70		1.54	1.66		–	–	
	Median	1189	353		–	–		–	–		118	67	
	Q1	889	294		–	–		–	–		6	6	
	Q4	2500	2499		–	–		–	–		2833	2500	
PAR+CIN (*SD*)	Average	1434	743	$p = 0.0005$	8.65	9.40	$p = 0.01$	5.60	6.25	$p = 0.08$	367	107	$p = 0.0001$
	SD	–	–		1.02	0.96		1.57	1.37		–	–	
	Median	1443	720		–	–		–	–		198	72	
	Q1	795	330		–	–		–	–		102	63	
	Q4	1958	1688		–	–		–	–		1324	563	
P-value		$p = 0.051$	$p = 0.0009$	–	$p = 0.006$	$p < 0.0001$	–	$p = 0.09$	$p = 0.002$	–	$p = 0.0007$	$p = 0.009$	–

on the basis of dosing and efficacy data from US patients involved to the IMPACT SHPT study, found that a strategy with paricalcitol administered intravenously was more cost effective than cinacalcet plus low-dose vitamin D in the management of PTH in patients with SHPT receiving hemodialysis.

Intravenous paricalcitol appears to be more cost-effective in hemodialysed patients with vascular access as well as has a better compliance. Oral cinacalcet would be more appropriate for patients with chronic kidney disease, including kidney transplant recipients treated conservatively and patients with CKD V treated with peritoneal dialysis. Adding cinacalcet to sHPT treatment protocol with iv paricalcitol can help to reduce hypercalcemic activity of paricalcitol however it was not showed in our study.

Data comparing head to head paricalcitol and cinacalcet are scarce. Chertow et al. (30) in open-label 16 weeks clinical trial, assess the effects of a treatment combined with low dose of active vitamin D derivatives and cinacalcet on mineral metabolism in patients on hemodialysis who had controlled PTH (iPTH 80–160 pg/ml) but remaining elevated Ca x P (>55 mg^2/dl^2) receiving paricalcitol >6 μg/week (or an equipotent dose of an alternative active vitamin D derivative). At the start of the trial, active vitamin D derivatives dose was decreased to average equivalent dose of paricalcitol 6 μg/week, and cinacalcet was titrated from 30 to 180 mg/day as a maximum possible dose. They concluded that treatment based on combination of low-dose active vitamin D derivatives and cinacalcet improved control of mineral metabolism. On the other hand, in this *post-hoc* analysis of ADVANCE study (31), coronary artery calcification progression was compared between 70 protocol-adherent subjects on cinacalcet and low doses of vitamin D (CPA) as specified in the study protocol and control group with 120 patients given vitamin D sterols. The study protocol stated specifically that the vitamin D dose was not to exceed the equivalent of 6 mcg of paricalcitol i.v. weekly among those receiving cinacalcet. Patients involved to the control group were treated with higher, varying doses of vitamin D sterols given intravenously during thrice-weekly hemodialysis sessions or orally every day. The authors found that the progression of CAC was weaker in the group treated with cinacalcet and small doses of vitamin D compared with the control group treated with higher doses of vitamin D sterols alone after 52 weeks. In the TARGET study on 444 hemodialysed patients with moderate to severe secondary parathyroidism (mean biointact PTH>160–430 pg/ml and approximately iPTH on the level 300–800 pg/ml or ng/l) the cinacalcet dose was titrated sequentially during an 8 weeks dose titration phase (30–180 mg/day) to get the bioPTH level below 160 pg/ml (iPTH 300 pg/ml or ng/l approximately) and the effectiveness was assessed over 8 weeks observation. At the second week of the trial, patients receiving vitamin S sterols get the reduced doses to the equivalent of 2 mcg of paricalcitol administered three times a week or 6 mcg administered in one dose weekly. Block et al. (32) concluded that the proportion of subjects with values of

biPTH, of calcium x phosphorus product and of both biPTH and Ca x P within the target range during the analyzed period didn't show the difference between group received cinacalcet and vitamin D together and the group who was treated with cinacalcet alone. However, they did not assess the cost of this therapy. FARO 2 study showed that paricalcitol administered intravenously significantly increased the number of patients at the target for the combined endpoint composed of PTH, calcium and phosphate ($P = 0.001$), although the intravenous calcitriol and paricalcitol iv and cinacalcet combination groups weren't assessed due to the low patients number (2). In the retrospective study, Schumock et al. (33) compared rates of surgical intervention (parathyreoidectomy) in secondary hyperparathyroidism patients treated with paricalcitol or cinacalcet. They found that long-term treatment with paricalcitol was connected with lower number of parathyroidectomies in comparison with the patients treated with cinacalcet. It was obscure why patients in the cinacalcet group were more likely to experience parathyroidectomy in comparison to those who were treated with paricalcitol. The paricalcitol group consisted patients with more comorbidities, seemed to be more sicker and with a shorter period between the start of hemodialysis treatment and start of the index drug, while the cinacalcet group contained more females. Even when these discreptances and other ones were adjusted for in the final analysis, the risk of parathyroidectomy was markedly higher in the cinacalcet group. In our pilot study, we analyzed the results of 3-months paricalcitol treatment of 36 patients receiving hemodialysis with sHPT (serum iPTH >500 pg/ml), including 11 patients who additionally received cinacalcet. Analysis of the results shows a statistically significant lowering in iPTH and alkaline phosphatase in the whole group (34). In 2017 we published data (35) on 64 hemodialyzed patients with unsuited control of serum PTH levels treated for 6-months with intravenous paricalcitol, including 16 patients simultaneously receiving oral cinacalcet. In the first paper we collected all the available patients who entered the therapeutic programme with paricalcitol in FMC units. Till that time only treatment with cinacalcet was available. We had only 3-months complete data of a pilot study. Then we collected all the 6-months data from the growing HD population benefiting from the therapeutic programme. In both papers we had two study groups receiving either paricalcitol or paricalcitol with cinacalcet. In this study we were able to collect the biggest population of paricalcitol treated patients in Poland. We also were able to have 3 study group for comparisons i.e., paricalcitol, cinacalcet, and paricalcitol with cinacalcet. Moreover, we collected 1-year data.

Our study has several limitations, one is small sample size, nevertheless statistical analysis was feasible, contrary to FARO-2 study where the combination therapy sample was too small for analysis. We assessed only biochemical parameters, but not FGF23, we did not assess vascular calcifications. However, this is a real-world data. Intravenous paricalcitol, as well as combination of two drugs were not available for dialysis units until recently, when therapeutic program for sHPT and reimbursement by national Health Found were introduced. Now opportunity arises to assess the effect of this therapy, including combination of two drugs, clinical parameters and costs of the therapy as well. The results of EVOLVE have proven controversial (36–40). The unadjusted primary composite endpoint showed a non-significant reduction (HR: 0.93; P 1/4 0.112) with cinacalcet use (36–40). Both ADVANCE (31) and EVOLVE (36–40) trials evaluated the activity of cinacalcet on cardiovascular calcificationand the risk of cardiovascular incidents and mortality, respectively. Albeit the primary assay of both trials didn't find significant impact of cinacalcet, the benefit of one was insinuated in the subanalyses in which the potential issues of the trials were taken into account.

However, due to subsequent series of next publications of the EVOLVE study (36–40) KDIGO decided that calcimimetics, calcitriol or vitamin D analogs as well are capable first line strategy in G5D patients as agents active in PTH lowering. Authors didn't prioritized any option listed above. In principle, this recommendation was supposed to be maintained as it was in the previous set of KDIGO guidelines from 2009 (41). It is also worth to mention that there are alternative pathways of vitamin D (42–44) and lumisterol activation (45) that may affect the final outcomes.

CONCLUSIONS

We report for the first time the long-term (1 year) efficacy data on combined therapy of sHPT in relation to either iv paricalcitol or oral cinacalcet. We are fully aware of limitation of our data, but they represent about 10% of Polish haemodialysed patients and therefore could be extrapolated to the larger population. The strength of the study is the same clinical protocol in all dialysis units, the same dialysers and same target goals on phosphate control, iPTH etc., possibility to access the data from the same system and to collect all available patients on combination therapy to make the comparison possible. Randomization of treatment allocation was not possible due to the retrospective analysis and this is clearly a limitation of this study. We are fully aware that allocation to either therapy would be the only way to convince readers and prevent from endless discussions about the results. However, there are no ongoing or registered studies on the comparison between cinacalcet and paricalcitol in the clinical.trials.gov. Thus, our not perfect, but real-life study show that both therapeutic strategies are effective in the treatment of sHPT, whereas simultaneous administration of the agents did not improve efficacy. It appears that low compliance of the patients in group treated with oral calcimimetics seems to be the main reason of weaker response in this group. In case of unsatisfactory results after 3-months treatment, the continuation of the therapy should be carefully considered. The treatment of sHPT with paricalcitol and cinacalcet is safe, however Ca concentration should be controlled during whole treatment period.

Cinacalcet is an effective agent in lowering iPTH level, but due to compliance issues should be considered mainly in the patients without vascular access (non-dialyzed CKD IV and V and patients receiving peritoneal dialysis) and

in the patients with high Ca serum concentration to avoid hypercalcemia and all consequences connected with high calcium level.

Paricalcitol, due to intravenous administration should be considered in patients with vascular access (haemodialysed patients). Serum calcium level should be observed carefully and in case of severe hypercalcemia the treatment should be stopped, or dose of paricalcitol administered should be decreased.

AUTHOR CONTRIBUTIONS

JZ, JM, and JSM conceived the idea for the study and contributed to the design of the research. JZ performed the statistical analysis of the collected data. JZ, JM, and TD-R were involved in the preparation of the manuscript. JZ, JM, WM, and TP were involved in data collection. All the authors analyzed the data, edited and approved the final version of the manuscript.

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The remaining 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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