VITAMIN D FOR OLDER ADULTS

Determinants of status, supplementation strategies and its role in muscle function

Anouk MM Vaes

PROPOSITIONS

1. When recommending vitamin D supplementation, emphasis should be placed on preventing deficiencies rather than raising 25(OH)D concentrations beyond current guidelines.
(this thesis)

2. Confirmatory trials on the health benefits of vitamin D supplementation are challenged by the fact that studying deficient populations in placebo-controlled settings is considered unethical.
(this thesis)

3. Increasing longevity requires healthcare to shift from cure-and-care to a more transdisciplinary approach that integrates aspects of social connection, self-perception and resilience *(based on: Beard et al., Lancet, 387:2145-54, 2016)*.

4. Informing the public on how to interpret date-labels on perishable foods will substantially reduce food waste *(based on: Wilson et al., Food Quality and Preference, 55:35-44, 2017)*.

5. Competition among researchers works counterproductively: while it might stimulate scientific excellence, it concurrently limits resource sharing and integration of knowledge.

6. People should learn to balance their information bubble with opposing views, similar to the way in which researchers balance scientific evidence.

Propositions belonging to the thesis, entitled:

'Vitamin D for older adults. Determinants of status, supplementation strategies and its role in muscle function'

Anouk MM Vaes
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CONTENTS

CHAPTER 1

General introduction

As the world population is increasingly living longer, research is mapping the risk factors for age-related diseases to define prevention strategies or therapies that will support healthy aging. In this quest, vitamin D has been identified as one of the factors receiving growing attention in clinical research. Vitamin D deficiency is prevalent in the older population and is suggested to have health implications beyond bone health. Deficiency has been linked to a broad range of diseases, and is especially relevant for the elderly given the potential effect on muscle function and fall risk. As such, the Institute of Medicine (IOM) and the Dutch Health Council re-evaluated the public health recommendations for vitamin D intake and supplementation. However, in their most recent guidelines, both institutes could base their advice only on the established benefits for bone health, as the evidence for other health outcomes was considered inconclusive [1, 2]. Further research on the optimal vitamin D treatment for other health benefits is needed to guide evidence-based policy making. To contribute to this field of research, this thesis aimed to gain insight into the prevalence and main determinants of a low vitamin D status, to investigate strategies to prevent or reverse vitamin D deficiency, and to study the effect of vitamin D supplementation on muscle strength and physical performance in Dutch older adults.

Vitamin D

Vitamin D was discovered in the beginning of the 20th century. However, it was already in the 1600s that, without being aware of the causal factor, rickets was reported as the poor mineralization, softening and bending of bones [3]. During the 1900s, the time of the Industrial Revolution, rickets became an epidemic among children living in industrialized cities in the US and Europe [4]. In that time, researchers experimented with cod-liver oil or ultraviolet radiation using quartz mercury-vapor lamps and noted that an unknown factor in both the diet and UV-light could cure the disease [5-8]. It was by the work of these scientists that, in 1922, Professor McCollum could ascribe the effect in cod-liver oil to the substance, he called 'vitamin D'[9]. However, it was not until 1980, that the cutaneous synthesis of vitamin D was fully unraveled by the identification of pre-vitamin D_3 in the skin and, shortly after, vitamin D was re-classified as a prohormone [10, 11]. Today, vitamin D is still considered essential for human health, and though the occurrence of rickets is rare, population based studies show that severe vitamin D deficiency is also a condition of modern times [12].

Vitamin D sources

Vitamin D belongs to a family of fat-soluble molecules that are all based on the same secosteroid structure. Vitamin D_3 (cholecalciferol) is mainly obtained through cutaneous synthesis after exposure to sunlight [13]. Exposure to UV-B radiation with wavelengths between 280-320 nm can activate vitamin D synthesis in the skin [14]. This is comparable to the sun intensity that occurs from March-October in the Netherlands [15]. Furthermore, vitamin D_3 or vitamin D_2 (ergocalciferol) is in limited amounts available in foods. Vitamin D_3 is present in animal based food sources, like fatty fish, egg yolks,

meat and dairy [16], whereas vitamin D_2 is the plant based source of vitamin D, and is mainly found in mushrooms [17]. On top of that, many countries fortify specific food products with vitamin D, of which milk, fruit juices, cereals and margarines are the most commonly fortified products. In the Netherlands, the Dutch Commodity Act allows fortification of margarines with extra vitamin D [18], and since 2007, fortification of other food products is allowed up to 4.5 μg per 100 kcal of product [19]. Lastly, supplements can be an important source contributing to the total vitamin D intake.

Metabolism of vitamin D

After exposure to UV-B radiation, the synthesis of vitamin D_3 is performed by converting 7-dehydrocholesterol into pre-vitamin D_3 by the enzyme 7-dehydrocholesterol reductase (DHCR7) (Figure 1.1). Pre-vitamin D_3 then isomerizes to form vitamin D_3. Ingeniously, the body has a feedback mechanism that degrades pre-vitamin D_3 into inactive photoproducts, like lumisterol and tachysterol, to prevent vitamin D intoxication in case of long-term sunlight exposure [20]. Both vitamin D_3 obtained after exposure to UV-B radiation, and vitamin D_3 or vitamin D_2 obtained from foods or supplements bind to vitamin D binding protein (DBP, also named group-specific component i.e. GC-globulin) for transport in the bloodstream [21]. To become biologically active, vitamin D must undergo two hydroxylation steps. First, vitamin D is hydroxylated in the liver to form 25-hydroxyvitamin D (25(OH)D), which is performed by several 25-hydroxylase enzymes that belong to the cytochrome P450 family, of which CYP2R1 is considered the key activator for 25-hydroxylation [22]. The 25(OH)D metabolite is the major circulating form of vitamin D and is, given its relatively long half-life of ~2 weeks, currently considered the best clinical marker to define vitamin D status. Next, 25(OH)D undergoes hydroxylation by the enzyme 1α-hydroxylase (CYP27B1). The activity of 1α-hydroxylase occurs mainly in the kidneys, as well as extra-renal tissues, to form the metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) [23]. This metabolite is kept under strict homeostatic control by parathyroid hormone (PTH), serum calcium and phosphate, fibroblast growth factor 23 (FGF23), and activation of the 24-hydroxylase by CYP24A1. Serum $1,25(OH)_2D$ has a half-life of 4-7 hours and its serum concentration is 500-1000 times lower than that of 25(OH)D [24]. The $1,25(OH)_2D$ metabolite acts on the vitamin D receptor (VDR) in many cell types throughout the body to modulate its biological responses, of which its main function is to regulate calcium homeostasis. Finally, both 25(OH)D and $1,25(OH)_2D$ can be metabolized by 24-hydroxylase to form 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) or 1,24,25-trihydroxyvitamin D ($1,24,25(OH)_3D$), which ensures catabolism of metabolites into inactive substrates [25, 26].

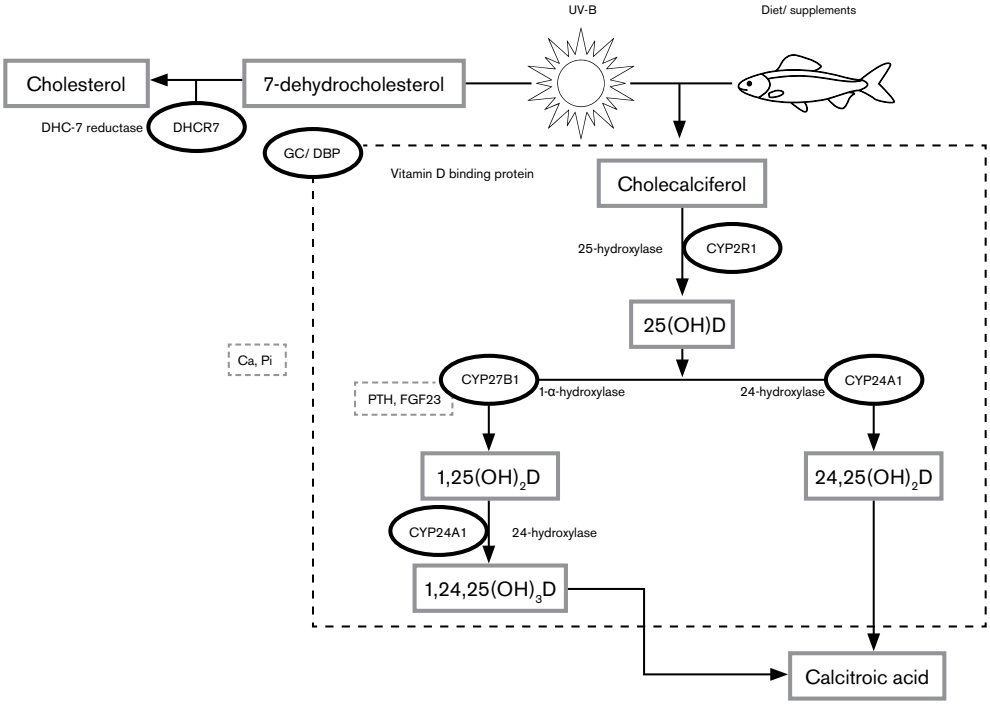

Figure 1.1 Schematic overview of vitamin D pathway.

Vitamin D supplementation regimens

When supplementing with vitamin D, several treatment regimens are available. The most common supplementation strategies include cholecalciferol, ergocalciferol, calcifediol or calcitriol. Furthermore, numerous vitamin D analogues have been synthesized, which represent effective pharmacological compounds, each having their distinct indications for choice of treatment.

Cholecalciferol or Ergocalciferol

Supplementation with vitamin D_2 or D_3 is most commonly used to increase 25(OH)D levels in case of insufficient dietary vitamin D intake and cutaneous synthesis of vitamin D. Several studies suggest that vitamin D_3 is more potent compared to vitamin D_2 in raising serum 25(OH)D concentrations [27]. This might be explained by the lower affinity of vitamin D_2 to bind to DBP, due to which it is cleared faster from the bloodstream [28]. Neither toxicity nor hypercalcemia are reported for intakes <250 μg/day, however, the upper limit is set at 100 μg/day due to limited data on adverse effects over the long-term [2].

Calcifediol

Calcifediol, also named calcidiol, is the 25(OH)D metabolite. Calcifediol is more hydrophilic, does not require hepatic hydroxylation and binds with higher affinity to its binding proteins, when compared to vitamin D_3 [29, 30]. As such, supplementation with calcifediol is characterized by fast absorption and requires only 1α-hydroxylation before becoming biologically active. This supplementation type is especially effective in patients with malabsorption syndromes or impaired hepatic function [31]. Hypercalcemia is the most common side-effect, though when compared to supplementation with calcitriol, it shows a relatively lower risk of hypercalcemic effects. Clinical studies have been performed in patient populations where the supplement was well tolerated [32]. However, limited data are available on pharmacokinetics of daily supplementation with different doses of calcifediol in healthy older adults [33, 34].

Calcitriol

Calcitriol is the active vitamin D hormone $1,25(OH)_2D$. A specific indication for supplementing with calcitriol, or its 1α-derivatives, involves patients with chronic kidney disease as the production of $1,25(OH)_2D$ is diminished, which causes secondary hyperparathyroidism. Besides, supplementation with calcitriol might be considered for the treatment of postmenopausal osteoporosis in order to correct mineral and bone homeostasis [35]. However, supplementation with calcitriol requires careful monitoring as direct supplementation with the active metabolite limits the feedback regulation in the system and side-effects, like hypercalcemia and hypercalciuria are more likely to occur [32].

Vitamin D for older adults

Aging is characterized by a gradual decline in muscle mass and bone density, which can result in musculoskeletal conditions such as sarcopenia and osteoporosis [36]. These conditions are related to an increased risk of falls and fractures, which may subsequently lead to disability or institutionalization. As the social and economic impact of these events is significant, research is looking for protective factors in these conditions [37, 38]. Bone and muscle tissue both act as endocrine organs in which vitamin D is suggested to play an integrated role. The main function of vitamin D is to stimulate calcium and phosphorus absorption from the intestine, and to interact with PTH to mobilize calcium from the skeleton to regulate serum calcium concentrations. As such, vitamin D is crucial for the mineralization of bone and the prevention of its related conditions; osteomalacia, osteoporosis and fractures [39]. However, vitamin D deficiency-related fractures may in part be explained by the observed associations with muscle weakness and increased risk of falling. As such, vitamin D presents an important regulator in the musculoskeletal health of older adults.

Prevalence of vitamin D deficiency

The prevalence of vitamin D deficiency in the older population varies between countries and depends on the cut-off levels that are used to define deficiency. Based on the serum 25(OH)D cut-off as defined by the Dutch Health Council of 50 nmol/L for older adults (≥70 years), the estimated prevalence of vitamin D deficiency among community-dwelling elderly ranges between 25-50% in Europe [40]. The Longitudinal Aging Study Amsterdam (LASA), showed a high prevalence of vitamin D deficiency, with 41% of adults (55-65 years) having a serum 25(OH)D status <50 nmol/L [41]. Moreover, the older cohort (65-88 years), showed an even higher prevalence of deficiency, with a prevalence estimate of 47% [42]. Furthermore, the Maastricht Sarcopenia Study (≥65 years) showed that the prevalence of deficiency was higher among sarcopenic seniors, with a prevalence of 51% compared to a prevalence of 25% in non-sarcopenic seniors [43].

Determinants of vitamin D status

Serum 25(OH)D concentrations depend on a number of internal and external factors, some of which are specific for the older population. First of all, general factors, like latitude, season and skin pigmentation are important determinants of cutaneous vitamin D production, and as such of vitamin D status. The Netherlands is located at a latitude of 50°N, and vitamin D can only be synthesized in the months March till October [1]. This means that, during the winter months, there is a dependency on dietary vitamin D intake, leaving many people deficient at the end of winter season. Other factors that contribute to vitamin D status are more behavioral in character, such as the time spend outdoors, clothing, sunscreen-use, but also dietary preferences. BMI or body fat percentage are also considered important determinants of vitamin D status as sequestration in adipose tissue has been described [44, 45]. In the last years, research on genetic factors also suggest a considerable heritable role on vitamin D status. A genome-wide association study showed that several single nucleotide polymorphisms (SNPs) were linked to serum 25(OH)D concentration [46].

Older adults, especially frail and institutionalized elderly, are at increased risk of vitamin D deficiency due to their limited time spent outdoors and ability to expose themselves to sunlight. Moreover, the cutaneous synthesis of vitamin D is suggested to decrease with age. Studies show a 30-50% reduced production of 7-dehydrocholesterol, or response in serum 25(OH)D concentration after standardized UV-B doses in individuals aged 60-80 years, compared to 20-30 year-old controls [47, 48]. Besides that, age-related declines in the 25-hydroxylation and 1α-hydroxylation capacity might occur due to an impaired hepatic or renal functioning [49], but also medication use or chronic diseases can interfere with the vitamin D metabolism [50-52]. All in all, vitamin D status is thus influenced by a broad scale of factors, however, the extent to which these factors contribute to the risk of vitamin D deficiency is not completely understood.

Current guidelines and recommendations

Reference values for vitamin D intake and status have been defined by several health authorities, however, different views are taken. The IOM recommends to maintain serum 25(OH)D concentrations >30-50 nmol/L, which corresponds with an Estimated Average Requirement (EAR) of 10 μg/day and Recommended Daily Allowance (RDA) of 15 μg/day for those 1-70 years of age. Above the age of 70 years, the EAR and RDA are set at 15 and 20 μg/day, respectively [2]. On the other hand, the Endocrine Society (ES) defines deficiency as having serum 25(OH)D concentrations <50 nmol/L, insufficiency as levels between 50-75 nmol/L, and sufficiency as levels >75 nmol/L, recommending supplementation up to 50 μg/day for adults >50 years [53]. The dietary reference guidelines of the Dutch Health Council recommend to maintain serum 25(OH) D concentrations >30 nmol/L for those 4 to 70 years of age, and >50 nmol/L for those ≥70 years of age. Daily supplementation with 10 μg/day is advised for women ≥50 years, and 20 μg/day for men and women ≥70 years of age [1]. In part, the different approach between policies can be explained by the fact that the IOM aims to provide guidance to the general population, whereas the ES specifically targets at-risk populations for the prevention and treatment of vitamin D deficiency. However, the optimal serum 25(OH)D concentration for specific health outcomes is under considerable debate. For example, the IOM based its recommendations on the beneficial effect on bone, whereas the ES advocates levels >75 nmol/L to maximize the effect of vitamin D on PTH suppression, fall risk reduction, and bone and muscle metabolism. All in all, there is controversy in these recommendations and what constitutes an 'optimal vitamin D status' requires further investigation.

Vitamin D and musculoskeletal health

Muscle strength and function are necessary for basic bodily movement and increasingly important to remain mobile and independently living when aging. There is growing evidence that vitamin D plays an important role in skeletal muscle. Already in times when rickets was prevalent, symptoms of myopathy, hypotonia or waddling gait were associated with this disease [54]. In adults with osteomalacia or extreme vitamin D deficiency, comparable symptoms, like diffuse muscle pain and difficulty in rising from a chair are described [55]. Moreover, early case-studies suggest that these complaints could be relieved after supplementation with vitamin D [56, 57] and thus, provide relevant leads for further research.

Muscle and vitamin D mechanism

While the exact effect of vitamin D on muscle metabolism remains to be elucidated, numerous cell-line and animal studies have attempted to address this question. Several mechanisms have been proposed, which either relate to systemic endocrine effects via mineral homeostasis or regulatory effects through the VDR in muscle tissue (Figure 1.2). The $1,25(OH)_2D$ metabolite is suggested to stimulate the accumulation and release of calcium from the sarcoplasmic reticulum, thereby influencing calcium influx in muscle

cells [58]. Besides, 1,25(OH)$_2$D can support phosphate transport through the cell membrane [59], both of which are necessary for muscle contraction [60]. Moreover, the identification of the VDR in muscle cells and the local expression of CYP27B1 and CYP24A1, support a direct role of 1,25(OH)$_2$D in skeletal muscle [61-64]. In the target cell, 1,25(OH)$_2$D binds to the VDR and heterodimerizes with the RXR receptor, after which the complex will bind to vitamin D response elements (VDREs) on target genes to induce gene expression involved in calcium handling, cell proliferation and differentiation [65, 66]. The expression of the VDR in muscle cells has been under debate as it could be detected by several studies [67-69], although not by all [70]. In mice models, ablation of the VDR results in reduced grip strength, abnormal muscle development and reduced size of both type I and II muscle fibers [71, 72]. In humans, the VDR expression in muscle is reported to decrease with age and supplementation with vitamin D appeared to reverse this process [73, 74].

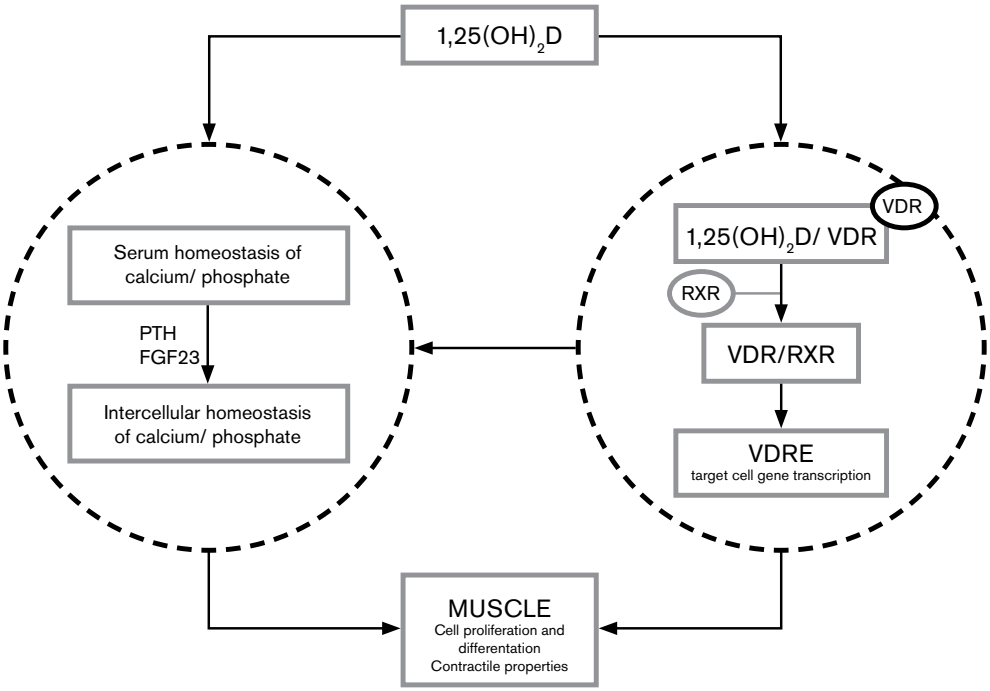

Figure 1.2 Suggested mechanisms by which vitamin D acts on muscle.

Evidence from epidemiological studies

Several observational studies have described associations between vitamin D status and measures of physical performance, muscle strength or postural balance, where serum 25(OH)D concentrations <50 nmol/L have been associated with poor physical performance [75, 76] or muscle strength [77-79]. However, the observed associations appear inconsistent. Several studies also observed higher cut-offs of >75 nmol/L or >100 nmol/L to be relevant [80, 81], whereas others report no association [82, 83]. Prospective observations from the LASA cohort showed that serum 25(OH)D concentrations <25 nmol/L were associated with an increased risk of losing muscle mass and grip strength over a 3 year period [84]. Likewise, an accelerated decline was observed over a 2,5 year period in performance on the Timed Up and Go test (TUG) in older women with a serum 25(OH)D status <50 nmol/L [85].

Evidence from intervention studies

A number of randomized trials investigated the effect of vitamin D supplementation on physical performance and muscle strength, with some reporting beneficial effects [86-92], whereas others did not [93-96]. While these studies vary considerably in design, several meta-analyses have attempted to pool the results. A meta-analysis in older adults with a serum 25(OH)D status <50 nmol/L, concluded that supplementation with 20-25 μg/day resulted in beneficial effects on the TUG test and balance performance [97]. In addition, a meta-analysis studying the effect of vitamin D supplementation on lower limb strength, observed a beneficial effect only in those with a serum 25(OH)D status <25 nmol/L [98]. A large meta-analysis including 30 studies and all age groups, indicated a small significant effect of supplementation on muscle strength, but no effect on muscle power or muscle mass [99]. Moreover, the beneficial effect on strength was more pronounced in subgroups with serum 25(OH)D status <30 nmol/L or those aged ≥65 years. In contrast, a recent meta-analysis on the effect of vitamin D supplementation in older adults, concluded no improvements on grip strength and observed even a small significant deterioration on the TUG test [100]. These meta-analyses indicate that the current evidence-base is inconsistent, but point towards a plausible beneficial effect of vitamin D supplementation, especially in older populations with low baseline 25(OH)D concentrations. When studying the effect of vitamin D supplementation, the goal is to correct deficiency and induce a significant increase in serum 25(OH)D concentrations towards the targeted therapeutic range. Up until now, most studies supplemented with vitamin D_3 to increase serum 25(OH)D concentrations. However, in this context, calcifediol might offer an effective supplementation strategy as well. The European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) reported in a recent position statement that other vitamin D compounds require investigation with respect to their efficacy and safety on extra-skeletal health [101]. A previous pilot study (n=20) in postmenopausal women indicated beneficial effects of calcifediol on knee-extension strength and gait speed when compared to vitamin D_3 [102]. However, whether these effects are explained by the rapid increase

in serum 25(OH)D concentration, the higher serum 25(OH)D thresholds obtained, or other characteristics of this metabolite remains to be determined. Therefore, further randomized trials, in placebo-controlled settings, using effective treatment regimens and taking into account the baseline 25(OH)D concentration, are needed to investigate the potential effect of vitamin D supplementation on muscle function in older adults.

This thesis – rationale and outline

This thesis aimed to address several topics related to vitamin D, with a focus on the community-dwelling, or pre-frail and frail older population. First of all, to identify those at risk of deficiency, we aimed to get insight in the prevalence and main determinants of a low vitamin D status. Next, we assessed how dietary intake and supplementation can contribute to prevent or overcome deficiency, and lastly, we studied whether improving serum 25(OH)D status might benefit muscle function in vitamin D deficient pre-frail and frail older adults. These study questions are addressed in the following chapters:

In **chapter 2**, the prevalence of vitamin D deficiency, and the importance of sunlight exposure, dietary vitamin D intake and genetic variance for adequate serum 25(OH) D concentrations is described. **Chapter 3** examines the dietary vitamin D intake and specific food sources that contribute most to vitamin D status. In **chapter 4**, a dose-response study was performed to explore the potential for calcifediol as a valuable supplementation strategy in the treatment of vitamin D deficiency. Moreover, **chapter 5** describes the association between serum 25(OH)D status, physical performance and frailty, and in **chapter 6**, a placebo-controlled trial was performed to study the effect of vitamin D_3 or calcifediol supplementation on muscle function in vitamin D deficient older adults. Finally, in **chapter 7**, the main findings are discussed, providing a critical methodological reflection and suggestions for future research.

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CHAPTER 2

Relative importance of summer sun exposure, vitamin D intake and genes to vitamin D status in Dutch older adults: the B-PROOF study

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ABSTRACT

Background & aims: The prevalence of vitamin D deficiency among seniors is high. Whereas sun exposure, vitamin D intake, genes, demographics, and lifestyle have been identified as being important determinants of vitamin D status, the impact of these factors is expected to differ across populations. To improve current prevention and treatment strategies, this study aimed to explore the main determinants of vitamin D status and its relative importance in a population of community-dwelling Dutch older adults.

Methods: Serum 25-hydroxyvitamin D (25(OH)D) was measured in 2857 adults aged ≥65 years. Sun exposure was assessed with a structured questionnaire (*n*=1012), vitamin D intake using a Food Frequency Questionnaire (*n*=596), and data on genetic variation that may affect 25(OH)D status was obtained for 4 genes, DHCR7 (rs12785878), CYP2R1 (rs10741657), GC (rs2282679), and CYP24A1 (rs6013897) (*n*=2530).

Results: Serum 25(OH)D concentrations <50 nmol/L were observed in 45% of the population; only 6% of these participants used vitamin D supplements. Sun exposure (being outside daily during summer: 66 ± 25 nmol/L versus not being outside daily during summer: 58 ± 27 nmol/L, *P*=0.02) and vitamin D intake (per unit μg/day during winter/spring: 3.1 ± 0.75 nmol/L, *P*<0.0001) were associated with higher 25(OH)D concentrations. Major allele carriers of SNPs related to DHCR7, CYP24A1, and GC, as well as CYP2R1 minor allele carriers had the highest 25(OH)D concentrations. Together, sun (R^2=0.29), vitamin D intake (R^2=0.24), and genes (R^2=0.28) explained 35% (R^2=0.35) of the variation in 25(OH)D concentrations during summer/autumn period, when adjusted for age, sex, BMI, education, alcohol consumption, smoking, physical activity, and self-rated health status (*n*=185).

Conclusion: The investigated determinants explained 35% of 25(OH)D status. Of the three main determinants under study, sun exposure still appeared to be an important determinant of serum 25(OH)D in older individuals, closely followed by genes, and vitamin D intake. Given the low frequency of vitamin D supplement use in this population, promoting supplement use may be an inexpensive, easy, and effective strategy to fight vitamin D deficiency.

INTRODUCTION

In the Netherlands about half of the community-dwelling older people have a vitamin D status (25(OH)D) below 50 nmol/L [1] and are classified as having an insufficient status according to guidelines of the Institute of Medicine (IOM) [2]. In order to tackle this issue of low 25(OH)D concentrations it is important to gain knowledge on its main determinants.

One of the sources of vitamin D is the diet, but only a limited number of foods contain vitamin D. Vitamin D is therefore mainly acquired through sunlight exposure, specifically ultraviolet-B radiation (UV-B), which activates the cutaneous synthesis of pre-vitamin D_3 in the skin [3]. The efficiency of sunlight exposure and vitamin D intake to increase 25(OH)D status depends on a variety of factors, including latitude, season, air pollution, sunscreen use, skin pigmentation, age, efficiency of absorption in the gut, liver and kidney disease, and medication use [4]. To illustrate this, at higher latitudes (e.g. >50°) the intensity of UV-B during the winter months is too low to activate the vitamin D synthesis in the skin [5]. It is also shown that 25(OH)D concentrations decrease with age due to a decrease in cutaneous vitamin D synthesis in the skin [6]. Genetic make-up has furthermore been associated with vitamin D metabolism and variations in 25(OH)D concentrations [7]. Thus, 25(OH)D concentrations depend on a broad variety of factors ranging from environmental and behavioral factors to genetics. Despite this knowledge, vitamin D deficiency is observed worldwide [8-11], of which particularly older populations are at increased risk [12].

This study is performed to assess the prevalence of vitamin D deficiency, and to examine to what extent vitamin D intake, frequency of vitamin D supplement use, sunlight exposure habits, and genetic variance are associated with 25(OH)D concentrations in a population of Dutch community-dwelling older adults. Identification of the relative contribution of these factors to vitamin D status in this particular age category might help to pinpoint important determinants in the prevention and treatment of vitamin D deficiency.

METHODS

Participants

This cross-sectional study was performed using baseline data of the B-PROOF study (B-vitamins for the PRevention Of Osteoporotic Fractures); a randomized, double-blind, placebo-controlled trial designed to assess the efficacy of daily oral supplementation with vitamin B_{12} (500 μg) and folic acid (400 μg) on fracture risk in mildly hyperhomocysteinemic (plasma homocysteine 12-50 μmol/l) older adults ≥65 years. Details of this study have been reported elsewhere [13]. Data on 25(OH)D concentration were available of 2857 participants. Genetic information on vitamin D related genes was obtained from 2530 participants. Sun exposure was assessed in 1012 participants, and vitamin D intake in 596 participants. The Medical Ethics Committee of Wageningen UR approved the study protocol and the Medical Ethics Committees of VUmc and Erasmus MC confirmed local feasibility. All participants gave their written informed consent.

Dietary assessment

Dieticians at the division of Human Nutrition at Wageningen University developed a 190-item Food Frequency Questionnaire (FFQ) to measure vitamin D intake and vitamin D supplement use. The questionnaire was developed based on two validated FFQs [14, 15], which was updated to include vitamin D intake by means of the Dutch FFQ-TOOL™. Specifically, food items contributing to ≥0.1% of total vitamin D intake were included, which was estimated to cover 80% of total vitamin D intake based on the Dutch National Food Consumption Survey of 1998 [16].

Sunlight exposure

Habitual sunlight exposure was assessed using a questionnaire, which was administered on the day of blood sampling, thus throughout the year depending on date of inclusion. Data were collected on the amount of time spent outdoors and in the sun during summer, use of sun protection and solariums, type of clothing worn during summer, and holidays with a sunny destination during the past three months.

Genotyping

DNA was isolated from buffy coats. Samples were genotyped for about 700,000 SNPs using the Illumina Omni-express array, covering >90% of all common variations in the genome. SNPs selected for this study were based on a genome-wide association study on relations between genes and serum 25(OH)D concentrations, and included rs12785878 (DHCR7), rs6013897 (CYP24A1), rs10741657 (CYP2R1), and rs2282679 (GC) [7].

Biochemical analyses

Blood samples were drawn throughout the year, and always in the morning, when participants were fasting or had consumed a restricted breakfast. Samples were stored at -80 ˚C until determination. Measurement of serum 25(OH)D occurred by releasing it from its binding protein(s) and by adding a denaturised internal standard IS: 25(OH)D_3-d6. Subsequently, serum 25(OH)D was measured by isotope dilution-online solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS) [17]. Inter-assay coefficient of variation was 9% at a level of 25 nmol/L and 6% at a level of 62 nmol/l. Analyses were performed at the Endocrine Laboratory of the VU University Medical Centre.

Covariates

Body height was measured at baseline with a stadiometer to the nearest 0.1 cm. Body weight was measured to the nearest 0.5 kg with a calibrated analogue scale, while wearing light clothes. Body Mass Index (BMI) was calculated as weight/height^2. Data on educational level (years), smoking status (never, current, former), physical activity (kcal/day) [18], and alcohol consumption (no and light, moderate, high and excessive) [19] were collected by means of questionnaires. Self-rated health was obtained from the Short-Form Health Survey (SF-12) [20]. Season of blood collection was dichotomized into summer/autumn (June-November) and winter/spring (December-May) [21].

Statistical Analyses

Participants characteristics are reported as mean with standard deviation (SD), or percentages. To compare baseline characteristics of participants having inadequate serum 25(OH)D concentrations (<50 nmol/L) with participants having adequate serum 25(OH)D concentrations (≥50 nmol/L), chi-squared tests were performed for categorical variables and independent t-tests for continuous variables. To assess the association between total vitamin D intake and serum 25(OH)D status, multiple linear regression analyses were conducted with adjustment for age, sex, BMI, years of education, alcohol consumption, smoking, physical activity, and self-rated health status, and stratified by season. Stratification for season was applied as we assumed that the impact of vitamin D intake may be higher during winter/spring than during summer/autumn, specifically larger effects of vitamin D supplementation are expected when 25(OH)D concentrations are lower. Analysis of Covariance (ANCOVA) was used to explore associations between sunlight exposure variables and 25(OH)D status with adjustment for age, sex and BMI, and stratified by season. Associations between vitamin D related genetic make-up and 25(OH)D status were tested using ANOVA, stratified by season. In order to further investigate the importance of summer sunlight exposure, vitamin D intake, and genes for serum 25(OH)D concentrations all three factors were individually and simultaneously added to the multiple linear regression model, and age, sex, BMI, years of education, alcohol consumption, smoking, physical activity, and self-rated health status were included as covariates. As at higher latitudes

the impact of sun exposure on 25(OH)D status is expected to be small during winter/spring, only data obtained during summer/autumn - and only for those with complete data of the determinants under study - were included in this model (n=185). Missing data were not imputed. All tests were two-sided (P<0.05). Analyses were performed using the statistical package SPSS, version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Descriptive data of the population are shown in Table 2.1. In this population of older individuals, 45% had serum 25(OH)D concentrations <50 nmol/L, 28% had serum 25(OH)D concentrations <40 nmol/L, and 14% had serum 25(OH)D concentrations <30 nmol/L. As expected, stratification for season showed that the prevalence of vitamin D deficiency was higher during the winter/spring (63%) than during the summer/autumn (37%). Participants with vitamin D deficiency were more likely to be women, older, have a higher BMI, have a lower vitamin D intake, and were more likely to be included during the winter/spring ($P<0.0001$). As depicted in Figure 2.1-A, a clear seasonal fluctuation in serum 25(OH)D was observed; Figure 2.1-B confirms the expected age-dependent differences in serum 25(OH)D.

Sunlight exposure

ANCOVA showed that all sunlight measures were significantly associated with serum 25(OH)D in participants who were enrolled during the summer/autumn months after adjustment for age, sex, and BMI, including "daily outside 2 weeks prior to blood sampling" ($F_{633}=5.6$, $P=0.02$), "daily outside during summer" ($F_{633}=4.9$, $P=0.03$), "clothing" ($F_{621}=19.5$, $P<0.0001$), "sun holiday" ($F_{631}=18.9$, $P<0.0001$), "sun lamps" ($F_{628}=13.6$, $P<0.0001$), and "sun cream use" ($F_{631}=5.8$, $P<0.01$) (Table 2.2) ($n=1012$). Associations for "daily outside 2 weeks prior to blood sampling" ($F_{362}=4.1$, $P=0.04$) and "sun lamp use" ($F_{360}=11.0$, $P<0.01$) with serum 25(OH)D were less strong, but still significant, in participants that were included during the winter/spring months. Other components were not significant anymore when participants were enrolled during the winter/spring months.

Vitamin D intake

Mean total vitamin D intake was 4.9 ± 2.9 μg per day. Vitamin D intake was significantly associated with serum 25(OH)D; stratification for season revealed that the association between vitamin D intake and 25(OH)D status was more pronounced during winter months (β 3.1 ± 0.8, $P<0.0001$) than during summer/autumn months (β 1.0 ± 0.4, $P=0.02$). These linear regression coefficients suggest that every μg increase in vitamin D intake increases serum 25(OH)D with about 3.1 nmol/L during winter/spring months and with 1.0 nmol/L during summer/autumn months.

Table 2.1 Descriptive statistics of 2857 Dutch men and women aged ≥65 years.

	25(OH)D <50 nmol/L	25(OH)D ≥50 nmol/L	*P*-value	*n*
25(OH)D, nmol/L	34 ± 10	74 ± 18	<0.0001	2857
Age, years	75.1 ± 7.1	73.2 ± 5.9	<0.0001	2857
Sex, n (% men)	597 (42)	831 (58)	<0.0001	2857
BMI, kg/m^2	27.5 ± 4.3	26.8 ± 3.6	<0.0001	2842
Physical activity level (kcal/day)	598 ± 440	691 ± 502	<0.0001	2842
Years of education	9.8 ± 3.9	10.3 ± 4.0	0.11	2855
Smoking			0.01	2857
Never	459 (36)	510 (32)		
Current	142 (11)	135 (9)		
Former	690 (53)	921 (59)		
Alcohol consumption			<0.0001	2855
Light	925 (72)	998 (64)		
Moderate	318 (25)	505 (32)		
Excessive	46 (3)	63 (4)		
Self-experienced health			<0.0001	2855
Excellent	79 (6)	130 (8)		
Very good	230 (18)	386 (25)		
Good	787 (61)	874 (56)		
Mediocre	183 (14)	170 (11)		
Poor	11 (1)	5 (0)		
Blood sampling			<0.0001	2857
December until May	813 (63)	543 (35)		
June until November	478 (37)	1023 (65)		
Vitamin D supplement use, n (%)	174 (6)	411 (14)	<0.0001	2857
Total vitamin D intake, μg/day	4.2 ± 2.1	5.2 ± 3.2	<0.0001	596
Vitamin D intake from foods, μg/day	4.0 ± 1.9	4.5 ± 2.1	0.002	596

Values are expressed as mean ± SD, median (IQR), or n (%).

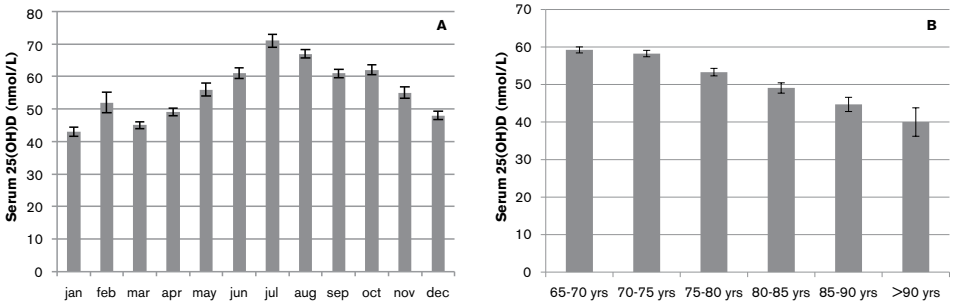

Figure 2.1 Serum 25(OH)D distribution (mean ± SEM) per month (A) and per age category (B) in Dutch men and women aged ≥65 years.

Table 2.2 Associations between sun exposure and serum 25(OH)D of 1012 Dutch men and women aged ≥65 years stratified for season.

	Summer/Autumn		Winter/spring	
	25(OH)D	F_{df}, *P*-value	25(OH)D	F_{df}, *P*-value
Daily outside 2 weeks before blood sampling		F_{633}=5.6, 0.02		F_{362}=4.1, 0.04
No	58 ± 27		41 ± 19	
Yes	66 ± 25		47 ± 21	
Daily outside during summer		F_{633}=4.9, 0.03		F_{359}=1.0, 0.32
No	57 ± 25		42 ± 20	
Yes	66 ± 25		46 ± 21	
Clothing		F_{621}=19.5, <0.0001		F_{355}=3.0, 0.09
Long sleeved	51 ± 27		42 ± 22	
Short sleeved	66 ± 25		47 ± 20	
Sun holiday 3 months before blood sampling		F_{631}=18.9, <0.0001		F_{358}=4.0, 0.05
No	62 ± 25		44 ± 21	
Yes	73 ± 23		51 ± 18	
Use of sunlamps		F_{628}=13.6, <0.0001		F_{360}=11.0, 0.01
No	64 ± 26		44 ± 20	
Yes	78 ± 20		59 ± 27	
Sun cream use		F_{631}=5.8, <0.01		F_{359}=3.0, 0.05
Always	69 ± 24		51 ± 22	
Sometimes	67 ± 26		45 ± 18	
Never	60 ± 25		43 ± 22	

Serum 25(OH)D levels (nmol/L) are displayed as mean ± SD. Models are adjusted for age, sex, BMI.

Vitamin D related genetic make-up

Vitamin D status varied significantly between allele carriers for all genes under study, except for the CYP24A1 gene (Figure 2.2). As expected, there were differences in 25(OH)D concentrations between summer/autumn and winter/spring, but differences between alleles were comparable. The gene GC, which encodes for the protein related to vitamin D transport in the circulation, was most strongly associated with 25(OH)D status ($P<0.0001$), indicating that during summer/autumn, minor allele carriers have the lowest 25(OH)D concentrations (53 ± 20 nmol/L) and major allele carriers the highest 25(OH)D concentrations (68 ± 25 nmol/L) (Figure 2.2).

Sunlight, vitamin D intake and vitamin D related genetic make-up combined

Finally, after considering individual associations of sunlight exposure, vitamin D intake, and vitamin D related genetic make-up with 25(OH)D concentrations, a multiple linear regression model was built using data of participants that were included during summer/autumn and had complete data of the determinants under study (n=185). Individually, vitamin D intake - while also taking into account age, sex, BMI, education, alcohol consumption, smoking, physical activity, and self-rated health status - explained 24% of the variance in 25(OH)D status, sunlight exposure 29%, and genes 28%. All together these factors explained 35% of the variance in 25(OH)D status, as reflected by an R^2 of 0.35 (adjusted R^2: 0.27) (Table 2.3 and Figure 2.3).

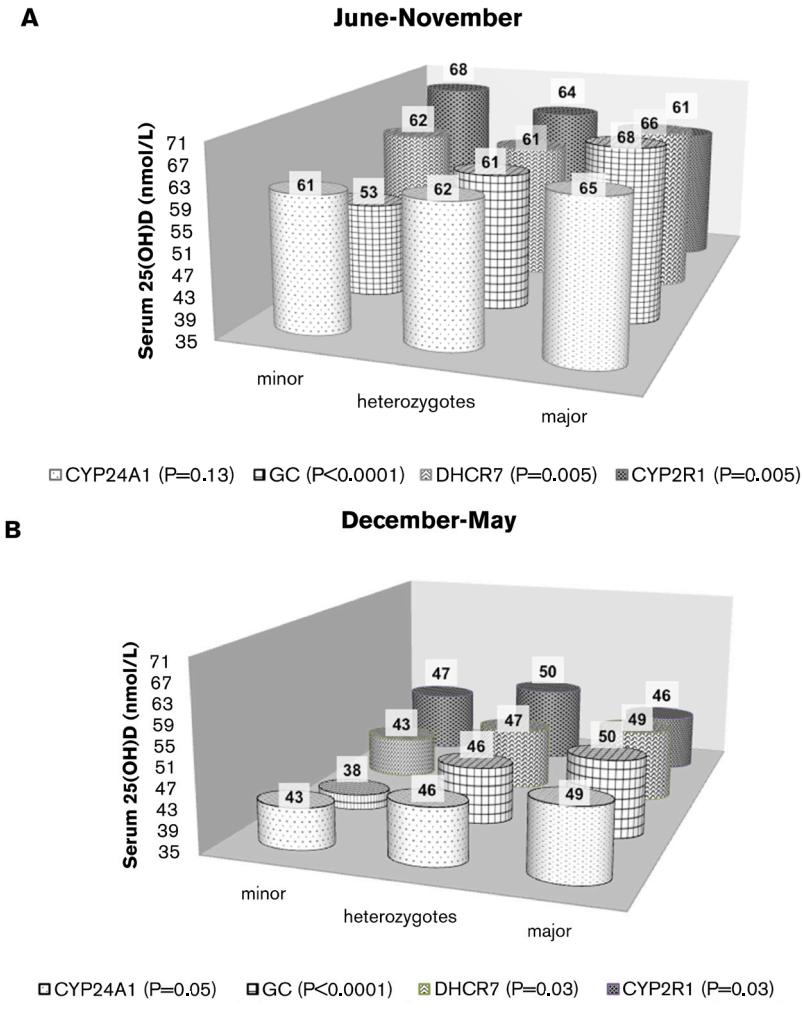

Figure 2.2 Associations between vitamin D related genetic make-up and serum 25(OH)D.

Analyzed using ANOVA in 2530 Dutch men and women aged ≥65 years, stratified by season of blood sampling. A) Summer/autumn: Bonferroni post hoc tests indicate significant differences for DHCR7 [heterozygotes vs. major], CYP2R1 [minor vs. major], and GC [minor vs. heterozygotes, heterozygotes vs. major, minor vs. major]. B) Winter/spring: Bonferroni post hoc tests indicate significant differences for DHCR7 [minor vs. major], CYP2R1 [heterozygotes vs. major], and GC [minor vs. heterozygotes, heterozygotes vs. major, minor vs. major].

Table 2.3 Estimates of the relative importance of vitamin D intake, sunlight exposure, and vitamin D related genetic make-up using data of Dutch men and women aged ≥65 years that were included during the summer/autumn months (*n*=185).

	β	**SE**	**s*β***	***P*-value**
(Constant)	147.7	34.1	-	<0.0001
CYP24A1	4.1	3.1	0.09	0.18
GC	7.4	2.6	0.18	0.005
DHCR7	2.5	2.9	0.06	0.39
CYP2R1	-4.2	2.3	-0.12	0.07
Outside past 2 weeks	15.6	6.2	0.24	0.01
Outside past summer	-9.2	6.5	-0.14	0.16
Clothing worn	2.4	6.7	0.03	0.72
Sun cream use	-2.2	2.6	-0.06	0.41
Sunlamp use	12.7	5.7	0.14	0.03
Sun holiday	3.7	4.4	0.06	0.40
Vitamin D intake	0.4	0.5	0.06	0.38

Model was adjusted for age (s*β* -0.21, *P*=0.003), sex (s*β* -0.15, *P*=0.06), BMI (s*β* -0.21, *P*=0.004), years of education (s*β* -0.12, *P*=0.08), smoking (s*β* -0.03, *P*=0.66), alcohol consumption (s*β* 0.11, *P*=0.12), physical activity level (s*β* 0.05, *P*=0.45), self-experienced health (s*β* -0.09, *P*=0.24). s*β*=standardized beta.

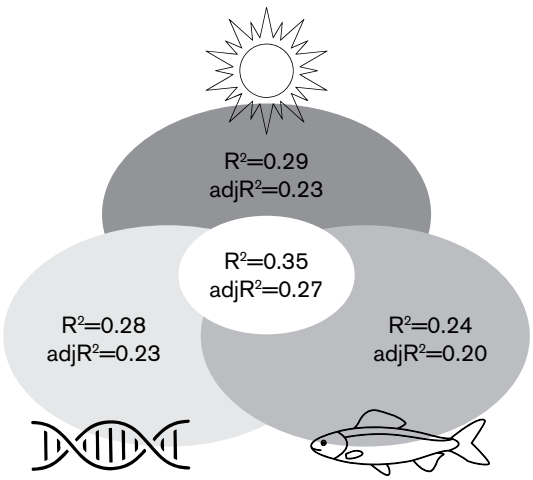

Figure 2.3 Explained variance per component.

Using data of the participants that were included during the summer/autumn months and had complete data of all determinants under study (n=185), adjusted for age, sex, BMI, years of education, alcohol consumption, smoking, physical activity, and self-rated health status.

DISCUSSION

In this Dutch community-dwelling older population, living at a latitude of 52˚N, 45% of the participants had 25(OH)D concentrations below 50 nmol/L. Total vitamin D intake was far below the recommended level of 20 μg/day as set by the Dutch Health Council for adults ≥70 years [22]; supplement use was reported by only 20% of the participants. Total vitamin D intake, sunlight exposure, and vitamin D related genetic make-up were all significantly associated with serum 25(OH)D. When exploring the contribution of the three factors to serum 25(OH)D status, vitamin D intake explained 24% of the variance, sunlight exposure 29%, and vitamin D related genetic make-up 28%. Including these three factors simultaneously, while accounting for other known relevant covariates, resulted in a 35% explained variance in serum 25(OH)D.

Methodological considerations

In order to appreciate these findings, several methodological issues of this study warrant further discussion. To the best of our knowledge, this is the first study examining the relative contribution of habitual sunlight exposure, vitamin D intake, and genetic make-up to the variation in serum 25(OH)D in a community-dwelling older population, while taking into account many potential important covariates. As participants were included throughout the year, we had the possibility to study the influence of sun exposure, vitamin D intake, and genetic make-up in association to serum 25(OH)D in winter/spring and summer/autumn. Unfortunately, we could not account for the potential role of diseases known to alter the absorption and metabolism of vitamin D. Another possible limitation is the use of a non-validated Food Frequency Questionnaire (FFQ). The FFQ used, however, was very detailed, composed using a validated method, and covered 80% of the total vitamin D intake according to the Dutch National Food Consumption Survey of 1998. In addition, our vitamin D intake data are in line with recent vitamin D intake data of this age group that were obtained from the Dutch Food Consumption Survey of 2013 [23]. This suggests that the reported vitamin D intake estimates were reasonably accurate. In both studies, however, vitamin D intake may be underestimated as evidence indicates that certain foods may contain 25(OH)D, while food consumption tables do not yet account for this [24]. Taking into account 25(OH)D in animal-based foods may result in vitamin D intake estimates that are about 1.7-2.9 μg/day higher than current estimates [24]. Finally, the assessment of habitual sun exposure can be considered suboptimal, which probably resulted in an underestimation of the explained variation in 25(OH)D concentrations resulting from UV-B exposure. For instance, sunlight questions did not account for time of day at which a participant was exposed to sunlight, while it has been shown that during summer ultraviolet-B is most efficient in producing vitamin D_3 between approximately 10 a.m. and 2 p.m. [25]. Using dosimeters might have resulted in more accurate UV-B exposure estimates [26].

Vitamin D deficiency

The high prevalence of deficient 25(OH)D concentrations observed in this population is in line with reports on 25(OH)D deficiency in other countries [8, 9]. Serum 25(OH)D concentrations of 50 nmol/L or higher are considered sufficient in order to prevent disturbances in calcium metabolism [2]. Recent insights also indicate that 25(OH)D may relate to cardiovascular problems, glucose homeostasis, inflammation, muscle strength, and cognitive function [9, 27-30]; where future well-designed large RCTs are needed to establish whether these links are actually causal. Thus, the low 25(OH)D concentrations in this older population are alarming and more knowledge on the determinants of 25(OH)D may help to steer guidelines.

Sunlight exposure and 25(OH)D concentrations

Despite the fact that the role of sunlight exposure to maintain serum 25(OH)D concentrations decreases with age [6], we observed significant associations between surrogate markers of habitual sun exposure and 25(OH)D status in this older population. This finding is in line with a study by Holick et al. (2007) that showed that exposing nursing home residents to 0.75 MED whole body exposure, using a tanning bed three times a week for five weeks, increased 25(OH)D concentrations up to 150% of its baseline concentration [25]. Interestingly, in our population, serum 25(OH)D was higher among participants reporting use of sun cream; Hyppönen and colleagues (2007) reported a similar finding when studying a nationwide cohort of British adults [21]. It may be that participants exposing themselves more abundantly to sunlight were more aware of their increased risk of for instance skin cancer and as such felt the necessity to use sun cream. As a result of their overall higher exposure to sunlight, these people were still the ones with the highest 25(OH)D concentrations.

Vitamin D intake and 25(OH)D concentrations

Total vitamin D intake - including both dietary intake and supplement use - in this population was on average 4.9 ± 2.9 μg/day. Vitamin D supplement use was reported by 20% of the population of which 6% still had a 25(OH)D deficient status. Studies in southern European countries as well as Australia have reported vitamin D intakes ranging from 1.2 to 1.4 μg/day [31-33]. In Scandinavian countries, where vitamin D fortified products are more common, substantially higher vitamin D intake levels have been observed, ranging from 6 to 8 μg/day [34, 35]. The vitamin D intake in this Dutch population is far from adequate [22], which can be explained by the fact that the Dutch diet does not contain many foods that are naturally rich in vitamin D, and fortified products are hardly available. Therefore, Dutch men and women ≥70 years are recommended to use 20 μg vitamin D daily via supplements [22]. However, based on our data and data from the Dutch Food Consumption Survey 2013 [23], it can be concluded that compliance to this recommendation is low. This suggests that more actively promoting the vitamin D recommendation may be important to reduce the

prevalence of 25(OH)D deficiency, particularly during winter months. When analyzing the dose-response relation between vitamin D intake and serum 25(OH)D levels in this study, data suggested a 1.0 nmol/L (summer/autumn) and 3.1 nmol/L (winter/spring) increase in 25(OH)D status with every unit increase in vitamin D intake. This finding is in line with previous studies that showed that dietary vitamin D intake was positively associated with 25(OH)D status during winter, but not in summer [32, 35-37].

Vitamin D related genetic make-up and 25(OH)D concentrations

In this population, three out of four investigated genes in the pathway of vitamin D metabolism (i.e. DHCR7, CYP2R1, and GC) were significantly associated with serum 25(OH)D concentrations. These results are in line with the findings of a large genome-wide association study by Wang and colleagues [7], and several smaller studies [38-40]. Our data suggest that major allele carriers of the DHCR7 gene have higher 25(OH)D concentrations. DHCR7 encodes for the enzyme 7-dehydrocholesterol reductase. This enzyme catalyzes the conversion of 7-dehydrocholesterol into cholesterol in the skin, and thus prevents that 7-dehydrocholesterol is metabolized into vitamin D. The minor allele of CYP2R1 was associated with higher 25(OH)D concentrations. CYP2R1 encodes for the hepatic enzyme 25-hydroxylase that converts vitamin D into 25(OH)D. Carriers of the major CYP24A1 and GC alleles were shown to have higher 25(OH)D concentrations. CYP24A1 encodes for an enzyme that initiates the degradation of 25(OH)D and 1,25(OH)D into calcitroic acid. GC is the major transport protein of vitamin D metabolites, such as 25(OH)D, to different target organs, tissues and cells [7].

Important determinants of serum 25(OH)D

Previous estimations indicate that sunlight accounts for 70-90% of the 25(OH)D supply of the body [4]. In this study, habitual summer sun exposure also explained most of the variance (29%) in serum 25(OH)D, closely followed by genetic make-up (28%), and vitamin D intake (24%), while taking into account other relevant covariates. Larger differences were expected regarding the importance of sun exposure and vitamin D intake. Assessing sun exposure habits, however, is challenging and measurement error is very likely to have occurred. The three factors, together with potential relevant covariates, explained 35% of the variance in serum 25(OH)D. When extending our findings to other studies that also calculated R^2 in order to identify determinants of serum 25(OH)D, we conclude that there are substantial differences with respect to predictors included in the models. To the best of our knowledge, this is the first study taking into account genetic factors in a population of community dwelling seniors. The explained variance in 25(OH)D status of our final model is in line with a comparable study in postmenopausal women, published by Engelman and colleagues, who accounted for 29% of the variation in the model when taking into account vitamin D intake, waist circumference, season, self-reported sun exposure, cholesterol, and genetic profile [41]. On the other hand Gilbert and colleagues did consider taking genetic information

into account, but concluded that this information did not improve the fit of the prediction score in their data, which explained 28% of the variation in 25(OH)D when sun exposure, vitamin D intake, anthropometrics, clinical factors, demographics, age, season, study center, and batch assay were included in the model [42]. Other previous studies that included vitamin D intake, a measure of UV-B exposure, demographic, and environmental factors have explained between 21 up to 33% of the variation in 25(OH)D status [33, 43, 44]. Studies considering vitamin D intake, demographic and environmental factors and season of blood sampling have shown an explained variation in 25(OH)D ranging from 19-28% [45-47]. All in all, based up on the current literature, our data do suggest that taking genetic factors into account does contribute to a better understanding of the variance in 25(OH)D concentrations.

Conclusion

In summary, the findings of this study acknowledge the previously reported inadequate vitamin D intake and the relatively high prevalence of 25(OH)D deficiency in the older population. Moreover, it was shown that UV-B exposure, vitamin D intake and vitamin D-related genetic make-up all substantially contribute to the variability in 25(OH)D concentrations. The high prevalence of vitamin D deficiency as well as the low intake of vitamin D supplements imply that more effort should be undertaken to encourage the use of vitamin D supplements in order to optimize the 25(OH)D concentrations in the Dutch older population. Moreover, given the suggested importance of genes involved in vitamin D metabolism, in combination with the on-going question on whether the associations found between 25(OH)D concentrations and non-skeletal health are causal [28-30, 48], these results plea for studies examining associations between vitamin D related genetic make-up and the health outcomes under debate, and large well-designed RCTs in populations with low vitamin D concentrations.

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CHAPTER 3

Food sources of vitamin D and their association with 25-hydroxyvitamin D status in Dutch older adults

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ABSTRACT

Background & aims: Various populations are at increased risk of developing a low vitamin D status, in particular older adults. Whereas sun exposure is considered the main source of vitamin D, especially during summer, dietary contributions should not be underestimated. This study aims to identify food sources of vitamin D that associate most strongly with serum vitamin D concentration.

Methods: Data of 595 Dutch adults, aged ≥65 years, were analysed. Vitamin D intake was assessed with a food frequency questionnaire and 25-hydroxyvitamin D (25(OH)D) was determined in serum. Associations of total vitamin D intake and vitamin D intake from specific food groups with serum 25(OH)D status were examined by *P*-for trend analyses over tertiles of vitamin D intake, prevalence ratios (PRs), and spline regression.

Results: The prevalence of vitamin D deficiency was high, with 36% of the participants having a 25(OH)D status <50 nmol/L. Participants with adequate 25(OH)D concentrations were more likely to be men and more likely to be younger than participants with vitamin D deficiency. Total median vitamin D intake was 4.3 μg/day, of which 4.0 μg/day was provided by foods. Butter and margarine were the leading contributors to total vitamin D intake with 1.8 μg/day, followed by the intake of fish and shellfish with 0.56 μg/day. Participants with higher intakes of butter and margarine were 21% more likely to have a sufficient 25(OH)D status after adjustment for covariates (T1 vs. T3: PR 1.0 vs. 1.21 (95% CI: 1.03-1.42), *P*-for trend 0.02). None of the other food groups showed a significant association with the probability of having a sufficient 25(OH)D status.

Conclusion: This study shows that vitamin D intake was positively associated with total serum 25(OH)D concentration, with butter and margarine being the most important contributors to total vitamin D intake.

INTRODUCTION

Various populations are at increased risk of developing a low vitamin D status, in particular older adults [1]. Recent studies show adverse associations between 25-hydroxyvitamin D (25(OH)D) deficiency and a broad range of health outcomes, e.g. cardiovascular and autoimmune function, neuropsychiatric health, diabetes and muscle function [2]. While more studies are needed to investigate the causality of these vitamin D-health associations, the effect on bone homeostasis is considered established [3]. Based on these classical effects of vitamin D on bone health, current dietary guidelines emphasize the need to prevent low serum 25(OH)D concentrations. Although vitamin D is primarily synthesized after sun exposure, particularly during summer months [4], dietary vitamin D intake can significantly contribute to higher serum 25(OH)D concentrations [5-8]. As such, the Institute of Medicine (IOM) and the Health Council of the Netherlands recommend a vitamin D intake between 10-20 μg/day to maintain serum 25(OH)D levels above a target value of 50 nmol/L [9, 10]. Vitamin D can be obtained as ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Limited amounts of ergocalciferol are obtained via UV-irradiated mushrooms, milk, and butter [11, 12]. Cholecalciferol is mainly obtained from fatty fish (e.g. salmon, mackerel, herring) and in lesser quantities via meat, egg yolks, milk and butter [13]. Nevertheless, dietary vitamin D intakes are far below the recommended reference intake in many countries [14-16]. To prevent these observed low dietary intakes, several countries fortify specific foods with vitamin D. Fortification of milk products in the USA, and the fortification of milk and fat spreads in Canada are mandatory [17]. In addition, many countries also fortify other foods, such as cereals and fruit juices. In Europe, fortification policies differ between countries, where fat spreads and some cereals are the most commonly fortified products; milk fortification is not customary, with the exception of Finland, Norway and Sweden [18]. Currently, in the Netherlands, vitamin D food fortification is not common practice, with the exception of margarines. Vitamin D intake data from the Dutch National Food Consumption Survey (DNFCS) published in 2013 show mean vitamin D intakes of 4.1 μg/day in a population ≥70 years [19]. This average is far below the current dietary recommendation for the older adults and while specific supplementation advice is in order, only 22% of the older adults reports to use a vitamin D supplement [19]. Therefore, the importance of an adequate dietary intake should not be underestimated, especially in case of modest vitamin D inadequacy. For that reason, the aims of this study were I) to investigate which food source contributes most to total vitamin D intake, and II) to examine which food source contributes most to higher serum 25(OH)D status and adequacy in older Dutch adults.

METHODS

Study population

Cross-sectional analyses were conducted using baseline data of the B-PROOF study, which is a multi-center, placebo-controlled, double-blind, randomized trial performed by three study centers in the Netherlands (Wageningen University, Erasmus MC and VUmc). The primary aim of this study was to investigate the effect of supplementation with folic acid and vitamin B-12 to prevent osteoporotic fractures in mildly hyperhomocysteinemic adults, aged 65 years or older. Participants were recruited between August 2008 and March 2011. Main exclusion criteria were: a low or high plasma homocysteine status (<12 μmol/L or >50 μmol/L), the use of vitamin-B supplements or injections in the past 4 months, being diagnosed with cancer in the past 5 years, renal dysfunction or being bed bound. Dietary intake was only measured in the Wageningen cohort of which reliable data on vitamin D intake and 25(OH)D status were available for 595 participants. More specific information on the research protocol and study population have been described elsewhere [20]. The study protocol was approved by the Medical Ethics Committees of Wageningen UR and VUmc and the medical ethics committee of Erasmus MC confirmed local feasibility. All participants gave their written informed consent. The study was registered at ClinicalTrials.gov as NCT00696514 since June 9, 2008.

Dietary assessment

To estimate dietary vitamin D intake, an extensive Food Frequency Questionnaire (FFQ) was used of which the methods are previously described [21]. FFQ food items were categorized as total vitamin D intake, and the vitamin D intake from meat, fish and shellfish, eggs, butter and margarine, total dairy, and dairy subgroups i.e. milk, yogurt, cheese. In addition, the FFQ included questions on vitamin D supplement use, and the type, dose and frequency of the supplement.

Biochemical analyses

Blood was drawn in the morning and participants were requested to remain fasted or only take a light breakfast (according provided instructions). Serum 25(OH)D concentrations were analyzed by tandem mass spectrometry (ID-XLC-MS/MS) at the VU University Medical Centre [22]. Inter-assay coefficient of variation was 9 and 6% at a serum 25(OH)D level of 25 and 62 nmol/L, respectively.

Covariates

Weight was measured with a calibrated analogue scale to the nearest 0.5 kg. Height was measured to the nearest 0.1 cm, using a stadiometer. Body Mass Index (BMI) was reported as kg/m^2. Furthermore, each participant filled out a questionnaire to report

data on education level (primary, secondary, higher), smoking (non, current, former), alcohol intake (light, moderate, excessive) [23], and physical activity (min/day) [24]. Date of blood collection was used to define a covariate for season (summer: June-November and winter: December-May).

Data analyses

General characteristics and dietary intake of the population are presented as mean (SD), median (25-75th percentile) or *n* (%) by subgroups. Subgroups were created based on serum 25(OH)D status (inadequate <50 nmol/L *versus* adequate ≥50 nmol/L) and age (<70 *versus* ≥70 years). Potential differences between subgroups were tested by ANOVA or Kruskal-Wallis test in case of continuous variables or the Chi-square test in case of categorical variables. ANCOVA analyses were used to calculate adjusted means (95% CI) per tertile of vitamin D intake from the total diet and specific food categories. *P*-for trend analysis was performed to analyze the association between vitamin D intake and serum 25(OH)D status across these tertiles. Additionally, Prevalence Ratios (PR) for serum 25(OH)D levels ≥50 nmol/L were determined by Cox proportional hazards regression with robust error variance and tertile 1 as a reference group. The hazard ratio obtained from this analysis is presented as a PR because a constant risk period was assigned to all study subjects [25]. All models were adjusted for appropriate covariates. The PRs were further investigated by restricted cubic spline regression, with knots set at the 1st, 5th and 9th decile of intake. Analyses were executed using SAS, version 9.2 statistical software (SAS Institute Inc., Cary, NC, USA) and a *P*-value of ≤0.05 (two-sided) was determined to be statistically significant.

RESULTS

Table 3.1 presents the participant characteristics of the study population. The mean age of the total study population was 72 ± 5 years and 58% were men. Mean (SD) BMI was 26.9 ± 3.6 kg/m^2, serum 25(OH)D was 61 ± 26 nmol/L, and 40% of the participants were included during winter/spring. Participants with an adequate serum 25(OH)D concentration (64%) were more likely to be men (62% *versus* 52%) and more likely to be younger (71 ± 5 *versus* 73 ± 6 years), compared to participants with an inadequate serum 25(OH)D status (<50 nmol/L). Participants included in the higher age category (≥70 years) had significantly lower serum 25(OH)D concentrations (59 ± 25 *versus* 64 ± 27 nmol/L) compared to those in the younger age category (<70 years).

Table 3.2 describes the dietary intake of the total study population stratified by serum 25(OH)D status and age. The study population had a mean fat intake of 36 ± 6 En%, protein intake of 15 ± 2, carbohydrate intake of 44 ± 7, and fiber intake of 24 ± 7 (data not shown in tables). Total median (25-75th percentile) vitamin D intake was 4.3 (3.2-5.8) μg/day, of which 4.0 (3.0-5.4) μg/day from foods. When the different food sources of vitamin D were examined, butter and margarine were the main contributors to total dietary vitamin D intake, with a median of 1.8 (0.9-2.9) μg/day (comprising 45% of dietary vitamin D intake). Fish and shellfish intake was the second most contributing dietary vitamin D source, with a median intake of 0.56 (0.22-1.04) μg/day, followed by meat intake, with a median intake of 0.40 (0.27-0.52) μg/day. Furthermore, participants with adequate serum 25(OH)D concentrations (≥50 nmol/L) had significantly higher vitamin D intakes compared to participants with inadequate serum 25(OH)D concentrations (<50 nmol/L), with a median vitamin D intake of 4.7 (3.4-6.3) versus 3.8 (3.0-5.2) μg/day. No significant differences were observed between age categories in total vitamin D intake or supplement use.

Table 3.3 and Table 3.4 show the associations of serum 25(OH)D concentrations by tertiles of total vitamin D intake or intake from specific food sources. A significant association was observed between total vitamin D intake and serum 25(OH)D status, with a 10 nmol/L difference in serum 25(OH)D concentration between the lowest (<3.55 μg/day) and highest (≥5.32 μg/day) tertile of vitamin D intake (Table 3.3). In line with the data indicating butter and margarine as the main contributors to vitamin D intake, these data show that there is also a significant association between butter and margarine and serum 25(OH)D status. Participants with higher butter and margarine intakes (T1 vs. T3: PR 1.0 vs. 1.21 (95%CI: 1.03-1.42), *P*-for trend 0.02) have a 21% higher probability of having an adequate serum 25(OH)D status after adjustment for covariates, compared to participants with lower butter and margarine intakes (Table 3.4). Associations between total vitamin D intake from foods and vitamin D intake from

butter and margarine (modelled continuously) with 25(OH)D adequacy (<50 vs. ≥50 nmol/L) are visualized in Figure 3.1. None of the other vitamin D-food sources were significantly associated with 25(OH)D status.

Table 3.1 Participant characteristics (*n*=595).

	Total	25(OH)D		Age	
		<50 nmol/L	**≥50 nmol/L**	**<70 years**	**≥70 years**
N	595	212	383	225	370
Sex, n men (%)	346 (58)	110 (52)	236 (62)[#]	132 (59)	214 (58)
Age, years	72 ± 5	73 ± 6	71 ± 5[#]	67 ± 2	75 ± 4[#]
BMI, kg/m^2	26.9 ± 3.6	26.9 ± 3.9	26.9 ± 3.4	26.9 ± 3.7	26.9 ± 3.6
Education, n (%)					
Primary	251 (42)	99 (47)	152 (40)	94 (42)	157 (42)
Secondary	144 (24)	52 (25)	92 (24)	56 (25)	88 (24)
Higher	200 (34)	61 (29)	139 (36)	75 (33)	125 (34)
Smoking, n (%)					
Non-smoker	184 (31)	61 (29)	123 (32)	76 (34)	108 (29)
Smoker	62 (10)	27 (13)	35 (9)	27 (12)	35 (10)
Former Smoker	349 (59)	124 (58)	225 (59)	122 (54)	227 (61)
Alcohol intake, n (%)					
Light	379 (64)	156 (74)	223 (58)[#]	135 (60)	244 (66)
Moderate	198 (33)	48 (23)	150 (39)	80 (36)	118 (32)
Excessive	18 (3)	8 (4)	10 (3)	10 (4)	8 (2)
Physical activity, min/day	128 (84-193)	123 (83-193)	131 (85-193)	127 (81-194)	129 (85-191)
Serum 25(OH)D, nmol/L	61 ± 26	35 ± 11	75 ± 20[#]	64 ± 27	59 ± 25[#]
Winter/Spring, n (%)	235 (40)	114 (54)	121 (32)	74 (33)	161 (44)

BMI, Body Mass Index; 25(OH)D, 25-hydroxyvitamin D. Values represent mean ± SD, or medians (25-75th percentile). [#]Significant difference between groups $P \leq 0.05$.

Table 3.2 Total vitamin D intake and vitamin D intake from specific food sources in a population of older Dutch adults (*n*=595).

	Total	25(OH)D		Age	
		<50 nmol/L	**≥50 nmol/L**	**<70 years**	**≥70 years**
N	595	212	383	225	370
Energy intake, kcal/day	2005 ± 475	1933 ± 425	2044 ± 496[#]	2016 ± 452	1998 ± 488
Total vitamin D intake, µg/day	4.3 (3.2-5.8)	3.8 (3.0-5.2)	4.7 (3.4-6.3)[#]	4.3 (3.0-5.9)	4.4 (3.3-5.8)
Vitamin D supplements, µg/day	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Vitamin D from food sources					
Total foods, µg/day	4.0 (3.0-5.4)	3.7 (2.8-4.8)	4.3 (3.0-5.7)[#]	3.9 (2.8-5.5)	4.1 (3.1-5.3)
Meat, µg/day	0.40 (0.27-0.52)	0.38 (0.25-0.49)	0.42 (0.28-0.53)[#]	0.43 (0.29-0.54)	0.39 (0.26-0.51)[#]
Fish and shellfish, µg/day	0.56 (0.22-1.04)	0.52 (0.16-0.97)	0.58 (0.28-1.06)	0.58 (0.26-1.11)	0.56 (0.19-1.01)
Eggs, µg/day	0.25 (0.13-0.25)	0.25 (0.13-0.25)	0.25 (0.13-0.38)[#]	0.25 (0.13-0.25)	0.25 (0.13-0.38)
Dairy, µg/day	0.29 (0.20-0.41)	0.29 (0.18-0.41)	0.29 (0.20-0.41)	0.31 (0.20-0.41)	0.27 (0.18-0.41)
Milk, µg/day	0.04 (0.01-0.09)	0.04 (0.01-0.08)	0.04 (0.02-0.09)	0.04 (0.02-0.08)	0.04 (0.01-0.09)
Yogurt, µg/day	0.02 (0.00-0.05)	0.01 (0.00-0.05)	0.02 (0.00-0.05)	0.02 (0.00-0.05)	0.02 (0.00-0.05)
Cheese, µg/day	0.14 (0.09-0.24)	0.14 (0.08-0.21)	0.15 (0.09-0.24)	0.16 (0.10-0.25)	0.14 (0.08-0.22)[#]
Butter and margarine, µg/day	1.8 (0.9-2.9)	1.6 (0.7-2.6)	1.9 (1.0-3.1)[#]	1.7 (0.8-2.9)	1.8 (0.9-2.8)

Values represent mean ± SD, or medians (25-75th percentile). [#]Significant difference between groups $P \leq 0.05$.

Table 3.3 The association between vitamin D intake from different food sources and serum 25-hydroxyvitamin D status in older Dutch adults.

	Tertile 1	**Tertile 2**	**Tertile 3**	***P* for trend**
Total vitamin D intake, µg/day	<3.55	3.55-5.31	≥5.32	
Serum 25(OH)D, nmol/L	57 (48 ; 65)	61 (53 ; 69)	67 (59 ; 76)	0.0004
Meat				
Total intake, g/day	44 ± 23	88 ± 16	122 ± 26	
Vitamin D intake, µg/day	<0.32	0.32-0.47	≥0.48	
Serum 25(OH)D, nmol/L	60 (52 ; 69)	61 (53 ; 69)	62 (54 ; 71)	0.73
Fish and shellfish				
Total intake, g/day	5 ± 6	14 ± 5	33 ± 24	
Vitamin D intake, µg/day	<0.34	0.34-0.83	≥0.84	
Serum 25(OH)D, nmol/L	59 (50 ; 67)	63 (54 ; 71)	63 (54 ; 71)	0.17
Eggs				
Total intake, g/day	3 ± 2	11 ± 3	33 ± 16	
Vitamin D intake , µg/day	<0.13	0.13-0.24	≥0.25	
Serum 25(OH)D, nmol/L	61 (52 ; 69)	61 (53 ; 69)	61 (52 ; 70)	0.66
Dairy				
Total intake, g/day	251 ± 147	342 ± 151	402 ± 176	
Vitamin D intake, µg/day	<0.23	0.23-0.36	≥0.36	
Serum 25(OH)D, nmol/L	61 (53 ; 70)	63 (55 ; 71)	59 (51 ; 68)	0.32
Milk				
Total intake, g/day	65 ± 100	146 ± 90	294 ± 123	
Vitamin D intake , µg/day	<0.02	0.02-0.05	≥0.06	
Serum 25(OH)D, nmol/L	62 (53 ; 70)	61 (52 ; 69)	62 (53 ; 70)	0.89
Yogurt				
Total intake, g/day	66 ± 75	77 ± 71	160 ± 87	
Vitamin D intake, µg/day	0	0.01-0.03	≥0.04	
Serum 25(OH), nmol/L	58 (50 ; 66)	64 (56 ; 73)	64 (56 ; 73)	0.02
Cheese				
Total intake, g/day	16 ± 10	31 ± 10	62 ± 26	
Vitamin D intake, µg/day	<0.11	0.11-0.19	≥0.20	
Serum 25(OH)D, nmol/L	60 (62 ; 69)	62 (53 ; 70)	62 (54 ; 70)	0.84
Butter and margarine				
Total intake, g/day	13 ± 11	27 ± 16	47 ± 15	
Vitamin D intake, µg/day	<1.16	1.16-2.50	≥2.51	
Serum 25(OH)D, nmol/L	58 (50 ; 66)	59 (51 ; 68)	66 (57 ; 74)	0.01

25(OH)D, 25-hydroxyvitamin D. Values represent adjusted means (95% CIs) calculated by ANCOVA, adjusted for age, sex, BMI, smoking, alcohol intake, education, physical activity level, season, energy intake and vitamin D intake from other food categories. Values for total food group intakes represent mean ± SD.

Table 3.4 Prevalence ratios (95% CIs) for vitamin D adequacy (25(OH)D ≥50 nmol/L) by tertiles of vitamin D food sources.

	25-hydroxyvitamin D ≥50 nmol/L (*n*=383)		
Food sources of vitamin D	**Model 1**	**Model 2**	**Model 3**
Total vitamin D intake, μg/day			
<3.55	1 (ref)	1 (ref)	1 (ref)
3.55-5.31	1.14 (0.96-1.34)	1.13 (0.97-1.32)	1.13 (0.97-1.32)
≥5.32	1.35 (1.16-1.57)	1.31 (1.13-1.52)	1.31 (1.13-1.52)
P for trend	<0.0001	0.0002	0.0004
Vitamin D intake from meat, μg/day			
<0.32	1 (ref)	1 (ref)	1 (ref)
0.32-0.47	0.98 (0.84-1.15)	1.01 (0.87-1.18)	0.98 (0.83-1.14)
≥0.48	1.13 (0.98-1.31)	1.14 (0.99-1.32)	1.09 (0.94-1.27)
P for trend	0.08	0.06	0.23
Vitamin D intake from fish and shellfish, μg/day			
<0.34	1 (ref)	1 (ref)	1 (ref)
0.34-0.83	1.06 (0.92-1.24)	1.02 (0.88-1.18)	1.03 (0.89-1.20)
≥0.84	1.10 (0.95-1.28)	1.05 (0.91-1.22)	1.06 (0.92-1.23)
P for trend	0.20	0.50	0.42
Vitamin D intake from eggs, μg/day			
<0.13	1 (ref)	1 (ref)	1 (ref)
0.13-0.24	1.14 (0.96-1.36)	1.09 (0.92-1.29)	1.08 (0.91-1.27)
≥0.25	1.17 (0.96-1.41)	1.11 (0.92-1.35)	1.11 (0.92-1.34)
P for trend	0.15	0.31	0.31
Vitamin D intake from dairy, μg/day			
<0.23	1 (ref)	1 (ref)	1 (ref)
0.23-0.36	1.01 (0.88-1.17)	1.00 (0.87-1.15)	1.00 (0.86-1.16)
≥0.36	1.00 (0.87-1.16)	0.99 (0.85-1.14)	0.96 (0.82-1.13)
P for trend	0.98	0.95	0.88
Vitamin D intake from milk, μg/day			
<0.02	1 (ref)	1 (ref)	1 (ref)
0.02-0.05	1.00 (0.87-1.16)	1.03 (0.89-1.18)	1.01 (0.88-1.17)
≥0.06	1.02 (0.88-1.18)	1.04 (0.91-1.19)	1.02 (0.88-1.17)
P for trend	0.79	0.58	0.83
Vitamin D intake from yogurt, μg/day			
0	1 (ref)	1 (ref)	1 (ref)
0.01-0.03	1.11 (0.96-1.29)	1.13 (0.98-1.30)	1.13 (0.98-1.30)
≥0.04	1.12 (0.96-1.29)	1.12 (0.97-1.29)	1.14 (0.98-1.31)
P for trend	0.19	0.17	0.12
Vitamin D intake from cheese, μg/day			
<0.11	1 (ref)	1 (ref)	1 (ref)
0.11-0.19	1.02 (0.88-1.18)	1.01 (0.87-1.17)	1.01 (0.87-1.17)
≥0.20	1.05 (0.91-1.22)	1.01 (0.88-1.16)	1.01 (0.87-1.17)
P for trend	0.46	0.93	0.93
Vitamin D intake from butter and margarine, μg/day			
<1.16	1 (ref)	1 (ref)	1 (ref)
1.16-2.50	1.09 (0.93-1.27)	1.09 (0.93-1.27)	1.08 (0.93-1.26)
≥2.51	1.20 (1.03-1.39)	1.22 (1.05-1.42)	1.21 (1.03-1.42)
P for trend	0.02	0.007	0.02

Model 1: incl. covariates for age and sex. Model 2: incl. covariates of model 1 plus BMI, smoking alcohol intake, education, physical activity level, and season. Model 3: incl. covariates of model 1 and 2 plus energy intake and vitamin D intake from other food categories.

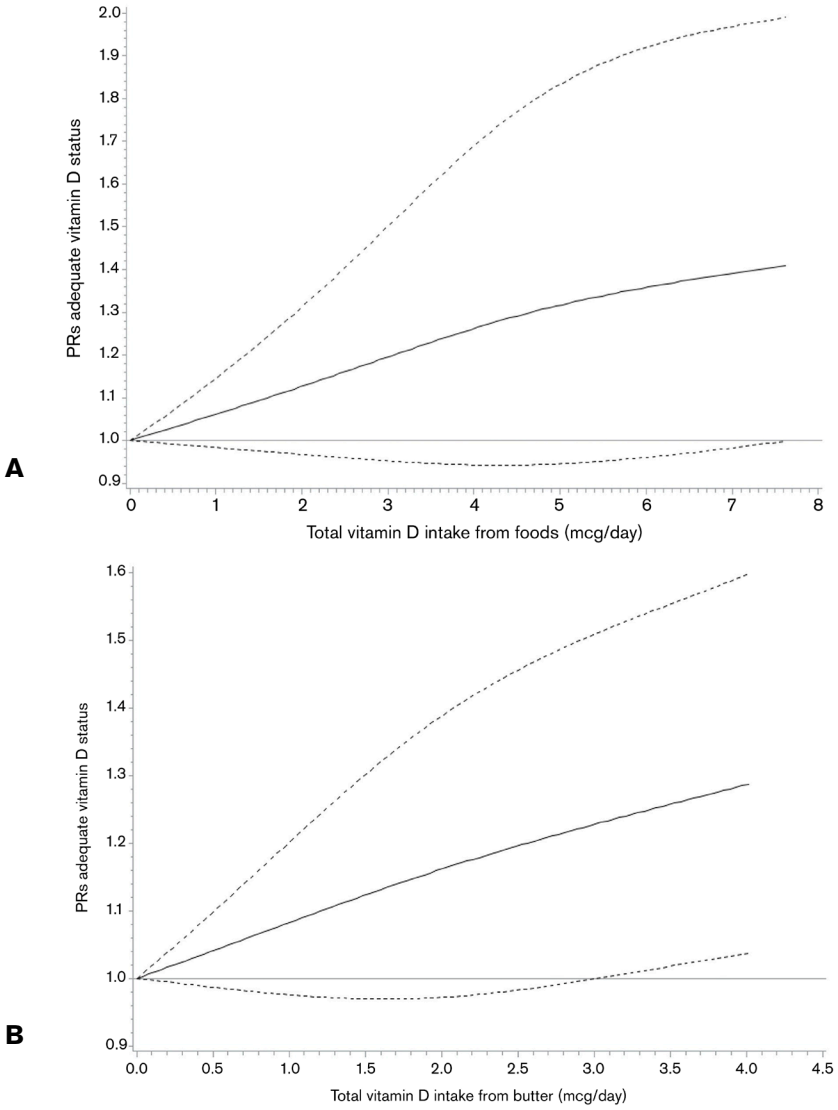

Figure 3.1 Associations between vitamin D-intake and serum 25-hydroxyvitamin D concentrations ≥50 nmol/L (i.e. defined as adequate vitamin D status).

Graphs represent Prevalence Ratios incl. 95% CIs. Models incl. covariates for age, sex, BMI, smoking, alcohol intake, education, physical activity level, season, energy intake and vitamin D intake from other food categories. A: P for non-linearity 0.37. B: P for non-linearity 0.59.

DISCUSSION

Our analyses showed that mainly butter and margarine contributed to the total vitamin D intake in this Dutch community-dwelling older population. Fish and shellfish intake was the second most important contributor to the total vitamin D intake, although comprising less than half the amount of vitamin D obtained from butter and margarine. Both total vitamin D intake as well as vitamin D intake from butter and margarine were positively associated with higher serum 25(OH)D concentrations after full adjustment for potential covariates.

Several methodological considerations should be addressed before further discussing these findings. Although the FFQ used in this study was not validated to estimate vitamin D intake, the method to compose the FFQ was validated [26, 27]. As our estimated vitamin D intakes are in agreement with data obtained by two 24-hour recalls of the Dutch Food Consumption Survey 2013, we assume an accurate estimate of total vitamin D intake [19]. A strength of our study includes the opportunity to not only analyze intake data, but also to link these data to serum 25(OH)D concentrations, while accounting for a broad set of potential covariates.

This study shows a high prevalence of inadequate vitamin D intake (median intake: 4.3 μg/day) in older Dutch adults. Of the total vitamin D intake reported in this study, 4.0 μg/day originated from the diet. This daily intake is intermediate in comparison to the intake of European countries, with intakes ranging between 2-15 μg/day [28]. The NHANES cohort showed total vitamin D intakes of 10.7 μg/day and 10.0 μg/day in American men and women aged >71y, respectively [29]. When vitamin D from supplements was excluded, mean vitamin D intakes were still higher compared to our population, that is 4.5 μg/day in women and 5.6 μg/day in men. Additionally, a Canadian cross-sectional study showed total vitamin D intakes of 8.2 μg/day and 13.6 μg/day in men and women, respectively [30]. Also in this study, higher vitamin D intakes predominantly related to higher supplemental vitamin D intakes. Specifically, supplements accounted for 56% of the total vitamin D intake, with 45% of women and 17% of men using a supplement. In our study population, only 12% of the population used a vitamin D supplement. Moreover, higher vitamin D intakes in the US and Canada may also be explained by higher intakes of fortified products. In the US and Canada, dairy products, especially fortified milk, are considered the main food source of vitamin D intake, followed by meat and fish [30-32].

Despite relatively low vitamin D intakes in our population total vitamin D intake was significantly associated with vitamin D status. Our data also indicated that butters or margarines are the most important sources to increase serum 25(OH)D status.

Participants in the highest tertile of butter and margarine intake, representing an intake of 47 g/day (equalling 4 sandwiches with fat spread), had a 21% higher probability of having a sufficient vitamin D status. Although fish intake was the second major contributor to dietary vitamin D intake, higher fish intake was not significantly associated with higher serum 25(OH)D levels. A recent meta-analysis published by Lehman et al. investigated the effect of fish intake on serum 25(OH)D concentrations [33]. The authors showed that the consumption of ± 300 g fish/week over a period of at least 4 weeks, was associated with a significant increase in serum 25(OH)D concentrations. The non-significant association observed in this study may relate to the relatively low intake of fish in this study group (median 13 (25 -75th percentile: 7-21) g fish/day).

In the presence of adequate cutaneous vitamin D synthesis, adequate vitamin D supplement intake, and consumption of fortified foods, the importance of vitamin D intake from foods is likely to be diminished. However, according to our data, the use of supplements and fortified products is limited among older Dutch adults. As such, the total vitamin D intake lies far below the Dutch dietary reference value, currently set at an Adequate Intake (AI) of 10 μg/day for adults <70 years, and Recommended Dietary Allowance (RDA) of 20 μg/day for adults ≥70 years (based on the assumption of insufficient sunlight exposure) [9]. In our population, only 4 participants consumed at least 10μg vitamin D day. Since 2007, the Dutch commodities act allows the addition of vitamin D to food products other than margarine (to 4.5 μg/100 kcal of product). However, food fortification is currently hardly practiced. A recent report by the National Institute for Public Health and the Environment (RIVM) shows that the fortification strategies in the Netherlands could be optimized without exceeding the tolerable upper intake level in the general Dutch population [34]. The scenario analysis indicated that the Dietary Reference Intake (DRI) could be met by >80% of the older Dutch adults when 5 μg of vitamin D would be added per 100 g of milk or yogurt, and 25 μg would be added per 100 g of margarines. Thus, food fortification in combination with the promotion of vitamin D supplement use may substantially improve the 25(OH)D status in older Dutch adults. However, as shown by our data, regular intake of foods high in vitamin D could also support an increase in vitamin D status across the general population, particularly in case of modest 25(OH)D insufficiency. Also the observed low fish/shellfish intake shows room for improving the dietary vitamin D intake. Nevertheless, for the older adults with more severe 25(OH)D deficiency, the habitual diet will not suffice in the total amount of vitamin D needed to meet the recommendations. Therefore, policies should focus on health messages regarding food fortification and vitamin D supplementation specifically targeted to this age group.

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Conflict of interest: The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results. EM Brouwer-Brolsma and LCPGM de Groot have filed a patent on the effect of vitamin D on cognitive executive function. P Lips and NM van Schoor received an unconditional grant of Merck and Co for the assessment of vitamin D in Longitudinal Aging Study Amsterdam (LASA). The other authors have no conflicts of interest to declare.

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CHAPTER 4

Dose-response effects of supplementation with calcifediol on serum 25-hydroxyvitamin D status and its metabolites: a randomized controlled trial in older adults

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ABSTRACT

Background & aims: Oral supplementation with vitamin D is recommended for older adults to maintain a sufficient 25-hydroxyvitamin D (25(OH)D) status throughout the year. While supplementation with vitamin D_2 or D_3 is most common, alternative treatment regimens exist which require further investigation with respect to increasing 25(OH)D concentration. We investigated the dose-response effects of supplementation with calcifediol compared to vitamin D_3 and assessed the dose which results in mean serum $25(OH)D_3$ concentrations between 75–100 nmol/L.

Methods: This randomized, double-blind intervention study included men and women aged ≥65 years (*n*=59). Participants received either 5, 10 or 15 μg calcifediol or 20 μg vitamin D_3 per day, for a period of 24 weeks. Blood samples were collected every four weeks to assess response profiles of vitamin D related metabolites; serum vitamin D_3, $25(OH)D_3$, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$). Further, serum calcium, plasma parathyroid hormone, and urinary calcium were evaluated.

Results: Supplementation with 20 μg vitamin D_3 increased $25(OH)D_3$ concentrations towards 70 nmol/L within 16 weeks. Supplementation with 10 or 15 μg calcifediol increased $25(OH)D_3$ levels >75 nmol/L in 8 and 4 weeks, respectively. Steady state was achieved from week 12 onwards with serum $25(OH)D_3$ levels stabilizing between 84-89 nmol/L in the 10 μg calcifediol group. A significant association was observed between the changes in $25(OH)D_3$ and $24,25(OH)_2D_3$ (R^2=0.83, P<0.01), but not between $25(OH)D_3$ and $1,25(OH)_2D_3$ (R^2=0.04, P=0.18). No cases of hypercalcemia occurred in any treatment during the study period.

Conclusions: Calcifediol supplementation rapidly and safely elevates serum $25(OH)D_3$ concentrations to improve vitamin D status in older adults. A daily dose of 10 μg calcifediol allows serum $25(OH)D_3$ concentrations to be maintained between 75–100 nmol/L.

INTRODUCTION

Vitamin D deficiency is common worldwide, and particularly prevalent in the elderly [1-4]. A deficiency can be caused by environmental and age-related factors, affecting vitamin D uptake or metabolism. Vitamin D can be obtained from the diet as vitamin D_2 (ergocalciferol) or D_3 (cholecalciferol). However, relatively few foods contain vitamin D, and therefore the dietary intake is considered low. As such, vitamin D_3 is mainly acquired after sun exposure, as it can be synthesized from 7-dehydrocholestrol after cutaneous exposure to ultraviolet-B radiation [5]. However, production of vitamin D_3 is often limited to the summer months [6], and depends on many behavioral factors, such as outdoor activities and clothing [7], as well as the capacity of the skin to synthesize vitamin D_3, which is suggested to be decreased in older adults [8, 9]. To be biologically active, vitamin D is hydroxylated by the liver into the prehormone 25-hydroxyvitamin D (25(OH)D) and converted primarily in the kidney to the active hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which acts upon a broad variety of cells in the body. These metabolites can be further hydroxylated in the kidney into the inactive metabolite 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) and as such regulate the available pool and synthesis of 25(OH)D and $1,25(OH)_2D$. However, several factors such as, a declined hepatic or renal function [10, 11] can affect the metabolism of vitamin D and can increase the risk of vitamin D deficiency. Current recommendations show no consensus with regard to the optimal vitamin D status, with the Institute of Medicine (IOM) defining serum 25(OH)D levels of 50 nmol/L as adequate and others advocating a threshold of 75 nmol/L [12-14]. However, all agree that vitamin D supplements are needed to meet requirements in the older population. Supplementation with vitamin D_2 or D_3 is currently most common. However, supplementation with calcifediol, the $25(OH)D_3$ metabolite, might be considered as well. As calcifediol is more hydrophilic and already hydroxylated, it can present an effective supplementation strategy in cases of malabsorption or impaired hepatic function [15]. Previous studies have demonstrated that calcifediol is more potent in increasing serum $25(OH)D_3$ status compared to native vitamin D_3 [15-19]. This makes it an interesting alternative to be considered in the older population. However, additional clinical trials are needed to establish the appropriate dosing and safety of calcifediol supplementation in this population. Therefore, we investigated the dose-response effects of calcifediol compared to vitamin D_3 on serum $25(OH)D_3$ and its metabolites in people aged 65 years or older.

METHODS

Trial design

This study was a double-blind trial including subjects randomly assigned to either 5, 10 or 15 μg calcifediol or 20 μg vitamin D_3 per day. The full study covered a screening visit and a 24 week intervention period including monthly visits to measure vitamin D metabolites and to monitor safety parameters. Randomization was carried out by an independent researcher using SAS software 9.20, with stratification on BMI (20–29, 30–35 kg/m^2) and permuted blocks of 4. All subjects and researchers remained blinded to treatment assignment until data collection and analyses were completed. The study was carried out in Wageningen, the Netherlands (latitude 51°N), between 26th of August 2013 and 30th of April 2014. The study protocol was approved by the Medical Ethics Committee of Wageningen UR and written informed consent was provided by all participants. The study was registered at clinicaltrials.gov as NCT01868945 and was performed according to ICH-GCP.

Participants

Subjects were recruited via registries of municipalities and invited for a screening visit to measure eligibility according inclusion and exclusion criteria. Subjects were included if they were 65 years or older, had a serum 25(OH)D_3 concentration between 25 and 50 nmol/L and a body mass index between 20 and 35 kg/m^2. Exclusion criteria were a serum calcium level >2.6 mmol/L, diagnosis with kidney stones in the past 10 years, renal insufficiency, liver failure, malabsorption syndromes, sarcoidosis and primary hyperparathyroidism. Use of medication that might interfere with vitamin D metabolism led to exclusion (e.g. thiazides, parathyroid hormone, bisphosphonates). In addition, subjects were excluded if they consumed >3 alcoholic beverages per day, used vitamin D supplements in the three months prior to the screening visit, were not willing to stop the use of multivitamins during the study, were expected to increase sun exposure (e.g. planned holiday to a sunny resort), were blood donor or had a surgery planned.

Intervention

Study supplements were hard gelatin capsules that were identical in appearance and taste. DSM Nutritional Products Ltd. provided calcifediol or vitamin D_3 in spray-dried form, and supplements were manufactured by Fisher Clinical Services GmbH. The Analytical Research Centre of DSM Nutritional Products tested the capsules using high performance liquid chromatography analysis (HPLC). The actual content of the capsules was: 5.1, 10.3 and 15.3 μg calcifediol or 22.3 μg vitamin D_3. At the start of the study, subjects were instructed to consume one capsule per day at breakfast. Compliance was assessed by capsule count every two months. Subjects were considered compliant when ≥80% of the supplements were taken during the intervention.

Measurements

Laboratory analyses

All blood samples were collected in a fasted state in the morning and stored at -80 ˚C until analysis. At screening, serum 25(OH)D_3 samples were analyzed using isotope dilution-online solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS) (VU Medical Centre, Amsterdam, the Netherlands) [20]. At baseline and every 4 weeks during the intervention period, a more comprehensive analysis was performed. Serum albumin and calcium were measured by colorimetric analysis to monitor albumin-corrected calcium [21]. EDTA blood samples were used to measure intact PTH by sandwich chemiluminescence immunoassay. In addition, morning spot-urine was collected to monitor urinary calcium levels (expressed as calcium/creatinine ratio) (SHO laboratory, Velp, the Netherlands). Vitamin D metabolites, i.e. serum vitamin D_3, 25(OH)D_3, 1,25$(OH)_2D_3$, 24,25$(OH)_2D_3$ were analyzed at the end of the study using LC/MS/MS (Analytical Research Center, DSM Nutritional Products, Kaiseraugst, Switzerland). The inter-assay and intra-assay CVs were ≤15%. Due to sensitivity reasons, the Lower Limit of Quantitation (LLQ) for the baseline measurement of vitamin D_3 had to be increased from 1.3 to 2.6 nmol/L in 56 out of 59 baseline blood samples. Besides, analysis of vitamin D_3 and 1,25$(OH)_2D_3$ showed several laboratory values below the calibration point, these values are set at the LLQ for data interpretation. The method of analysis lacked sensitivity to accurately measure low concentrations of 25(OH)D_2 as 37 out of 59 samples were below the detection limit at baseline. Therefore, we restrict our analysis to the reporting of D_3-related metabolites. All laboratory analyses were performed blinded to treatment allocation.

Questionnaires

Participants filled out a comprehensive questionnaire during the screening visit. Medical history, medication, dietary supplement use, alcohol consumption (number of alcoholic drinks per week) and smoking habits (current, former, never) were assessed. During the intervention phase, subjects filled out a questionnaire every 4 weeks to monitor changes in health status or medication use. Dietary vitamin D and calcium intake were recorded using a Food Frequency Questionnaire (FFQ) at baseline. This FFQ was developed using validated FFQs that were updated to facilitate the reporting of habitual vitamin D and calcium intake [22-24].

Anthropometrics

Weight was measured during each study visit, using a calibrated analogue scale and without wearing heavy clothing. Weight was reported to the nearest 0.5 kg. Height was measured at screening, baseline and at the end of the study. Height was measured using a stadiometer and reported to the nearest 0.1 cm. Body mass index (BMI) was reported as weight/height^2.

Statistical methods

Sample size was based on a publication by Cashman et al., 2012, of 56 adults, aged ≥50 years who completed a 10-week intervention receiving either 20 μg vitamin D_3, 7 or 20 μg calcifediol or a placebo [17]. From this publication, we derived the serum 25(OH)D response per μg calcifediol to estimate the mean response after 10 weeks with doses of 5, 10 and 15 μg calcifediol. The primary study objective was to determine which of these doses would result in mean serum 25(OH)D concentrations between 75–100 nmol/L. For a calcifediol dose of 10 μg/day, a mean ± SD serum 25(OH)D level of 84 ± 18 nmol/L was predicted. When including 14 subjects per group, the expected standard error of the mean was therefore 4.8 nmol/L (95% CI of ± 10 nmol/L). This was considered an acceptable degree of uncertainty and 60 subjects were randomized. Baseline characteristics were described as mean, SD or percent of categorical class and compared between treatment groups using one-way ANOVA or Chi-Square test. Linear regression was used to quantify the association between variables. Analyses of the dose-response in vitamin D metabolites were performed as pre-specified in the study protocol, i.e. by using subjects who completed the trial and had no major protocol deviations (per-protocol). Safety parameters were analyzed with all available data (intention-to-treat). Response profiles of each outcome variable were analyzed using mixed model analysis. Fixed effects were treatment, time (week) and the interaction of treatment x time. All models included a random effect for subject. The baseline level of the response variable and BMI were included as covariates in all models. Results were expressed as model predicted means including 95% CIs. In addition, steady state of serum 25(OH)D_3 concentration was examined in each group by ANOVA contrasts, comparing each time point versus the final time point by Bonferroni post-hoc tests, using 5 contrasts. Steady state was determined by the last non-significant contrast. Statistical tests were all two-sided and carried out at the 5% level of significance. Data analyses were performed using SPSS (version 19) and graphs by using Graphpad Prism (version 5).

RESULTS

In total, 481 subjects were screened for study participation and 60 subjects were randomized (Figure 4.1). However, one of these randomized subjects did not receive treatment due to violation of eligibility criteria and thus 59 subjects started the intervention. After enrolment, 5 subjects discontinued their participation and 54 subjects completed the study. In addition, 3 subjects were excluded from the per-protocol analysis due to major protocol deviations (Figure 4.1).

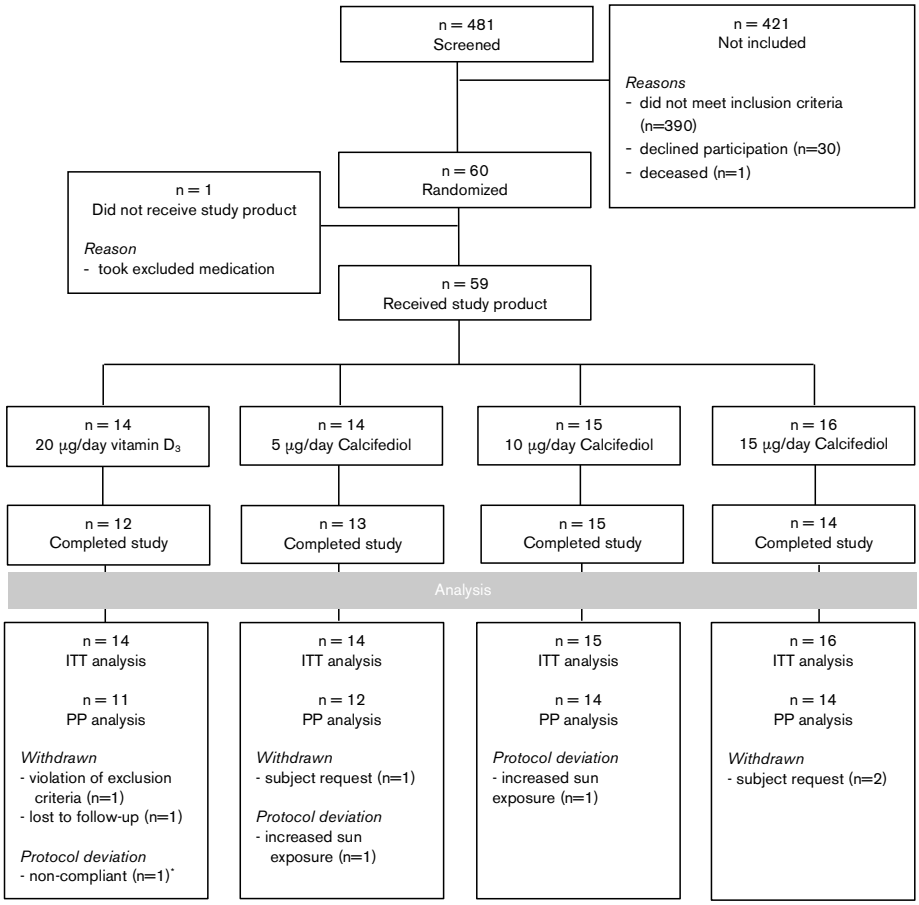

Figure 4.1 Flow-chart of subjects.

*Non-compliant: <80% of the supplements were taken during the intervention. ITT, intention-to-treat; PP, per-protocol.

Table 4.1 shows the population characteristics per treatment group. The mean age of the total study population was 79 ± 7.1 years and 53% were men. Mean baseline serum 25(OH)D_3 concentration was 39.4 ± 11.9 nmol/L and there were no significant differences in baseline 25(OH)D_3 concentration between treatment groups (*P*=0.56). Mean dietary vitamin D and calcium intakes were 3.6 ± 1.4 μg/day and 1087 ± 402 mg/day, respectively. Average compliance of the study population was 97%, 58 subjects had a compliance of 80% or higher.

Table 4.1 Baseline characteristics by treatment group.[1]

	Vitamin D_3 20 μg/d (*n*= 14)	Calcifediol 5 μg/d (*n*= 14)	Calcifediol 10 μg /d (*n*= 15)	Calcifediol 15 μg/d (*n*= 16)	Total (*n*= 59)	*P* [2]
Demographics						
Age (y)	78 (7.7) [3]	80 (7.3)	79 (7.0)	80 (7.0)	79 (7.1)	0.81
Gender (M), % (n)	36 (5)	57 (8)	60 (9)	56 (9)	53 (31)	0.55
Weight (kg)	78 (11.1)	74 (11.8)	74 (11.0)	76 (10.7)	76 (11.0)	0.73
BMI (kg/m^2)	27.6 (3.5)	26.0 (4.4)	26.6 (3.7)	26.8 (3.9)	26.8 (3.8)	0.74
Alcohol intake, % (n)[4]						
Light	71 (10)	79 (11)	93 (14)	75 (12)	80 (47)	
Moderate	29 (4)	21 (3)	7 (1)	25 (4)	20 (12)	0.47
Excessive	-	-	-	-	-	
Smoking						
Non-smokers	43 (6)	36 (5)	40 (6)	31 (5)	37 (22)	
Current smokers	7 (1)	-	-	-	2 (1)	0.68
Ex-smokers	50 (7)	64 (9)	60 (9)	69 (11)	61 (36)	
Laboratory parameters						
Vitamin D_3 (nmol/L)[5]	<LLQ	<LLQ	<LLQ	<LLQ	<LLQ	
25(OH)D_3 (nmol/L)	37.7 (7.0)	43.4 (15.8)	38.3 (10.5)	38.6 (12.9)	39.4 (11.9)	0.56
1,25$(OH)_2D_3$ (pmol/L)	79.3 (17.2)[6]	68.0 (19.2)[7]	77.5 (22.2)[6]	79.4 (19.6)[6]	76.2 (19.7)	0.36
24,25$(OH)_2D_3$ (nmol/L)	5.5 (2.1)	7.9 (3.8)	6.2 (3.0)	6.6 (2.8)	6.5 (3.0)	0.20
PTH (pmol/L)	5.2 (1.9)	5.7 (1.7)	4.9 (1.3)	4.9 (1.8)	5.2 (1.7)	0.55
Calcium (mmol/L)[8]	2.2 (0.1)	2.2 (0.1)	2.2 (0.1)	2.2 (0.1)	2.2 (0.1)	0.92
UCa/Cr ratio (mmol/mmol)	0.5 (0.3)	0.3 (0.2)	0.3 (0.2)	0.4 (0.2)	0.4 (0.2)	0.29
Dietary intake						
Vitamin D (μg/day)	3.7 (1.2)	4.2 (1.6)	3.3 (1.3)	3.5 (1.5)	3.6 (1.4)	0.36
Calcium (mg/day)	985 (438)	1204 (487)	1041 (293)	1111 (386)	1087 (402)	0.53

[1]LLQ, Lower Limit of Quantitation; UCa/Cr, Urinary Calcium/Creatinine. [2]Between group differences explored by one-way ANOVA or chi-square test. [3]Mean; SD in parentheses (all such values). [4]Light: ≤7 drinks, moderate: 8-21 drinks, severe >21 drinks per week. [5]All measured laboratory values were below the calibration point of 1.3 nmol/L (or 2.6 nmol/L for samples with sensitivity issues). [6]Laboratory value of 1 subject was below the calibration point, and this value was set at the LLQ of 48 pmol/L for data interpretation. [7]Laboratory values of 2 subjects were below the calibration point, and thus these values were set at the LLQ of 48 pmol/L for data interpretation. [8]Serum albumin-corrected calcium by the formula (plasma Ca-(0.02x[Alb-40]).

Changes in serum 25(OH)D_3 concentration

Figure 4.2-A presents the changes in serum 25(OH)D_3 status by treatment group throughout the 24 week intervention period. On average, all treatments resulted in an increase of serum 25(OH)D_3 levels >50 nmol/L with a significant treatment x time interaction (*P*=0.00). One month of supplementation already showed large differences in achieved serum 25(OH)D_3 levels, with a mean of 52.4 nmol/L (CI 44.4, 60.5), 67.9 nmol/L (CI 60.5, 75.3), 84.8 nmol/L (CI 77.4, 92.1) and 58.7 nmol/L (CI 50.2, 67.1) in

the 5 μg, 10 μg, 15 μg calcifediol and 20 μg vitamin D_3 group, respectively. Thereafter, serum 25(OH)D_3 levels continued to rise in the 10 μg and 15 μg calcifediol group and 20 μg vitamin D_3 group, while the group receiving 5 μg of calcifediol did not show significant changes over subsequent time points, with an average between 52 and 55 nmol/L. The other treatments all plateaued from week 12 onwards with serum 25(OH)D_3 stabilizing between 69 and 72 nmol/L in the 20 μg vitamin D_3 group, between 84 and 89 nmol/L in the 10 μg calcifediol group and between 106 and 110 nmol/L in the 15 μg calcifediol group over subsequent time points.

Changes in vitamin D related metabolites and PTH

By the end of the study, significantly higher serum vitamin D_3 concentrations were observed in the vitamin D_3 group compared to the calcifediol groups, confirming treatment allocation (Table 4.2). During the study, serum 1,25$(OH)_2D_3$ levels fluctuated in all treatment groups with a gradual increase towards a peak concentration in week 20 (Figure 4.2-B). By the end of the study, there were no significant differences between groups in serum 1,25$(OH)_2D_3$ concentration (Table 4.2). Serum 24,25$(OH)_2D_3$ concentrations increased over time, with a significant treatment x time interaction (P=0.00) (Figure 4.2-C). There was a significant association between the change in 25(OH)D_3 and 24,25$(OH)_2D_3$ (R^2=0.83, P<0.01), but not between 25(OH)D_3 and 1,25$(OH)_2D_3$ (R^2=0.04, P=0.18). During the study, plasma PTH levels fluctuated in all treatment groups, with no significant treatment x time interaction (P=0.39) (Figure 4.2-D). By the end of the study, plasma PTH levels were significantly lower in the 15 μg versus 5 μg calcifediol group (Table 4.2).

Safety results and adverse events

Serum calcium concentrations remained below the reference value of 2.6 mmol/L and no cases of hypercalcemia occurred in any treatment during the study period. Furthermore, there were no significant differences in serum calcium levels or urinary calcium/ creatinine ratios between groups after 24 weeks of supplementation (Table 4.2). A total of 76 adverse events (AEs) occurred in 39 subjects and 8 serious adverse events (SAEs) occurred in 6 subjects. The number of AEs and SAEs did not differ significantly between groups. None of the AEs or SAEs led to discontinuation of the study or changes in supplementation regimen. All SAEs were reviewed by the Ethics Committee and were not related to the study products.

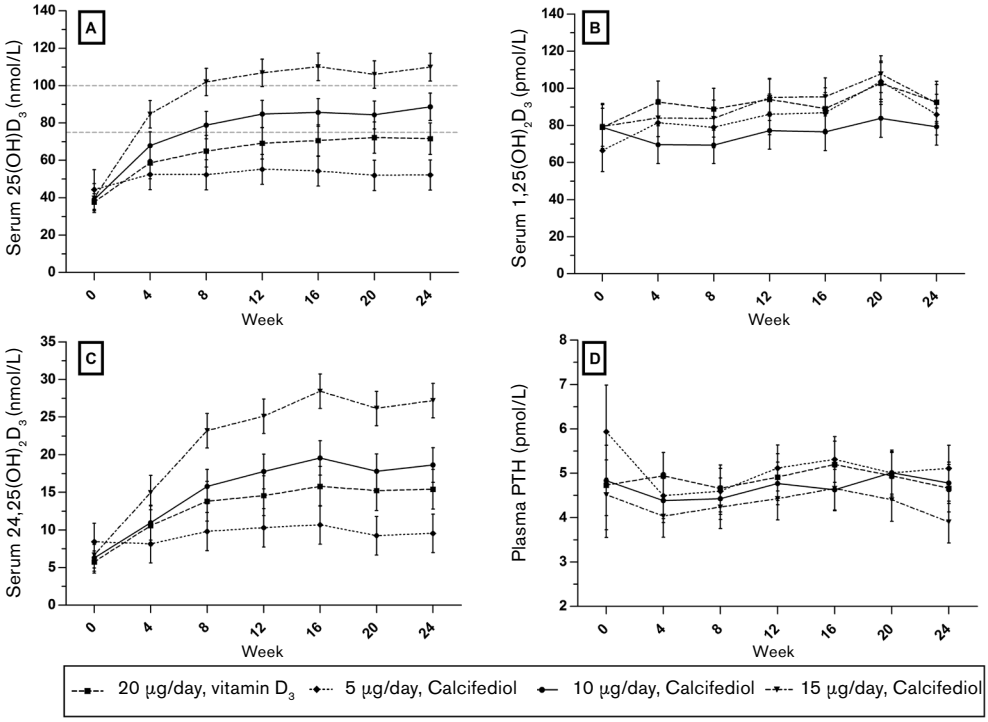

Figure 4.2 Serum concentration time curves of vitamin D metabolites and PTH.

Graph represents unadjusted baseline and model predicted means including 95% CIs (per-protocol). Models are adjusted for BMI and baseline value of the response variable. A) Mean serum $25(OH)D_3$ (nmol/L). Grey dashed lines indicate the reference at 75 and 100 nmol/L. B) Mean serum $1,25(OH)_2D_3$ (pmol/L). 4% of the laboratory results were below the calibration point, and thus these values were set at the LLQ of 48 pmol/L for data interpretation. C) Mean serum $24,25(OH)_2D_3$ (nmol/L). D) Mean plasma PTH (pmol/L).

Table 4.2 End-of-study comparison of laboratory values between groups.[1]

	Vitamin D_3 20 μg/d	**Calcifediol 5 μg/d**	**Calcifediol 10 μg /d**	**Calcifediol 15 μg/d**
Vitamin D_3 (nmol/L)	7.0 (6.4-7.6) [2, 3]	<LLQ[4]	<LLQ[4]	<LLQ[4]
25(OH)D_3 (nmol/L)	71.6 (63.2-80.0)[a]	52.2 (44.4-60.2)[b]	88.7 (81.4-96.1)[c]	109.9 (102.5-117.2)[d]
1,25$(OH)_2D_3$ (pmol/L)	92.4 (81.1-103.7)[a]	85.8 (75.0-93.6)[a, 5]	79.3 (69.3-89.3)[a]	92.0 (82.1-102.0)[a]
24,25$(OH)_2D_3$ (nmol/L)	15.4 (12.8-17.0)[a]	9.5 (7.0-12.1)[b]	18.6 (16.3-20.9)[a]	27.2 (24.9-29.5)[c]
PTH (pmol/L)	4.7 (4.1-5.2)[a, b]	5.1 (4.6-5.6)[a]	4.8 (4.3-5.3)[a, b]	3.9 (3.4-4.4)[b]
Calcium (mmol/L)[6]	2.3 (2.3-2.3)[a]	2.3 (2.3-2.3)[a]	2.3 (2.3-2.3)[a]	2.3 (2.3-2.3)[a]
UCa/Cr ratio (mmol/mmol)	0.4 (0.3-0.5)[a]	0.4 (0.3-0.5)[a]	0.5 (0.4-0.6)[a]	0.5 (0.4-0.6)[a]

[1]LLQ, Lower Limit of Quantitation; UCa/Cr, Urinary Calcium/Creatinine. [2]Model predicted means; 95% CIs in parentheses (all such values). [3]Laboratory value of one subject was below the calibration point, and this value was set at the LLQ of 1.3 nmol/L for data interpretation. [4]Laboratory values of all subjects were below the calibration point of 1.3 nmol/L. [a, b, c, d]Values that have no superscript letter in common are significantly different, $P<0.05$ (Bonferroni-adjusted tests). [5]Laboratory values of 2 subjects were below the calibration point, and thus these values were set at the LLQ of 48 pmol/L for data interpretation. [6] Serum albumin-corrected calcium by the formula (plasma Ca-(0.02x[Alb-40]).

DISCUSSION

This study shows clear differences in response to supplementation with three different dosages of calcifediol. First of all, supplementation with 10 or 15 μg calcifediol resulted in a prompt increase in 25(OH)D_3 concentrations, with serum levels increasing above the threshold of 75 nmol/L after 8 and 4 weeks, respectively. In contrast, a significant longer period (16 weeks) was needed to increase status levels towards 70 nmol/L with 20 μg vitamin D_3, whereby mean concentrations of 75 nmol/L were not achieved. Overall, these data support the results of previous studies with repeated dosing of calcifediol, showing a fast increase in serum 25(OH)D early in the supplementation phase [16, 17, 25]. Supplementation with 5 μg calcifediol appeared to be insufficient to reverse deficiency, as about 50% of the subjects remained below the 50 nmol/L threshold throughout the intervention period. Nevertheless, as the study was mainly performed in the winter months, during which 25(OH)D_3 status normally decreases due to insufficient UV-B exposure, the 5 μg calcifediol dose might have compensated at least this expected seasonal decrease in 25(OH)D_3 status.

Steady state attainment is an important aspect of dose determination when aiming at achieving certain serum concentrations. In a study published by Cashman et al., older adults were supplemented with 7 or 20 μg calcifediol per day over a period of 10 weeks [17]. Serum 25(OH)D concentration increased with 28 nmol/L and 96 nmol/L, respectively. Besides, in a study published by Bischoff-Ferrari et al., postmenopausal women were supplemented with 20 μg calcifediol per day or 140 μg calcifediol per week over a period of 16 weeks [16]. Dose-response effects were comparable for both the daily and weekly supplementation strategy, and results of the calcifediol groups were combined. In this study, serum 25(OH)D concentration increased with 143 nmol/L after supplementation with calcifediol. However, both studies could not confirm a steady state in serum 25(OH)D_3 concentrations, which might relate to the shorter study duration and higher doses of calcifediol (20 μg). In our study, daily supplementation with 10 μg or 15 μg calcifediol, increased status levels with 50 nmol/L and 71 nmol/L, respectively. Average steady state was tested aiming at serum 25(OH)D_3 concentrations between 75–100 nmol/L. Steady state was reached at 12 weeks of supplementation in both the 20 μg vitamin D_3 as in the 10 and 15 μg calcifediol group. However, only the 10 μg calcifediol group plateaued within the target range of 75–100 nmol/L. Treatment with 20 μg vitamin D_3 plateaued at 72 nmol/L and 15 μg calcifediol exceeded the upper reference of 100 nmol/L after 8 weeks.

As suggested by a recent report from the European Society for Clinical and Economic Aspects of Osteoporosis (ESCEO) and the International Osteoporosis Foundation (IOF), equipotent doses of calcifediol and vitamin D_3 should be tested to allow

comparison of target level effectiveness [26]. When describing the relative potency of calcifediol compared to vitamin D_3, Cashman et al. reported conversion factors between 1.4 and 5.0 based on results of previous studies [17]. Because of variability in study design, baseline levels and dosing regimens, direct comparison of these conversion factors should be perceived with caution. Nevertheless, when considering the effective doses of 10 and 15 μg calcifediol in the current study, conversion factors were 2.8 and 3.0, indicating that, per microgram supplemented, calcifediol was about 3 times more effective to increase serum 25(OH)D_3 status when compared to vitamin D_3. Furthermore, Zittermann et al. published a formula to calculate the expected increase in serum 25(OH)D concentration when supplementing with vitamin D while taking into account the age, baseline 25(OH)D status and body weight of the study population [27]. Using this formula, the predicted increase in serum 25(OH)D concentration when supplementing with 20 μg vitamin D_3, was in line with the actual increase as observed in our study (34 nmol/L actual increase versus 40 nmol/L predicted increase). Moreover, this formula indicates that much higher doses, of about 40 μg and 125 μg vitamin D_3, would be required to establish the increase in serum 25(OH)D concentration as observed in the 10 μg and 15 μg calcifediol groups. Along with its higher potency, our study shows that the calcifediol doses appeared to be safe for use over a 24-week period as no cases of hypercalcemia occurred. Nevertheless, this safety evaluation is limited to the timeframe under study and further research is needed to investigate the long-term daily use of calcifediol.

Serum 25(OH)D is currently considered the best biomarker to reflect vitamin D status, as it has a longer half-life and correlates better with PTH suppression compared to the active hormone 1,25$(OH)_2$D [28]. Nevertheless, recent studies suggest that other metabolites might also provide clinically relevant information [29]. Serum 1,25$(OH)_2$D is under tight homeostatic control by PTH and serum concentrations of calcium and phosphorus. Our study shows no association between the change in 25(OH)D_3 and 1,25$(OH)_2D_3$ concentrations after supplementation with either form of supplementation. This is consistent with findings of previous randomized trials supplementing with either vitamin D_3 or calcifediol [25, 30]. Serum 24,25$(OH)_2$D is suggested as an index of vitamin D deficiency and catabolism. Our study showed a strong positive correlation between the change in 25(OH)D_3 and 24,25$(OH)_2D_3$ after supplementation. Serum 24,25$(OH)_2D_3$ showed similar dose-response patterns as serum 25(OH)D_3, which suggests stimulation of the catabolic pathway to regulate 1,25$(OH)_2D_3$ [29].

Strengths of this study are the monthly measurements of multiple vitamin D metabolites, providing comprehensive data on vitamin D status and repletion. Besides, all vitamin D metabolites were measured using chromatography-based techniques, which are now considered the research gold standard [31]. Other strengths are the high subject compliance and good adherence to the study visits which resulted in few missing data. Our study also has limitations. First of all, results in this study were restricted

to the reporting of D_3-related metabolites to accurately reflect serum 25(OH)D_3 dose-response relationships. However, total serum 25(OH)D status is mostly used for clinical diagnosis of deficiency. Nevertheless, the contribution of serum 25(OH)D_2 to total serum 25(OH)D status in our study is expected to be low, as indicated by the high number of samples with undetectable values. Furthermore, the study started in late summer which might induce confounding due to endogenous generation of vitamin D in all treatment arms. However, the main study period fell within the season of minimal endogenous vitamin D synthesis (October-April when latitudes above 40°N) which might limit this confounding [32]. Period of inclusion was considered as a covariate in the dose-response models but did not affect the study results. Lastly, the findings may not be generalizable to patients with an impaired renal functioning, as those were excluded from participation. An impaired renal functioning is known to affect vitamin D metabolism and can alter the regulation of calcium and phosphorus levels [33]. Therefore, the efficacy and safety of calcifediol supplementation in this specific population requires further research.

Scientific findings of the possible biologic actions of vitamin D and epidemiological studies linking vitamin D to a broad spectra of diseases have led to guidelines to increase the recommended status levels. For example, the Endocrine Society suggests serum 25(OH)D concentrations >75 nmol/L for at risk populations, including the elderly, to support the possible effect on bone and muscle metabolism [13]. Although the optimal serum 25(OH)D status remains a subject of ongoing debate and needs further investigation, higher target ranges require higher doses of vitamin D_3 per day. Therefore, calcifediol might be a potential strategy to rapidly increase serum 25(OH)D levels towards desired levels.

To conclude, this study adds to the characterization of dose-response effects with calcifediol in an older population. Our results show that a dose of 10 μg/day resulted in sustained serum 25(OH)D_3 concentrations between 75–100 nmol/L.

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Conflict of interest: LdG declares to have filed a patent related to vitamin D and cognitive executive function. JW is an employee of DSM. LvL, MT, MdR and AV have no disclosures.

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CHAPTER 5

The association between 25-hydroxyvitamin D concentration, physical performance and frailty status in older adults

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Submitted

ABSTRACT

Background & aims: Sufficient 25-hydroxyvitamin D (25(OH)D) concentrations might prevent a decline in physical performance, and are considered important for the prevention of frailty. This study investigates the association of serum 25(OH)D concentration with physical performance and frailty status in Dutch older adults.

Methods: This cross-sectional study included 756 men and women, aged ≥65 years. Serum 25(OH)D) concentration and frailty status (Fried criteria) were assessed in the total population. Screening for frailty status included functional tests of gait speed and hand grip strength. In a subgroup (*n*=494), the Timed-Up and Go test (TUG) and knee-extension strength were measured. Associations of serum 25(OH)D status with physical performance were examined by multiple linear regression. Prevalence ratios (PR) were used to quantify associations between serum 25(OH)D deficiency (<50 nmol/L) and frailty.

Results: In total, 45% of the participants were vitamin D deficient. Participants with vitamin D status <50 nmol/L and 50-75 nmol/L had significantly lower scores on the TUG and gait speed test, compared to participants with vitamin D status >75 nmol/L. No significant associations with serum 25(OH)D concentrations were observed for handgrip strength or knee-extension strength. Participants with serum 25(OH)D status <50 nmol/L were about 2 times more likely to be frail compared to participants with serum 25(OH)D status ≥50 nmol/L.

Conclusion: In this study, serum 25(OH)D concentrations were significantly associated with frailty status and measures of physical performance, including gait speed and TUG, but not with strength related outcomes.

INTRODUCTION

Frailty is a geriatric syndrome associated with adverse health outcomes, such as physical disability, increased risk of falls, institutionalization, hospitalization and mortality [1]. To identify older people at risk, Fried et al. proposed a characterization of a frail state, using a clinical phenotype [2]. The definition consists of five physical components (weakness, slow walking speed, exhaustion, physical inactivity, and unintentional weight loss) and is now commonly applied in clinical research. The prevalence of frailty is relatively high among community-dwelling elderly, with 44% of seniors being pre-frail, and 10% being frail [3]. In view of the ageing population, the prevalence of the frailty syndrome will increase, which in turn will result in higher rates of hospitalization, and considerably burden the public health care costs [4]. As such, the need for interventions, supporting older people to remain healthy and independent, increases. One of the key features of frailty is profound muscle weakness and a decline in functional capabilities [2]. The cause of this loss in strength and function is multifactorial, and a low vitamin D status is suggested to be one of the risk factors [5, 6]. Vitamin D stimulates calcium absorption in the intestine and is responsible for the mineralization of bone and general functioning of cells throughout the body [7]. Deficient vitamin D concentrations (serum 25(OH)D <50 nmol/L) [8] are common in frail older adults, with a prevalence reported up to 62% [9]. Low vitamin D concentrations have been associated with an impaired muscle function and an increased risk of being frail [9-11]. However, the strength and shape of these associations, and the ability to control for confounding factors differs between studies. Further characterization of the association between serum 25(OH)D concentration and frailty, but also the closely related functional parameters, might help to define consensus about the optimal vitamin D status for these health outcomes. Therefore, the aim of this study was to determine the association of serum 25(OH)D concentrations with physical performance and frailty status.

METHODS

Study sample

In this study, we report data of 756 older adults that attended a screening visit for participation in the D-DOSE or D-FIT trial (clinicaltrial.gov registration: NCT01868945 or NCT02349282). These studies used similar recruitment strategies, inclusion criteria and measurement protocols, which allowed combining of datasets. Both studies were performed by the Division of Human Nutrition, Wageningen University, the Netherlands. Recruitment took place via the university database, or municipality registers of Wageningen and surroundings. Participants were invited to the screening visit if they were 65 years or older. Visits took place between May 2013 and April 2015. All participants provided data on general characteristics, serum 25(OH)D status and frailty criteria (gait speed, handgrip strength, physical activity, weight loss and self-reported exhaustion). Additional measures of muscle strength and physical performance were performed in a subgroup of 494 participants. Before screening, all participants signed informed consent and study protocols were approved by the ethical committee of Wageningen University.

Serum 25-hydroxyvitamin D

Serum blood samples were collected to measure 25(OH)D concentration. Samples were centrifuged, stored at -80 ºC and thereafter analyzed using LC-MS/MS. Samples collected for the D-DOSE study (*n*=259) were analyzed at the Endocrine Laboratory of the VU University Medical Centre, Amsterdam, the Netherlands [12]. The intra-assay and inter-assay coefficients of variation were below 6% and 8%, respectively. Serum 25(OH)D samples collected for the D-FIT study (*n*=497) were analyzed at the Department of Clinical Chemistry, Canisius Wilhelmina Hospital, Nijmegen, the Netherlands. The intra-assay and inter-assay coefficients of variation were below 4% and 7.5%, respectively [13]. Both laboratories are DEQAS-certified and the comparability of the LC-MS/MS methods between these two laboratories has been published previously, which indicated good agreement between methods [14].

Physical performance

Handgrip strength (HGS) was measured on the dominant hand by taking the mean of 3 attempts (Jamar® hydraulic hand-held dynamometer, Patterson Medical, IL, USA). Mean gait speed was assessed by taking the average time, of 2 attempts, to walk a course of 15 feet. In a subgroup (*n*=494), the Timed Up and Go test (TUG) and maximal isometric knee- extension strength were assessed. The TUG test is a test of functional ability to rise from a chair, walk 3 meters, make a turn, and walk back to the chair to sit down again. The average time to complete this test, out of 2 attempts, was recorded. Knee-extension strength was measured using the MicroFET hand-held dynamometer

(Hoggan Health Inc., West Jordan, UT, USA). Participants were asked to sit upright with their knees in a 90˚ angle. Maximal strength (Newton) was measured 3 times per leg with 5 seconds of muscle contraction and 60-seconds of rest between repetitions. The average muscle strength of the right leg was used for analysis. All measurements were performed by examiners trained to regularly perform these tests according study protocol and standardized verbal encouragement was provided.

Fried frailty criteria

Frailty status was assessed using the criteria published by Fried et al. [2]. These consist of the following five criteria: unintentional weight loss (in the past year, by questionnaire), self-reported exhaustion (CES-D questionnaire) [15], weakness (handgrip strength), slow walking speed (gait speed), and low physical activity levels (Short version of the Minnesota questionnaire) [16]. According the frailty definition of Fried et al., a participant scores non-frail when no criteria are present, pre-frail when one or two criteria are present and frail when three or more criteria are present [2].

Covariates

Questionnaires were used to record general participant characteristics such as, age, sex, ethnicity (caucasian, other), physical activity (short version of the Minnesota questionnaire) [16], vitamin D supplement use, smoking status, alcohol intake, and the number of chronic diseases (including heart failure, hypertension, diabetes mellitus, renal insufficiency, liver disease or cancer). A stadiometer was used to measure the height of the participants, and a calibrated analog scale was used to measure their weight. BMI was calculated as kg/m^2. In addition, laboratory site and season of blood collection (winter: December-February, spring: March-May, summer: June-August, autumn: September-November) were recorded.

Statistical analyses

Characteristics of the study population are described as mean (SD), median (25 – 75^{th} percentile) or number (%) of categorical class. Serum 25(OH)D concentrations <50 nmol/L are generally considered deficient [8, 17], and a status between 50-75 nmol/L or >75 nmol/L is suggested for optimal muscle health and physical performance [18, 19]. Serum 25(OH)D was categorized accordingly, with the latter (>75 nmol/L) being the reference category. Differences between categories of serum 25(OH)D concentration were examined by One-way ANOVA for continuous variables, Kruskal-Wallis test in case of skewed variables and Chi-square tests for categorical variables. The association between serum 25(OH)D concentration and measures of physical performance (TUG and gait speed) and muscle strength (handgrip strength and knee-extension strength) were explored for nonlinearity by restricted cubic spline regression. As associations with TUG and hand grip strength tended to be nonlinear, all outcomes were further explored across categories of serum 25(OH)D. Multiple linear regression models were adjusted for factors known to be related to both serum 25(OH)D

and physical performance. Model 1 was adjusted for age, sex and laboratory site. Model 2 was additionally adjusted for BMI and season of blood collection, and model 3 was additionally adjusted for ethnicity, physical activity, alcohol intake, smoking and number of diseases. A Cox Proportional Hazards analysis with robust error variance was performed to calculate Prevalence Ratios (PR) of participants being pre-frail or frail across categories of serum 25(OH)D status. By assigning a constant risk period to all participants, the obtained hazard ratio can be considered a PR [20]. Models including frailty as dependent variable were not corrected for physical activity, as this measure is also included in the definition of frailty status. Previous studies identified sex as a possible effect modifier in the association between vitamin D and physical performance [21]. Therefore, interaction terms including sex were added to the final models. A *P*-value of ≤0.1 was considered significant to retain an interaction term in the model. All analyses were performed using statistical software package SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) or using the R software package version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). A two-sided *P*-value of ≤0.05 was considered statistically significant.

RESULTS

Table 5.1 shows the general characteristics of the study population in total, and by categories of serum 25(OH)D status. The mean ± SD age of the study population was 74 ± 6 years and 55% were men. Mean BMI was 27.1 ± 3.5 kg/m^2 and median serum 25(OH)D status was 54 (38-72) nmol/L, irrespective of season. Participants in the deficient serum 25(OH)D category (<50 nmol/L) were more likely to be men and more likely to have a higher BMI compared to participants in the higher categories of serum 25(OH)D status. Season of blood collection was significantly different between the vitamin D categories, with 81% of the vitamin D deficient participants measured in the winter/ spring. Of all participants, 12% reported to use a vitamin D supplement. A significant difference was observed in the number of supplement users across categories, with 4% in the deficient category and 18% and 19% in the two higher categories. Most participants scored non-frail according the Fried criteria, namely 57%, followed by 39% scoring pre-frail and 4% scoring frail.

Table 5.2 shows the association between serum 25(OH)D concentration and measures of physical performance. There was an inverse association between serum 25(OH)D and TUG test scores, which remained significant after full adjustment for confounders. Compared with the reference category (>75 nmol/L), participants with serum 25(OH)D concentrations <50 nmol/L (β 0.73, 95% CI 0.14; 1.32) and 50-75 nmol/L (β 0.83, 95% CI 0.21; 1.45) had significantly higher TUG scores, indicating more time needed to complete the test. Likewise, participants with serum 25(OH)D status <50 nmol/L (β -0.04, 95% CI -0.08; -0.01) and status between 50-75 nmol/L (β -0.04, 95% CI -0.07; -0.01) had significantly lower gait speed scores, compared with the reference category. Serum 25(OH)D categories were not associated with handgrip strength and knee-extension strength. The effect of vitamin D supplement use was explored but did not change the interpretation of results. Furthermore, interaction analyses did not suggest significant modification of the associations by sex.

Table 5.3 shows the association between serum 25(OH)D concentrations and frailty status. As only 2 participants scored frail in the >75 nmol/L category, the 50-75 nmol/L and >75 nmol/L categories were combined to further explore the association between serum 25(OH)D and frailty status. Participants with serum 25(OH)D status <50 nmol/L were about 2 times more likely to be frail (PR=2.30, 95% CI 1.11; 4.76, *P*=0.02), compared to participants with serum 25(OH)D status ≥50 nmol/L. The effect of vitamin D supplement use was explored, which attenuated the prevalence ratio but the association remained significant (PR=2.16, 95% CI 1.04; 4.52, *P*=0.04). When comparing non-frail versus pre-frail older adults (or pre-frail and frail combined), no significant associations were observed with serum 25(OH)D status.

Table 5.1 Participant characteristics.

		Serum 25-hydroxyvitamin D			
	Total	**< 50 nmol/L**	**50-75 nmol/L**	**> 75 nmol/L**	***P***
	n=756	*n*=340	*n*=254	*n*=162	**value**
Men, n (%)	416 (55)	217 (64)	125 (49)	76 (46)	<0.01
Age, y	73.8 ± 6.4	74.1 ± 6.6	74.0 ± 6.2	72.9 ± 5.9	0.08
BMI, kg/m^2	27.1 ± 3.5	27.5 ± 3.7	27.0 ± 3.3	26.2 ± 3.1	<0.01
Caucasian, n (%)	736 (98)	327 (97)	250 (98)	159 (98)	0.49
Independent living, n (%)[a, §]	723 (96)	320 (95)	243 (96)	160 (99)	0.10
Non-smokers, n (%)[a]	705 (94)	311 (92)	239 (94)	155 (96)	0.31
Alcohol consumers, n (%)[a]	598 (79)	269 (80)	197 (78)	132 (82)	0.61
25(OH)D, nmol/L	54 (38-72)	36 (29-42)	62 (58-67)	91 (84-100)	<0.01
VitD suppl. users, n (%)[b]	88 (12)	13 (4)	45 (18)	30 (19)	<0.01
Season, n (%)[†]					
Summer - autumn	259 (34)	64 (19)	101 (40)	94 (58)	<0.01
Winter - spring	497 (66)	276 (81)	153 (60)	68 (42)	
Number of diseases, n (%)[c]					
0	380 (51)	159 (47)	130 (52)	91 (56)	0.36
1-2	353 (47)	167 (50)	117 (46)	69 (43)	
≥ 3	17 (2)	10 (3)	5 (2)	2 (1)	
Physical activity, MJ/wk[d]	8.4 (4.5-13.0)	7.8 (4.5-13.1)	8.5 (4.3-12.0)	9.6 (5.4-15.1)	0.11
TUG, s[#, d]	9.8 ± 2.4	9.9 ± 2.5	9.9 ± 2.3	9.1 ± 2.2	0.04
Gait, m/s[e]	1.06 ± 0.20	1.05 ± 0.21	1.05 ± 0.19	1.10 ± 0.20	0.03
Knee-extension, N[#]	328 ± 104	336 ± 103	324 ± 104	301 ± 106	0.04
HGS, kg[f]	28.9 ± 9.5	29.4 ± 9.3	28.1 ± 9.6	28.9 ± 9.7	0.23
Frailty, n (%)[g]					
Non-frail	425 (57)	183 (55)	142 (57)	100 (62)	0.16
Pre-frail	289 (39)	131 (39)	98 (39)	60 (37)	
Frail	33 (4)	20 (6)	11 (4)	2 (1)	

25(OH)D, 25-hydroxyvitamin D; BMI, Body Mass Index; VitD suppl. users, vitamin D supplement users; TUG, Timed Up and Go; HGS, Hand grip strength; N, Newton. Values presented are mean ± SD or median (25 -75th percentile). [a]3 missing values. [b]5 missing values. [c]6 missing values. [d]1 missing value. [e]7 missing values. [f]4 missing values. [g]9 missing values. [§]Assisted living includes: home care or service flat. [†]Winter-spring: Dec-May, summer-autumn: Jun-Nov. [#]Subgroup *n*=494.

Table 5.2 Association between serum 25-hydroxyvitamin D status and physical performance.

	Serum 25-hydroxyvitamin D			
	< 50 nmol/L	**50-75 nmol/L**	**> 75 nmol/L**	
	β (95% CI)	*β* (95% CI)	Reference group	*n*
TUG, s				
Model 1	0.85 (0.24; 1.45)**	0.83 (0.19; 1.47)*	0 (ref)	493
Model 2	0.77 (0.18; 1.36)*	0.84 (0.22; 1.47)**	0 (ref)	493
Model 3	0.73 (0.14; 1.32)*	0.83 (0.21; 1.45)**	0 (ref)	488
Gait, m/s				
Model 1	-0.06 (-0.10; -0.02)**	-0.05 (-0.09; -0.01)**	0 (ref)	749
Model 2	-0.05 (-0.09; -0.01)**	-0.04 (-0.08; -0.01)*	0 (ref)	749
Model 3	-0.04 (-0.08; -0.01)*	-0.04 (-0.07; -0.01)*	0 (ref)	745
HGS, kg				
Model 1	-0.93 (-2.25; 0.38)	-0.71 (-2.02; 0.61)	0 (ref)	752
Model 2	-1.06 (-2.38; 0.26)	-0.84 (-2.15; 0.47)	0 (ref)	752
Model 3	-0.92 (-2.25; 0.40)	-0.78 (-2.10; 0.53)	0 (ref)	748
Knee-extension, N				
Model 1	7.74 (-15.03; 30.50)	12.23 (-11.95; 36.42)	0 (ref)	494
Model 2	7.70 (-15.10; 30.50)	13.09 (-11.11; 37.29)	0 (ref)	494
Model 3	9.89 (-12.82; 32.60)	12.71 (-11.37; 36.80)	0 (ref)	489

**$P<0.01$, *$P<0.05$. Model 1: adjusted for age, sex and laboratory site. Model 2: adjusted for age, sex, laboratory site, BMI and season. Model 3: adjusted for age, sex, laboratory site, BMI, season, ethnicity, physical activity, alcohol, smoking and number of diseases. TUG, Timed Up and Go; HGS, Hand grip strength; N, Newton.

Table 5.3 Prevalence ratios (PR) and 95% CIs for frailty status of participants with serum 25-hydroxyvitamin D concentrations <50 nmol/L versus ≥50 nmol/L.

	Serum 25-hydroxyvitamin D		
	< 50 nmol/L	**≥ 50 nmol/L**	
	PR (95% CI)	Reference group	*n*
Frail vs. non-frail			
Model 1	2.24 (1.06; 4.75)*	1 (ref)	458
Model 2	2.07 (1.02; 4.20)*	1 (ref)	458
Model 3	2.30 (1.11; 4.76)*	1 (ref)	453
Pre-frail vs. non-frail			
Model 1	1.10 (0.91; 1.32)	1 (ref)	714
Model 2	1.08 (0.90; 1.29)	1 (ref)	714
Model 3	1.06 (0.88; 1.26)	1 (ref)	711
Pre-frail or frail vs. non-frail			
Model 1	1.14 (0.97; 1.35)	1 (ref)	747
Model 2	1.13 (0.96; 1.32)	1 (ref)	747
Model 3	1.10 (0.93; 1.29)	1 (ref)	742

*$P<0.05$. Model 1: adjusted for age, sex and laboratory site. Model 2: adjusted for age, sex, laboratory site, BMI and season. Model 3: adjusted for age, sex, laboratory site, BMI, season, ethnicity, alcohol, smoking and number of diseases. Models are not corrected for physical activity, as this measure is also included in frailty status.

DISCUSSION

In this study, serum 25(OH)D concentrations were significantly associated with physical performance and frailty status in a population of community-dwelling older adults. To date, no generally accepted recommendation on the optimal serum 25(OH)D status for muscle function is present, with the IOM proposing concentrations of 30-50 nmol/L for older adults [8], and others supporting thresholds of 75 nmol/L or higher [18, 19]. Our results indicated that serum 25(OH)D status <50 nmol/L, but also between 50-75 nmol/L, were associated with lower functioning on TUG and gait speed tests, when compared to serum 25(OH)D status >75 nmol/L. Similar associations were observed in two large cohorts, where low serum 25(OH)D was associated with physical performance, and the strongest associations were observed on walking tests [22, 23]. In addition, comparable effect estimates were reported in a study of older adults at-risk of disability, with slower walking speed (mean difference 0.04 m/s) in vitamin D deficient older adults (<50 nmol/L) compared to those with a sufficient status [24]. The association with TUG was also observed in previous studies, where higher vitamin D concentrations were associated with a faster performance on the TUG test [21, 25]. In a study by van Dam et al., women with serum 25(OH)D concentrations ≤80 nmol/L showed slower TUG scores (mean difference 0.77 sec) than those who had a serum 25(OH)D status ≥115 nmol/L, and the lower 25(OH)D category appeared predictive of a greater decline in function over a period of 2.5 years [21]. In addition, several studies report an association between vitamin D deficiency and reduced handgrip strength [23, 26], or leg extension strength [27, 28], albeit not all [29]. In our study, no significant association was observed between serum 25(OH)D concentration and measures of muscle strength. Mechanistically, the link between vitamin D and muscle function is explained via the regulation of calcium and phosphate, necessary for muscle contraction, or via the activation of the vitamin D receptor (VDR) in muscle cells [30]. However, the presence of the VDR is also observed in neurons and glial cells in several regions of the brain, which suggests a role of vitamin D in the neuromuscular system [31, 32]. Vitamin D deficiency is associated with an increased postural sway and greater risk of falling [33]. Moreover, a previous trial showed that vitamin D supplementation improved balance with 9% in vitamin D deficient older adults [34]. It is thus plausible that vitamin D status is more strongly associated with complex functional parameters rather than muscle strength due to its suggested role in neurological processes of motor performance. However, more research is needed in this field.

Vitamin D deficient participants (<50 nmol/L) were ~2 times as likely to score frail, compared to those with sufficient serum 25(OH)D concentrations. This is in line with most previous studies investigating this association [9, 35, 36]. In the Longitudinal Aging Study Amsterdam (LASA), participants with 25(OH)D status between 25-50

nmol/L were 1.7 times as likely to be frail, and those with serum status below 25 nmol/L were 2.6 times as likely to be frail compared to the reference group with vitamin D status >50 nmol/L [37]. Likewise, in the NHANES III study, older adults with vitamin D deficiency (<37 nmol/L) were 3.7 times as likely to score frail on the Fried criteria compared to the reference group (≥75 nmol/L) [11]. In our study, serum 25(OH)D concentrations were not associated with the pre-frail state. However, a prospective study reported that serum 25(OH)D status ≥50 nmol/L tended to prevent a decline from the pre-frail to frail state over a period of 3 to 6 years [38], which might indicate that prevention of vitamin D deficiency is also relevant in this stage. Furthermore, sex has been reported as an effect modifier in the association between 25(OH)D and frailty [39]. In our study, the prevalence of frailty was relatively low (4%), limiting reliable testing of effect modification across such a small sample of cases.

Overall, the association between vitamin D status and frailty appears to be consistent, with lower serum 25(OH)D status associated with an increased likelihood of being frail. However, the causality of the association remains to be determined, given the cross-sectional design of these reported associations. An important determinant of vitamin D status is sun-exposure, which closely relates to being outdoors and physically active. As frail older adults might stay more indoors, reverse causation is plausible. Autier et al. recently discussed that the serum 25(OH)D status might not be a cause of adverse health outcomes, but a marker of ill health [40]. While we controlled for a broad range of lifestyle and health related factors, correcting for confounding remains challenging. While we used questionnaires to estimate physical activity status and season to correct for sun-exposure, residual confounding cannot be excluded. Besides the factors inherent to the cross-sectional design of this study, other limitations should be noted. The prevalence of frailty was relatively low when compared to the reported prevalence of 10% for physical frailty in community-dwelling older adults [3]. The low prevalence might relate to the fact that this population consists of older adults that were willing to participate in an intervention trial and had likely a better health status or were more mobile compared to the general population, limiting the generalizability of the study findings. Also, parathyroid hormone (PTH), could potentially mediate the association between serum 25(OH)D status and physical performance or frailty, but was not measured in this study. Nevertheless, Pabst et al. investigated the mediating effect of PTH on frailty, but the attenuation of the odds ratio was small, suggesting an independent association with 25(OH)D [36]. Strengths of this study include the broad range of vitamin D concentrations measured in this population, the relatively high prevalence of vitamin D deficiency, and the fact that, besides the measurement of frailty, we included measures of lower extremity strength and TUG to reflect overall body function and strength.

With only 12% of the participants using a vitamin D supplement, 45% of our study population was vitamin D deficient. Identifying older adults at risk of vitamin D

deficiency might be important given the possible predisposed risk of frailty. In this study, associations were observed between 25(OH)D status and the performance on the TUG and gait speed test. Both tests represent the ability of motor performance and balance control, supporting the plausible modulatory role of vitamin D in fall prevention [41]. Although the observed associations represent only small clinically meaningful changes [42, 43], if causal, these findings might be relevant for public health.

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Conflict of interest: This study was funded by TI Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition, and DSM Nutritional Products Ltd., R&D Human Nutrition and Health. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion. LG and EB declare to have filed a patent related to vitamin D and cognitive executive function. AV, MR, NT, MT and LL have nothing to declare.

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CHAPTER 6

The effect of calcifediol or vitamin D3 supplementation on muscle strength and physical performance in pre-frail and frail older adults: a randomized placebo-controlled trial

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Submitted

ABSTRACT

Objectives: Vitamin D supplementation is proposed as a potential treatment strategy to counteract functional decline in older adults. However, data from randomized trials are either limited or inconsistent. This study investigates the effect of daily supplementation with calcifediol or vitamin D_3 on muscle strength and physical performance in older adults.

Design: A randomized, double-blind, placebo-controlled trial of 6 months.

Setting and participants: Seventy-eight pre-frail or frail, community-dwelling older adults, aged 65 years or older, with a baseline 25-hydroxyvitamin D (25(OH)D) status between 20-50 nmol/L.

Intervention: 10 μg calcifediol, 20 μg vitamin D_3 or a placebo capsule per day.

Measurements: Serum 25(OH)D was measured by liquid chromatography-mass spectrometry (LC-MS/MS). Outcome measures included: maximal isometric knee-extension and knee-flexion strength (Biodex System 4), hand grip strength, Short-Physical Performance Battery (SPPB), Timed Up and Go (TUG), postural sway, muscle mass (DXA) and muscle fiber type and size.

Results: Mean baseline serum 25(OH)D concentrations were 37.7 nmol/L (95% CI 35.4 to 39.9). After 6 months of supplementation, status levels increased towards 98.7 nmol/L (95% CI 93.1 to 104.4) in the calcifediol group, and to 72.0 nmol/L (95% CI 66.1 to 77.8) in the vitamin D_3 group, compared to 47.5 nmol/L (95% CI 41.8 to 53.3) in the placebo group. Knee-extension strength did not significantly change in the calcifediol group (5.9 Nm; 95% CI -6.2 to 18.0), nor in the vitamin D_3 group (5.5 Nm; 95% CI -6.8 to 17.8), or placebo group (1.8 Nm; 95% CI -10.7 to 14.4) (treatment x time interaction P=0.74). Further, no significant differences were observed in the mean change on physical performance tests, muscle mass, or muscle fiber type and size between groups.

Conclusion: Improving serum 25(OH)D concentration over a period of 6 months did not significantly change muscle strength and physical performance in pre-frail and frail older adults.

INTRODUCTION

Aging is characterized by a gradual decline in skeletal muscle mass and muscle strength, which increases the risk of falls, disability, and frailty [1]. Vitamin D is suggested to be one of the factors that can moderate the age-related decline in muscle function. Low vitamin D levels have been linked to an impaired physical performance, and vitamin D deficiency is highly prevalent in frail older adults [2]. Therefore, frail elderly might represent an important target group for interventions including vitamin D supplementation.

Vitamin D is a prohormone that plays a key role in the regulation of calcium and phosphate for the maintenance of bone tissue [3]. The hypothesis that vitamin D plays a role in muscle function originates from early case reports of proximal muscle weakness and complaints of muscle pain in patients with severe vitamin D deficiency [4]. Treatment with vitamin D appeared to relief these symptoms [5, 6]. The mechanism by which vitamin D acts on muscle tissue is suggested to work either direct, by binding the active metabolite 1,25-dihydroxyvitamin D to the vitamin D receptor (VDR) in muscle cells, or indirect, through its effect on intracellular calcium and phosphate handling [7].

In the last decade, observational studies have shown that low serum 25-hydroxyvitamin D (25(OH)D) levels (<50 nmol/L) are associated with impaired physical performance [8]. In addition, several prospective studies reported that adequate serum 25(OH) D levels are associated with reduced risks of functional limitations [9-11]. As such, supplementation is proposed as a potential treatment strategy to counteract functional decline. Several randomized trials showed positive effects of vitamin D supplementation on lower extremity strength, balance and physical performance in older adults [12-17], however, the evidence is inconsistent, as a number of studies also reported null-findings [18-21].

Besides supplementation with vitamin D_3, calcifediol might provide an alternative supplementation strategy. Previous studies have shown that, compared to vitamin D_3, calcifediol has a higher potency in raising serum 25(OH)D towards desired concentrations [22-24]. Interestingly, a pilot study in postmenopausal women showed beneficial effects of calcifediol over vitamin D_3 on knee extension strength and gait speed after 4 months of supplementation [25, 26]. This study, however, had a small sample size and did not include a placebo arm.

More evidence from placebo-controlled trials is needed to further define the causality and determine the magnitude of the effect of vitamin D on muscle function. Therefore, the aim of this study was to evaluate the effect of supplementation with either vitamin D_3 or calcifediol on muscle strength and physical performance in pre-frail and frail, vitamin D deficient older adults.

METHODS

Study design and objectives

This study was a six-month, double-blind, placebo-controlled trial, including subjects randomly allocated to a treatment, receiving supplements with either 10 μg calcifediol (CAL), 20 μg vitamin D_3 (VD3) or a placebo (PLA) capsule per day. The primary outcome measure was change in knee-extension strength. Secondary outcome measures included change in knee-flexion strength and hand grip strength, physical performance (Timed Up and Go test, TUG; Short Physical Performance Battery, SPPB; and postural balance), muscle mass (Dual-energy X-ray Absorptiometry, DXA), muscle fiber type and size (muscle biopsy). Measurements were performed at baseline, after 3 months, and 6 months of intervention, with the exception of the DXA scans and muscle biopsies, which were performed at baseline and after 6 months only. The study was carried out at Wageningen University and Hospital Gelderse Vallei, Ede, the Netherlands (latitude 51°N), between December 2014 and December 2015. The Medical Ethics Committee of Wageningen UR approved the study protocol and hospital Gelderse Vallei approved local feasibility. All participants gave their written informed consent. The study was registered at clinicaltrials.gov as NCT02349282.

Study population

Participants were recruited via registries of municipalities in Wageningen and surroundings. A total of 78 men and women volunteered to partake in this study. Participants were included if they were ≥65 years of age, had a serum 25(OH)D level between 20-50 nmol/L, a BMI between 18.5 and 35 kg/m^2 and were pre-frail or frail based on the frailty criteria of Fried et al. [27]. Exclusion criteria were: a serum calcium level >2.6 nmol/L or uncontrolled hypocalcaemia, diagnosed malabsorption disorders, sarcoidosis, lymphoma, primary hyperparathyroidism, kidney stones (in past 10 years), renal insufficiency, cancer or the use of medication that may influence vitamin D metabolism (e.g. bisphosphonates, PTH treatment, tuberculostatica, anti-epileptica, bile acid sequestrate or lipase inhibitors). Furthermore, participants were excluded if they consumed >21 alcoholic beverages per week, were not willing or able to stop the use of vitamin D containing supplements during the study, were expected to increase their sun exposure (e.g. planned holiday) or had a surgery planned.

Intervention

An independent investigator randomly allocated subjects to one of the 3 intervention groups by a computer-generated list (SAS software 9.20). Randomization was carried out in permuted blocks (block size 3) and stratified by sex and BMI (18.5-29.9, 30-35 kg/m^2). The 3 groups received supplements with either 10 μg/day CAL, 20 μg/day VD3 or PLA. Both the participants and investigators were blinded to treatment allocation, and

study supplements were identical in appearance and taste. DSM Nutritional Products Ltd. provided CAL (calcifediol 0.25% SD/S), VD3 or placebo (microcrystalline cellulose, Avicel PH-102) in spray-dried form. Capsules were manufactured by Fisher Clinical Services GmbH. The actual content of the CAL capsules was 9.9 μg, which was tested using High-Performance Liquid Chromatography (HPLC). The actual content of the VD3 capsules was 22.9 μg, which was tested using Liquid Chromatography-Mass Spectrometry (LC-MS/MS) (Analytical Research Centre of DSM Nutritional Products). Participants were instructed to consume one capsule per day at breakfast. Treatment compliance was reported at 3 and 6 months by capsule count of returned capsules, taking into account the number of days active in the study. Participants were considered compliant when ≥80% of the study supplements were taken.

Measurements

Strength tests and physical performance

Lower extremity strength was measured as maximal knee-extension and knee-flexion (Nm) using the Biodex System 4 dynamometer (Biodex Medical Systems, Shirley, NY, USA). Subjects were seated upright with their chest and waist secured by belts. The lateral epicondyle of the femur was aligned with the rotation axis of the lever arm and the ankle was secured in the ankle attachment. Experiments were performed with knee angle of 60^0 and hip angle of 90^0. Subjects performed 3 maximal voluntary isometric contractions for five seconds, with 30 seconds of rest between trials and five minutes of rest between knee-extension and knee-flexion trials. Researchers provided standardized verbal encouragement during the strength tests. Upper extremity strength was determined by hand-held dynamometer (Jamar) and recorded to the nearest 1.0 kg as the mean hand grip strength of 3 consecutive trials with the dominant hand. The TUG-test and SPPB were performed to include a measure of functional mobility. For the TUG-test, subjects had to rise from a chair of standardized height, walk a distance of 3 meters in normal speed, turn, walk back to the chair, and sit down again [28]. The average of two trials was recorded. The SPPB consists of 3 components: balance, gait speed and chair rise time [29]. Each of the components was scored on its test-specific scale and on a 0 to 4 point scale, resulting in a total score between 0-12. In addition, postural body sway was measured using a force plate (AMTI Accusway Plus Balance Platform, Version 2.02.01). Subjects were asked to stand as still as possible for 30 seconds on the force plate under four conditions; with their feet together (closed base) and eyes open (CBEO), feet together and eyes closed (CBEC), feet hip-width apart (open base) and eyes open (OBEO), feet hip-width apart and eyes closed (OBEC). Each stand was performed twice and the average area ellipse was used as a measure of sway. The area ellipse represents 95% of the center of pressure points distributed in both the x-axis and y-axis. Higher values indicate increased sway and as such poorer balance. If a participant was unable to complete the stand (by stepping off the force plate or touching the handles), the test was stopped.

Blood samples

Blood samples were collected in a fasting state in the morning and stored at -80 ^{0}C until analysis. At screening, serum 25(OH)D was measured by LC-MS/MS at the Department of Clinical Chemistry, Canisius Hospital, Nijmegen, the Netherlands (DEQAS-certified laboratory). During the intervention, intact plasma parathyroid hormone (PTH) was measured in EDTA blood by sandwich chemiluminescence immunoassay. To monitor calcium concentrations, serum calcium and albumin were measured by colorimetric analysis, and morning spot-urine was collected to monitor urinary calcium levels (expressed as calcium/creatinine ratio) (SHO laboratory, Velp, the Netherlands) [30]. Serum 25(OH)D concentration during the intervention was analyzed using LC-MS/MS (Analytical Research Center, DSM Nutritional Products, Kaiseraugst, Switzerland). Serum 25(OH)D concentration reflects the sum of serum 25(OH)D_2 and 25(OH)D_3. The analysis of serum 25(OH)D_2 showed several laboratory values below the Lower Limit of Quantitation (LLQ) of 1.2 nmol/L, and these values were set at the detection limit for data interpretation. To assess laboratory performance of the method, dedicated standard and quality control samples were analyzed daily to ensure the accuracy and precision of the method (inter-assay and intra-assay CVs were ≤15 % and accuracy 85 - 115 % according FDA and EMEA guidelines).

Biopsy samples

Muscle biopsies were collected from a subgroup (n=35) as subjects taking anticoagulant medication (except platelet inhibitors) or subjects not willing to undergo the biopsy were excluded from this procedure. Biopsies were collected from the middle region of the vastus lateralis muscle by percutaneous needle biopsy technique, as described previously [31]. Muscle biopsies were carefully freed from any visible fat and blood and embedded in Tissue-Tek (Sakura Finetek Europe BV, the Netherlands) and frozen in liquid nitrogen cooled isopentane. Samples were stored at -80 ^{0}C until analysis.

Immunohistochemistry

Frozen muscle biopsies were cut into 5 μm thick cryosections using a cryostat at −20 ˚C. Histochemical methods are previously described in more detail [32]. In brief, muscle cross sections were stained with antibodies against laminin (polyclonal rabbit antilaminin, dilution 1:50; Sigma, Zwijndrecht, the Netherlands) and myosin heavy chain (MHC)-I (A4.840, dilution 1:25; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Secondary antibodies were goat anti-rabbit IgG Alexa555 and goat anti-mouse IgM Alexa488 (dilution 1:500 and 1:400, respectively; Molecular Probes, Invitrogen, Breda, the Netherlands). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 0.238 μM; Molecular Probes). Imaging was performed with 10x magnification using an Olympus BX51WI spinning disk confocal fluorescence microscope coupled to a Hamamatsu EM-CCD C9100 digital camera. Micromanager 1.4 software was used for image acquisition [33] and ImageJ software (version 1.50b, National Institute of Health,

MD) for quantitative analyses [34]. To assess fiber cross-sectional area (CSA), laminin was used to (semi)automatically detect the outline of the individual muscle fibers; corrections were made by hand where necessary. Based on these indications, fiber size was measured for each muscle fiber to calculate mean type I and type II muscle fiber size. Mean (SD) number of muscle fibers analyzed was 264 ± 146 and 328 ± 179 at baseline and after 6 months, respectively.

Body composition

At baseline, weight was measured using an analogue scale and reported to the nearest 0.5 kg. Height was measured using a stadiometer and reported to the nearest 0.1 cm. BMI was reported as weight/height^2. Body composition was assessed by DXA (Lunar Prodigy Advance; GE-Healthcare, Madison, WI, USA) scan and Appendicular Lean Mass (ALM) was calculated as the lean mass of arms and legs [35]. In 7 subjects, the specific regions of interest (ROI) for ALM could not be accurately defined due to overlap of upper limbs and trunk.

Questionnaires

Participants filled out a comprehensive questionnaire during the screening visit. Medical history, medication, and dietary supplement use were assessed. During the intervention phase, subjects filled out a questionnaire to monitor changes in health status or medication use. Dietary intake of vitamin D and calcium were assessed by a Food Frequency Questionnaire (FFQ), as described previously [36-38].

Statistical methods

The sample size was based on a previous trial studying the effect of CAL or VD3 supplementation on knee extension strength [25]. Based on the effect size of 47.6 N (*P*-value 0.03), the corresponding variability was calculated as the pooled estimate of the SD (49.0 N) [39]. Considering a power of 80% and an alpha level of 0.05, 54 subjects were needed. Taking into account a drop-out rate of 30%, 78 subjects were included. Baseline characteristics were described as mean, SD or percent of categorical class and compared between treatment groups using one-way ANOVA for continuous variables and Chi-Square test or Fisher's Exact test in case of categorical variables. Changes in study outcomes between groups over time were analyzed using linear mixed models. Models included fixed effects of treatment, time, and the interaction between treatment and time, with subject defined as random effect. Covariates (age, sex, BMI) were included based on model fit. Model assumptions were checked by visual inspection of residual plots and the TUG time and chair rise time were log-transformed. Results are described as model adjusted means and mean changes over time including 95% confidence intervals. Log-transformed variables were transformed back to their original scale using the anti-log to present the geometric means at baseline, and ratios of geometric means to describe changes over time (baseline set as a reference 1.0). Analyses were performed based on the intention to treat principle. Statistical tests

were all two-sided and carried out at the 5% level of significance. Data analyses were performed using SPSS (version 22, IBM Corp., Armonk, NY, USA) and Graphpad Prism (version 5).

RESULTS

Figure 6.1 presents the participant flow from recruitment and randomization to study completion. In total, 500 subjects were screened for study participation and 78 subjects were randomized. After baseline, 3 subjects withdrew and 75 subjects completed the study. Two subjects withdrew due to a serious adverse event not related to study treatment and one subject because of personal reasons. Overall compliance to treatment was ≥80% in all participants, with an average compliance of 98%. At baseline, the mean age of the study population was 74 ± 6 years, and 55% were men (Table 6.1). Participants scored predominantly pre-frail on the Fried criteria (91%) and had a mean serum 25(OH)D concentration of 37.7 nmol/L (CI 35.4 to 39.9), with no baseline differences between groups (P=0.69). Figure 6.2 illustrates the change in serum 25(OH)D concentration over time, which was significantly different between all groups (treatment x time interaction P<0.01). After 6 months of supplementation, mean serum 25(OH)D concentration had increased with 60.6 nmol/L (CI 53.7 to 67.5), 35.7 nmol/L (CI 28.6 to 42.7) and 8.9 nmol/L (CI 2.0 to 15.9) in the CAL, VD3 and PLA group, respectively (Table 6.2). Furthermore, PTH concentrations were not significantly different between groups at baseline (P=0.28). However, during the study, PTH concentrations decreased significantly in both the CAL (-1.7 pmol/L, CI -2.5 to -0.9) and VD3 (-1.4 pmol/L, CI -2.2 to -0.6) group, compared to placebo (0.3 pmol/L, CI -0.5 to 1.1) (treatment x time interaction P<0.01).

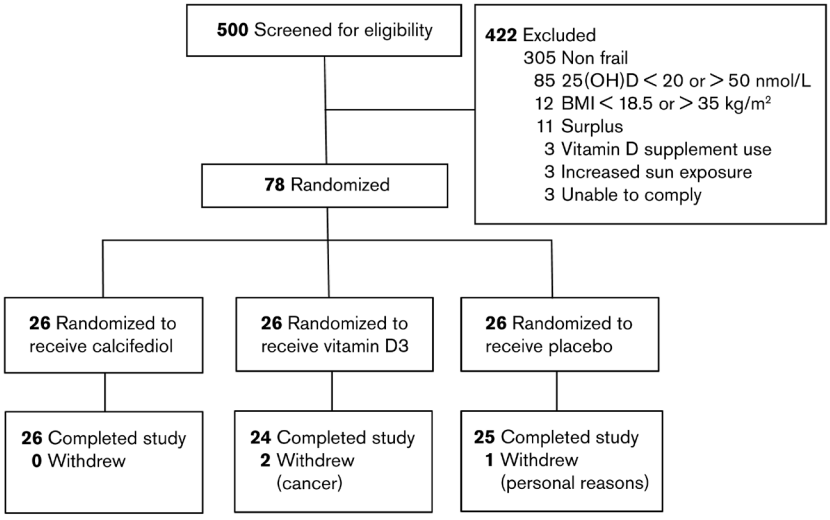

Figure 6.1 Flowchart of recruitment and inclusion of study participants.

Table 6.1 Participant characteristics.

	Calcifediol, 10μg/d (*n*=26)	**Vitamin D3, 20μg/d** (*n*=26)	**Placebo** (*n*=26)	*P*-value[†]
Demographics				
Sex, male	54 (14)	58 (15)	54 (14)	0.95
Age, y	73.1 ± 6.0	74.8 ± 6.7	73.7 ± 6.2	0.64
Height, cm	167.8 ± 9.6	167.7 ± 7.9	167.5 ± 9.2	0.99
Weight, kg	77.9 ± 12.8	77.0 ± 12.0	78.2 ± 13.6	0.94
BMI, kg/m^2	27.6 ± 3.5	27.4 ± 3.6	27.8 ± 3.7	0.92
Frailty status				
Pre-frail	81 (21)	96 (25)	96 (25)	0.20
Frail	19 (5)	4 (1)	4 (1)	
Dietary intake				
Vitamin D, μg/d	3.5 ± 1.6	3.6 ± 1.1	3.6 ± 1.5	0.97
Calcium, mg/d	1105 ± 481	985 ± 304	1014 ± 555	0.62

Values are mean ± SD or % (n). [†]Between group differences were analyzed by one-way ANOVA, Chi-Square test, or Fisher's Exact test.

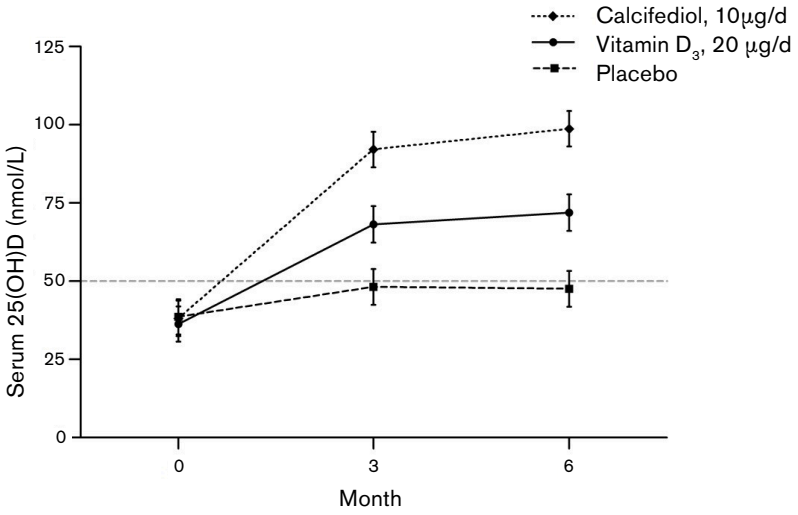

Figure 6.2 Changes in serum 25-hydroxyvitamin D concentration per treatment group.

Graph represents mean and 95% confidence intervals. Grey dashed line indicates the reference at 50 nmol/L. To convert 25(OH)D to ng/mL divide by 2.496.

Muscle strength and physical performance

Results on muscle strength and physical performance tests are described in Table 6.3. At baseline, there was no significant difference in knee-extension strength between treatment groups (P=0.67). Over time, knee-extension strength did not significantly change in the calcifediol group (5.9 Nm, CI -6.2 to 18.0), the vitamin D_3 group (5.5 Nm, CI -6.8 to 17.8) or placebo group (1.8 Nm, CI -10.7 to 14.4) (treatment x time interaction P=0.74). Likewise, no significant treatment x time interactions were observed for other strength measures (knee-flexion strength or hand grip strength). Baseline SPPB scores did not differ between groups (P=0.68), with an average score of 10.6 points. The SPPB score decreased on average during the intervention period (time effect P<0.003), with no differential change between groups (treatment x time interaction P=0.23). The time to complete the TUG test increased on average during the intervention period (time effect P=0.02), with no differences in mean change among the treatment groups (treatment x time interaction P=0.94). Likewise, no significant treatment x time interactions were observed on the gait speed and chair stand tests. Results on postural sway of the foot positions CBEC and OBEC are presented as these conditions are most distinctive for balance performance. At baseline, balance performance did not significantly differ between groups (P>0.05 for all stands), and the degree of postural sway did not change differently between groups over time.

Table 6.2 Laboratory results at baseline and changes after 3 and 6 months per treatment group.

	Calcifediol, 10μg/d	**Vitamin D3, 20μg/d**	**Placebo**	
	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	*P*-value*
25(OH)D (nmol/L)				
Baseline	38.1 (32.5; 43.8)	36.3 (30.6; 42.0)	38.1 (32.5; 43.8)	<0.01
Δ 3 months	54.0 (47.1; 60.8)	31.8 (24.8; 38.9)	9.6 (2.6; 16.5)	
Δ 6 months	60.6 (53.7; 67.5)	35.7 (28.6; 42.7)	8.9 (2.0; 15.9)	
PTH (pmol/L)				
Baseline	7.5 (6.6; 8.4)	7.6 (6.7; 8.5)	6.5 (5.6; 7.4)	<0.01
Δ 3 months	-2.0 (-2.8; -1.2)	-1.0 (-1.8; -0.2)	0.1 (-0.7; 0.9)	
Δ 6 months	-1.7 (-2.5; -0.9)	-1.4 (-2.2; -0.6)	0.3 (-0.5; 1.1)	
Calcium (mmol/L)†, §				
Baseline	2.35 (2.32; 2.38)	2.32 (2.29; 2.35)	2.34 (2.31; 2.37)	0.39
Δ 3 months	0.04 (0.00; 0.07)	0.02 (-0.02; 0.05)	0.00 (-0.03; 0.04)	
Δ 6 months	0.00 (-0.03; 0.04)	0.02 (-0.02; 0.05)	-0.03 (-0.06; 0.00)	
UCa/Cr ratio (mmol/mmol)‡, ‖				
Baseline	0.31 (0.23; 0.40)	0.39 (0.27; 0.44)	0.38 (0.30; 0.46)	0.07
Δ 3 months	0.12 (0.04; 0.21)	0.04 (-0.04; 0.13)	0.03 (-0.06; 0.11)	
Δ 6 months	0.10 (0.02; 0.07)	-0.03 (-0.12; 0.16)	-0.01 (-0.10; 0.07)	

Values are model adjusted means and 95% confidence intervals, including all three treatments and time points. *P-values represent the treatment x time interaction. †Adjusted for BMI. ‡Adjusted for BMI and sex. §Values are corrected for albumin according the following formula (plasma Ca-(0.02x[Alb-40]). ‖Urinary Calcium/ Creatinine ratio. To convert 25(OH)D to ng/mL divide by 2.496.

Table 6.3 Muscle strength and physical performance results at baseline and changes after 3 and 6 months per treatment group.

	Calcifediol, 10μg/d Mean (95% CI)	**Vitamin D3, 20μg/d** Mean (95% CI)	**Placebo** Mean (95% CI)	*P*-value*
Knee-extension strength (Nm) ‡				
Baseline	134.9 (121.5; 148.4)	127.1 (113.6; 140.6)	126.1 (112.6; 139.5)	0.74
Δ 3 months	-4.0 (-15.9; 8.0)	-1.9 (-14.1; 10.2)	1.7 (-10.7; 14.0)	
Δ 6 months	5.9 (-6.2; 18.0)	5.5 (-6.8; 17.8)	1.8 (-10.7; 14.4)	
Knee-flexion strength (Nm) ‡				
Baseline	67.7 (61.8; 73.7)	62.1 (56.1; 68.0)	62.7 (56.8; 68.7)	0.22
Δ 3 months	2.8 (-2.3; 7.9)	-0.6 (-5.7; 4.6)	1.6 (-3.6; 6.9)	
Δ 6 months	4.0 (-1.2; 9.1)	-3.3 (-8.7; 2.0)	0.3 (-5.0; 5.6)	
SPPB total (points 0-12) §				
Baseline	10.4 (9.6; 11.2)	11.0 (10.4; 11.8)	10.5 (9.7; 11.3)	0.23
Δ 3 months	-0.1 (-0.6; 0.4)	0.1 (-0.5; 0.6)	-0.6 (-1.2; -0.1)	
Δ 6 months	-0.3 (-0.9; 0.2)	-0.4 (-0.9; 0.2)	-0.6 (-1.2; -0.1)	
Timed Up and Go test (sec) †, §				
Baseline	10.4 (9.6; 11.3)	9.5 (8.7; 10.3)	10.4 (9.5; 11.3)	0.94
Δ 3 months	1.04 (0.98; 1.11)	1.02 (0.95; 1.08)	1.03 (0.97; 1.10)	
Δ 6 months	1.06 (0.98; 1.13)	1.06 (0.99; 1.11)	1.04 (0.98; 1.12)	
Gait speed, (m/sec) §				
Baseline	1.02 (0.95; 1.09)	1.08 (1.00; 1.15)	1.05 (0.98; 1.13)	0.32
Δ 3 months	-0.05 (-0.13; 0.02)	-0.06 (-0.13; 0.02)	-0.13 (-0.20; -0.06)	
Δ 6 months	-0.10 (-0.17; -0.03)	-0.11 (-0.19; -0.04)	-0.12 (-0.19; -0.04)	
Chair rise, 5 stands (sec) †, §				
Baseline	12.5 (11.4; 13.8)	11.9 (10.7; 13.1)	12.2 (11.0; 13.4)	0.33
Δ 3 months	0.96 (0.91; 1.09)	0.98 (0.90; 1.08)	1.06 (0.97; 1.16)	
Δ 6 months	1.00 (0.92; 1.10)	1.06 (0.97; 1.16)	1.07 (0.98; 1.17)	
Hand grip strength (kg) ‡				
Baseline	25.8 (23.3; 28.2)	25.5 (23.1; 27.9)	24.0 (21.6; 26.4)	0.99
Δ 3 months	0.9 (-0.4; 2.3)	0.7 (-0.7; 2.1)	0.7 (-0.7; 2.1)	
Δ 6 months	1.1 (-0.3; 2.4)	1.1 (-0.3; 2.5)	1.3 (-0.1; 2.7)	
95% area ellipse, OBEC (cm^2) ‡				
Baseline	4.24 (3.16; 5.32)	3.33 (2.26; 4.40)	3.53 (2.46; 4.61)	0.32
Δ 3 months	-0.68 (-1.85; 0.48)	0.16 (-1.01; 1.34)	0.06 (-1.15; 1.26)	
Δ 6 months	-0.75 (-1.93; 0.43)	0.61 (-0.59; 1.80)	0.45 (-0.74; 1.63)	
95% area ellipse, CBEO (cm^2) ‡				
Baseline	3.92 (3.13; 4.71)	3.61 (2.84; 4.38)	3.68 (2.90; 4.46)	0.95
Δ 3 months	-0.39 (-1.20; 0.42)	-0.43 (-1.25; 0.39)	-0.18 (-1.02; 0.65)	
Δ 6 months	-0.42 (-1.24; 0.39)	-0.65 (-1.47; 0.17)	-0.30 (-1.12; 0.53)	

Values are model adjusted means and 95% confidence intervals, including all three treatments and time points. **P*-values represent the treatment x time interaction. †Values were log-transformed and represent geometric means at baseline, and ratios of geometric means to describe changes over time (baseline set as a reference 1.0). ‡Adjusted for age and sex. §Adjusted for age. Nm, Newton meter; SPPB, Short Physical Performance Battery; OBEC, Open Base Eyes Closed; CBEO , Closed Base Eyes Open.

Muscle mass, muscle fiber type and size

Muscle mass and muscle fiber type characteristics are presented in Table 6.4. At baseline, no significant differences were observed in total lean mass (P=0.97) or ALM between groups (P=0.89). The total lean mass and ALM decreased on average during the intervention period (time effect $P \leq 0.01$). However, the changes in lean mass or ALM did not significantly differ between groups (treatment x time interaction $P > 0.05$). At baseline, no significant differences were observed in muscle fiber type distribution (P=0.20) or muscle fiber size (type I fiber CSA: P=0.42, type II fiber CSA: P=0.92) between groups. Overall, subjects showed specific type II muscle fiber atrophy, with smaller type II versus type I fiber size (P=0.03). However, over time, there were no differences in mean change between groups in muscle fiber type distribution (treatment x time interaction P=0.19) or muscle fiber size (treatment x time interaction $P > 0.05$).

Table 6.4 Body composition and biopsy results at baseline and changes after 6 months per treatment group.

	Calcifediol, 10μg/d	Vitamin D3, 20μg/d	Placebo	
	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	*P*-value*
Total lean mass (kg)†				
Baseline	49.2 (47.1; 51.2)	48.2 (46.2; 50.3)	49.0 (47.0; 51.1)	0.10
Δ 6 months	-0.35 (-0.88; 0.18)	-1.18 (-1.73; -0.63)	-0.68 (-1.22; -0.14)	
ALM (kg)†				
Baseline	21.0 (20.0; 22.0)[a]	20.3 (19.4; 21.3)[b]	21.0 (20.0; 22.0)[c]	0.43
Δ 6 months	-0.27 (-0.56; 0.03)	-0.36 (-0.68; -0.05)	-0.08 (-0.38; 0.25)	
Fiber type distribution (% type II fibers)‡, §, ‖				
Baseline	62 (52; 72)	49 (40; 59)	53 (43; 62)	0.19
Δ 6 months	1 (-8; 10)	1 (-8; 9)	11 (2; 19)	
Type I muscle fiber CSA (μm^2)‡, §				
Baseline	6595 (5523; 7667)	6476 (5490; 7463)	5717 (4645; 6789)	0.80
Δ 6 months	284 (-670; 1238)	-138 (-1016; 740)	105 (-849; 1059)	
Type II muscle fiber CSA (μm^2)‡, §				
Baseline	5223 (3672; 6775)	5404 (3978; 6832)	4993 (3442; 6545)	0.22
Δ 6 months	615 (-344; 1573)	-375 (-1457; 507)	598 (-361; 1557)	

Values are model adjusted means and 95% confidence intervals, including all three treatments and time points. *P-values represent the treatment x time interaction. †Adjusted for sex and BMI. ‡Adjusted for sex. [a]1 missing, [b]2 missing, [c]4 missing. §Biopsy measures were performed in a subgroup of n=11 CAL, n=13 VD3, n=11 PLA. ‖Percent type I fibers is the inverse of percentage shown in the table. CSA, Cross-sectional Area.

Safety evaluation

During the intervention, serum calcium concentrations remained below the reference value of 2.6 mmol/L, except for one subject in the CAL group, who had a serum calcium level of 2.61 mmol/L at 3 months. However, these values normalized by the end of the study. No cases of hypercalcemia occurred during the study period and changes in serum calcium did not significantly differ between groups (treatment x time interaction P=0.39) (Table 6.2). Urinary calcium/ creatinine ratios tended to increase in the CAL group compared to VD3 and PLA (treatment x time interaction P=0.07), however

remained below 1.0 in all groups [40]. In total, 43 participants reported one or more adverse events (AE), with 39% occurring in the VD3 group, 30% in the CAL group, and 30% in the PLA group. The type of reported AE were not related to a specific treatment (P=0.44).

DISCUSSION

In this study, 6 months of daily supplementation with either 10 μg CAL or 20 μg VD3 increased serum 25(OH)D concentration towards status levels of 97 nmol/L and 71 nmol/L respectively, whereas the PLA group remained on average below the 50 nmol/L threshold during the entire study period. Despite these significant contrasts in serum 25(OH)D levels achieved, no significant differential effects were observed on muscle strength or physical performance.

These findings are in line with several previous studies [18-21], though conflict with a comparable number of studies that did establish a beneficial effect on muscle strength after vitamin D supplementation [14-17, 25]. The broad variation in study designs, treatment comparator, baseline 25(OH)D levels and study measures, may in part explain these inconclusive results. Therefore, we aimed to include participants who were pre-frail or frail, and vitamin D deficient at baseline (20-50 nmol/L), as those might benefit most from supplementation [41]. Moreover, we used a broad battery of functional tests and included the Biodex System, which is considered the gold standard in assessing muscle strength [42].

In this study, one treatment arm was supplemented with CAL as it can rapidly restore serum 25(OH)D levels. Similar to previous trials, CAL was ~3 times more potent (per microgram supplemented) in raising serum 25(OH)D levels compared to native VD3 [22]. To date, only two previous trials have examined the effect of CAL on muscle function [18, 25]. The first trial was a pilot study among 20 postmenopausal women, supplemented with either CAL (20 μg/d or 140 μg/wk) or VD3 (20 μg/d) for 16 weeks, increasing serum 25(OH)D levels from an average of 30-35 nmol/L to 173 nmol/L in the CAL group and to 77 nmol/L in the VD3 group [25]. In that study, CAL supplementation resulted in a significant 17% improvement in knee extension strength compared to supplementation with VD3. The superior effect of CAL over VD3 could not be confirmed in our study, which might relate to the lower dose and status levels achieved. However, previous studies have also indicated a possible U-shaped distribution, where higher status levels not always equal positive results [43]. This was observed in a recent trial among 200 older adults, comparing a low monthly dose (600 μg VD3) with high monthly doses of either VD3 (1500 μg) or VD3 plus CAL (600 μg VD3 + 300 μg CAL) [33]. One year supplementation did not improve physical performance, but reduced the number of falls at the lower 25(OH)D threshold of 53 to 76 nmol/L, while an increased risk of falls was observed in the highest 25(OH)D quartile, reaching status levels between 112-247 nmol/L.

The optimal serum 25(OH)D concentration for muscle health is a matter of ongoing debate. Although the IOM recommends serum 25(OH)D levels between 30-50 nmol/L based on bone health [44], higher thresholds are suggested for muscle function. Observational studies suggest that physical performance and strength outcomes tend to increase with serum 25(OH)D concentrations between 60-115 nmol/L [11, 45-47]. A recent one-year trial, supplementing a daily dose of 20 μg VD3 and high monthly dose of 1250 μg VD3 compared with PLA, achieved status levels ≥50 nmol/L and ≥75 nmol/L with daily and monthly treatment, respectively [34]. Although participants were not vitamin D deficient at baseline, this study showed that increasing status levels up to these specific thresholds did not improve functional parameters. In our study, comparable status levels were achieved after 6 months of supplementation with VD3 (72 nmol/L) or CAL (99 nmol/L) and the study duration allowed serum 25(OH) D concentrations to reach a plateau, along with significant suppression of PTH levels. Nevertheless, it is plausible that profound vitamin D deficiency must exist to elicit an effect of supplementation on muscle function. Two meta-analyses indeed reported that subjects with serum 25(OH)D levels <25-30 nmol/L show greater improvements in muscle strength than older adults with baseline levels above these thresholds [41, 48]. In our study, it was considered unethical to randomize and include participants with severe vitamin D deficiency. Therefore, subjects with serum 25(OH)D levels <20 nmol/L were excluded from participation and mean baseline 25(OH)D status was 38 nmol/L. In addition, although we screened according the Fried criteria [27], participants were in general good health, and physical performance levels were relatively high, indicated by average SPPB scores between 10.4 and 11.0 points. As such, participants might have been less likely to show improvements in strength and functioning.

Myopathy and atrophy of type II muscle fibers is described in severe vitamin D deficient states [7]. However, not many randomized studies have assessed the impact of vitamin D supplementation on morphological changes in muscle fibers. In a study among 21 mobility-limited older women, 4 months of VD3 supplementation (100 μg/day) tended to induce a selective type II muscle fiber hypertrophy compared to PLA, although, significance was only reached for total fiber size (mean type I and II fibers) [49]. In contrast, we did not observe any indication of increased muscle fiber size in response to the 6 months of supplementation with either VD3 or CAL. An increase in fiber size may have been more easily detected in the study by Ceglia et al. [49], since fiber size at baseline was substantially smaller than in our population of relatively healthy, predominantly pre-frail older adults; a difference that cannot only be explained by the inclusion of both men and women in the current study. Given the relatively low number of subjects included in the biopsy analyses of both studies, as well as the substantial intra-individual variation inherent to the muscle biopsy and histochemical procedures, the effects of vitamin D supplementation on myocellular characteristics in humans remains to be further investigated.

In our study, the placebo group remained on average vitamin D deficient (end of study serum 25(OH)D of 48 nmol/L), with only minor changes in serum 25(OH)D concentration related to the seasonal changes. Considering the well-established effect of vitamin D on bone health, this emphasizes the need for supplementation in older adults. Current literature points to a role of vitamin D in muscle function, especially in cases of extreme deficiency. However, results of randomized trials are inconclusive, and meta-analysis of these trials conclude either a small beneficial effect [41, 50] or no effect of vitamin D supplementation [51]. Therefore, it remains to be elucidated whether vitamin D supplementation can effectively improve or maintain muscle function in older adults. In conclusion, improving serum 25(OH)D concentration over 6 months did not significantly change muscle strength or function in a population of predominantly pre-frail older adults.

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Authors' contributions: AV, MT, LG and LL were involved in study design; AV, MT, NT conducted the study; RN and LV analyzed biopsy samples; AV analyzed the data; all authors contributed to the writing and approval of the manuscript.

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CHAPTER 7

General discussion

This thesis aimed to address three topics related to vitamin D in the older population. First, we examined the prevalence and the main determinants of a low vitamin D status. Next, we investigated strategies to improve serum 25(OH)D concentrations by exploring the contribution of dietary vitamin D intake and the potential of calcifediol as a supplementation strategy. Finally, we examined the role of vitamin D in muscle health, by exploring the associations of serum 25(OH)D status with measures of physical performance and frailty, and by performing a placebo-controlled trial to study the effect of vitamin D_3 or calcifediol supplementation on muscle strength and physical performance in pre-frail and frail older adults.

Findings in view of current literature

Prevalence and determinants of vitamin D deficiency

Year-round measures of serum 25(OH)D concentration in a large study sample of community-dwelling Dutch older adults indicated a high prevalence of vitamin D deficiency, with serum 25(OH)D concentrations <50 nmol/L in 45%, and <30 nmol/L in 14% of the population (chapter 2). These prevalence rates are in line with estimates across Europe, with 40% and 13% having serum 25(OH)D levels <50 nmol/L and <30 nmol/L, respectively [1]. From a public health perspective, these numbers are alarming and action is needed to protect against vitamin D deficiency. However, identifying those at risk of a low vitamin D status requires a good understanding of its main determinants. Therefore, we examined to what extent vitamin D intake, frequency of supplement use, sun exposure and genetic factors are associated with serum 25(OH)D concentrations. Daily vitamin D intake from dietary sources showed a median (25-75th percentile) intake of 4.0 (3.0-5.4) μg/day and only 12-20% of older adults reported to take vitamin D supplements. This result clearly indicates that the vast majority of the older population does not meet the current recommendations of vitamin D intake [2]. Furthermore, stratification for season indicated a higher prevalence of serum 25(OH)D levels <50 nmol/L during winter/spring (63%). However, low vitamin D status was also evident during summer/autumn (37%), suggesting that many older adults are unable to overcome deficiencies even during the seasons when cutaneous vitamin D synthesis is at its highest. Previous studies indeed suggest that the cutaneous synthesis of pre-vitamin D decreases with age [3, 4] and that older adults, especially frail older adults, tend to go outside less. However, we still observed positive associations between behavioral factors, such as, 'being outside daily' and serum 25(OH)D concentrations, suggesting that habitual summer sun exposure remains an important determinant of vitamin D status in the older population (chapter 2). Moreover, we learned that taking genetic factors into account resulted in a better understanding of the variation in serum 25(OH)D concentrations. Single nucleotide polymorphisms (SNPs) of the genes encoding for the enzymes 7-dehydrocholesterol reductase (DHCR7), cytochrome P450 2R1 (CYP2R1) and Group-specific Component (GC) were significantly associated with serum 25(OH)D concentrations, with differences between allele carriers ranging from 4-15 nmol/L. Most strongly associated with serum 25(OH)D status was the gene

GC, which encodes for the vitamin D binding protein (DBP). This protein plays an important role in vitamin D metabolism as most circulating metabolites are transported to target cells while bound to DBP [5]. These genetic variations can be of clinical relevance as risk-allele carriers have been associated with a reduced response to vitamin D supplementation [6, 7] and a greater risk of adverse health outcomes, such as fractures [8]. Taking genetic factors into account, contributed to identifying those at predisposed risk of a low vitamin D status. However, genetic factors never work individually and creating combined risk scores with environmental and behavioral factors might further advance our understanding of the variation in vitamin D status across the older population.

Strategies to improve serum 25(OH)D status

When aiming to prevent vitamin D deficiency, several strategies are possible to achieve adequate serum 25(OH)D concentrations in the older population. In chapter 3, we observed that despite relatively low intakes, dietary vitamin D intake was positively associated with serum 25(OH)D concentration. Here, the food group 'butter and margarines' mainly contributed to total vitamin D intake, which is not surprising as margarines are commonly fortified with vitamin D. As a result, older adults with higher intakes of butter and margarines were 21% more likely to have a sufficient 25(OH)D status (≥50 nmol/L). We concluded that while regular intake of foods rich in vitamin D can support the prevention of modest 25(OH)D insufficiency, fortified foods or supplements are essential to meet current requirements and to substantially improve serum 25(OH)D concentrations across the general population. However, it is important to note that food fortification is not that common in the Netherlands. A previous study showed that only 17% of the total vitamin D intake in older adults is attributable to fortified foods [9]. Furthermore, a recent simulation study in the general Dutch population indicated that while an increased fortification of margarines and milk products can double the dietary vitamin D intakes in the Netherlands, additional supplementation would still be required to reach current recommendations [10]. Thus, the Dutch Health Council recommends women 50-70 years of age, and men and women ≥70 years to take a vitamin D supplement of 10 and 20 μg/day, respectively [2]. Supplementation with vitamin D_3 is most common, and although alternative supplementation strategies do exist, these require further investigation.

In chapter 4, we compared the dose-response effects of three different doses of calcifediol (5, 10 and 15 μg/day) to vitamin D_3 (20 μg/day) over a 6-month period. Supplementation with calcifediol might result in a predictable serum response as it is more readily absorbed and does not require hepatic 25-hydroxylation, resulting in a rapid increase in serum 25(OH)D concentrations [11, 12]. In our study, a daily dose of 10 μg calcifediol was able to correct deficiency (<50 nmol/L) within 4 weeks after start of supplementation, which resulted in a sustained increase towards serum 25(OH)D levels between 75-100 nmol/L after 8 weeks. These effects occurred along with a

significant suppression of parathyroid hormone (PTH). Daily supplementation with 20 μg vitamin D_3 increased serum 25(OH)D concentrations above the >50 nmol/L threshold within 4 weeks as well, however, the 75 nmol/L threshold was not reached during the entire study period. Calcifediol supplementation was 3 times more potent compared to vitamin D_3 (per μg supplemented) in raising serum 25(OH)D concentration. In previous clinical studies, calcifediol induced a 1.5-5 times higher increase in serum 25(OH)D concentrations when compared to vitamin D_3 [13-15]. The observed variation between studies can be explained by the fact that the pattern of increase in serum 25(OH)D depends on the dosage and degree of deficiency at the start of supplementation.

Vitamin D and muscle health

Vitamin D supplementation is suggested to prevent or to alleviate the age-related loss in muscle strength and function in older adults [16]. In chapter 5, we observed that community-dwelling older adults with deficient serum 25(OH)D concentrations (<50 nmol/L) were more likely to be frail compared to their sufficient counterparts, and serum 25(OH)D concentrations >75 nmol/L were associated with better performance on the gait speed and the Timed Up and Go test. These findings are largely in line with the current literature [17-20] and might suggest that higher thresholds than 50 nmol/L are required to optimize muscle function in older adults. However, while the associations between low vitamin D status and physical performance are well documented, the impact of supplementation on these outcomes is uncertain. Several studies observed slight positive effects of vitamin D supplementation on strength and balance [21-24], whereas others report no effect after supplementation [25-28]. A previous pilot trial (*n*=20) studied the effect of calcifediol supplementation in postmenopausal women and reported beneficial effects on lower extremity function when compared to vitamin D_3 supplementation [13]. In chapter 6, we performed a placebo-controlled trial to investigate the effect of either vitamin D_3 or calcifediol supplementation in vitamin D deficient (20-50 nmol/L), pre-frail and frail older adults, over a 6-month period. While both supplementation regimens resulted in significant increases in serum 25(OH)D status compared to placebo, no effect was observed on lower extremity strength after supplementation. Meta-analyses reflect the inconsistent findings from randomized trials, suggesting either null-effects [29], or small beneficial effects of vitamin D supplementation on muscle strength [30, 31]. Meanwhile, two additional randomized trials have been published, with daily or monthly supplementation in older adults without apparent deficiencies at baseline (serum 25(OH)D 47-52 nmol/L). In both studies, one-year supplementation did not affect lower extremity function (sit-to-stand, timed walk, or SPPB test) [25, 26]. Moreover, one of these studies even observed adverse effects on fall risk after high monthly intermitted doses (600 μg vitamin D_3 + 300 μg calcifediol) [25]. Similar findings were observed in a previous large trial, where a high annual dose (12,500 μg vitamin D_3) over a period of 3-5 years increased the risk of falls and fractures [32]. The underlying mechanism for these adverse results are not yet understood but may relate to induced catabolic effects of vitamin D, increasing

24,25$(OH)_2$D metabolite production and decreasing 1,25$(OH)_2$D [33, 34]. All in all, these studies suggest a specific therapeutic window where both the baseline serum 25(OH)D status and the achieved target levels need to be taken into consideration when evaluating the effect of vitamin D supplementation.

The contradictory findings among published studies are likely caused by the broad variation in dosing regimens, baseline serum 25(OH)D levels, participant characteristics, and the variety of tests that were used to assess muscle strength. However, part of the inconclusive findings might also relate to the fact that both direct and indirect effects of vitamin D can be expected. Vitamin D deficiency might indirectly affect muscle function by causing a number of metabolic changes, including secondary hyperparathyroidism, hypocalcemia or hypophosphatemia [16]. A recent study in mice showed that deficiency of both calcium and vitamin D resulted in poorer performance on tasks than vitamin D deficiency alone [35]. In addition, a study in rats showed that muscle force was affected only when vitamin D deficiency was accompanied by hypophosphatemia and muscle contraction could be normalized by restoring serum phosphate concentrations [36]. Nevertheless, this was contradicted by a recent study, showing that vitamin D depleted mice had weaker grip strength compared to replete controls, while maintained on an adequate calcium and phosphate diet [37]. Furthermore, several studies report independent associations between PTH and muscle function as an impaired muscle strength is also observed in older adults with mild secondary hyperparathyroidism [38, 39]. In addition, plausible synergistic effects between vitamin D and other nutrients, e.g. protein have been suggested. Exposure of murine C2C12 muscle cells to 1,25$(OH)_2$D enhanced the stimulating effect of leucine on protein synthesis rates [40]. In humans, a recent study indeed suggested that both sufficient serum 25(OH)D concentrations (>50 nmol/L) and protein intakes (>1.0 g/kg/day) were essential to increase muscle mass in sarcopenic older adults [41]. As such, vitamin D and its related co-factors might elicit distinct and complementary functions on muscle health, which require further investigation.

Methodological considerations

Study designs

The studies described in chapter 2, 3 and 5 had a cross-sectional design and thus, no inferences can be made on causality of the observed associations. This may be especially true for our analysis on serum 25(OH)D concentrations and physical performance or frailty (chapter 5) as many of the factors that affect serum 25(OH)D concentrations (e.g. old age, BMI, physical inactivity) are also considered risk factors of an impaired physical functioning or frailty. Although we were able to include these factors into our models, along with a broad range of other lifestyle and health related factors, residual confounding cannot be excluded. For example, we had no or limited data on chronic diseases, medication use or PTH, all of which are important factors to consider. Also for the randomized trials, some methodological considerations are

noteworthy (chapters 4 and 6). To accurately estimate the dose-response relationship of vitamin D supplementation, one of the challenges is limiting seasonal effects (chapter 4). Especially when investigating supplementation over a relatively long timespan (6 months), seasonal variation is inevitable, and might reduce the external validity of the dose-response findings. In our study, randomization assured comparability between groups and participants that had planned a sunny holiday were excluded. Moreover, the main study period fell in winter season and as such, possible endogenous vitamin D synthesis was minimized. In chapter 6, we aimed to test the effect of calcifediol and vitamin D_3 supplementation on muscle strength, compared to a placebo treatment. Based on our dose-response data, we chose a dosing regimen that would induce a significant increase of serum 25(OH)D towards the range of 75-100 nmol/L. No concomitant supplementation with calcium was chosen as to specifically test the individual effect of vitamin D and the dietary calcium intake was considered sufficient (1015 ± 450 mg/d).

Study populations

The study populations included in the studies described in this thesis all represent community-dwelling older adults. Nevertheless, the fact that the participants in the cross-sectional studies were willing to volunteer in randomized trials might have resulted in the inclusion of adults who were more health conscious than the general population. Besides, the B-Proof study (chapter 2 and 3) only included participants with (mildly) elevated homocysteine levels [42]. Inverse associations have been described between serum 25(OH)D and homocysteine [43], which might have resulted in the inclusion of older adults with relatively low serum 25(OH)D concentrations. However, the observed prevalence of deficiency in this study sample, was comparable to previous Dutch cohorts of community-dwelling older adults [44, 45]. The study populations in the randomized trials (chapter 4 and 6), were explicitly selected to support the primary study objectives, taking into account factors that could affect the metabolism of the supplement, e.g. age, BMI, baseline 25(OH)D levels, clinical conditions and medication use. Furthermore, we aimed to include frail elderly with baseline 25(OH)D between 20-50 nmol/L (chapter 6), as the positive effects of vitamin D supplementation on muscle strength are particularly shown in vulnerable populations with low baseline serum 25(OH)D levels [23, 46-48]. However, it appeared challenging to recruit the frailest seniors, and despite being pre-frail or frail according the Fried definition, our participants were in a relatively good physical state [49]. Moreover, the inclusion of participants in more severely deficient states i.e. <20 nmol/L, was considered unethical. These restrictions may have limited the inclusion of participants that might have benefited the most from supplementation.

Serum 25(OH)D measurements

In all chapters, vitamin D was assessed using the gold standard, liquid chromatography-mass spectrometry (LC-MS) [50]. In chapter 5, analyses were performed in two different laboratories, however, measurement variation between these laboratories has

been reported to be acceptable [51] and will not substantially affect the ranking of the individuals. Moreover, laboratory site was included as a covariate in all models. Serum 25(OH)D concentration is currently considered the best marker of vitamin D status. However, the accurate measurement of serum 25(OH)D status remains a challenge as it can interact with other vitamin D metabolites, such as 3-epi-25(OH)D (C3-epimer) which is known to interact with LC-MS methods [52]. In addition, the impact of varying DBP levels on the measurement of protein-bound 25(OH)D concentrations, as compared to 'free' available 25(OH)D concentrations is debated [53, 54]. Serum 25(OH)D binds up to 90% to DBP, around 10% to albumin, and less than 1% remains unbound in the circulation [55]. This 'free' fraction of 25(OH)D is suggested to enter target-cells without its protein-carrier and as such, is suggested as a potential marker of status and biological effect. However, the exact role of these metabolites and their impact on 25(OH)D assays is still under investigation.

Measuring determinants of intake and status

Self-reported methods were used to assess dietary intake and habitual sun exposure. The associations observed between these proxy measures and serum 25(OH)D concentrations, but also the clear seasonal trends add face validity to our findings. However, methods are suboptimal and could have resulted in an underestimation of the explained variation in 25(OH)D concentrations. Sun exposure questionnaires for example did not account for time of the day and UVB-intensity of exposure. The use of dosimeters in combination with a questionnaire can improve precision of these estimates, yet these might be costly in large study populations [56, 57]. Besides, vitamin D intake may be underestimated as certain foods also contain 25(OH)D while food consumption tables do not yet account for this metabolite. Food sources that contain 25(OH)D include meat and poultry (0.2-0.4 μg/100g) and egg yolk (~1.0 μg/100g) [58]. Adding this metabolite to the dietary intake estimates as assessed by a food frequency questionnaire may result in vitamin D intake estimates that are ~1.7-2.9 μg/day higher and may improve the association-analyses between intake and status [59].

Measuring muscle strength and function

In chapter 5 and 6, we measured physical performance using an extensive test battery to investigate various aspects of muscle strength and function. When aging, muscle strength decreases particularly in the lower extremities, and based on previous studies [30], lower limb strength was considered a relevant study endpoint. However, testing muscle strength in older adults is challenging as measures might be limited due to fear or pain complaints in the frailest elderly to perform maximum strength. Therefore, we performed sensitive tests according standardized procedures including a measure of isometric muscle strength (Biodex System), but also a measure to assess subtle changes in postural sway (Accusway platform). In addition, functional assessments such as the SPPB, TUG and gait-speed test were included to aid comparison of trial results with previous studies.

Translation of findings to public health

Prevention of deficiency

Considering the acknowledged role of vitamin D on bone health, the high prevalence of vitamin D insufficiency and the low supplement use, due attention is warranted to prevent vitamin D deficiencies. As most factors that contribute to a low vitamin D status in older adults are behavioral, small changes in sun exposure and dietary habits might support the prevention of deficiencies. Nevertheless, raising awareness not only about the behavioral factors and foods that contribute to vitamin D status, but also about the fortified foods and current supplementation advice is key to effectively prevent vitamin D deficiency in the majority of the older population [2].

Supplementation strategies

Both the Dutch Health Council, and international clinical guidelines endorse the use of vitamin D supplementation in older adults without prior screening of serum 25(OH)D status, although screening is supported in at-risk populations, such as patients with osteoporosis, or those at risk of fractures or recurrent falling [2, 60, 61]. In clinical practice, calcifediol could provide a valuable supplementation alternative, ensuring rapid correction of deficiency without the necessity to use loading-doses. Particularly in patients with impaired liver function, but also in cases of obesity, calcifediol might more effectively improve serum 25(OH)D status [62]. In previous trials among older adults, daily doses of calcifediol ranged between 5-20 μg/day and showed good acceptability in all participants, with no cases of hypercalcemia [13, 14, 63, 64]. Nevertheless, when supplementing with higher dosages over longer time-periods, monitoring of serum calcium levels might be warranted.

Vitamin D and muscle health

There is considerable debate on what constitutes an optimal vitamin D status. The Institute of Health (IOM) and Dutch Health Council both consider serum 25(OH)D concentrations of 50 nmol/L sufficient [2, 65]. The International Osteoporosis Foundation (IOF) and Endocrine Society (ES) consider a target of 75 nmol/L sufficient to support musculoskeletal health and the prevention of falls and fractures [44, 45]. These higher thresholds are mainly supported by the observation that PTH levels start to increase when serum 25(OH)D concentrations fall below 75 nmol/L. However, the question remains whether these higher thresholds will truly benefit public health. Current literature indicates that older adults with severe deficiencies (serum 25(OH)D <25-30 nmol/L) tend to benefit most from vitamin D supplementation when aiming to support muscle health and reduce the risk of falls and fractures. However, the evidence from intervention studies in less extreme cases of deficiency appears too inconsistent to support the contention that an impaired muscle function can be prevented or improved by raising serum 25(OH)D concentrations above the threshold of >50 nmol/L. Moreover, caution is needed with high intermitted dosing regimens of vitamin D, given the possible adverse effects on fall risk [32, 46]. Thus, until more evidence becomes available, it is

considered best to advise a cautious approach towards vitamin D supplementation and place more emphasis on preventing deficiencies rather than promoting higher serum 25(OH)D levels that go beyond current guidelines. While on-going research continues to provide evidence-based guidance, public health practitioners could however, pay specific attention to at-risk populations, such as frail, institutionalized older adults or persons with functional limitations by determining their vitamin D status and advising vitamin D supplements.

Future directions

Preventing vitamin D deficiency and defining the optimal serum 25(OH)D status for muscle health remains challenging. Future studies should therefore continue to examine the effectiveness of current strategies to promote adequate vitamin D intakes in older adults and evaluate how these strategies can be improved. The dietary reference values for vitamin D require regular evaluation with regard to their validity according most recent insights. In this context, performing individual patient data (IPD) meta-analyses might offer a valuable approach to better define the dose-response relationship between intake and status, as well as to link individual response data to health outcomes. A recent study indicated that the use of IPD analyses better captured the between-person variability compared to the use of aggregate data, resulting in intake estimates of 26 μg/day to maintain serum 25(OH)D status >50 nmol/L in 97.5% of the general population [66]. Besides that, more evidence is needed from randomized trials specifically designed to test the hypothesis that vitamin D supplementation benefits muscle health. Until now, most studies have had a small sample size, and thus, the chance of under-powering these studies was high. Moreover, included subjects were often not vitamin D deficient at the start of the study, while those might respond most to supplementation [30, 31]. However, performing future trials is challenged by the fact that studying deficient populations in placebo-controlled settings is considered unethical. As such, researchers might miss the 'window of opportunity' to perform confirmatory trials on vitamin D and muscle health. Currently, several mega-trials (n=2159-25,875) are underway to investigate the effect of vitamin D supplementation on multiple health outcomes, which often include physical performance measures as secondary outcomes [67-69]. Although these trials do not have a deficient status as inclusion criteria, sample sizes are expected to be large enough to stratify on baseline status and specify subgroups. Moreover, the observed associations between genetic factors and serum 25(OH)D concentrations plea for new research opportunities. Several studies indicate that genetic variants of the VDR are linked to muscle strength and the risk of sarcopenia in older adults [70-73]. Mendelian randomization studies, including large population based datasets (e.g. biobanks), might help to identify the complex role of genes in the susceptibility to developing low vitamin D status and its related health outcomes. Lastly, there is a continuous need for mechanistic studies to better understand both the direct and indirect pathways between 25(OH)D, the active metabolite $1,25(OH)_2D$ and muscle tissue, as well as to identify the role of possible co-factors.

Conclusion

In an ageing society, preventing vitamin D deficiency and promoting adequate vitamin D intakes are important considering their beneficial effects on bone health. Besides, frail older adults with vitamin D deficiencies (<25-30 nmol/L) are likely to benefit from vitamin D supplementation with regard to muscle function. However, further trials are needed to ascertain these reference ranges and the magnitude of effect on functional outcomes before changing the recommendations on vitamin D supplementation. Until then, focus should be placed on the prevention and identification of deficiency.

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SUMMARY

Vitamin D has been identified as an important factor in healthy aging and is receiving growing attention in clinical research. Vitamin D is a fat-soluble molecule, which is synthesized by hepatic and renal or extra-renal hydroxylation into the active hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). The main function of this metabolite is to regulate calcium and phosphorus homeostasis and to support bone mineralization. In the circulation, the 25-hydroxyvitamin D metabolite (25(OH)D) is most stable and thus, considered the best marker of vitamin D status. A serum 25(OH)D concentration <30-50 nmol/L is considered deficient. Given the increased risk of deficiency and the potential beneficial effect of supplementation on musculoskeletal health, older adults present a specific target group for vitamin D interventions. However, the optimal serum 25(OH)D concentration is a matter of ongoing debate as randomized trials show conflicting results.

With the research presented in this thesis, we aimed to gain insight in the prevalence and main determinants of a low vitamin D status, to investigate strategies to prevent or reverse vitamin D deficiency, and to study the effect of vitamin D supplementation on muscle strength and physical performance in Dutch older adults.

In **chapter 2**, we examined the prevalence and the main determinants of a low vitamin D status in a large population of community-dwelling older adults (*n*=2857). Vitamin D deficiency was highly prevalent, with serum 25(OH)D concentrations <50 nmol/L in 45%, and <30 nmol/L in 14% of the population. When exploring the main determinants of serum 25(OH)D status, significant associations were observed with age, BMI, dietary intake, sun exposure behavior, and genetic polymorphisms encoding for enzymes in the vitamin D pathway. Combined, these factors explained 35% of the variation in serum 25(OH)D concentrations.

To explore potential strategies that prevent vitamin D deficiency, we investigated the contribution of dietary vitamin D intake and specific food groups to serum 25(OH) D concentration in **chapter 3**. Daily vitamin D intake from dietary sources showed a median (25-75th percentile) intake of 4.0 (3.0-5.4) μg/day (*n*=595) and only 12-20% of older adults reported to take vitamin D supplements. These findings are in sharp contrast with the current nutrient guidelines and show that the vast majority of older adults do not meet the reference intakes for vitamin D. Nevertheless, significant associations were observed between the highest tertile of dietary vitamin D intake and serum 25(OH)D concentration, suggesting that regular intake of foods rich in vitamin D can support the prevention of modest insufficiency.

For the majority of older adults, supplementation is required to ensure sufficient serum 25(OH)D concentrations throughout the year. Currently, supplementation with vitamin D_3 is the most common strategy. However, alternative treatment regimens exist that require further investigation. In **chapter 4**, we report on a dose-response trial (*n*=59)

that investigated the efficacy of calcifediol (5, 10 or 15 μg/d) as a supplementation strategy. Compared to vitamin D_3, calcifediol is more hydrophilic, does not require hepatic hydroxylation, and binds with higher affinity to its binding proteins. In our study, we observed that calcifediol was safe and well tolerated in the supplemented doses over the entire study period of 6-months. We concluded that a dose of 10 μg/day resulted in sustained serum 25(OH)D concentrations between 75-100 nmol/L. Furthermore, calcifediol had a ~3 times higher potency when compared to vitamin D_3, in increasing serum 25(OH)D concentrations. All in all, calcifediol may offer a valuable supplementation regimen to rapidly correct deficiency.

Vitamin D presents an important endocrine regulator in the musculoskeletal health of older adults. Besides its role in bone health, low serum 25(OH)D concentrations have been linked to impaired physical performance and increased risk of falling. The active metabolite 1,25-dihydroxyvitamin D is suggested to act upon a wide variety of cells throughout the body, including muscle cells. Although the exact mechanisms by which vitamin D acts on muscle are unclear, several indirect or direct regulatory pathways have been described, including effects of 1,25-dihyroxyvitamin D on intracellular calcium and phosphate homeostasis, or via activation of transcription factors when binding to the vitamin D receptor in muscle cells.

In **chapter 5** we observed significant associations between low serum 25(OH)D concentrations, physical performance and frailty in community-dwelling older adults (*n*=494-756). However, randomized trials are needed to define the causality of the observed associations. A previous pilot study indicated plausible beneficial effects of calcifediol over vitamin D_3 on performance and strength. As such, we aimed to further explore the potential role of calcifediol or vitamin D_3 on muscle function in **chapter 6**. We performed a placebo-controlled trial in pre-frail and frail, vitamin D deficient older adults, supplementing either 10 μg/d calcifediol or 20 μg/d vitamin D_3, compared to placebo over a 6-month period (*n*=78). Again, calcifediol induced a faster and higher increase in serum 25(OH)D status when compared to vitamin D_3. However, we observed no effect of either supplementation regimen on lower extremity strength or physical performance. Current literature suggests positive effects on strength and balance when supplementing with vitamin D, however, results are inconsistent. Meta-analyses of randomized trials indicate that the beneficial effects of vitamin D supplementation might be more pronounced in vulnerable populations with more severe vitamin D deficiencies.

All in all, the high prevalence of vitamin D deficiency is alarming. Promoting adequate vitamin D status is important considering the beneficial effects on bone health. In the last decade, research has come a long way in exploring the role of vitamin D in muscle function. However, the evidence base remains uncertain and further research on the optimal vitamin D status for older adults is needed to guide clinical practice. Until then, focus should be placed on prevention and identification of deficiency.

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Anouk

ABOUT THE AUTHOR

CURRICULUM VITAE

Anouk Vaes was born on the 1st of March, 1988 in Maastricht, the Netherlands. After having obtained her secondary school diploma at the 'Sintermeerten College' in Heerlen, she started the BSc program 'Nutrition and Dietetics' at HAN University of Applied Sciences in 2005 and graduated in 2009. She proceeded with a MSc program in 'Nutrition and Health' at Wageningen University. In 2011 she conducted her MSc thesis at the B-Proof cohort at Wageningen University and her internship at the Radboud Medical Centre. After having obtained her MSc diploma, she started working as a research assistant in several large scale cohort studies at Wageningen University. Besides, she worked as a lecturer at the HAN University of Applied Sciences, where she gave a course in Evidence Based Practice for BSc students in Nutrition and Dietetics. In October 2012, she started her PhD program at the department of Human Nutrition at Wageningen University. She executed her PhD research under the supervision and guidance of Prof. Lisette de Groot and Dr. Michael Tieland. This project was partly funded by the Top Institute of Food and Nutrition where she worked in a project team called Muscle Health and Function. Besides, she collaborated with researchers from the B-Proof consortium to write two additional publications. The results of these projects are described in this PhD thesis 'Vitamin D for older adults'. In addition to her PhD research, she worked in a research group on a project for the European Food Safety Authority (EFSA) for the setting of Dietary Reference Values for vitamin D. In 2016 she was selected to participate in the 22nd seminar of the European Nutrition Leadership Platform in Luxembourg. Besides her research activities, Anouk was involved as a tutor for the course Clinical Nutrition and she supervised 20 students with their MSc thesis. During the last year of her PhD she was involved in educational development for the distance learning master Nutritional Epidemiology and Public Health. Currently, Anouk works as an advisor at the Knowledge Institute of the Federation Medical Specialists in Utrecht.

LIST OF PUBLICATIONS

Peer reviewed publications

Vaes AMM, Tieland M, de Regt MF, Wittwer J, van Loon LJC, de Groot LCPGM (2017). "Dose-response effects of supplementation with calcifediol on serum 25-hydroxyvitamin D status and its metabolites: a randomized controlled trial in older adults." Clinical Nutrition. DOI 10.1016/j.clnu.2017.03.029.

Vaes AMM, Brouwer-Brolsma EM, van der Zwaluw NL, van Wijngaarden JP, Berendsen AA, van Schoor NM, van der Velde N, Uitterlinden AG, Lips P, Dhonukshe-Rutten RA, de Groot LCPGM (2016). "Food sources of vitamin D and their association with 25-hydroxyvitamin D status in Dutch older adults." Journal of Steroid Biochemistry and Molecular Biology. DOI 10.1016/j.jsbmb.2016.10.004.

Brouwer-Brolsma, EM, **Vaes AMM**, van der Zwaluw NL, van Wijngaarden JP, Swart KM, Ham AC, van Dijk SC, Enneman AW, Sohl E, van Schoor NM, van der Velde N, Uitterlinden AG, Lips P, Feskens EJM, Dhonukshe-Rutten RA, de Groot LCPGM (2016). "Relative importance of summer sun exposure, vitamin D intake, and genes to vitamin D status in Dutch older adults: The B-PROOF study." Journal of Steroid Biochemistry and Molecular Biology. DOI 10.1016/j.jsbmb.2015.08.008.

Brouwer-Brolsma EM, Berendsen AM, **Vaes AMM**, Dullemeijer C, de Groot LCPGM, Feskens EJM (2016). Collection and analysis of published scientific information as preparatory work for the setting of Dietary Reference Values for Vitamin D: External Scientific Report; EFSA supporting publication. DOI 10.2903/sp.efsa.2016.EN-766.

Expected publications

Vaes, AMM, Brouwer-Brolsma EM, Toussaint N, de Regt MF, Tieland M, van Loon LJC, de Groot LCPGM (*submitted*). "The association between 25-hydroxyvitamin D concentration, physical performance and frailty status in older adults."

Vaes, AMM, Tieland M, Toussaint N, Nilwik R, Verdijk LB, van Loon LJC, de Groot LCPGM (*submitted*). "The effect of calcifediol or vitamin D_3 supplementation on muscle strength and physical performance in pre-frail and frail older adults: a randomized placebo-controlled trial."

Vaes, AMM, van Dijk M, van Norren K, Schuurman T, Feskens EJM, de Groot LCPGM, Steegenga WT, Brouwer-Brolsma EM (*in preparation*). "Vitamin D deficiency adversely affects grip strength but not ex vivo muscle parameters in C57BL6/J mice."

Hangelbroek RWJ, **Vaes AMM**, Kersten S, Boekschoten MV, Verdijk LB, van Loon LJC, de Groot LCPGM (*in preparation*). "No effect of calcifediol supplementation on skeletal muscle transcriptome in vitamin D deficient frail older adults."

Grootswagers P, **Vaes AMM**, Hangelbroek RWJ, Tieland M, van Loon LJC, de Groot LCPGM (*in preparation*). "Isometric lower extremity strength assessed by hand-held dynamometer: its reliability, validity and its practical use to identify frailty in a population of community dwelling seniors."

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Wet- en Regelgeving Wetenschappelijk Onderzoek – ZGV *(Ede, 2012)*
Dutch Nutritional Science days – NAV *(Deurne, 2013)*
TI Food and Nutrition Conference – TIFN *(Amsterdam, Vlaardingen, Wageningen 2013, 2015, 2016)*
25 jaar Ouderenonderzoek – WUR *(Wageningen, 2013)*
International Conference on Frailty and Sarcopenia Research – IANA *(Barcelona, 2014)*
The 18th Vitamin D Workshop – Scientific Conference *(Delft, 2015)*
The 19th Vitamin D Workshop – Scientific Conference *(Boston, 2016)*
Voedingswetenschap over grenzen heen – NAV *(Maastricht, 2016)*
Geriatriedagen – NVKG *('s-Hertogenbosch, 2016)*

General courses

Teaching an supervising thesis students – VLAG *(Wageningen, 2012)*
Masterclass longitudinal data analysis – WUR *(Wageningen, 2013)*
Basic intellectual property for researchers – TIFN *(Wageningen, 2013)*
MBTI workshop – TIFN *('s-Hertogenbosch, 2013)*
Data management – VLAG *(Wageningen, 2013)*
PhD week – VLAG *(Baarlo, 2013)*
Masterclass confounding – *WUR (Wageningen, 2014)*
Linear mixed model analysis – Biometris *(Wageningen, 2014)*
Scientific writing – Wageningen in'to Languages *(Wageningen, 2016)*
Essentials Seminar – European Nutrition Leaderships Platform *(Luxembourg, 2016)*

Optional activities

PhD study tour Division of Human Nutrition – WUR *(USA, 2015)*
Expert meetings MH001 – TIFN *(Wageningen, 2012-2017)*
Staff seminars: LSG, EPIC, Paperclip, ouderenoverleg – WUR *(Wageningen, 2012-2017)*
Preparation of research proposals – WUR *(Wageningen, 2012-2013)*

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