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Vitamin D status and effects of food fortification in families

VitmaD

Katja Howarth Madsen
PhD Thesis
2013

DTU Food
National Food Institute

Preface

This PhD study was conducted at the Division of Nutrition, National Food Institute, Technical University of Denmark between November 2009 and November 2013 including maternity leave. The PhD thesis is based on three papers written on data from the VitmaD study.

The work was supported by grants from the Danish Dairy Research Fund, Centre for Advanced Food Studies and The European Regional Development Fund. Arla Foods A/S, Lantmännen Cerealia A/S and The Association of Danish Trade Mills partially sponsored the study foods.

First of all I would like to thank my supervisors Lone Banke Rasmussen, Rikke Andersen, Elisabeth Wreford Andersen and Christian Mølgaard for always taking their time, invaluable support and discussions.

I gratefully acknowledge the work done by the VitmaD project team (Heddie Mejborn, Lone Banke Rasmussen, Rikke Andersen, Janna Nissen, Anna Klöcker and Ida Ipsen), for whom it would not have been possible to run the study. I also appreciate the colleagues at the Division of Nutrition with special thanks to Professor Inge Tetens for great inspiration. I thank my office partner Janna Nissen for the many talks in ups and downs.

I am grateful to all the families for their active and enthusiastic participation in the VitmaD study. I thank the students from Metropolitan University College for assistance with examination of the families and master student Johanne Kierkegaard Severinsen from University of Copenhagen for her work with the evaluation of the FFQ.

I also value the great co-operations with our colleague Jette Jakobsen from Division of Food Chemistry, Arla Foods A/S, Lantmännen Cerealia A/S and their cooperative baker, the association of Danish Consumer Co-operatives and their supermarkets Dagli Brugsen in Mørkhøj, former Super Brugsen in Bagsværd and Kvickly in Buddinge, Copenhagen´s General Practitioners Laboratory in Søborg and the Biochemical Department at Holbæk Hospital.

English summary

Background and aims: The importance of vitamin D in bone health is recognised and low concentrations have been associated with increased risk of disease. Cutaneous synthesis is considered the major source of vitamin D, but during winter where sufficient sun exposure is restricted at Northern latitudes, intake from food and dietary supplements become essential. Vitamin D intakes are lower than dietary recommendations in most populations and low vitamin D status is common.

The PhD thesis is based on the VitmaD study in which a realistic and model derived fortification strategy was investigated in a real-life setting. The aim was to investigate the effect of increasing vitamin D intake by fortification of milk and bread to the amount recommended in the Nordic Nutrition recommendations (NNR) on serum 25(OH)D concentration in families during winter in Denmark (**paper 1**). Secondly, the aim was to assess vitamin D status and its determinants at baseline of the study (**paper 2**). Further, to model the relationship between total vitamin D intake and serum 25(OH)D taking into account potential effect modifiers and estimate required vitamin D intake during winter (**paper 3**).

Methods: The VitmaD study was a randomized controlled trial in 782 children and adults (4-60 years) recruited as 201 families. Families were randomly assigned to vitamin D fortified or nonfortified milk and bread for 6 months starting from September. The milk and bread replaced the subjects´ usual consumptions of products. Information on dietary intake, supplement use, health and lifestyle was obtained by self-administered web-based questionnaires. Serum 25(OH)D was analysed by liquid chromatography-tandem mass spectrometry (LC/MS-MS). Mixed models with family as a random factor were applied in all the statistical analyses.

Results: At baseline of the study (late summer) the geometric mean (IQR) serum 25(OH)D concentration was 72.1 (61.5-86.7) nmol/L with no overall differences between age (P=0.190), gender (P=0.332) or age and gender groups (P=0.223) (**paper 2**). The prevalence of serum 25(OH)D <50 nmol/L was 9 %. In the multiple analysis of all subjects, vitamin D status was negatively associated with BMI (P<0.001) and positively associated with dietary vitamin D (P=0.008), multivitamin use (P=0.019), solarium use (P=0.006), outdoor stay in light clothes (P=0.001), sun preference (P=0.002) and sun vacation (P<0.001). The intra-family correlation was

stronger in children (0.42) compared with adults (0.24). Thus children within a family seemed to be more alike than adults within a family with respect to vitamin D status.

The planned fortification strategy was to increase the vitamin D intake to 7.5 µg/day. This succeeded in 66 % of the subjects in the fortification group with a median vitamin D intake (habitual diet plus fortified milk and bread) of 9.4 µg/day compared with 2.2 µg/day in the control group (**paper 1**). During winter the serum 25(OH)D concentration decreased from 73.1 to 67.6 nmol/L (-Δ5.5 nmol/L) in the fortification group ($P<0.001$) and from 71.1 to 41.7 nmol/L (-Δ29.4 nmol/L) in the control group ($P<0.001$). The final serum 25(OH)D concentration was significantly higher in the fortification group compared with in the control group ($P<0.001$, interpreted estimate 1.59) and the treatment effect was not affected by BMI, multivitamin use and sun vacation. The prevalence of serum 25(OH)D <50 nmol/L remained low in the fortification group (16 %) whereas it increased to 65 % in the control group.

The relationship between total vitamin D intake from natural foods, fortified milk and bread and dietary supplements and serum 25(OH)D concentration in winter was best fitted by a non-linear curve (**paper 3**). The effect of total vitamin D intake on serum 25(OH)D concentration was 4 % higher in men compared with women ($P<0.014$) and 10 % higher in the group with lowest initial 25(OH)D concentration (<61.5 nmol/L) compared with the group with highest initial 25(OH)D concentration (>86.9 nmol/L) ($P<0.001$). It was not modified by age ($P=0.132$) or BMI ($P=0.884$). Estimated required vitamin D intake was 5, 11, 23 and 39 µg/day for 50, 75, 90 and 95 % of the population to maintain vitamin D status >50 nmol/L during winter. These figures were higher for the group with lowest initial 25(OH)D concentration (11, 18, 34 and >34 µg/day) and lower for the group with highest initial 25(OH)D concentration (<1, 3, 8 and 17 µg/day).

Conclusions: In the population of Danish families, serum 25(OH)D concentration was above 50 nmol/L in late summer and it was associated with both dietary and sun related factors. Children within a family seemed to be more alike than adults within a family with respect to vitamin D status. Vitamin D fortification of milk and bread reduced the decrease in serum 25(OH)D concentration during winter and ensured concentrations above 50 nmol/L. The relationship between total vitamin D intake and vitamin D status was non-linear. Estimated total vitamin D intake to maintain serum 25(OH)D above 50 nmol/L was largely dependent on the initial vitamin D status.

Dansk resumé

Baggrund og formål: Betydningen af D-vitamin for knoglesundhed er anerkendt og lave koncentrationer er forbundet med øget risiko for visse sygdomme. Syntese af D-vitamin i huden betragtes som den primære kilde til D-vitamin, men om vinteren på nordlige breddegrader, hvor solen ikke er tilstrækkelig til at danne D-vitamin, bliver indtag fra fødevarer og kosttilskud essentielt. D-vitaminindtaget er lavere end næringsstofanbefalinger i de fleste befolkninger og lav D-vitaminstatus er almindeligt.
Ph.d.-afhandlingen er baseret på VitmaD studiet, hvor en realistisk og model baseret berigelsesstrategi blev undersøgt i et real-life setting. Formålet var at undersøge effekten af at øge D-vitaminindtaget ved berigelse af mælk og brød til det anbefalede indtag i de nordiske næringsstofanbefalinger (NNR) på serum 25(OH)D koncentrationen hos familier om vinteren i Danmark (**artikel 1**). For det andet var formålet at vurdere D-vitaminstatus og dens determinanter ved baseline af studiet (**artikel 2**). Endvidere var formålet at modellere forholdet mellem det totale D-vitaminindtag og serum 25(OH)D under hensyn til eventuelle effektmodifikatorer og at estimere krævet D-vitaminindtag om vinteren (**artikel 3**).

Metoder: VitmaD studiet var et randomiseret kontrolleret forsøg i 782 børn og voksne (4-60 år) rekrutteret som 201 familier. Familierne blev randomiseret til D-vitaminberiget eller ikke-beriget mælk og brød i 6 måneder startende fra september. Det udleverede mælk og brød erstattede deltagernes sædvanlige forbrug af produkterne. Information om deltagernes kostindtag, kosttilskudsbrug, sundhed og livsstil blev indsamlet med selvadministrerede webbaserede spørgeskemaer. Serum 25(OH)D blev analyseret ved væskechromatografi tandem masse spektrometri (LC/MS-MS). Mixede modeller med familie som en tilfældig faktor blev anvendt i alle de statistiske analyser.

Resultater: Ved baseline af studiet (sensommer) var den geometriske middel (IQR) serum 25(OH)D koncentration 72,1 (61,5-86,7) nmol/L med ingen overordnede forskelle mellem alder (P=0,190), køn (P=0,332) eller alder- og kønsgrupper (P=0,223) (**artikel 2**). Prævalensen af serum 25(OH)D <50 nmol/L var 9 %. I den multiple analyse af alle deltagerne var D-vitaminstatus negativt associeret med BMI (P<0,001) og positivt associeret med D-vitaminindtag fra kosten (P=0,008), multivitaminbrug (P=0,019), solariumbrug (P= 0,006), udendørsophold i let påklædning (P=0,001), solpræference (P=0,002) og solferie (P<0,001). Intra-familie korrelationen var stærkere

hos børn (0,42) sammenlignet med voksne (0,24). Således var børn indenfor en familie tilsyneladende mere ens end voksne indenfor en familie i forhold til D-vitaminstatus.

Den planlagte berigelsesstrategi var at øge D-vitaminindtaget til 7,5 µg/dag. Dette lykkedes i 66 % af deltagerne i berigelsesgruppen med et mediant D-vitaminindtag (sædvanlige kost plus beriget mælk og brød) på 9,4 µg/dag sammenlignet med 2,2 µg/dag i kontrolgruppen (**artikel 1**). Henover vinteren faldt serum 25(OH)D koncentrationen fra 73,1 til 67,6 nmol/L (- Δ5.5 nmol/L) i berigelsesgruppen (P<0,001) og fra 71,1 til 41,7 nmol/L (-Δ29.4 nmol/L) i kontrolgruppen (P<0,001). Den endelige serum 25(OH)D koncentration var signifikant højere i berigelsesgruppen sammenlignet med i kontrolgruppen (P<0,001, fortolket estimat 1,59), og berigelseseffekten blev ikke påvirket af BMI, multivitaminbrug og solferie. Prævalensen af serum 25(OH)D <50 nmol/L forblev lav i berigelsesgruppen (16 %), mens den steg til 65 % i kontrolgruppen.

Sammenhængen mellem det totale D-vitaminindtag fra naturlige fødevarer, beriget mælk og brød og kosttilskud og serum 25(OH)D koncentration om vinteren var bedst modelleret med en ikke-lineær kurve (**artikel 3**). Effekten af det totale D-vitaminindtag på serum 25(OH)D koncentrationen var 4 % højere hos mænd sammenlignet med kvinder (P<0,014) og 10 % højere i gruppen med lavest initial 25(OH)D koncentration (<61,5 nmol/L) sammenlignet med gruppen med højest initial 25(OH)D koncentration (>86,9 nmol/L) (P<0,001). Effekten var ikke modificeret af alder (P=0,132) eller BMI (P=0,884). Det estimerede D-vitaminindtag var 5, 11, 23 og 39 µg/dag for at opretholde D-vitaminstatus >50 nmol/L i 50, 75, 90 og 95 % af populationen henover vinteren. Disse indtag var højere for gruppen med lavest initial 25(OH)D koncentration (11, 18, 34 og >34 µg/dag) og lavere for gruppen med højest initial 25(OH)D koncentration (<1, 3, 8 og 17 µg/dag).

Konklusioner: I populationen af danske familier var serum 25(OH)D koncentrationen over 50 nmol / L i sensommeren og den var associeret til både kost-og solrelaterede faktorer. Børn indenfor en familie syntes at være mere ens end voksne indenfor en familie med hensyn til D-vitaminstatus. D-vitaminberigelse af mælk og brød reducerede faldet i serum 25(OH)D koncentrationen henover vinteren og sikrede koncentrationer over 50 nmol/L. Sammenhængen mellem det totale D-vitaminindtag og D-vitaminstatus var ikke-lineær. Estimeret totalt D-vitaminindtag for at opretholde serum 25(OH)D over 50 nmol/L var afhængig af den initiale D-vitaminstatus.

List of papers

Paper 1:

Randomized controlled trial of the effects of vitamin D-fortified milk and bread on serum 25-hydroxyvitamin D concentrations in families in Denmark during winter: the VitmaD study.
Madsen KH, Rasmussen LB, Andersen R, Mølgaard C, Jakobsen J, Bjerrum PJ, Andersen EW, Mejborn H, Tetens I. *American Journal of Clinical Nutrition* 2013, 98: 374-382.

Paper 2:

Vitamin D status and its determinants in children and adults among families in late summer in Denmark.
Madsen KH, Rasmussen LB, Mejborn H, Andersen EW, Mølgaard C, Nissen J, Tetens I, Andersen R. *Resubmitted to British Journal of Nutrition*

Paper 3:

Relationship between winter vitamin D intake and status in Denmark.
Madsen KH, Mejborn H, Tetens I, Andersen EW, Mølgaard C, Rasmussen LB, Andersen R. *Submitted to American Journal of Clinical Nutrition*

Abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
BMI	Body mass index
CI	Confidence interval
CV	Coefficient of variation
DBP	Vitamin D binding protein
EAR	Estimated average requirement
FFQ	Food frequency questionnaire
IQR	Inter quartile range
LC/MS-MS	Liquid chromatography tandem mass spectrometry
NIST	National institute of standards and technology
NNR	Nordic nutrition recommendation
PTH	Parathyroid hormone
RCT	Randomized controlled trial
RDA	Recommended daily allowance
RI	Recommended intake
UL	Upper tolerable intake level
UVB	Ultraviolet B
VDR	Vitamin D receptor

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1 Rationale and aims of the study

Vitamin D is a hormone like vitamin that is provided by both intakes and endogen synthesis after skin exposure to sun light. Cutaneous synthesis is considered the major source of vitamin D, but during winter where sufficient sun exposure is restricted at Northern latitudes, intake from food and dietary supplements become essential. It is still discussed which vitamin D status is optimal for health and how to reach this target. Vitamin D intake in Denmark and other populations is lower than the recommendation and low vitamin D status is common. As relatively few foods contain vitamin D naturally and sun exposure should be limited due to risk of skin cancer, it is hard to meet the vitamin D requirement by a normal, healthy lifestyle. Therefore it is relevant to investigate methods to increase the vitamin D intake and there are different ways to consider [1] : advice on sun exposure, dietary diversification e.g. increase fish intake, dietary supplements and food fortification. It may not be ethical to recommend an increased sun exposure due to the risk of skin cancer. Dietary diversification or supplementation may not be fully implemented in a population and may be dependent on social and financial status [2, 3]. Furthermore, public advice is mostly followed by the health conscious and paradoxically dietary supplements are often taken by those that have the least need for it [4]. Food fortification is a strategy that targets the entire population [1] at least if it is implemented on a mandatory basis. The focus of the present research was a targeted vitamin D fortification strategy based on models on usual food consumption in the Danish population and the effects of implementing it in a real-life setting. The outcome of the research is the three attached papers with the following aims:

Paper 1: To investigate the effects of increasing vitamin D intake by fortification of milk and bread to the amount recommended in the Nordic Nutrition Recommendation (NNR) on serum 25(OH)D concentration in families during a 6-mo winter period in Denmark.

Paper 2: To assess serum 25(OH)D concentration and its determinants in children and adults among families in late summer in Denmark.

Paper 3: To investigate the relationship between total vitamin D intake from natural foods, fortified foods and dietary supplements and vitamin D status in children and adults in late winter in Denmark taking into account the initial vitamin D status, age, gender and BMI class. Furthermore, we estimated the vitamin D intake needed to maintain 25(OH)D concentration >25 and >50 nmol/L in specified proportions of the population.

2 Background

2.1 Vitamin D

2.1.1 Metabolism and function

Vitamin D is provided by both endogen synthesis and as a nutrient through food or supplements and it exists in two native forms ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). In the following vitamin D refers to both forms unless otherwise stated as they are metabolized in an identical manner (**Figure 1**). Vitamin D is synthesized when the skin is exposed to ultraviolet B (UVB) light in the wavelengths 290-315 nm [5]. In the epidermis 7-dehydrocholesterol is converted to previtamin D_3 that is rapidly converted to vitamin D_3 in a heat dependent process [6]. This synthesis is dependent on the amount of solar UVB photons reaching the surface of the earth which is influenced by the ozone layer, smog and the zenith angle of the sun and thus on latitude, season and time of the day [5-9]. This is why when the zenith angle is increased during wintertime and in the early morning and late afternoon, little if any vitamin D is synthesized in the skin at latitudes above 40 °N [5, 8, 9]. Aging and skin pigmentation also reduce the skin´s capacity to produce vitamin D as well as blocking of the skin with clothing and proper applied sunscreen reduces the synthesis of vitamin D [5, 10, 11]. After synthesis vitamin D_3 diffuses into the circulation and is transported to the liver bound to vitamin D binding protein (DBP). Consumed vitamin D, either as vitamin D_2 or vitamin D_3, is absorbed in the intestine and transported via chylomicrons and the lymphatic system to the liver. From here vitamin D from both sources undergoes two hydroxylation steps in order to become active [12].

In the liver vitamin D is hydroxylated to 25-hydroxyvitamin D (25(OH)D, calcidiol) by the enzyme vitamin D-25-hydroxylase. This conversion seems to be only loosely regulated and to depend primarily on the concentration of vitamin D [13]. Bound to DBP 25(OH)D is the major circulating form of vitamin D with a relatively long half-life of 2-3 weeks [14] and its concentration is used as a biomarker of vitamin D status [15]. 25(OH)D is further hydroxylated to its biologically active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) by the 1α-hydroxylase enzyme primarily in the kidney [15-17]. Several extrarenal tissues also express 1α-hydroxylase [18, 19], and the $1,25(OH)_2D$ produced here acts locally in an autocrine or paracrine fashion to regulate the cell´s own proliferation and differentiation and generally does not reach the circulation [14]. In contrast to 25(OH)D, circulating $1,25(OH)_2D$ has a short half-life of around 4-6 hours and its concentration is tightly homeostatic

regulated by calcium and phosphate concentrations via a negative feedback mechanism with parathyroid hormone (PTH) [16, 20]. From the kidney 1,25$(OH)_2$D enters the circulation and travels bound to DBP to its target tissues, where it mediates its action by binding to the vitamin D receptor (VDR) [19, 21, 22]. The major target tissues are the intestine and bone [15], but several other tissues have VDR activity [21, 22]. Biological responses are generated both by regulating gene transcription (genomic response) and by rapidly activating a variety of signal transduction pathways at or near the plasma membrane (rapid response) [14]. Vitamin D can be stored in fat and other tissues [23] and although it is presently unknown, these stores may not be readily available when needed [24]. If not stored vitamin D is excreted from the body via the bile in the form of water-soluble, inactive metabolites [25].

The principal function of vitamin D is to maintain blood concentrations of calcium and phosphorus within the normal range [13] (**Figure 1**). Calcium is essential for development and maintenance of bone and numerous cellular functions including muscle contraction and nerve conduction [26]. Calcium sensing receptors are present in the parathyroid gland, and low blood calcium concentrations induce the release of PTH that in return rapidly stimulates the production of 1,25$(OH)_2$D which increases the serum calcium (and phosphorus) concentration through three separate activities [12]: directly enhances intestinal absorption and interacts with PTH to stimulate renal reabsorption and mobilization from bone. In this endocrine system dietary calcium is favoured over mobilization from bone to support serum concentrations under normal conditions [12]. It has also been suggested that bone cells can convert 25(OH)D to 1,25$(OH)_2$D to have a direct anabolic function on bone when calcium supply is adequate [27, 28]. Higher serum calcium concentrations suppress the PTH secretion and induces 24-hydroxylase in the kidney to convert 25(OH)D and 1,25$(OH)_2$D to 24,25-dihydroxyvitamin D [25, 29]. Although this metabolite may also have a biological function [15], it is less potent than 1,25$(OH)_2$D and it is generally considered as the first step to inactivate and degrade 25(OH)D [15]. In addition to this negative feedback mechanisms with PTH, 1,25$(OH)_2$D also down-regulates its own production by inducing the 24-hydroxylase [14].

As 1α-hydroxylase and VDR are expressed in numerous cell types [30] additional non-classic functions of vitamin D have been suggested. These include regulation of cell differentiation and proliferation, cellular growth and hormone secretion [31]. For example vitamin D may play a role in the immune system, growth of cancer cells and blood pressure regulation [32].

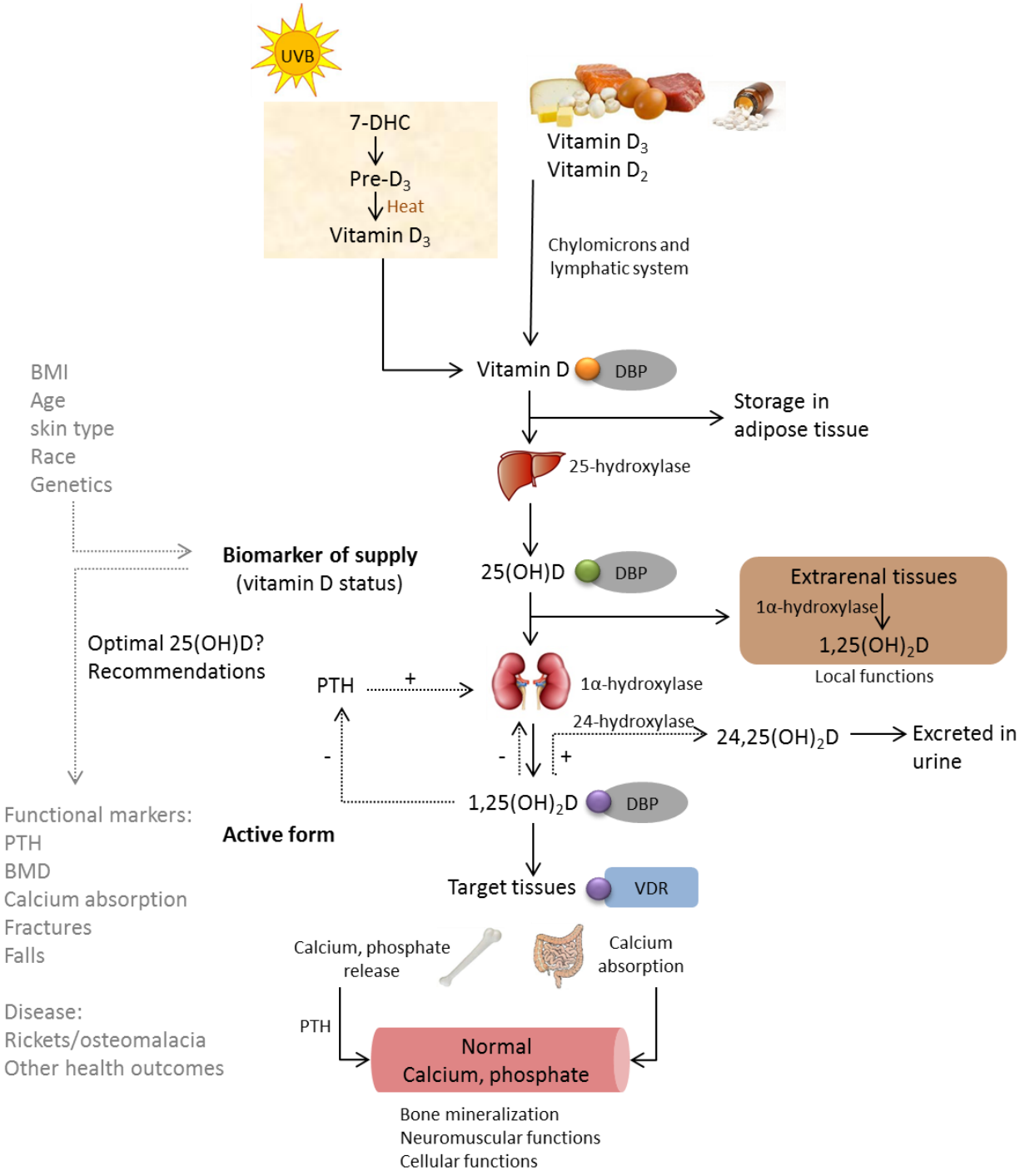

Figure 1. Vitamin D metabolism, regulation and primary functions. BMD, bone mineral density; DBP, vitamin D binding protein; 7-DHC, 7-dehydrocholesterol; PTH, parathyroid hormone; UVB, ultraviolet B; VDR, vitamin D receptor.

2.1.2 Deficiency and toxicity

Low vitamin D concentrations will often be accompanied by elevated PTH concentrations known as secondary hyperparathyroidism with normal to high bone turnover [32, 33]. The classic disease of vitamin D deficiency is rickets in children and osteomalacia in adults caused by an impaired mineralization of bone [32, 34]. Rachitis is characterized by growth retardation, pain, soft bone and skeletal deformities and in some children also muscle weakness [32, 35]. The symptoms in adults are less pronounced and may be diffuse pain in bone and muscles. Prolonged and less severe vitamin D deficiency may lead to weak bone and contribute to development of the longer latency disease osteoporosis by reducing calcium absorption [13, 29, 36]. Osteoporosis is characterized by low bone mass and structural deterioration of bone tissue that increases bone fragility and the risk of fractures [29]. As these symptoms are irreversible, a high peak bone mass early in life is prudent [36] and thus sufficient vitamin D is essential throughout the life cycle even though symptoms of severe deficiency are not present. Rickets and osteomalacia occur seldomly in Denmark and mostly among immigrants [37, 38], but osteoporosis has substantial public health implications [29, 39]. The estimated prevalence of osteoporosis in persons aged 50 years or more in Denmark is 41 % among women and 18 % among men [40]. Low vitamin D concentrations have also been associated with a range of health outcomes [25, 41] including an increased risk of cardiometabolic disease [42], certain types of cancer [43], autoimmune diseases [44] and mortality [45].

As vitamin D can be stored in the body it is potential toxic. However, vitamin D intoxication is rare and most likely to occur from very high intakes of dietary supplements as endogenously synthesized vitamin D is limited by photodegradation in the skin after prolonged UVB exposure [5, 13]. Acute toxic effects of vitamin D are hypercalcemia and hyperphosphatemia and chronic toxicity can lead to internal organ calcification and failure [46]. Vitamin D intoxication is usually not seen with serum 25(OH)D concentrations <375 nmol/L and daily intakes <250 µg/day [47, 48]. However, serum 25(OH)D concentrations lower than what causes acute toxicity may potentially have adverse health effects [46], and the consequences of prolonged higher vitamin D intakes (>25 µg/day) on health are not known [49]. Due to the findings of U-shaped or reverse-J-shaped associations between 25(OH)D concentration and some health outcomes for example mortality, the US dietary committee suggested that potential adverse effects may occur at concentrations >125 nmol/L [47]. The U-shaped associations have been critiqued for being due to statistical interference [50]. The Tolerable Upper Intake Level (UL) defines the maximum level of total chronic daily intake of a nutrient

judged to be unlikely to pose a risk of adverse health effects to humans [51]. The new UL for vitamin D was set at 100 µg/day for adults and children aged 11-17 years and at 50 µg/day for children aged 1-10 years based on hypercalcaemia and impaired growth in children [52].

2.1.3 Measurement of status

Circulating concentration of 25(OH)D is generally accepted as the best marker of vitamin D status as it reflects the sum of vitamin D from intakes and cutaneous synthesis [53]. Several methods for quantification of 25(OH)D have been developed over the years. These have moved from competitive protein binding assays that could not be automated, to automated immunoassay (radio, enzyme-linked or chemiluminescence based) and liquid chromatography techniques (HPLC or LC-MS/MS) [54, 55]. In the present research we used the LC-MS method that might be considered the golden standard [55-57]. Immunoassays are the most common method due to their ease of use, relative cost and high sample throughput, but they are limited in their ability to detect vitamin D_2 [55]. The chromatographic methods are more specific and allow determination of several vitamin D metabolites [58, 59]. However, they are also more expensive and labour-extensive and the use of different in-house procedures has been criticized [55]. Therefore a LC-MS candidate reference method has been developed by the National Institute of Standards and Technology (NIST) [60].

Partly due to lack of standardization, several studies have shown that different assays as well as laboratories can yield markedly differing results [57, 61-67]. In 2010, an International standard reference material was introduced by NIST [68] which may improve method-related variability. This calibrator has been used for analysis of serum 25(OH)D in the present research. The Vitamin D External Quality Assessment Scheme (DEQAS) supports the evaluation of different assays and individual laboratory performances [69]. In DEQAS the LC-MS method is positively biased for the All-Laboratory Trimmed Mean (ALTM) whereas the immunoassays are mostly negatively biased [62]. Furthermore, standardization of results from National surveys is on-going in the VDSP with the purpose to be able to compare results with liability [70, 71].

2.1.4 Status and cut-off limits

Low vitamin D status with seasonal variations has been found in various groups in the Danish [72-77] and other populations [75, 78-87]. Several factors such as age, BMI, sun habits, ethnicity, dietary intake and supplement use have been associated with vitamin D status so that pigmented skin, limited sunlight exposure, obesity and increasing age are risk factors for vitamin D deficiency [88]. There is no International standard for definition of deficient and optimal vitamin D concentrations and cut-off values are still discussed. The traditional approach was to determine vitamin D sufficiency as the point where clinical symptoms of rickets and osteomalacia were avoided [29]. It is recognized that a 25(OH)D concentration <12.5 nmol/L can result in rickets and osteomalacia and that concentrations <20-25 nmol/L may also result in these diseases [32, 35, 89]. However, prevention of clinical symptoms is not the same as optimal 25(OH)D concentration since less severe degrees of deficiency may produce longer-latency diseases such as osteoporosis [29, 90]. Current research focuses on defining optimal vitamin D concentrations for health outcomes rather than the lack of explicit clinical disease [91].

To better understand the adequacy of vitamin D supply for biological responses, serum 25(OH)D concentrations have been associated with functional markers such as PTH, calcium absorption, bone mineral density (BMD), bone resorption and fracture rates [53, 90-95]. PTH has received the most attention to date. An inverse relation between 25(OH)D and PTH concentration has been found in numerous studies with maximal PTH suppression around 75-100 nmol/L [13, 33, 96-100]. However, a large cross-sectional study among 20-60 year olds did not find a threshold for PTH suppression and that the relation was strongly age-dependent so that PTH concentrations were elevated at higher age for the same concentration of 25(OH)D [101]. Most of the research focused on adults, and in children there was not enough evidence to support the use of BMD and PTH as functional markers [102]. Despite the limited evidence for the use of functional markers in children, it has mostly been assumed that children have the same requirements as adults.

In Denmark, the following values have commonly been used to define deficient, insufficient and sufficient vitamin D status: <25, 25-50, >50 nmol/L [103]. These are the limits that were used in the present research for discussion except in paper 1 where the American limit for deficiency was used. In the US dietary reference intake for vitamin D, 30 nmol/L was set as the limit beyond which adverse effects on bone might increase and 50 nmol/L was used as the sufficient level to maintain bone in 97.5 % of the population. Some argue that 25(OH)D concentrations in the interval 75-125

nmol/L should provide the benefit of vitamin D while limiting the risk of potential harm [95, 98, 104-106].

2.1.5 Dietary intake

The general population derives most of its vitamin D from endogen synthesis after sun exposure [24]. However, when sufficient sun exposure is restricted, for example during winter at higher latitudes [5, 9], dietary vitamin D becomes essential [107, 108]. Furthermore, due to limited sun exposure as a consequence of changes in life style such as being more inside and increased awareness about skin cancer [30], dietary intakes may play a larger role than previously assumed [109]. Vitamin D in the diet is mainly provided in the form of vitamin D_3 which is found in animal sources such as fish, meat, milk and eggs [10, 110]. Vitamin D_2 is found in certain plants and in mushrooms exposed to UVB light [111-113]. Both vitamin D_2 and D_3 are used in dietary supplements, however, in Denmark it is mostly in the form of vitamin D_3. It appears that vitamin D_2 and D_3 possess equal bioavailability, but vitamin D_3 may be more potent to sustain the 25(OH)D response [114-119]. However, this is controversial [120, 121] and the meaning of the prompting of a higher clearance by vitamin D_2 on the function of vitamin D is unclear [115]. 25(OH)D may also be present in foods and it is around five times more potent than vitamin D_3 to raise serum 25(OH)D [122, 123]. Although there is uncertainty about this conversion factor [110, 124], it has been suggested that the impact of 25(OH)D in foods is highly underestimated [109].

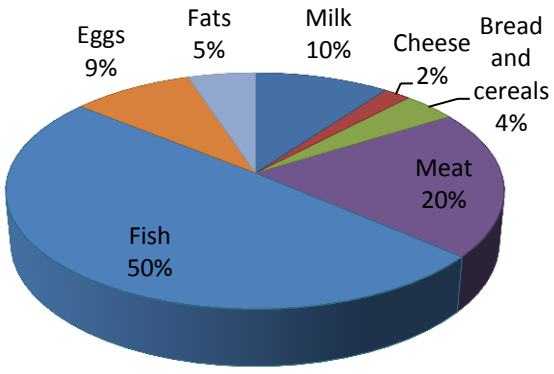

Figure 2. Relative contributions of food groups to the vitamin D intake in Denmark [129].

Relatively few foods contain vitamin D naturally and concentrations may vary depending on among other things the breeding circumstances and the feed [125]. For example the vitamin D content in wild caught salmon has been found to be four times higher compared to farmed salmon [10] and to range from 2.5 to 25 µg/100 g in various species [10]. Fish is the major contributor to the vitamin D intake in Denmark (**Figure 2**). In other countries where food fortification is more widespread than in Denmark for example in Ireland, the UK, Spain, USA and Canada fortified foods are a significant contributor as well [126, 127]. Milk is mandatorily fortified with vitamin D in Canada and provides almost half of the vitamin D intake and contributes even more than fish to the total intake [128].

The mean vitamin D intakes from foods in Denmark are 2.3 and 3.4 µg/day (median 1.8 and 2.5 µg/day) for children aged 4-17 years and adults aged 18-75 years [129]. These intakes are lower than the recommended intake (RI) by the Nordic Nutrition Recommendation (NNR) that was recently increased from 7.5 to 10 µg/day [130, 131]. However, dietary supplement use is common in Denmark, and the median total vitamin D intake in supplement users was 7.6-8.4 µg/day [132]. The US dietary guidelines set the Recommended Daily Allowance (RDA) (corresponding to the RI) for vitamin D to 15 µg/day [47]. Vitamin D recommendations in Europe range from 2.5 to 10 µg/day [133]. Generally, vitamin D intakes are low and lower than the dietary recommendations in most populations [3, 86, 87, 134-137].

2.2 Dietary assessment methods

Different dietary assessment methods exist including the food frequency questionnaire (FFQ), dietary record (eventually weighed) and dietary recalls either 24-hour or history [138, 139]. They all have their strengths and limitations and choice of method should be based on the food group or nutrient of interest and the target group. In the present study we used the FFQ which retrospectively report the frequency of consumption of selected food items and it is designed to estimate usual intake over a specified period of time [138]. Dietary history is a long-term method similar to FFQ, but it provides more details. 24-hour recall is a detailed registration of all foods and beverages consumed the last day. Dietary record is the only prospective method and it uses a detailed protocol of all foods and beverages consumed over typically 3-7 days with weighed or estimated portion sizes. The short-term methods (dietary record and 24-hour recall) allow greater specificity for

describing foods and food preparation methods than FFQ, and the dietary record has the advantage of not relying on the memory of the respondent, but they also have a high respondent burden, are unrepresentative of usual intake if only a few days are assessed and they are generally expensive as they require a large effort to collect and process data [138]. Recording the diet may also influence the usual dietary pattern of the respondent which is not the case with the retrospective methods [138, 139]. The dietary record is self-administered whereas the 24-hour recall requires highly trained interviewers. These methods are often used to validate FFQ which is the most commonly used method for measuring dietary exposures in epidemiologic studies [138, 140]. For vitamin D the dietary record is the most frequently used reference method for validating FFQ [140]. The FFQ is simpler than other methods and has the advantage of easier administration, low cost and burden on respondents, but may be limited through poor design and inappropriate use [141]. The limitations of the FFQ are the reliance on memory, the ability of the subjects to accurately report and the use of estimated portion sizes. When designing a FFQ, it is important to consider the length of the reference period, frequency categories, number of food items, how portion sizes are described (self-defined versus fixed and eventually use of pictures), format (food list versus meal based) and administration (interviewer-based versus self-administered, paper and pen versus computerized). The highest correlations in validation studies were seen when respondents were able to describe their own portion sizes, with the highest number of food items and when the administration was interviewer-based [141].

An advance in dietary assessment is the increasing use of digital technologies such as computers, the internet, cameras and mobile phones [142, 143] as was also applied in the present study. It is must faster and cheaper to collect and process data in this way. Its limitations is that respondents with no access to the digital instrument used will not be included if the method is self-administered. The interviewer based approach can still be computerized to optimize data collection time. Furthermore, web-based methods have given higher compliance due to the flexibility of completion at any time and location, personalized feedback and interactive help, it required less time and the respondents felt less observed and more independent and it enables communication with dispersed populations [142]. This requires of course a high internet access in the population of interest.

As the main dietary source of vitamin D is fish, which may not be consumed frequently or on a regular, daily basis, it is important to assess the intake of a longer period of time. Even a 7-day dietary record may not capture an eventual fish intake, and it would be too demanding for the

subjects to complete a dietary record >7 days. Generally, it is said that the intake over approximately one month would be necessary to estimate a subjects´ usual fish intake. Thus, FFQ seemed to be the best choice to estimate vitamin D intake in the present study. Validation studies on FFQs designed to assess vitamin D intake generally showed acceptable correlations between FFQ and other methods, though FFQs tend to overestimate the vitamin D intake compared with the dietary record [144-148].

2.3 Fortification

Food fortification is not widely used in Denmark due to a previously restrictive regulation viewing fortification as unnecessary and a conservative attitude in the population [127]. It was only allowed to fortify a food product if it could be justified that there was a nutritional need in the population. In 2006 the regulation on addition of vitamins and minerals to foods was harmonised within the European Union in order to facilitate the free movement of foods [149]. If a company wants to market a fortified food product they need an approval from the Danish Veterinary and Food Administration. They consider if the product possesses a risk for any groups in the population and if not an approval is given. This authority can also give general authorizations to fortify a group of foods with a vitamin in a given amount or they can state mandatory fortification practices.

Foods can be fortified by different methods either directly added to a food product for example milk or indirectly during processing for example added to the flour for baking of bread and cakes. Another method is bio-fortification where the natural content of the vitamin in the food is enhanced for example during the feed to cows and thus the resulting milk or meat can have an increased concentration of vitamin D. Another example of bio-fortification is mushrooms exposed to UVB light [111-113]. When the present study was initiated in 2010 some margarine were fortified with vitamin D in Denmark. Just after the data collection was finished in spring 2011 the first vitamin D fortified milk was introduced. In the following period another vitamin D fortified milk, a rye bread and a mineral water became available in the stores. Generally, the consumption of fortified foods in Europe has increased over the years [127]. It is mostly margarines that are voluntarily fortified with vitamin D and/or milk and in some cases also breakfast cereals [91, 150, 151]. In Island, Sweden and Finland some milks and margarines are vitamin D fortified. Since 2003 almost all margarines and milk in Finland except for the organic milk have been fortified with vitamin D (0.5 µg D_3/100 mL

milk and 10 µg D_3/100 g margarine) on the recommendation from the authorities. Therefore this country is a case for evaluating the impact of a national vitamin D optimisation policy. The initiative improved vitamin D intake and status in some groups [152-154] whereas it had minor effects in other groups [155]. In USA almost all milk is fortified with vitamin D, as are many ready-to-eat cereals, some yogurt, margarines and orange juices [156]. Other dairy products made from milk, such as cheese and ice cream, are generally not fortified. In Canada milk and margarines are mandatorily fortified with vitamin D. Despite widespread vitamin D fortification in USA and Canada and that fortified foods constitute a large part of the dietary vitamin D, the vitamin D intake is still lower than the RDA in most groups [126, 128, 156, 157]. Although food fortification has improved vitamin D status in some groups [127], current practices do not seem to be optimal for improving vitamin D status in the general population. It might be due to the skewness of milk intake across population groups [155, 156].

It illustrates the importance of targeting the fortification strategy and finding the right foods to fortify and the appropriate levels. It has been suggested to fortify more food vehicles in lower concentrations to reach broader in the population [158]. Previous fortification studies showed that the efficiency of vitamin D in fortified foods to increase serum 25(OH)D concentration is good (**Table 1**) and equal to that of dietary supplements [159-162]. Most of the studies used milk as the fortification vehicle and none of the studies used a combination of foods. Some used doses higher than what would be realistic in real-life and only few included children.

Table 1. Overview of studies investigating the effects of vitamin D fortified foods on serum 25(OH)D concentration.

Type	Location	Population	*n*	Food	Dose (µg/day)	Duration	Assay	Change in 25(OH)D (control and fortification)		Effect compared with control	Reference
RCT	India	Girls, boys 10-14 y	713	Milk	15, 25	12 wk	CIA	29.4-27.1 28.6-57.2 29.9-69,2	↑	↑	(163)
RT	New Zealand	Toddlers 12-20 mo	181	Milk	~3.7	20 wk	RIA	48.8-52.6 52.8-70.5	↑	↑	(164)
RCT	Ireland	Women, men 17-54 y	102	Milk	~3.4	4-5 mo	RIA	85-54 77-62	↓	↑	(165)
RCT	New Zealand	Women 18-47 y	73	Milk powder	5	12 wk	RIA	74-53 76-65	↓	↑	(166)
RCT	UK	Women, men	40	Milk drinks	5, 10	4 wk	LC-MS	33.5-30.1 31.3-43.2 30.9-50.6	↑	↑	(167)
RCT	Mongolia	Girls, boys 9-11 y	579	Milk	7.5	7 wk	LC-MS	20-50 25-72.5	↑	↑	(162)
RCT	China	Women >55 y	63	Milk	6.4	12 wk	CIA	29.3-28.2 33.1-39.5	↑	↑	(168)
RCT	Australia	Men 50-87 y	167	Milk	20	2 y	RIA	76.1-56.2 77.2-84.6	↑	↑	(169)
Cross-sectional	New Zealand	Girls, boys mean 7.4 y	89	School milk	1.5	-	RIA	->43.8 ->49.6	-	↑	(170)
RCT	Iran	Diabetics 30-60 y	90	Youghurt drink	25	12 wk	HPLC	41.6-37.2 44.4-77.7	↑	↑	(171)
RCT	Iran	Diabetics 30-60 y	100	Youghurt drink	12.5	12 wk	HPLC	38.0-33.4 38.5-72.0	↑	↑	(172)
RCT	Greece	Women 55-65 y	101	Dairy products	7.5	5 mo	CIA	63.5-55.3 69.8-63.5	no	no	(173)
RCT	Greece	Women 55-65 y	66	Dairy products	22.5	30 mo	CIA	59.0-45.0 53.8-63.0	↑	↑	(174)
RCT	Greece	Women 55-65 y	173	Dairy products	10	12 mo	CIA	58.3-56.5 57.0-61.0	↑	↑	(175)
RCT	Canada	Women, men 18-60 y	80	Cheese	100	8 wk	RIA	55-51 55-110	↑	↑	(161)
RCT	France	Women 50-65 y	71	Cheese	2.5	6 wk	RIA	57.3-65.8 58.8-67.8	no	no	(176)
RCT	USA	Women, men 18-84 y	105	Orange juice	25	11 wk	LC-MS	49.5-45.3 44.8-76.8	↑	↑	(159)
RCT	USA	Women, men 22-60 y	26	Orange juice	25	12 wk	CPBA	50.0-73.0 37.0-94.0	↑	-	(177)
Single-arm	Romania	Nursing-home 58-89 y	45	Bread	125	12 mo	RIA	28.5-125.6	↑	-	(178)
RCT	Finland	Women 25-45 y	41	Bread	10	3 wk	HPLC	-	↑	↑	(160)

CIA, chemiluminescence immunoassay; CPBA, competitive protein binding assay; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; RCT, randomized controlled trial; RIA, radioimmunoassay

3 Methods

3.1 Study design

My thesis and the three papers are based on the VitmaD study. The principle in the study design was to investigate a realistic fortification strategy in a real-life setting. VitmaD was a double-blind, randomized, placebo-controlled intervention trial with children and adults recruited as families. The families received either vitamin D_3-fortified milk and bread or nonfortified placebo milk and bread for 6 months during the winter. Milk and bread were distributed to the families twice per week through three local supermarkets. A personal identification card informed each family which supermarket to go to and obtain products. The card ensured that families received the correct products and that products were handed out only to families who were participating in the study. Subjects were instructed to replace their usual consumption of milk and bread with the products provided in the study. In all other respects, subjects were requested to live their lives as normal without changing any habits. Adult subjects were seen three times during the study period (month 0, 3 and 6), and children (4-17 years) were seen twice (month 0 and 6). Blood samples were drawn at all 3 visits, and anthropometric measures and blood pressure were recorded at month 0 and 6 (**Figure 3**). The primary endpoint was serum 25(OH)D concentration, and secondary endpoints were plasma PTH and serum total calcium. Subjects were asked to complete questionnaires at months 0 and 6. The study protocol was approved by the Research Ethics Committee of the Capital Region of Denmark (record H-4-2010-020).

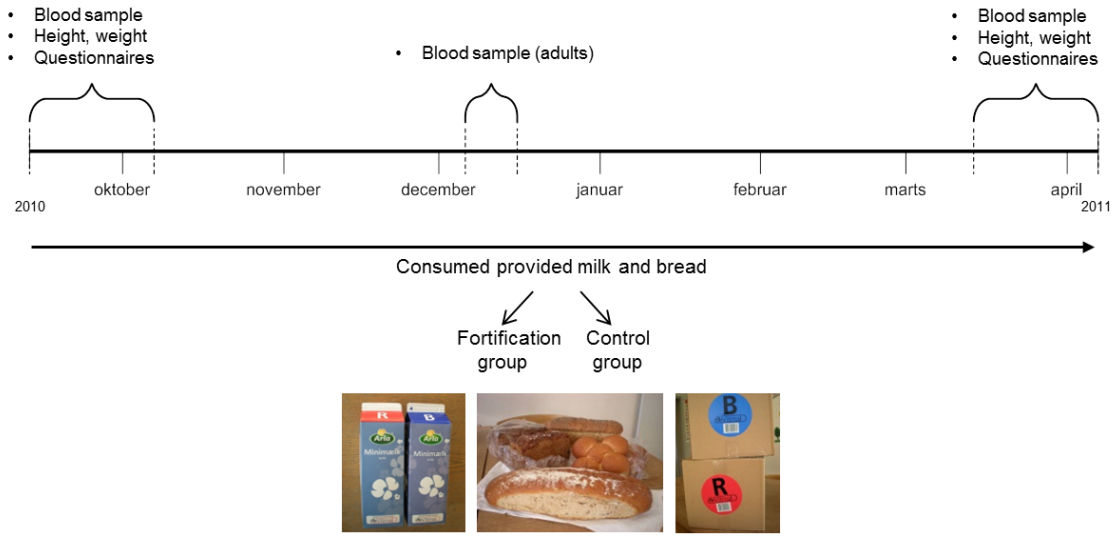

Figure 3. Outline of the VitmaD study.

3.2 Subjects

Sample size was calculated at 88 per group to detect a mean (±SD) difference in serum 25(OH)D concentration of 11 ± 26 nmol/L [165] between treatment groups at the 5 % significance level and with 80 % power. To be able to analyze female and male children and adults separately and allow for dropouts, we aimed to recruit 800 subjects who represented around 200 families. Subjects were recruited in Gladsaxe Municipality in Denmark (56 °N) drawn randomly from the Danish Civil Registration System. Families with a permanent address in Gladsaxe and 3-6 members in the household, including at least one child aged 4-17 years, were invited by letter to participate in the study. Inclusion criteria were age between 4 and 60 years and a permanent address in the Gladsaxe Municipality. Exclusion criteria were pregnancy and disease or use of a medication that would influence vitamin D metabolism (including dietary supplements with >10 µg vitamin D/day for children and >5 µg vitamin D/day for adults). There was an immense response to the letters, and registration was closed after 10 days. At that time, 429 families had responded to the invitation. After screening for eligibility, 230 families were selected, and invited to an information meeting. When the families hereafter decided on their participation, not all members of the household necessarily participated. All adult subjects and custody holders of children gave their written informed consent to participate, which resulted in a total of 782 subjects (201 families). One family withdrew before the study started, five families withdrew during the study, some of the children refused blood sampling, some of the subjects did only have one blood collection or insufficient blood to perform the analyses and not all of them had full questionnaire data (**Figure 4**).

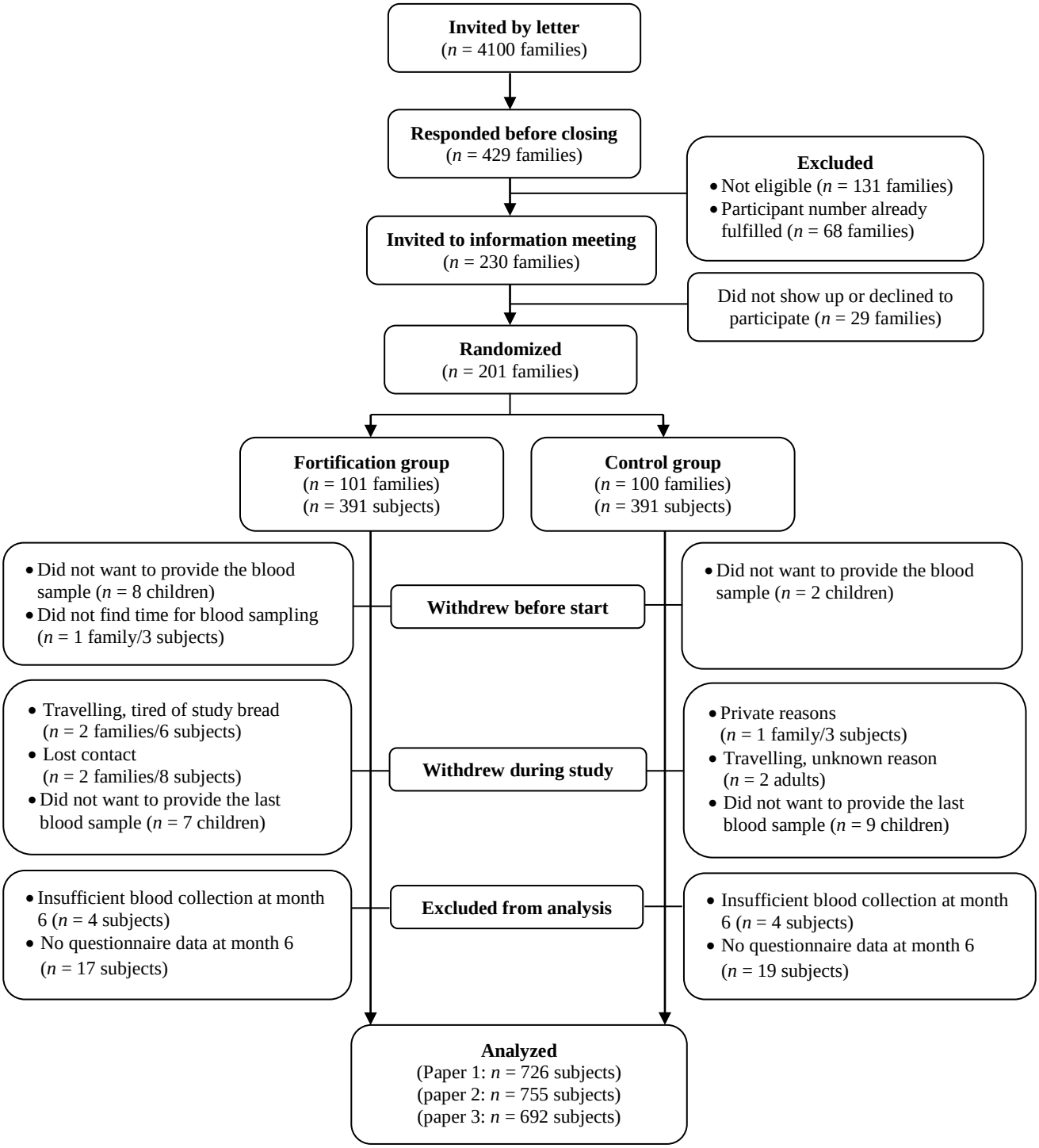

Figure 4. Flow of subjects throughout the VitmaD study.

3.3 Fortification model

The fortification model was built on the following 3 criteria: the model should include a combination of foods, foods should be consumed regularly in the chosen age group and foods should be low in fat and sugar. On the basis of these criteria, milk and bread were chosen as foods to include in the model.

The fortification strategy was to increase the vitamin D intake to 7.5 µg/day (the RI at that time [131]) in as many subjects as possible while avoiding an intake above the UL (25 µg/day for children and 50 µg/day for adults at that time [51]). The choice of fortification amounts was based on models generated from dietary intake data from the Danish National Survey of Dietary Habits and Physical Activity (7-day consecutive dietary record) [129]. These models used the vitamin D intake from natural foods and the consumption of milk and bread fortified in varying amounts of vitamin D (**Figure 5**). We selected the model where the largest proportion of the population was within the interval 7.5-15 µg/day thus allowing for the use of multivitamin supplements with 10 µg vitamin D per day which would add up the total vitamin D intake to the UL of 25 µg/day. The most vulnerable groups for excess intake was evaluated to be boys aged 6-9 years and girls aged 4-5 years, and special attention was paid for these groups in the selection of fortification model.

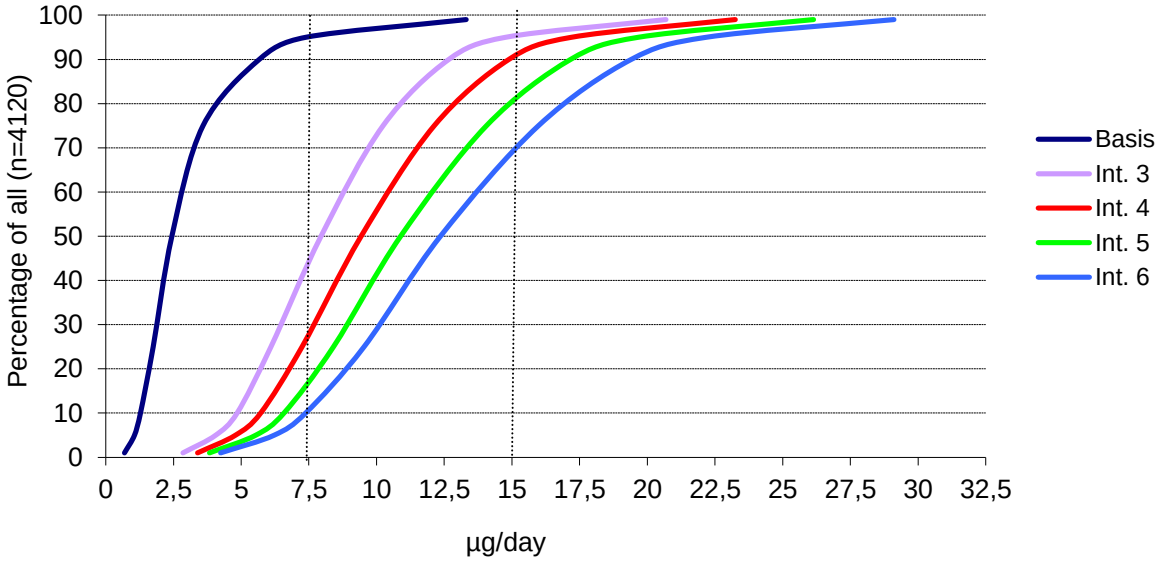

Figure 5. Cumulated vitamin D intake for different fortification models based on data from the Danish National Survey of Dietary Habits and Physical Activity [129].

Study milks were organic homogenized 0.5 % fat milk produced by Arla Foods A/S. The fortified milk had a vitamin D_3 (cholecalciferol; Kemikalia) concentration of 0.38 µg/100 mL, which was confirmed by analysis in triplicate during the study period (0.40±0.01 µg/100 mL). The placebo milk did not contain added vitamin D (<0.004 µg/100 mL). Study breads included white bread, brown bread, rye bread, and buns that were baked from wheat- and rye-flour blends produced by Lantmännen Cerealia A/S. Vitamin D_3 (cholecalciferol; DSM Nutritional Products) was added to flour blends to bake fortified breads. Before and after the study period, samples from 3 different flour blends of both types were analyzed, and the added vitamin D was recovered. Twice during the study period, breads of each type were collected on 3 consecutive baking days and analyzed in pools of 4 breads. We aimed to produce fortified breads with 6 mg vitamin D_3/100 g bread, but the analyses showed a concentration of 5.2±0.3 µg vitamin D_3/100 g in the wheat bread and 4.3±0.3 µg vitamin D_3/100 g in the rye bread. The placebo bread did not contain added vitamin D. Concentrations of vitamin D in study milks and breads were analyzed at the National Food Institute, Technical University of Denmark, by using a reverse-phase HPLC method described elsewhere [179]. Analyses were accredited according to the standard ISO 17025 [180].

3.4 Questionnaires

At baseline (month 0), subjects completed a detailed self-administered Web-based questionnaire that assessed their background, health, sun exposure and lifestyle including use of dietary supplements and a semi-quantitative FFQ that assessed their vitamin D and calcium intakes before the study. By the end of the study (month 6), questionnaires were repeated to assess eventual changes in health or lifestyle and vitamin D and calcium intakes during the study. The two FFQs were also used to evaluate if subjects changed their consumption of milk and bread during the study. Vitamin D and calcium intakes from the habitual diet were calculated on the basis of consumption frequencies and vitamin D and calcium concentrations in food items given in the Danish Food Composition Databank [181]. Vitamin D contributions from the fortified milk and bread were calculated on the basis of frequencies of milk and bread consumptions and measured vitamin D concentrations in the study milk and bread. The contribution of vitamin D from dietary supplements was calculated as the frequency of use times the self-reported content of vitamin D in the supplement used.

The FFQ was adapted from an FFQ used in the European Union project Towards a strategy for Optimal Vitamin D Fortification [75]. The composition of the questions was adjusted the present study with self-administration and Web-based setup. The necessity of food groups in relation to their vitamin D contribution in the target group was evaluated, and it was decided to add fats and cold meat. The FFQ included the following food categories: milk, milk products, cheese (on top of bread and as part of a dish), bread, eggs, cold meat, poultry, meat, offal, fish (on top of bread and as part of a dish) and fats (on top of bread and used for cooking). As the recording of the participants´ bread intake was important and as bread may be consumed several times a day, the eleven frequency categories covered from never to >8/day. Portion sizes were estimated in collaboration with an experienced Dietician.

The ease ability of the Web-based setup and understand ability of the FFQ was pilot-tested among colleagues and friends with no prerequisite in dietary assessment. No major adjustments were made hereafter. The FFQ was evaluated with a simple record covering the food groups in the FFQ combined with weighing of fish and meat in the main meal in 49 of the participating children. The frequencies of milk and rye bread consumption were overestimated in the FFQ compared with the daily registering whereas the frequencies of meat and cheese in the main meal were underestimated [182]. Portion sizes were overestimated in the FFQ compared with the weighted portion sizes in the 4-7 year-old children.

Compliance to the intervention was registered 4 times during the study. In a short self-administered Web-based questionnaire, subjects reported their consumption of milk and bread other than the products provided in the study. Compliance was estimated by dividing the number of portions of milk or bread consumed per day other than the products provided in the study by the total number of portions of milk or bread consumed per day as reported in the FFQ.

3.5 Examination and blood collection

Subjects were examined, and blood samples were collected, in an authorized laboratory (Copenhagen´s General Practitioners Laboratory). Weight was measured to the nearest 0.1 kg in normal clothes without shoes (1 kg was withdrawn from the measured weight) by using a body-composition analyzer (Tanita BC-418; Tanita Europe BV). Height was measured to the nearest centimeter without shoes by using an ultrasonic height measure (Soehnle 5001; Soehnle

Professional GmbH & Co). BMI (in kg/m^2) was calculated on the basis of measured weight and height, and subjects were categorized into normal weight, overweight and obese classes according to standards for children [183] and the World Health Organization International standard for adults [184]. Local anesthetic patches were offered to the children before blood sampling. A trained biomedical laboratory technician drew nonfasting venous blood samples from subjects. Blood samples were held at room temperature for 30 min before centrifugation (1800×g for 10 min), and serum and plasma were collected and stored at -80 °C until analysis.

3.6 Biochemical analyses

All blood samples were analyzed as single determinations in a random order at the Clinical Biochemical Department, Holbæk Hospital, Denmark, after completion of the study. Measurements of serum 25(OH)D concentration relied on the determination of both $25(OH)D_2$ and $25(OH)D_3$ and were conducted by isotope-dilution liquid chromatography-tandem mass spectrometry (LC/MS-MS) by using principles described elsewhere [185]. The standard reference material vitamin D in human serum (SRM 972) [68] was used as the primary calibrator. In DEQAS, the mean bias for our method compared with the mean of the DEQAS LC-MS group during the period of the present analyses was -3.2 %. Interassay CVs for our method were 2.2 % and 2.8 % at 30 and 180 nmol/L, respectively, for $25(OH)D_3$ and 7.6 % and 4.6 % at 43 and 150 nmol/L, respectively, for $25(OH)D_2$. Serum total calcium (CV: 1.2 %) was measured by using a chemistry analyzer Cobas c501 (Roche Diagnostics), and plasma PTH (CV: 3.4 %) was measured by using an immunology analyzer Cobas e601 (Roche Diagnostics) following standard procedures from the manufacturer.

3.7 Statistics

Due to the study design and logistics, we wanted an equal number of families in the control and the fortification group within each of the three distribution supermarkets. Therefore the random allocation was stratified by distribution supermarket, the number of female and male children and adults in the family, use of multivitamin supplements and week of blood sampling by using the minimization method [186].

Mixed models with family as a random factor were applied in all the statistical analyses to account for the familial component. When the effect of the fortification was analysed (**paper 1**), the model included age, gender, baseline serum 25(OH)D concentration and treatment group as categorical variables and final serum 25(OH)D concentration as the outcome. When the determinants of serum 25(OH)D concentration at baseline were identified (**paper 2**), variables significant ($P < 0.05$) in their simple models were included in the multiple model together with age and gender as fixed variables and baseline serum 25(OH)D concentration as the outcome. The strength of the familial component was considered by calculating the intra-class correlation for each model. The closer the value is to 1 the more alike are the subjects within a family with respect to vitamin D status. The relationship between total vitamin D intake and serum 25(OH)D concentration (**paper 3**) was fitted with both linear and non-linear models. Possible effect modifiers (age, gender, BMI class and baseline serum 25(OH)D concentration) were included in the model each at a time to see their influence on the relationship.

Further details on the statistical analyses are described in each paper.

4 Results

Below is given a summary of the results. Details are found in the three papers.

The majority of the study population was of normal weight according to their BMI class (94 % of the children and 55 % of the adults), and the genders were evenly distributed in both children and adults and between the treatment groups (**Table 3**). The median dietary vitamin D intake was similar across age groups (range 2.3-2.6 µg/day) and around 30 % reported multivitamin use both in summer and winter (41 % of the children and 25 % of the adults). During summer, 48 % had a sun vacation abroad and 23 % had a sun vacation during winter. Approximately half of the adults had a medium-long to long higher education, and their lifestyles were generally healthy.

Table 3. Characteristics of subjects in the fortification and control group.

	Fortification group	Control group
Gender [*n* (%)]		
Female	179 (50)	193 (52)
Male	176 (50)	178 (48)
Age [*n* (%)]		
4-10 year	80 (23)	83 (22)
11-17 year	74 (21)	84 (23)
18-40 year	106 (30)	86 (23)
41-60 year	95 (27)	118 (32)
BMI [*n* (%)]		
Normal weight	258 (73)	262 (71)
Overweight	75 (21)	80 (22)
Obese	22 (6)	29 (8)
Vitamin D intake[1] (µg/day)		
Habitual diet	2.4 (1.6, 3.4)	2.2 (1.4, 3.1)
Fortified milk and bread	6.8 (4.2, 9.5)	0
Total from foods	9.2 (7.0, 12.3)	2.2 (1.4, 3.1)
Dietary supplements[2]	3.6 (2.0, 7.1)	3.6 (2.1, 7.1)
Multivitamin users [*n* (%)]		
Summer	244 (32)	
Winter	105 (30)	109 (29)
Sun vacation [*n* (%)]		
Summer	363 (48)	
Winter	100 (28)	70 (19)

[1]Median (SD).
[2]Supplement users only.

At baseline of the study (late summer) the individual serum 25(OH)D concentrations ranged from 9.3 to 161.9 nmol/L with an overall geometric mean (IQR) of 72.1 (61.5-86.7) nmol/L. There was no overall age (P=0.190), gender (P=0.332) or age and gender (P=0.223) differences (**paper 2**). In the adults, geometric mean PTH concentration (95 % CI) for the 25(OH)D groups of <25, 25-49, 50-75 and >75 nmol/L was 59·8 (43.2-82.8), 39·9 (35·8, 44·3), 36·2 (34·3, 38·2) and 32·7 (31·1,

34·4) ng/L (*P* trend<0.001). The same trend was seen in children aged 4-10 years (*P* trend=0.012), but not in children aged 11-17 years (*P* trend=0.067).

In the multiple analysis of all subjects (**paper 2**) serum 25(OH)D was negatively associated with BMI (*P*<0.001) and positively associated with dietary vitamin D (*P*=0.008), multivitamin use (*P*=0.019), solarium use (*P*=0.006), outdoor stay in light clothes (*P*=0.001), sun preference (*P*=0.002) and sun vacation (*P*<0.001). When children and adults were analysed separately serum 25(OH)D was only associated with gender, outdoor stay in light clothes and sun vacation in the children. In the adults the associations remained the same as in the model with all subjects but the significances for dietary vitamin D, multivitamin use and sun vacation were weakened. In the further exploration of the adults serum 25(OH)D concentration was not associated with lifestyle related factors when these were included in the multiple analysis together with the vitamin D source related variables.

The intra-family correlation was calculated from these multivariate models to assess the strength of the familial component. It was 0.27 in all subjects, 0.42 in children and 0.24 in adults, thus children within a family seemed to be more alike than adults within a family with respect to their vitamin D status (**paper 2**).

At baseline, there was no difference between the treatment groups in the prevalence of serum 25(OH)D <30 nmol/L (*P*=0.158) and <50 nmol/L (*P*=0.814) (**paper 1**). By the end of the study, <1% of subjects in the fortification group compared with 25 % of subjects in the control group had 25(OH)D concentrations <30 nmol/L (*P* <0.001), and 16 % of subjects in the fortification group compared with 65 % of subjects in the control group had 25(OH)D concentrations <50 nmol/L (*P* <0.001) (**Figure 6**).

In the adult fortification group, 25(OH)D decreased from months 0 to 3 (*P*<0.001) and increased from months 3 to 6 (*P*=0.014); nevertheless, the final concentration was lower than the baseline concentration (*P* <0.001). In the adult control group, 25(OH)D decreased from months 0 to 3 (*P*<0.001) and continued decreasing from months 3 to 6 (*P*=0.001). In the children, 25(OH)D decreased from months 0 to 6 in both the fortification and control groups (both *P*<0.001) (**Table 3**).

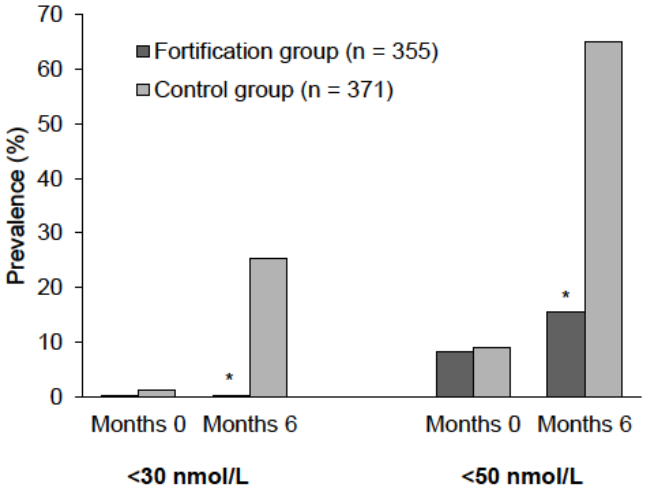

Figure 6. Prevalence of subjects with serum 25(OH)D concentration <30 and <50 nmol/L at baseline (month 0) and by the end of the study (month 6). *Significant difference between fortification and control group (P<0.001).

The final serum 25(OH)D concentration for all subjects was significantly higher in the fortification group (67.6 (56.2, 79.4) nmol/L) compared with in the control group (41.7 (29.5, 58.9) nmol/L) despite that 25(OH)D decreased in both groups (-5.5 and -29.4 nmol/L) (P<0.001, interpreted estimate 1.59, linear mixed model) (**paper 1**). Additional adjustment of the model for BMI (P=0.782) multivitamin usage (P<0.001) and sun vacation (P<0.001) did not change the magnitude of the effect of treatment (P<0.001, interpreted estimate 1.58).

Table 3. Biochemical measures in children and adults in the fortification and control group at month 0, 3 and 6[1].

	Children 4-17 years			Adults 18-60 years		
	Fortification group (n=154)	Control group (n=167)	P	Fortification group (n=201)	Control group (n=204)	P
25(OH)D (nmol/L)						
month 0	72.8 (64.0, 88.9)	72.8 (61.8, 83.9)	0.524	73.3 (61.9, 88.3)	70.0 (60.0, 86.7)	0.286
month 3	-	-	-	63.1 (52.5, 77.6)	44.7 (32.4, 63.1)	<0.001
month 6	67.6 (56.2, 79.4)	42.7 (30.9, 58.9)	<0.001	66.1 (56.2, 79.4)	40.7 (28.8, 60.3)	<0.001
PTH (ng/L)						
month 0	35.0 (28.8, 41.5)	35.3 (27.6, 43.3)	0.969	35.5 (27.8, 44.2)	34.2 (26.9, 41.4)	0.268
month 6	36.3 (27.5, 47.9)	41.7 (32.4, 52.5)	0.028	36.3 (29.5, 45.7)	38.9 (30.9, 46.8)	0.049
Total calcium (mmol/L)						
month 0	2.45 (2.40, 2.49)	2.47 (2.42, 2.51)	0.035	2.43 (2.37, 2.47)	2.43 (2.37, 2.49)	0.268
month 6	2.45 (2.40, 2.50)	2.45 (2.40, 2.50)	0.428	2.42 (2.37, 2.46)	2.41 (2.36, 2.47)	0.423

[1]Values are geometric means (IQR). P-values are for the comparison of treatment groups in biochemical measures at each time point.

The final PTH concentration was lower in the fortification group than in the control group in both children and adults, whereas there was no difference in final total calcium concentrations between

treatment groups. The combined analysis of children and adults in linear mixed models adjusted for baseline values of the endpoint, gender and age showed a treatment effect on PTH (P<0.001, interpreted estimate 0.91) and no treatment effect on total calcium (P=0.237).

During the study, daily median (IQR) milk consumptions were 532 (262, 712) mL in the children and 266 (139, 563) mL in the adults. Daily bread consumptions were 117 (80, 164) g in the children and 101 (63, 157) g in the adults (**paper 1**). The planned fortification strategy was to increase the vitamin D intake from foods to 7.5 µg/day. This succeeded in 66 % of the subjects in the fortification group with a median (IQR) intake of 9.2 (7.0, 12.3) µg/day compared with 2.2 (1.5, 3.0) µg/day in the control group. With contributions from dietary supplements, the median total vitamin D intake was 14.7 (10.6, 18.2) µg/day in supplement users in the fortification group and 6.2 (4.1, 9.0) µg/day in supplement users in the control group (**paper 3**).

The relationship between total vitamin D intake from natural foods, fortified foods and dietary supplements and the serum 25(OH)D concentration was best fitted by a log-model yielding a non-linear curve (**Figure 7**).

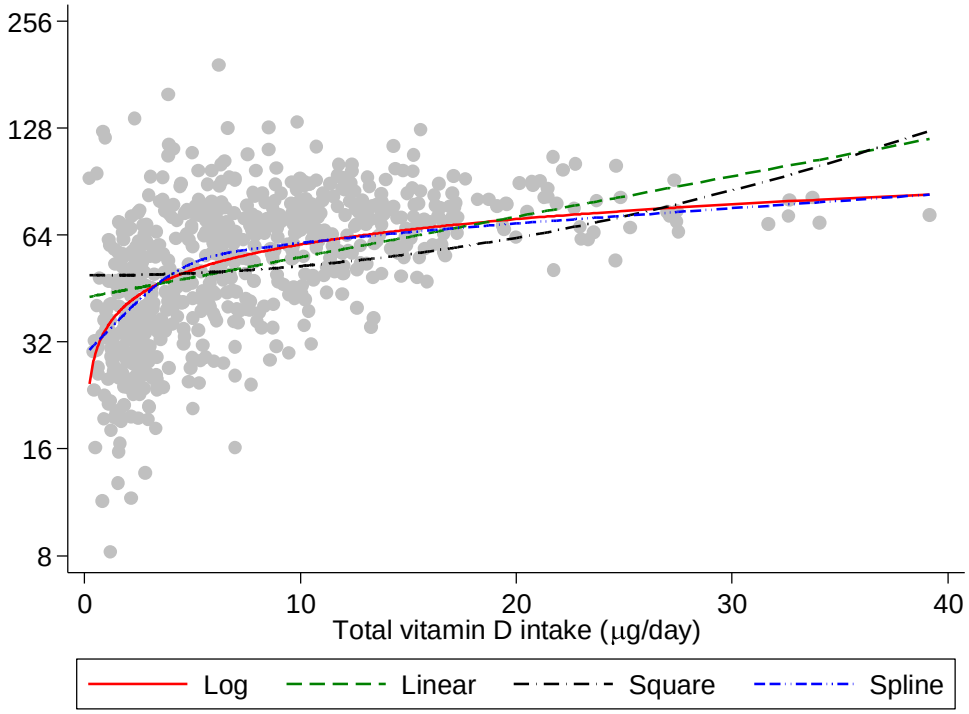

Figure 7. Models for the relationship between total vitamin D intake and serum 25(OH)D concentration (n=692).

The effect of the total vitamin D intake on serum 25(OH)D concentration was modified by gender (P = 0.014) and initial 25(OH)D group (P<0.001), but not by age group (P=0.132) or BMI class (P=0.884) (**paper 3**). For each doubling of vitamin D intake, 25(OH)D in men increased by 4 % more than in women, and vitamin D intake had a 10 % higher effect on participants with lowest initial 25(OH)D than on participants with highest initial 25(OH)D. Estimated total vitamin D intakes of 5, 11, 23 and 39 µg/day were required to maintain a winter vitamin D status above 50 nmol/L in 50, 75, 90 and 95 % of the population. These figures were higher for the group with lowest initial 25(OH)D concentration and lower for the group with highest initial 25(OH)D concentration (**Table 4**).

Table 4. Estimated vitamin D intake to maintain serum 25(OH)D concentration >25 and >50 nmol/L in specified proportions of the population and for the four initial 25(OH)D groups (n=688).

Vitamin D intake (µg/day)	97.5 % limit	95 % limit	90 % limit	75 % limit	50 % limit
Aim >50 nmol/L					
Overall		39	23	11	5
Initial <61.5 nmol/L	-	-	34	18	11
Initial 61.5-73.4 nmol/L	-	37	22	1	6
Initial 73.4-86.9 nmol/L	-	37	19	7	4
Initial >86.9 nmol/L	39	17	8	3	<1
Aim >25 nmol/L					
Overall	6	4	3	1	<1
Initial <61.5 nmol/L	11	7	5	3	2
Initial 61.5-73.4 nmol/L	7	4	3	2	<1
Initial 73.4-86.9 nmol/L	4	2	<1	<1	<1
Initial >86.9 nmol/L	<1	<1	<1	<1	<1

5 Discussion

5.1 Vitamin D status

Vitamin D status in the Danish children and adults by the end of the summer was >50 nmol/L with a mean of 72.1 nmol/L (**paper 2**). During winter serum 25(OH)D concentration remained above this limit in the majority (84 %) of children and adults receiving the vitamin D fortified milk and bread with a mean of 67.6 nmol/L whereas the prevalence >50 nmol/L decreased to 35 % in the control group with a mean of 41.7 nmol/L (**paper 1**). The found vitamin D status both in summer and winter and the prevalence >50 nmol/L were generally higher than previous findings in similar age and ethnicity groups in Denmark [72-75] and other countries [75, 78-87]. These differences could be attributed differences in dietary habits, use of dietary supplements, sun habits, lifestyle, socio-economic status and composition/characteristics of the study population. For example most of our subjects were of normal weight which could favourable impact the serum 25(OH)D concentration compared with the high rate of obesity in other groups for example in the USA [187]. Another explanation would be that the method used in our study (LC/MS) has often been found to yield higher results compared with other methods [55, 57, 64, 66]. In DEQAS the LC-MS methods is also positively biased for the ALTM whereas the often used immunoassays are mostly negatively biased [62]. However, the LC-MS method might be considered the golden standard [55, 57], and our method was standardized and calibrated against the International reference material by NIST [68]. In a study of the German population standardization to the LC-MS/MS method reduced the prevalence of vitamin D deficiency (<30 nmol/L) from approximately 48 % to 16 % [188]. Methods are continuously improving and standardization and cross-calibration seem to be possible now with the NIST standard that was used in the present study. Hopefully this will improve method related variability in the future.

A novelty in the VitmaD study was the quantitatively assessment of the familial component by calculation of the intra-class correlation. The intra-family correlation was almost the double in the children as in the adults (**paper 2**), which indicates that the children within a family were more alike than the adults within a family with respect to their vitamin D status. This might be an indication of the influence of genetic factors on vitamin D status or more similar habits in children than in adults from the same family. It is likely that children within a family share activities and dietary/supplementation habits to a larger extent than adults within a family. Another speculation could be the gender factor with a mother and a father in each family although the statistical analyses

did not show a gender difference in vitamin D status in the adults (**paper 2**). When the children were analysed separately, the boys had a statistically higher vitamin D status compared with girls. Thus, if one family included two boys or two girls they could be more similar than if the family included one of each gender. The apparently greater similarity in vitamin D status in children compared with in adults within a family may also be attributed a greater difference in adiposity between the adults. This hypothesis was expressed by BMI being negatively associated with serum 25(OH)D in the adults but not in the children (**paper 2**). However, BMI did not affect the effect of the fortification (**paper 1**) or the response in 25(OH)D to total vitamin D intake (**paper 3**). Several studies have investigated the relation between adiposity and serum 25(OH)D or the response in serum 25(OH)D. The use of BMI as a measure of adiposity in relation to vitamin D status is controversial and some studies did not find an association between BMI and 25(OH)D [85, 189-193] whereas a recent meta-analysis found an inverse relationship between BMI and 25(OH)D [194] like several other studies [78, 84, 195-198]. More direct measures of adiposity such as body fat mass, waist circumference and skin folds have also been associated with serum 25(OH)D or its response to supplementation [195, 196, 199-201] and might be alternative predictors of vitamin D status. Several mechanisms have been suggested for the apparently lower serum 25(OH)D levels associated with obesity and one explanation could be sequestration of vitamin D in fat tissue [198]. Although there is limited evidence, it has been speculated if low serum 25(OH)D levels may predispose to obesity rather than the obesity in itself predisposing for low serum 25(OH)D levels [199, 202, 203].

Vitamin D status in late summer was both associated with sun exposure factors (sun behaviour, outdoor stay in light clothes, sun vacation and solarium use), intake from the diet and use of multivitamins (**paper 2**). This finding substantiates the relevance of dietary intakes in addition to sun exposure not only during winter. Most suggestive may be that vitamin D status was associated to sun vacation (abroad) despite that the hours of sunshine the preceding summer (2010) in Denmark was only slightly below (3 %) the average hours of sunshine for the preceding ten years [204]. To our knowledge, this association between vitamin D status and sun vacation during summer season has not been shown previously.

After all, what is actually the optimal serum 25(OH)D concentration? And optimal for what? A serum 25(OH)D concentration of 50 nmol/L was considered sufficient for bone health in the NNR [103] and by the US Dietary Committee [47]. Some argue that 25(OH)D concentrations in the interval 75-125 nmol/L should provide the benefit of vitamin D while limiting the risk of potential harm [95,

[98, 104-106] based on the associations of serum 25(OH)D with functional markers or the incidence of certain diseases [25, 41]. For example PTH is a surrogate marker of bone resorption in adults [100]. We found a negative trend between PTH and serum 25(OH)D groups in the adults and in the children aged 4-10 years (**paper 2**). However, a comprehensive review of the health effects of vitamin D and calcium concluded that the evidence for non-skeletal effects of vitamin D was inconclusive [205]. In a socio-economic perspective it has been estimated that a 10-50 % reduction in specific disease rates, a 17-18 % reduction in mortality rates (range for Denmark 11-24 %) and a 2-3 year increase in life expectancy could be possible by increasing the population mean serum 25(OH)D concentration to 100-105 nmol/L and thereby the economic burden of disease would be reduced [206-209]. However, the causality remains yet unproven in randomized controlled trials (RCT) [210]. Studies have associated 25(OH)D concentrations with socio-demographic and lifestyle related factors [78, 211] and thus it might be that vitamin D is simply a marker of general lifestyle/health. I find it plausible that there might be extra-skeletal functions of vitamin D that we do not know a lot about yet, but I am skeptic about the unnatural high serum 25(OH)D concentrations that have been suggested which would be difficult to meet by a normal lifestyle.

Another question is if vitamin D status should be constant year-round? We still do not know the impact of seasonal fluctuations in vitamin D status and what it means for long-term health that a significant part of the population has a vitamin D status between 25 and 50 nmol/L during winter. It might be that people at Northern latitudes have adapted to these circumstances [24], so that some decline in serum 25(OH)D concentration during winter is acceptable and that a chronically low concentration is the condition more critical for long-term health.

5.2 Vitamin D intake

As well as optimal vitamin D concentrations are discussed, there is little agreement on the intake needed to maintain a specified vitamin D status year-round. It is complicated by the fact that vitamin D is provided by both endogen synthesis and intakes. The contribution of vitamin D from fortified foods resulted in a total vitamin D intake of 14.7 µg/day in dietary supplement users and 9.6 µg/day in non-users (**paper 3**) and maintained serum 25(OH)D concentration above 50 nmol/L in 84 % of the subjects with usual supplementation and sun vacation habits during winter in Denmark (**paper 1**). The corresponding figure from the dose-response relationship between total

vitamin D intake and serum 25(OH)D concentration was 11-23 µg/day for 75-90 % of the population (**paper 3**). This estimated required intake was increased to 39 µg/day if 95 % of the population should meet the goal of 50 nmol/L, which is higher than the new RI of 10 µg/ that is based on 97.5 % of the population. It was also higher than previous estimates in children, adults and elderly 21.8 [193, 212, 213]. Interestingly, a meta-analysis found that a vitamin D intake of only 9-12 µg/day was needed to sustain 97.5 % of the population above 50 nmol/L during winter [214]. Based on calculations of the metabolic clearance of 25(OH)D, a study estimated that an intake of 12.5-17.5 µg/day was sufficient to maintain serum 25(OH)D at 50 nmol/L [211]. The estimated required vitamin D intake was lower for the group with highest initial vitamin D status compared with the group with lowest initial vitamin D status. Thus, our results support the concept of initial vitamin D status and/or cutaneous synthesis during the summer probably offsets the dietary requirement for vitamin D during winter [193, 212]. Our calculation of required vitamin D intake for different levels of initial vitamin D status is novel in relation to previous studies that have found the initial vitamin D status to be associated with the response in 25(OH)D [192, 215, 216].

In the VitmaD study the relationship between total vitamin D intake and status was best fitted by a non-linear curve (**paper 3**) similar to previous observations [189, 191, 205, 212, 214, 215, 217]. Others have reported response estimates based on linear analyses [49, 53, 164, 191, 193, 213, 214, 218-221]. When considering required vitamin D intake in populations for example in the setting of dietary recommendations, the effect in serum 25(OH)D per microgram of ingested vitamin D is often considered. In a meta-analysis of the efficiency of vitamin D fortified foods in adults the effect was evaluated as the difference between the treatment groups in their 25(OH)D change in relation to the vitamin D intake from the fortification [219]. The treatment effect was 1.2 $nmol \cdot L^{-1} \cdot \mu g^{-1}$, which is lower than our finding of 3.4 $nmol \cdot L^{-1} \cdot \mu g^{-1}$ (**paper 1**). In studies with dietary supplements the effect has been calculated as the change in 25(OH)D or the final 25(OH)D as a function of vitamin D dose. These studies yielded constants of 0.4-5.0 $nmol \cdot L^{-1} \cdot \mu g^{-1}$ [49, 53, 190, 191, 217, 218, 222]. Thus, the figures are calculated by different means which should be kept in mind when using the figures for discussion of required vitamin D intake. Systematic reviews also concluded that studies were heterogeneous in their design, population and 25(OH)D assay [205, 214, 220] which may contribute to observed differences between studies.

5.3 Fortification model and its effects

The fortification strategy in the current study resulted in a higher serum 25(OH)D concentration in the fortification group (67.6 nmol/L) compared with the control group (41.7 nmol/L) at the end of the winter season, despite the fact that 25(OH)D decreased in both groups during the study (-Δ5.5 nmol/L in the fortification group and -Δ29.4 nmol/L in the control group) (**paper 1**). The planned fortification strategy was to increase the vitamin D intake to the amount of 7.5 µg/day recommended in the NNR at that time [131]. This strategy succeeded in 66 % in the fortification group. A difference in our results compared with most of the previous fortification studies was that the beneficial effect was expressed as a reduction in the seasonal decline in 25(OH)D compared with in the control group. Our observations were similar to those of 2 other RCTs in 17-54 year old men and women with baseline 25(OH)D and fortification amounts comparable to those in our study [102, 221]. Most previous studies had a lower initial starting point than that of our study and observed an increase in 25(OH)D after supplementation with vitamin D fortified foods [159-162, 164, 167-169, 171, 172, 175, 177]. An important conclusion from systematic reviews was the large heterogeneity between fortification studies [205, 220]. The studies varied in design, study population, baseline 25(OH)D, food vehicle used, and fortification amounts. Therefore, the treatment effects could not be directly compared and should be interpreted with caution. A novelty in the design of the current study was the combined use of two food vehicles consumed during the day according to the participants´ usual habits. This design was different from that in the previous studies which used single vitamin D fortified foods, mostly milk but also yoghurt drinks, cheese, orange juice and bread consumed in a predefined serving or amount [102, 159-162, 164, 165, 167-169, 171, 172, 175, 177].

This research demonstrated that a targeted vitamin D food fortification is an efficient way to improve vitamin D intake and status in the general population. The combination of fortified milk and bread proved suitable for 4-60 year-old Danes, partly because Danish children and, to some extent, adults drink more milk than do other population groups. Milk is not widely consumed in all populations, whereas bread is a primary food in many countries. Therefore, the present study suggested that including bread in the current selection of vitamin D fortified foods could be an effective way to attenuate seasonal fluctuations in vitamin D status. Others have investigated the effect of fortifying single food items with vitamin D (references). These were mostly dairy products but also orange juice and bread have been investigated. Orange juice may not be consumed on a regular basis and there might be large variations in the amounts of milk consumed in different

groups so that the vitamin D intake becomes skewed. Bread in different forms is a food that is consumed daily for most people. Vitamin D can be added the flour like in this study as well as during baking like in a previous study [160]. An important finding in the present study was that the analyses of bread did not fully recover all of the added vitamin D. We suspect that some of the vitamin D was lost during baking. This loss was unexpected because, in a previous fortification study, the added vitamin D was recovered in both dough and baked wheat and rye breads [160].

The present study is an example of modelling on usual food consumptions that can be applied in other populations as well. It is recommendable as the current diffuse fortification practices in many countries do not seem optimal to improve vitamin D status in the general population [127, 155, 156]. It is a political decision if the fortification should be on a voluntary or mandatory basis. The voluntary solution gives the consumers a choice to select or deselect fortified products, whereas the mandatory approach targets the entire population. It is often the most vulnerable groups that have the need for supplementation and they are targeted with mandatory food fortification.

The dose-response study (**paper 3**), however, showed that the required vitamin D to maintain vitamin D status above 50 nmol/L in the majority of the population should be higher than the new RI [130]. If this is actually the case, then the fortification concentrations should also be higher than those applied in the study. On the other hand, it can be discussed how large a proportion of the population the fortification strategy should target as the dose-response study also showed that the required vitamin D intake during winter varied largely between the selected proportion of the population (5-23 µg/day for 50-90 % of the population) and was dependent on the initial vitamin D status. When the present study was designed, the fortification concentrations should be effective without possessing a risk for any of the subjects reaching an intake above the UL. Since that time, the UL has been doubled [52] thus allowing for a greater interval for fortification. However, having the opportunity to do it is not the same as it is reasonable to do it. We still do not know the long-term effects of prolonged higher vitamin D intakes [49], and it is relevant to discuss how precaution in a future fortification model should be considered. The aim with a fortification strategy is to move the distribution curve for vitamin D intake towards right. It is just a question about how far it should be moved. Those with the most need (the left of the distribution curve) is benefited, but the vitamin D intake in those with the least need (the right part of the distribution curve) is also increased and moved closer to the UL. Do we want as large a proportion of the population as possible to have the recommended intake or higher? or just the median? Should a fortification strategy ensure a

minimum intake in all thus ensuring no severe deficiency rather than securing the optimal? Should the better part be protected against high intakes although they are not close to the UL?

Generally, it is a relevant discussion how to improve vitamin D intake and status in the population and if it is really needed. It is suggestive that the RI for vitamin D in the NNR has gone from 5 to 7.5 to 10 µg/day from 1996 to 2012 thus being doubled over 16 years. Have we gained that much new knowledge? Have our sun exposure behaviours changed that much so the dietary intake should be doubled? Then I question: Is it the recommendations that are set too high in relation to what is actually needed? Or is the intake too low in relation to the need?

5.4 Considerations about the study design

It is a challenging task to execute RCTs in a real-life setting and it is the art of balancing control, restriction and the wish of not to interrupt the natural behaviour of subjects. As with all other studies, it is also a balance between how demanding it can be for the subjects and the information you want to collect as well as the time frame and budget are important for what is feasible. If the demands are too high it can be difficult to recruit subjects and the ones you get might be the very dedicated and health-aware people. We wanted a sample of typical Danish families with children and minimal interruption of their daily life and therefore we had few inclusion criteria and restrictions. On the other hand, when approaching real-life, there is a chance of the intervention effect being blurred by confounders.

When designing the fortification study, several points were considered such as: how to deliver fresh milk and bread to 800 persons through a half year? How to minimize the interruption of the subjects´ daily life? How to monitor the subjects? How to register their food intake and compliance?

As children families usually have busy week days and as they were going to participate in the study for a relatively long period, we wanted the distribution of milk and bread to be easy for the subjects. This was logistically challenging as we also wanted the milk and bread to be fresh instead of for example frozen. The solution with distribution through three local supermarkets worked well with minimal issues and eased the participation for the families as they could pick up their study

products while doing their usual grocery shopping. Due to this logistics, a minor limitation of the study was that it was conducted at a single site in Denmark.

A motivation factor for the families was the free food. They said that it was “a nice offer” with free milk and bread through six months which might have been a large saving for some families. Others were interested in vitamin D and had previously been diagnosed with vitamin D deficiency and experienced symptoms during the winter. Therefore they wanted to see if fortification could help them improving their vitamin D status. Some of the families stated that they felt specially selected.

We believe that the family and real-life based design was one of the main reasons for the low dropout rate and high compliance. Informal feedback from subjects indicated that they made their participation a joint family project, which they could discuss at dinner times, and they encouraged and reminded one another to consume the study products provided. Thus, the results confirmed our assumption that it would ease the compliance to the intervention if the whole family participated instead of just one member of the family as it interrupts the routines less. The family-based design also ensured a broad spread of age and gender in both children and adults and it eased the recruitment of many subjects.

However, it might also be more challenging to adjust the information and to collect and use data on an individual level in diverse groups. For example, how differentiated should the information material be? Do you want the same information for children and adults? Which information do you need for each of the groups for example tanner stage in children and lifestyle in adults. Do you want the questionnaires to be differentiated or do you want them to use the same questionnaire? Or should it only be targeted the parents? Do you want the information to be addressed each of the family members or only one from the family? Should the family be examined at the same time or at different times? It was a balance between what was practically durable and what was wanted for the study design. For example the family could not start consuming the study products before all the family members had their blood drawn, but for some of them it was difficult to manage blood sampling the same day.
We decided to keep it simple and therefore we developed one questionnaire and FFQ as the basis. Some of the questions were then taken out for the children for example smoking habits and occupation. The same FFQ was used for children and adults including the use of portion sizes although portion sizes might be different for different age and gender groups. Evaluation of the FFQ also showed that the portion sizes were overestimated for the smallest children. The recording of the

compliance and how much the subjects had consumed of the study products was also challenging as the subjects should consume the milk and bread during the whole day according to their usual habits instead of consuming them in fixed amounts. It would have been too demanding for the subjects to register their milk and bread consumption every day during a half year. Instead we asked them to register their consumption of milk and bread other than what was given in the study in a frequency format. That resulted in a roughly estimate of the compliance which was considered sufficient in this study. We could also have given them a record covering a few days, but we did not want to put focus on their consumption so that the possibility of influencing their usual dietary pattern was opened.

6 Conclusion and perspectives

Serum 25(OH)D concentration in children and adults in late summer in Denmark were above 50 nmol/L in the majority with no overall differences between gender and age groups. Both dietary and sun related factors as well as BMI were determinants of vitamin D status, and the familial component was stronger for the children than for the adults. Vitamin D fortification of milk and bread, to an amount that increased the intake to recommended amount, reduced the decrease in serum 25(OH)D concentration during winter and maintained 25(OH)D above 50 nmol/L. The relationship between total vitamin D intake from natural foods, fortified foods and dietary supplements and serum 25(OH)D concentration was non-linear. Estimated required total vitamin D intake to maintain 25(OH)D above 50 nmol/L during winter was largely dependent on the initial vitamin D status. The family-based approach showed to be a good way to heighten compliance and ease the inclusion of a broad age group of both genders.

Overall, the present study demonstrated that it is feasible to conduct RCTs in real-life settings without compromising the quality of the study and the chance to see an effect of the intervention. Furthermore, the study showed the use of model data from usual food-consumption intakes to establish an effective fortification strategy targeted at the intended population. The investigated fortification model was efficient in a population of Danish families. It is possible that the response to the fortification would have been different in other population groups such as elderly or dark-skinned groups. These groups would probably have a lower initial vitamin D status than the families in the VitmaD study, and it is likely that a fortification strategy with bread would also work in these groups. This could be investigated. If implementation of the fortification strategy was mandatory, the choice of using fortified products or not could not be influenced by factors such as age, gender, education, social status and ethnic affiliation. It would be a matter of the consumer eating the type of fortified product or not and the frequency of consumption. The results are likely to be generalizable, and including bread in the current selection of vitamin D fortified foods could be an effective way to improve vitamin D status of the general population.

The stability of vitamin D in different types of bread should be further investigated, and the fortification model should be adjusted to the new RI in the NNR for future applications. Other foods could be considered for fortification, and awareness on how to ensure safety is recommended, not only in relation to the UL.

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8 Papers

Paper 1:

Randomized controlled trial of the effects of vitamin D-fortified milk and bread on serum 25-hydroxyvitamin D concentrations in families in Denmark during winter: the VitmaD study. Madsen KH, Rasmussen LB, Andersen R, Mølgaard C, Jakobsen J, Bjerrum PJ, Andersen EW, Mejborn H, Tetens I. *American Journal of Clinical Nutrition* 2013, 98: 374-382.

Randomized controlled trial of the effects of vitamin D–fortified milk and bread on serum 25-hydroxyvitamin D concentrations in families in Denmark during winter: the VitmaD study[1–3]

Katja H Madsen, Lone B Rasmussen, Rikke Andersen, Christian Mølgaard, Jette Jakobsen, Poul J Bjerrum, Elisabeth W Andersen, Heddie Mejborn, and Inge Tetens

ABSTRACT

Background: Vitamin D intakes are lower than dietary recommendations in most populations, and thus, a low vitamin D status is widespread, especially during winter.

Objective: We investigated the effects of increasing vitamin D intake to the recommended amount by fortification of milk and bread on serum 25-hydroxyvitamin D [25(OH)D] concentrations in families during winter in Denmark.

Design: The study was a randomized controlled trial in 782 children and adults (4–60 y old) recruited as 201 families. Families were randomly assigned to vitamin D–fortified or nonfortified milk and bread for 6 mo starting in September. The milk and bread replaced the participants' usual consumptions of products.

Results: Median (IQR) vitamin D intakes (habitual diet plus fortified products) were 9.4 μg/d (6.5, 12.3 μg/d) and 2.2 μg/d (1.5, 3.0 μg/d) in fortification and control groups, respectively. Geometric mean (IQR) serum 25(OH)D concentrations decreased from 73.1 nmol/L (61.9, 88.5 nmol/L) to 67.6 nmol/L (56.2, 79.4 nmol/L) in the fortification group and from 71.1 nmol/L (61.2, 85.9 nmol/L) to 41.7 nmol/L (29.5, 58.9 nmol/L) in the control group (both $P <$ 0.001). The final 25(OH)D concentration was significantly higher in the fortification group than in the control group ($P < 0.001$). By the end of the study, <1% of subjects in the fortification group and 25% of subjects in the control group had 25(OH)D concentrations <30 nmol/L and 16% and 65% of subjects, respectively, had 25(OH)D concentrations <50 nmol/L.

Conclusion: Vitamin D fortification of milk and bread reduces the decrease in serum 25(OH)D concentrations during winter and ensures 25(OH)D concentrations >50 nmol/L in children and adults in Denmark. This trial was registered at clinicaltrials.gov as NCT01184716. *Am J Clin Nutr* 2013;98:374–82.

INTRODUCTION

At latitudes above 40°N, vitamin D is not synthesized in the skin during the winter (1) and therefore, the vitamin D supply during this period depends on dietary intakes. Relatively few foods contain vitamin D (2) naturally, and vitamin D intakes are lower than dietary recommendations in most populations (3–5). Consequently, vitamin D deficiency is a public health concern in many countries (6–10).

The recently updated American dietary reference values for vitamin D from the Institute of Medicine (11) have stimulated scientific debate about the effects of different strategies to improve vitamin D status, which is measured as circulating 25-hydroxyvitamin D [25(OH)D][4] concentrations (12), at the population level. Food fortification is one preventive strategy to reach the entire population, and several countries fortify milk or margarine with vitamin D. Nonetheless, this fortification practice does not seem optimal for improving vitamin D status in the general population because of the skewness of milk intake across population groups (13, 14). A recently published systematic review emphasized the need for randomized controlled trials (RCTs) to ascertain the impact of potential vitamin D food-fortification strategies to ensure effectiveness at the population level (15). In Denmark, there are few fortified foods on the market, and thus, this country provides a unique opportunity to investigate the effects of a fortification strategy in a real-life environment.

Previous RCTs have investigated the effects of single vitamin D–fortified foods such as milk (16–24), yoghurt drink (25, 26), cheese (27, 28), orange juice (29, 30), and bread (31). In all but 2 of these RCTs (24, 27), the foods were shown to be effective in improving serum 25(OH)D concentrations. However, to reach the entire population, a strategy has been suggested (32) in which several food vehicles are fortified at lower amounts rather than one food item at a higher fortification amount. To our knowledge, no

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[4] Abbreviations used: FFQ, food-frequency questionnaire; NNR, Nordic Nutrition Recommendation; PTH, parathyroid hormone; RCT, randomized controlled trial; 25(OH)D, 25-hydroxyvitamin D.

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previous studies have investigated combinations of fortified foods, and only a few studies have targeted children (19, 22).

Our strategy was to build a realistic fortification model that could be used in a real-life setting on the basis of models generated from high-quality dietary intake data from the Danish National Survey of Dietary Habits and Physical Activity (33).

The objective of this RCT was to investigate the effects of increasing vitamin D intake by fortification of milk and bread to the amount recommended in the Nordic Nutrition Recommendations (NNRs) (34) on serum 25(OH)D concentration in families during a 6-mo winter period in Denmark.

SUBJECTS AND METHODS

Study design

The study was a double-blind, randomized, placebo-controlled intervention trial with children and adults recruited as families. An external statistician randomly allocated the families to either vitamin D_3–fortified milk and bread or nonfortified placebo milk and bread for 6 mo during the winter (September 2010 to April 2011). Milk and bread were distributed to the families 2 times/wk through 3 local supermarkets. The random allocation was stratified by the number of female and male children and adults in the family, use of multivitamin supplements, distribution supermarket, and week of blood sampling by using the minimization method (35). The milk and bread were blinded by a color and letter code so that neither the participants nor researchers knew which products contained vitamin D. A personal identification card informed each family which supermarket to go to and obtain products. The card ensured that families received the correct products and that products were handed out only to families who were participating in the study. Participants were instructed to replace their usual consumption of milk and bread with the products provided in the study. In all other respects, participants were requested to live their lives as normal without changing any habits. The principle in the study design was to investigate a realistic fortification strategy in a real-life setting. Adult participants were seen 3 times during the study period (months 0, 3, and 6), and children (4–17 y old) were seen twice (months 0 and 6). Blood samples were drawn at all 3 visits, and anthropometric measures were recorded at months 0 and 6. The primary endpoint was the serum 25(OH)D concentration, and secondary endpoints were plasma parathyroid hormone (PTH) and serum total calcium.

The study protocol was approved by the Research Ethics Committee of the Capital Region of Denmark (record H-4-2010-020).

Participants

The sample size was calculated at 88 per group to detect a mean (±SD) difference in serum 25(OH)D concentration of 11 ± 26 nmol/L (21) between treatment groups at the 5% significance level and with 80% power. To be able to analyze female and male children and adults separately and allow for dropouts, we aimed to recruit 800 participants who represented ~200 families. Participants were recruited in Gladsaxe Municipality in Denmark (56°N) by random withdrawal from the Danish Civil Registration System. Families with a permanent address in Gladsaxe and 3–6 members in the household, including at least one child aged 4–17 y, were invited by letter to participate in the study (**Figure 1**). Inclusion criteria were age between 4 and 60 y and a permanent address in the Gladsaxe Municipality. Exclusion criteria were pregnancy and disease or use of a medication that would influence vitamin D metabolism (including dietary supplements with >10 μg vitamin D/d for children and >5 μg vitamin D/d for adults, which corresponded to the typical amounts in multivitamin supplements in Denmark). There was an ample response to the letters, and registration was closed after 10 d. At that time, 429 families had responded to the invitation. After screening for eligibility, 230 families were selected, and invited to an information meeting. When the families hereafter decided on their participation, not all members of the household necessarily participated. All adult participants and custody holders of children gave their written informed consent to participate, which resulted in a total of 782 participants (201 families).

Fortification model

The fortification model was built on the following 3 criteria: the model should include a combination of foods, foods should be consumed regularly in the chosen age group, and foods should be low in fat and sugar. On the basis of these criteria, milk and bread were chosen as foods to include in the model. The fortification strategy was to increase the vitamin D intake to 7.5 μg/d as recommended in the NNRs (34) in as many participants as possible while avoiding an intake above the tolerable upper intake level [ie, 25 μg/d for children and 50 μg/d for adults (34)]. The choice of fortification amounts was based on models generated from dietary intake data from the Danish National Survey of Dietary Habits and Physical Activity (7-d consecutive dietary record) (33), which took into account the possible use of multivitamin supplements.

Study milks were organic homogenized 0.5% fat milk produced by Arla Foods A/S. The fortified milk had a vitamin D_3 (cholecalciferol; Kemikalia) concentration ~0.38 μg/100 mL, which was confirmed by analysis in triplicate during the study period (0.40 ± 0.01 μg/100 mL). The placebo milk did not contain added vitamin D (<0.004 μg/100 mL). Study breads included white bread, brown bread, rye bread, and buns that were baked from wheat- and rye-flour blends produced by Lantmännen Cerealia A/S. Vitamin D_3 (cholecalciferol; DSM Nutritional Products) was added to flour blends to bake fortified breads. Before and after the study period, samples from 3 different flour blends of both types were analyzed, and the added vitamin D was recovered. Twice during the study period, breads of each type were collected on 3 consecutive baking days and analyzed in pools of 4 breads. We aimed to produce fortified breads with 6 μg vitamin D_3/100 g bread, but the analyses showed a concentration of 5.2 ± 0.3 μg vitamin D_3/100 g in the wheat bread and 4.3 ± 0.3 μg vitamin D_3/100 g in the rye bread. The placebo bread did not contain added vitamin D. Concentrations of vitamin D in study milks and breads were analyzed at the National Food Institute, Technical University of Denmark, by using a reverse-phase HPLC method described elsewhere (36). Analyses were accredited according to the standard ISO 17025 (37).

Dietary assessment and compliance

At baseline (month 0), participants completed a self-administered Web-based questionnaire that assessed their background, health, and lifestyle and a semi-quantitative food-frequency questionnaire

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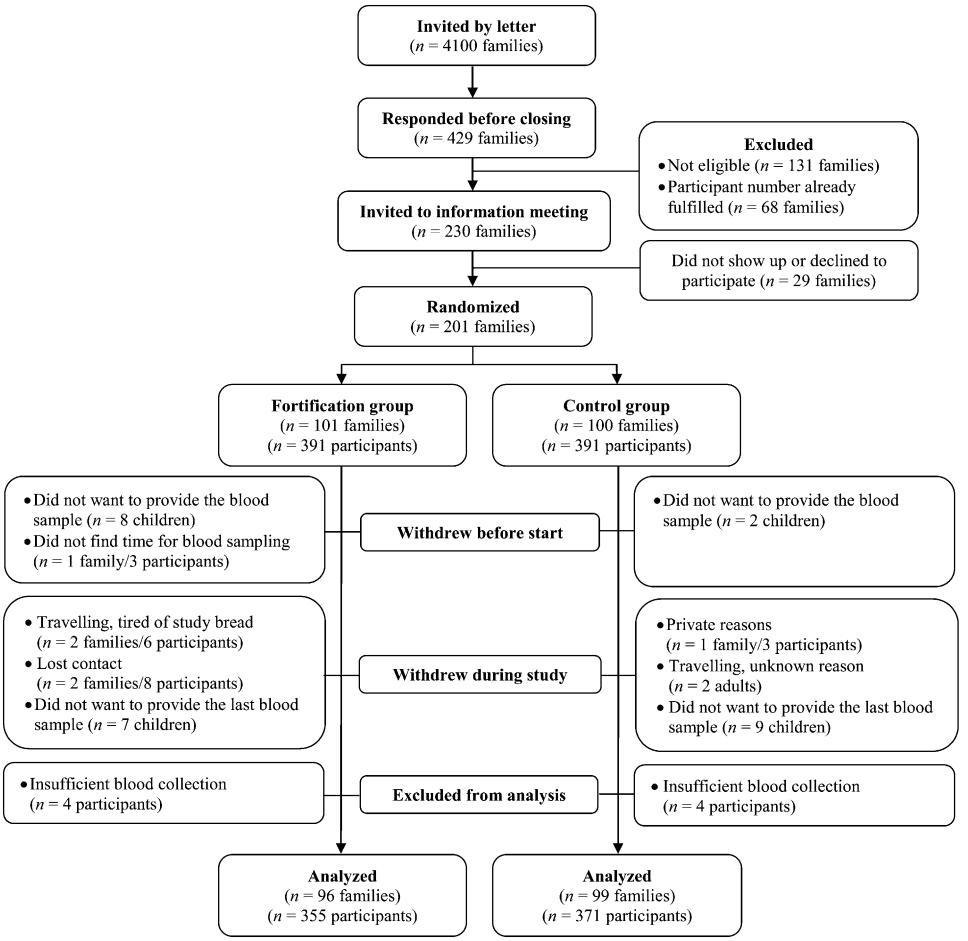

FIGURE 1. Flow of participants throughout the study.

(FFQ) that assessed their vitamin D and calcium intakes before the study. The FFQ was adapted from an FFQ used in the European Union project Towards a strategy for Optimal Vitamin D Fortification (38). By the end of the study (month 6), questionnaires were repeated to assess eventual changes in health or lifestyle and vitamin D and calcium intakes during the study. The 2 FFQs were also used to evaluate if participants changed their consumption of milk and bread during the study.

Vitamin D and calcium intakes from the habitual diet were calculated on the basis of consumption frequencies and vitamin D and calcium concentrations in food items given in the Danish Food Composition Databank (39). Vitamin D contributions from the fortified milk and bread were calculated on the basis of frequencies of milk and bread consumptions and measured vitamin D concentrations in the study milk and bread.

Compliance to the intervention was registered 4 times during the study. In a short self-administered Web-based questionnaire, participants reported their consumption of milk and bread other than the products provided in the study. Compliance was estimated by dividing the number of portions of milk or bread consumed per day other than the products provided in the study by the total number of portions of milk or bread consumed per day as reported in the FFQ.

Examination and blood collection

Participants were examined, and blood samples were collected, in an authorized laboratory (Copenhagen's General Practitioners Laboratory). Weight was measured to the nearest 0.1 kg in normal clothes without shoes (1 kg was withdrawn from the measured weight) by using a body-composition analyzer (Tanita BC-418; Tanita Europe BV). Height was measured to the nearest centimeter without shoes by using an ultrasonic height measure (Soehnle 5001; Soehnle Professional GmbH & Co). BMI (in kg/m^2) was calculated on the basis of measured weight and height, and participants were categorized into normal weight, overweight, and obese classes according to standards for children (40) and the WHO International standard for adults (41). Local anesthetic patches were offered to the children before blood sampling. A trained biomedical laboratory technician drew nonfasting venous blood samples from participants. Blood samples were held at room temperature for 30 min before centrifugation ($1800 \times g$ for 10 min), and serum and plasma were collected and stored at $-80°C$ until analysis.

Biochemical analyses

All blood samples were analyzed as single determinations in a random order at the Clinical Biochemical Department, Holbæk Hospital, Holbæk, Denmark, after completion of the study. Measurements of serum 25(OH)D relied on the determination of both $25(OH)D_2$ and $25(OH)D_3$ and were conducted by isotope-dilution liquid chromatography–tandem mass spectrometry by using principles described elsewhere (42). The standard reference material vitamin D in human serum (SRM 972) from the

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National Institute of Standards and Technology (43) was used as the primary calibrator. The analytic quality of the 25(OH)D assay was assured by Vitamin D External Quality Assessment Scheme certification. In this validation scheme, the mean bias for our method compared with the mean of the Vitamin D External Quality Assessment Scheme liquid chromatography–mass spectrometry group during the period of the present analyses was −3.2%. Interassay CVs for our method were 2.2% and 2.8% at 30 and 180 nmol/L, respectively, for 25(OH)D_3 and 7.6% and 4.6% at 43 and 150 nmol/L, respectively, for 25(OH)D_2. Serum total calcium (CV: 1.2%) was measured by using a chemistry analyzer Cobas c501 (Roche Diagnostics), and plasma PTH (CV: 3.4%) was measured by using an immunology analyzer Cobas e601 (Roche Diagnostics) following standard procedures from the manufacturer.

Statistical analyses

Statistical analyses were conducted with participants who completed the study and from whom baseline and final biochemical data were obtained (Figure 1). Data were analyzed with SPSS statistical software (version 20.0; IBM SPSS Inc), and statistical significance was determined at $P < 0.05$. Intakes are presented as medians (IQRs) and biochemical measures as geometric means (IQRs) unless otherwise stated. Linear mixed models with the family as a random factor were applied in the following analyses to account for the nonindependency of the participants. All continuous variables were log transformed before analysis to meet model requirements. Eventual changes in milk and bread consumptions during the study period were evaluated by testing the difference between the 2 registered consumptions (months 0 and 6) against the null hypothesis. Likewise, in children and adults separately, differences in biochemical measures [25(OH)D, PTH, and total calcium) between months 0, 3, and 6 within treatment groups were tested against the null hypothesis to determine eventual changes. Linear trend analyses for serum 25(OH)D in adults were applied. Treatment groups (children and adults separately) were compared for biochemical measures at the different time points in mixed models with the group as a categorical variable and serum 25(OH)D as the dependent variable. Treatment effects on biochemical endpoints were tested in mixed models adjusted for age and sex (categorical variables) and for baseline values of the endpoint (covariate). A second model with the primary endpoint 25(OH)D was further adjusted for BMI, multivitamin usage, and sun vacation to evaluate the potential influence on the treatment effect. Interactions between the group and baseline, group and sex, and group and age were assessed in the model with 25(OH)D as the endpoint. Binary logistic regression by using the generalized estimating equation (to allow for dependence within families) was applied to compare treatment groups in their prevalence of serum 25(OH)D concentrations <30 and <50 nmol/L at baseline (month 0) and at the end of the study (month 6).

RESULTS

Characteristics and intakes of participants

Of a total of 201 families (782 participants), 6 families (20 participants) and 28 single participants did not complete the study for various reasons (Figure 1). An additional 8 participants were excluded from the final analysis because of insufficient blood collection. Baseline characteristics and intakes are shown in **Table 1**.

Neither milk nor the bread consumption changed during the study (data not shown; $P = 0.551$ for milk and $P = 0.580$ for bread). Daily median (IQR) milk consumptions were 532 mL (262, 712 mL) in children and 266 mL (139, 563 mL) in the adults. Daily bread consumptions were 117 g (80, 164 g) in children and 101 g (63, 157 g) in adults. Because children consumed more milk and bread than did adults, fortified products provided median (IQR) vitamin D intakes of 8.0 μg/d (5.4, 10.0 μg/d) in children and 5.8 μg/d (3.5, 8.5 μg/d) in adults in the fortification group, with an average of 6.8 μg/d (4.2, 9.5 μg/d). Total median (IQR) vitamin D intakes (habitual diet plus fortified products) were 9.2 μg/d (7.0, 12.3 μg/d) in the fortification group and 2.2 μg/d (1.5, 3.0 μg/d) in the control group (with the contribution from dietary supplements not included).

In the fortification group, 78% of children and 56% of adults (average for children and adults: 66%) reached the vitamin D–intake goal of 7.5 μg/d. By comparison, only 2% in the control group had a vitamin D intake ≥7.5 μg/d. None of the participants exceeded the tolerable upper intake level.

Approximately 90% of participant total intake of milk and bread was the study milk and bread (Table 1). Primary reasons for participants drinking or eating other foods than study products were children having school milk or eating in the day care center and adults eating in the canteen at work.

Biochemical measures

The overall geometric mean (IQR) serum 25(OH)D concentration changed from 73.1 nmol/L (61.9, 88.5 nmol/L) to 67.6 nmol/L (56.2, 79.4 nmol/L) (difference: −Δ5.5 nmol/L) for all participants in the fortification group and from 71.1 nmol/L (61.2, 85.9 nmol/L) to 41.7 nmol/L (29.5, 58.9 nmol/L) (−Δ29.4 nmol/L) in the control group. The final serum 25(OH)D concentration was higher in the fortification group than in the control group in both children and adults (both $P < 0.001$) (**Table 2**). The combined analysis of children and adults with the primary linear mixed model, which was adjusted for baseline 25(OH)D, sex, and age, showed a treatment effect on the serum 25(OH)D concentration ($P < 0.001$) so that the final 25(OH)D was estimated to be 59% higher in the fortification group than in the control group. Additional adjustment of the model for BMI, multivitamin usage, and sun vacation did not change the magnitude of the effect of treatment (**Table 3**). No interaction effects were observed between group and baseline serum 25(OH)D ($P = 0.080$), group and age ($P = 0.082$), or group and sex ($P = 0.317$).

In the adult fortification group, 25(OH)D decreased from months 0 to 3 ($P < 0.001$) and increased from months 3 to 6 ($P = 0.014$); nevertheless, the final concentration was lower than the baseline concentration ($P < 0.001$). In the adult control group, 25(OH)D decreased from months 0 to 3 ($P < 0.001$) and continued decreasing from months 3 to 6 ($P = 0.001$). There was a linear trend for the serum 25(OH)D concentration in adults in both treatment groups (both P-trend < 0.001). In children, 25(OH)D decreased from months 0 to 6 in both the fortification and control groups (both $P < 0.001$) (Table 2).

At baseline, <1% of participants had a 25(OH)D concentration <30 nmol/L, and 8–9% of participants had a 25(OH)D

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TABLE 1
Characteristics and intakes in children and adults by treatment group

	Children aged 4–17 y		Adults aged 18–60 y	
	Fortification group (n = 154)	Control group (n = 167)	Fortification group (n = 201)	Control group (n = 204)
Sex [n (%)]				
F	79 (51.3)	89 (53.3)	100 (49.8)	104 (51.0)
M	75 (48.7)	78 (46.7)	101 (50.2)	100 (49.0)
Age [n (%)]				
4–10 y	80 (51.9)	83 (49.7)	—	—
11–17 y	74 (48.1)	84 (50.3)	—	—
18–40 y	—	—	106 (52.7)	86 (42.2)
41–60 y	—	—	95 (47.3)	118 (57.8)
BMI [n (%)][1]				
Normal weight	144 (93.5)	154 (92.2)	114 (56.7)	108 (52.9)
Overweight	9 (5.8)	13 (7.8)	66 (32.8)	67 (32.8)
Obese	1 (0.6)	0 (0.0)	21 (10.4)	29 (14.2)
Food consumption[2]				
Milk (mL/d)	583 (357, 762)[3]	516 (212, 703)	289 (139, 550)	254 (139, 582)
Compliance (%)[4]	84	89	91	96
Bread (g/d)	124 (89, 240)	106 (72, 157)	92 (58, 151)	107 (67, 170)
Compliance (%)[4]	93	94	91	96
Vitamin D intakes (μg/d)[2]				
Habitual diet	2.3 (1.7, 3.3)	2.2 (1.5, 2.9)	2.4 (1.6, 3.4)	2.2 (1.4, 3.1)
Fortified milk	2.3 (1.4, 3.1)	0	1.2 (0.6, 2.2)	0
Fortified bread	5.6 (3.5, 7.5)	0	4.2 (2.3, 6.6)	0
Total	10.2 (7.8, 13.1)	2.2 (1.5, 2.9)	8.1 (6.1, 11.5)	2.2 (1.4, 3.1)
≥7.5 μg/d [n (%)]	114 (78.1)	1 (0.6)	103 (55.7)	7 (3.6)
Calcium intake (mg/d)[2]	979 (628, 1261)	921 (554, 1136)	695 (454, 979)	680 (437, 1035)
Supplement users [n (%)][5]				
Multivitamins	58 (39.2)	64 (39.3)	47 (25.1)	45 (23.1)
Vitamin D	3 (2.0)	5 (3.1)	3 (1.6)	11 (5.6)
Sun vacation [n (%)][5]	43 (29.1)	32 (19.6)	57 (30.5)	38 (19.5)

[1] BMI categorization based on standards for children (40) and the WHO International standard for adults (41).
[2] Intakes during the study. Sample sizes: n = 146 in the children fortification group and n = 162 in the control group and n = 185 in the adult fortification group and n = 195 in the control group.
[3] Median; IQR in parentheses (all such values).
[4] Percentage of total milk and bread consumptions that come from study products. Sample sizes: for milk compliance, n = 130 in the children fortification group and n = 156 in the control group and n = 166 in the adult fortification group and n = 183 in the control group; for bread compliance: n = 140 in the children fortification group and n = 156 in the control group and n = 179 in the adult fortification group and n = 191 in the control group.
[5] Supplement usage and vacation in places where dermal vitamin D production was expected during the study. Sample sizes: n = 148 in the children fortification group and n = 163 in the control group and n = 187 in the adult fortification group and n = 195 in the control group.

concentration <50 nmol/L with no differences between treatment groups in their prevalence (P = 0.158 for <30 nmol/L and P = 0.814 for <50 nmol/L). By the end of the study, <1% of subjects in the fortification group compared with 25% of subjects in the control group had 25(OH)D concentrations <30 nmol/L ($P < 0.001$), and 16% of subjects in the fortification group compared with 65% of subjects in the control group had 25(OH)D concentrations <50 nmol/L ($P < 0.001$) (**Figure 2**).

PTH and total calcium did not change during the study in the fortification group (PTH: P = 0.099 for children and P = 0.156 for adults; calcium: P = 0.799 for children and P = 0.321 for adults). By contrast, PTH increased and total calcium decreased in the control group (PTH: $P < 0.001$ for both children and adults; calcium: P = 0.041 for children and P = 0.001 for adults) (Table 2).

The final PTH concentration was lower in the fortification group than in the control group in both children and adults, whereas there was no difference in final total calcium concentrations between treatment groups (Table 2). The combined analysis of children and adults in linear mixed models adjusted for baseline values of the endpoint, sex, and age showed a treatment effect on PTH ($P < 0.001$) so that the final PTH was estimated to be 9% lower in the fortification group than in the control group. There was no treatment effect on total calcium (P = 0.237).

DISCUSSION

The fortification strategy in the current study resulted in a higher serum 25(OH)D concentration in the fortification group (67.6 nmol/L) than in the control group (41.7 nmol/L) at the end of the winter season, despite the fact that 25(OH)D decreased in both treatment groups during the study ($-\Delta$5.5 nmol/L in the fortification group and $-\Delta$29.4 nmol/L in the control group). The planned fortification strategy was to increase the vitamin D intake to the amount of 7.5 μg/d recommended in the NNRs (34). This strategy succeeded in 78% of children and 56% of the adults in the fortification group. As a result of the increased intake, 84% of subjects in the fortification group had

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TABLE 2
Biochemical measures in children and adults by treatment group at months 0, 3, and 6[1]

	Children aged 4–17 y			Adults aged 18–60 y		
	Fortification group (n = 154)	Control group (n = 167)	P	Fortification group (n = 201)	Control group (n = 204)	P
25(OH)D (nmol/L)						
Month 0	72.8 (64.0, 88.9)	72.8 (61.8, 83.9)	0.524	73.3 (61.9, 88.3)	70.0 (60.0, 86.7)	0.286
Month 3[2]	—	—	—	63.1 (52.5, 77.6)**	44.7 (32.4, 63.1)**	<0.001
Month 6	67.6 (56.2, 79.4)**	42.7 (30.9, 58.9)**	<0.001	66.1 (56.2, 79.4)**,†	40.7 (28.8, 60.3)**,††	<0.001
P-trend	—	—		<0.001	<0.001	
PTH (ng/L)						
Month 0	35.0 (28.8, 41.5)	35.3 (27.6, 43.3)	0.969	35.5 (27.8, 44.2)	34.2 (26.9, 41.4)	0.268
Month 6	36.3 (27.5, 47.9)	41.7 (32.4, 52.5)**	0.028	36.3 (29.5, 45.7)	38.9 (30.9, 46.8)**	0.049
Total calcium (mmol/L)						
Month 0	2.45 (2.40, 2.49)	2.47 (2.42, 2.51)	0.035	2.43 (2.37, 2.47)	2.43 (2.37, 2.49)	0.268
Month 6	2.45 (2.40, 2.50)	2.45 (2.40, 2.50)*	0.428	2.42 (2.37, 2.46)	2.41 (2.36, 2.47)**	0.423

[1] All values are geometric means; IQRs in parentheses. P values are for the comparison of treatment groups in biochemical measures at each time point with linear mixed models with family as a random factor, group as a categorical variable, and the logarithm of the biochemical measure as the dependent variable. Significant change within treatment groups analyzed in mixed models with family as a random factor and the difference between the logarithm of the biochemical measure at time points of interest as the dependent variable: from month 0, $^{*}P < 0.05$, $^{**}P \leq 0.001$; from month 3, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P \leq 0.001$. P-linear trend values in 25(OH)D are given for adults. PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D.

[2] Sample sizes: n = 186 in the adult fortification group and n = 195 in the control group.

a serum 25(OH)D concentration >50 nmol/L by the end of the study.

The recently updated American Recommended Dietary Allowance for vitamin D was set at 15 μg/d by the Institute of Medicine (11). This value was estimated to ensure a target serum 25(OH)D concentration of 50 nmol/L under conditions of minimal exposure to the sun, such as under the circumstances in the current study. The target value of 50 nmol/L was considered to cover the requirement of the majority of the population in relation to the optimal calcium absorption and related bone health.

TABLE 3
Effects of different variables on serum 25(OH)D concentrations at month 6[1]

	Model 1 (n = 726)		Model 2 (n = 693)	
Variable	Interpreted estimate (95% CI)[2]	P	Interpreted estimate (95% CI)[2]	P
Treatment		<0.001		<0.001
Fortification group	1.59 (1.49, 1.69)		1.58 (1.49, 1.67)	
Control group	1		1	
Baseline 25(OH)D	5.73 (4.84, 6.77)[3]	<0.001	5.43 (4.60, 6.40)[3]	<0.001
Sex		0.045		0.033
F	1.04 (1.00, 1.07)		1.04 (1.00, 1.08)	
M	1		1	
Age		<0.001		0.011
41–60 y	0.93 (0.88, 0.99)	0.013	0.97 (0.92, 1.03)	0.367
18–40 y	0.91 (0.87, 0.96)	0.001	0.95 (0.90, 1.00)	0.057
11–17 y	0.88 (0.83, 0.94)	<0.001	0.91 (0.86, 0.97)	0.003
4–10 y	1		1	
BMI				0.782
Normal weight	—		0.97 (0.90, 1.05)	0.484
Overweight	—		0.98 (0.90, 1.06)	0.579
Obese	—		1	
Sun vacation				<0.001
Yes	—		1.16 (1.10, 1.23)	
No	—		1	
Multivitamin use				<0.001
Yes	—		1.18 (1.12, 1.24)	
No	—		1	

[1] Analyzed in linear mixed models with family as a random variable, variables as categorical variables, and the logarithm of the serum 25(OH)D concentration as the dependent variable. 25(OH)D, 25-hydroxyvitamin D.

[2] Regression coefficients have been transformed exponentially (10^{β}) so that values represent ratio estimates and corresponding confidence limits.

[3] By a 10-fold increase in baseline 25(OH)D.

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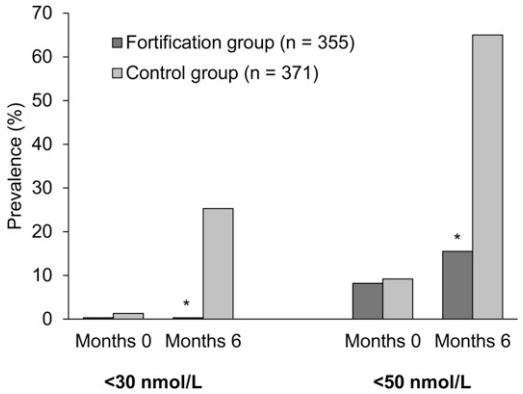

FIGURE 2. Prevalence of participants with serum 25-hydroxyvitamin D concentrations <30 and <50 nmol/L at months 0 and 6 by treatment group. *Significant difference between treatment groups as analyzed by using binary logistic regression with a generalized estimating equation, $P < 0.001$. Cutoffs defined the value below which adverse effects on bone health may be expected and the value that would meet the requirement for optimal bone health of the majority of the population according to the use in the American dietary reference values for vitamin D (11).

A meta-regression analysis with supplemental vitamin D estimated that 9–12 μg/d would be required for the general population to maintain 25(OH)D concentrations >50 nmol/L (44). Our results suggested that ~9 μg vitamin D/d would be sufficient to ensure 25(OH)D concentrations >50 nmol/L in the majority of 4–60-y-olds under conditions with usual habits during a winter in Denmark including multivitamin supplement use and sun vacation.

In the current study, the fortified milk and bread provided a median vitamin D intake ~7 μg/d. The difference between the treatment groups in their absolute changes in the geometric mean 25(OH)D concentration was ~24 nmol/L. This amount corresponded to a 3.4-nmol/L difference for each microgram of vitamin D ingested from fortified foods. A recent meta-analysis of the efficacy of vitamin D food fortification in adults showed an average treatment effect of 1.2 nmol $\cdot$ L^{-1} $\cdot$ μg^{-1} (15). This value is considerably lower than our result. It is to be noted that a higher percentage of our participants compared with those in the meta-analysis studies reported multivitamin supplement use and sun vacation during the study. Furthermore, our study had a relatively high mean baseline 25(OH)D and a relatively low fortification amount, and the beneficial effect was expressed as a reduction in the seasonal decline in 25(OH)D compared with in the control group. Our observations were similar to those of 2 other RCTs in 17–54-y-old men and women with baseline 25(OH)D and fortification amounts comparable to those in our study (18, 21). These studies showed treatment effects of ~2 nmol $\cdot$ L^{-1} $\cdot$ μg^{-1}. Most studies had a lower initial starting point than that of our study and observed an increase in 25(OH)D after supplementation with vitamin D–fortified foods (16, 17, 19, 20, 22, 23, 25, 26, 28–31). An important conclusion from the meta-analysis (15) and a systematic review by the NIH (45) was the large heterogeneity between fortification studies. The studies varied in design, study population, baseline 25(OH)D, food-vehicle used, and fortification amounts. Therefore, the treatment effects could not be directly compared and should be interpreted with caution.

To our knowledge, a novelty in the design of the current study was the combined use of 2 food vehicles consumed during the day according to the participants' usual habits. This design was different from that in other randomized studies that used the effects of single vitamin D–fortified foods consumed in a predefined serving or amount. It has been shown that the efficiency of vitamin D in fortified foods to improve serum 25(OH)D concentrations is equal to that of dietary supplements (22, 28, 29, 31), and daily dosing with supplements was shown to be more efficient than seasonal dosing in maintaining 25(OH)D (22). Therefore, we consider that the larger treatment effect observed in the current study compared with in other fortification studies suggested that the response to vitamin D fortification in circulating 25(OH)D also depends on the daily frequency of ingestion.

One of the strengths of this study was the family and real-life based design, which we believe was one of the main reasons for the low dropout rate and high compliance. Informal feedback from participants indicated that they made their participation a joint family project, which they could discuss at dinner times, and they encouraged and reminded one another to consume the study products provided. It is important to consider this compliance aspect in the design of future studies. The family-based design also ensured a broad spread of age and sex in both children and adults. Another strength was the use of a specific analytic method to measure serum 25(OH)D concentrations.

An important finding was that the analyses of bread did not fully recover all of the added vitamin D. We suspect that some of the vitamin D was lost during baking. This loss was unexpected because, in a previous fortification study, the added vitamin D was recovered in both dough and baked wheat and rye breads (31). This issue should be further investigated.

A minor drawback of the study was that the study population may not have been fully representative of the general population in Denmark. It is possible that the response to the fortification would have been different in other population groups such as elderly or dark-skinned groups. This possibility should be investigated in the future. However, if implementation of the fortification strategy was mandatory, the use of fortified products would not depend on the consumer's deliberate choice, which could be influenced by factors such as age, sex, education, social status, and ethnic affiliation.

This study showed that it is feasible to conduct RCTs in real-life settings without compromising the treatment effect. Furthermore, we showed the use of model data from usual food-consumption intakes to establish an effective fortification strategy targeted at the intended population. The combination of fortified milk and bread proved suitable for 4–60-y-old Danes, partly because Danish children and, to some extent, adults drink more milk than do other population groups. Milk is not widely consumed in all populations, whereas bread is a primary food in many countries. Therefore, this study suggested that including bread in the current selection of vitamin D–fortified foods could be an effective way to attenuate seasonal fluctuations in vitamin D status.

In conclusion, vitamin D fortification of milk and bread, to an amount that increased the intake to recommended amount, reduced the decrease in serum 25(OH)D concentrations during winter and ensured 25(OH)D concentrations >50 nmol/L in children and adults in Denmark.

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The authors' responsibilities were as follows—IT, RA, HM, KHM, LBR, and CM: designed the study; HM, KHM, LBR, and RA: conducted the study; JJ and PJB: were responsible for laboratory analyses; EWA, KHM, LBR, and RA: analyzed data; KHM: wrote the first draft of the manuscript; and all authors: critically reviewed the manuscript and approved the final manuscript. None of the authors reported a conflict of interest.

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Paper 2:

Vitamin D status and its determinants in children and adults among families in late summer in Denmark.

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Vitamin D status and its determinants in children and adults among families in late summer in Denmark

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Short title

Vitamin D status in families

Keywords

Serum 25(OH)D, determinants, family

Abbreviations

25(OH)D, 25-hydroxyvitamin D; ALTM, all laboratory trimmed mean; DEQAS, vitamin D External Quality Assessment Scheme; EAR, estimated average requirement; FFQ, Food Frequency Questionnaire; LC-MS, liquid chromatography mass spectrometry; NIST, National Institute of

Standards and Technology; NNR, Nordic Nutrition Recommendations; PTH, parathyroid hormone; RDA, recommended daily allowance.

Abstract

The importance of vitamin D in bone health is recognized and low concentrations have been associated with increased risk of disease. The impact of the family relation on vitamin D status has not been presented previously. The present cross-sectional study assessed serum 25-hydroxyvitamin D (25(OH)D) concentration and its determinants in children and adults among families in late summer in Denmark (56°N). Data from 755 apparently healthy children (4-17 years) and adults (18-60 years) recruited as families (*n* 200) in the VitmaD study were analysed. Blood samples were collected in September-October, and serum 25(OH)D was measured by LC-MS/MS. Information on potential determinants was obtained by questionnaires. Geometric mean (interquartile range) serum 25(OH)D concentration was 72·1 (61·5-86·7) nmol/l (range 9-162 nmol/l) with 9 % having 25(OH)D <50 nmol/L. The intra-family correlation was 0.27 in all subjects, 0.24 in adults and 0.42 in children. Serum 25(OH)D was negatively associated with BMI (P<0·001) and positively associated with dietary vitamin D (P=0·008), multivitamin use (P=0·019), solarium use (P=0·006), outdoor stay (P=0·001), sun preference (P=0·002) and sun vacation (P<0·001) but not with lifestyle factors in the adults when these were assessed together with the other determinants. In conclusion, the majority of children and adults among families had serum 25(OH)D concentrations >50 nmol/L in late summer in Denmark. Both dietary and sun related factors were determinants of vitamin D status, and the familial component was stronger for the children than for the adults.

Introduction

The importance of vitamin D in bone health is recognized with rickets in children and osteomalacia and osteoporosis in adults being the traditional clinical conditions linked with vitamin D deficiency [1, 2]. Furthermore, the expression of vitamin D receptors in different tissues [3] suggests additional biological functions of the vitamin. Low vitamin D status has been associated with a range of health outcomes [4, 5] including an increased risk of cardiometabolic disorders [6], some cancers [7], autoimmune diseases [8] and mortality [9]. However, it is still unclear if these associations are causal. Studies have found vitamin D status to be associated with socio-demographic and lifestyle related factors [10, 11], thus, it may be that vitamin D status serves as an indicator of general health and/or lifestyle.

The accepted biomarker of vitamin D status is 25-hydroxyvitamin D (25(OH)D) concentration in the blood [12]. In Denmark the following values are used to define deficient, insufficient and sufficient serum 25(OH)D concentration: <25, 25-50, >50 nmol/l [13]. The US Dietary Committee has defined 30 nmol/l as the limit beyond which adverse effects on bone might occur [14]. A serum 25(OH)D concentration of 40 and 50 nmol/L was used for the Estimated Average Requirement (EAR) and the Recommended Daily Allowance (RDA), which are assumed to meet the requirement in the average and the majority of the population for bone health. A threshold of >125 nmol/l was considered at-risk for harm by the US Dietary Committee.

The use of cut-off values and the comparison of vitamin D status between studies is complicated by variations in measurements between methods as well as among laboratories using the same 25(OH)D assay [15-17]. In 2010 the National Institute of Standards and Technology (NIST) introduced a standard reference material for measurements of vitamin D in human serum [18] which is expected to improve the analytical performance of 25(OH)D measurements and to facilitate harmonization across 25(OH)D assays [15].

Thus, there is a need for comparative data on vitamin D status yield by standardised and calibrated methods to better compare vitamin D status between population groups and evaluate the current situation of vitamin D deficiency. Vitamin D status has been measured in different population groups in Denmark [10, 19-23]. None of the previous studies assessed a broad span in age and gender in both children and adults. Especially there is a lack of information on vitamin D status in young boys. One of the studies presented vitamin D status in men, women and girls from Danish

immigrant families [23]. However, to our knowledge, no previous studies have quantified the impact of the family relation for vitamin D status. This knowledge on vitamin D status within families will be helpful when considering strategies to improve vitamin D status.

The objective of the present study was to assess serum 25(OH)D concentration and its determinants in children and adults among families in late summer in Denmark.

Subjects and methods

Study population

The present cross-sectional study used baseline data from the VitmaD study [24] conducted in Denmark (56°N). Children and adults were recruited as families randomly drawn from the Danish Civil Registration System. Inclusion criteria were age between 4 and 60 years and a permanent address in Gladsaxe municipality. Exclusion criteria were pregnancy and disease or use of medication influencing vitamin D metabolism (including dietary supplements with vitamin D levels >10 µg/d for children and >5 µg/d for adults, which corresponds to the typical levels in multivitamin supplements in Denmark). Of the 782 recruited children and adults. 755 (representing 200 families) had serum 25(OH)D concentration measured and complete questionnaire data at baseline. The present analyses were conducted with these subjects. Written informed consent was obtained from all adult subjects and from the guardians of the children. The study was conducted according to the guidelines in the Declaration of Helsinki and the protocol was approved by the Research Ethics Committee of the Capital Region of Denmark (record no. H-4-2010-020) and registered in ClinicalTrials.gov (NCT01184716).

Methods

The subjects were examined and blood samples were collected in September-October 2010 in an authorized laboratory (Copenhagen´s General Practitioners Laboratory, Søborg, Denmark). Weight was measured to the nearest 0·1 kg in normal clothes without shoes (1 kg was withdrawn from the measured weight) with a body composition analyzer (Tanita BC-418, Tanita Europe B. V., Hoofddorp, The Netherlands). Height was measured to the nearest centimeter without shoes with an ultrasonic height measure (Soehnle 5001, Soehnle Professional GmbH & Co, Backnang, Germany). BMI was calculated based on the measured weight and height, and subjects were categorized into normal weight, overweight and obese classes according to standards for children [25] and the WHO International standard for adults [26]. Non-fasting venous blood samples were drawn from the subjects, and serum and plasma were collected and stored at -80 ˚C until analysis.

Serum 25(OH)D concentration was measured at the Clinical Biochemical Department, Holbæk Hospital, Denmark by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) using the principles described elsewhere [27]. The method was calibrated against the NIST standard for analysis of vitamin D in human serum (SRM 972) [16], and the interassay CVs for our method were 2.2 % and 2.8 % at 30 and 180 nmol/L, respectively, for 25(OH)D_3 and 7.6 % and 4.6

% at 43 and 150 nmol/L, respectively, for 25(OH)D_2. The analytical quality of our method was assured by participation in the Vitamin D External Quality Assessment Scheme (DEQAS). In this validation scheme, the mean bias for our method compared with the mean of the DEQAS LC-MS group during the period of the present analyses was -3·2 %. Plasma parathyroid hormone (PTH) concentration was measured by using an immunology analyser (Cobas e601, Roche Diagnostics, Mannheim, Germany), following standard procedures from the manufacturer (CV = 3·4 %).

Information on background, health, sun exposure and lifestyle, including use of multivitamin and vitamin D supplements of the subjects, was obtained in detailed self-administered web-based questionnaires. Dietary vitamin D intakes were registered in a semi-quantitative food frequency questionnaire (FFQ) adapted from a FFQ used in the EU project OPTIFORD [23]. The vitamin D intakes were calculated based on the reported consumption frequencies and the vitamin D concentrations in the food items given in the Danish Food Composition Databank [28].

Statistics

Data were analysed with the SPSS statistical software (version 20.0, IBM SPSS Inc., Armonk, NY) and statistical significance was evaluated at a level of $P<0·05$ (two-sided). Linear mixed models with family as a random variable were applied in all analyses to account for the non-independency of the subjects. Before analysis serum 25(OH)D and PTH concentrations were logarithmically transformed to meet the model requirements. Trend analyses were performed to test for linear relations between PTH concentrations and 25(OH)D groups. Univariate models were used to assess the association between serum 25(OH)D and each of the following sun related variables: outdoor transport to school/work (<15, 15-30, 31-60, >60 min/d), solarium use at least once a week (yes, no), sun preference (prefer sun, sometimes in sun, avoid sun), outdoor stay in light clothes (most of the time, often, sometimes, seldom/never), sunscreen use (always, most of the time, sometimes, seldom/never), and sun vacation the preceding summer June-September (yes, no). Sun related variables with $P<0·05$ significance in their univariate model were included in a multiple analysis (linear mixed model) together with the following categorical variables: age (4-10, 11-17, 18-40, 41-60 years), gender (female, male), BMI (normal weight, overweight, obese), dietary vitamin D (quartiles: <1·7, 1·7-2·4, 2·5-3·3, >3·3 µg/d), and multivitamin use (yes, no). The interaction between age and gender was tested. This multiple analysis was performed in all subjects and in children and adults separately. The strength of the familial component was considered by calculating the intra-class correlation for each model: $\rho = \omega^2/(\omega^2+ \sigma^2)$, where ρ is the intra-family

correlation, σ is the within family standard deviation, and ω the between family standard deviation. The lower the variation within classes the higher the intra-class correlation which in this case means that the closer the correlation is to 1, the more alike are the subjects within a family with respect to their vitamin D status.

The relation between serum 25(OH)D and each of the following lifestyle related variables was explored in univariate models in adults only: smoking status (current, former, never), alcohol consumption (never, <1 time/month, 1-3 times/month, 1 time/week, 2-4 times/week, 5-6 times/week, daily), leisure-time physical activity (mainly sedentary, light to moderate activity, regular sport and exercise, athletic training), self-rated physical shape (really good, good, fairly good, bad, really bad), self-rated health (excellent, really good, good, less good/bad), effort to eat healthily (very often, often, sometimes, seldom/never), education after state and/or upper secondary school (none or technical education, higher education <3 years, higher education 3-4 years, higher education >4 years). Lifestyle related variables with $P<0{\cdot}05$ significance in their univariate model were included in the multiple model described above.

Results

Characteristics of the subjects are given in Table 1 and Table 2. The median (interquartile range) ages of the youngest children (4-10 y), the oldest children (11-17 y), the youngest adults (18-40 years) and the oldest adults (41-60 years) were 7 (6-9), 13 (12-15), 37 (33-39) and 45 (43-48) years. The genders were evenly distributed in both children and adults, and the majority of the subjects were of normal weight (Table 1). The median dietary vitamin D intakes were similar across age groups (range 2·3-2·6 µg/d). In the multivitamin users (41 % of the children and 25 % of the adults) the total median (interquartile range) vitamin D intake was 6·7 (4·5, 10·2) µg/d. Approximately half of the adults had a medium-long to long higher education, and their lifestyles were generally healthy (Table 2).

The individual serum 25(OH)D concentrations (sum of 25(OH)D_2 and 25(OH)D_3) ranged from 9·3 to 161·9 nmol/l with an overall geometric mean (interquartile range) of 72·1 (61·5-86·7) nmol/l. Serum 25(OH)D_2 was found in 11 % of the samples in the range 3-29 nmol/L. Serum 25(OH)D concentration in the different age and gender groups and the distribution across its ranges is seen in Table 3. The overall prevalence of serum 25(OH)D <30, <40 and <50 nmol/l was 1, 2 and 9 % with no children being <30 nmol/l. In the adults, geometric mean PTH concentration (95 % CI) for the 25(OH)D groups of <25, 25-49, 50-75 and >75 nmol/l was 59·8 (43.2-82.8), 39·9 (35·8, 44·3), 36·2 (34·3, 38·2) and 32·7 (31·1, 34·4) ng/l (*P* trend<0·001). The same trend was seen in children aged 4-10 years (*P* trend=0·012), but not in children aged 11-17 years (*P* trend=0·067).

No differences were found in serum 25(OH)D between age (*P*=0·190), gender (*P*=0·332) or age and gender groups (*P*=0·223) in the multiple analysis of all subjects (Table 4). When children were analysed separately serum 25(OH)D was associated with gender (*P*=0·034) so that 25(OH)D was estimated 5 % lower in the girls compared with the boys. In the univariate models outdoor transport to work/school (*P*=0·972) and sun screen use (*P*=0·154) were not associated to 25(OH)D and thus not included in the multiple models. In the multiple analysis of all subjects serum 25(OH)D was negatively associated with BMI (*P*<0·001) and positively associated with dietary vitamin D (*P*=0·008), multivitamin use (*P*=0·019), solarium use (*P*=0·006), outdoor stay in light clothes (*P*=0·001), sun preference (*P*=0·002) and sun vacation (*P*<0·001) (Table 4). When children and adults were analysed separately serum 25(OH)D was not associated to BMI and sun preference in the children. In the adults the associations remained the same as in the model with all subjects but the significances for dietary vitamin D, multivitamin use and sun vacation were weakened.

From these multivariate models the variations in serum 25(OH)D concentration were higher within than between families with an intra-family correlation of 0·27 in all subjects. The intra-family correlation was higher in the children than in the adults (Table 5).

In the further exploration of the adults serum 25(OH)D concentration was associated to leisure-time physical activity (P<0·001), self-rated physical shape (P=0·001) and self-rated health (P=0·003) in their univariate models. When these variables were included in the multiple analysis together with the vitamin D source related variables none remained significant, although leisure-time physical activity was borderline significant (P=0·054). Serum 25(OH)D was not associated to smoking status (P=0·722), alcohol consumption (P=0.070, effort to eat healthily (P=0·193) or education (P=0·219).

Discussion

The overall geometric mean serum 25(OH)D concentration among the families in the present study was 72·1 nmol/l with no differences between age and gender groups in the analysis of all subjects. The prevalence of 25(OH)D concentrations <25, <50 and <75 nmol/l were 1, 8 and 54 % with no children being <25 nmol/l. A novelty in our study was the quantitatively assessment of the familial component by calculation of the intra-class correlation. The intra-family correlation for all subjects was 0·27, which indicates that a subjects´ vitamin D status was not strongly related to the family relation. However, to our knowledge, no previous studies have quantified the familial component for vitamin D status and thus we do not have a number for comparison of the family relation. The intra-family correlation was almost the double in the children (0·42) as in the adults (0·24), which indicated that the children within a family were more alike than the adults within a family with respect to their vitamin D status. This might be an indication of the influence of genetic factors on vitamin D status or more similar habits in children than in adults from the same family. It is likely that children within a family share activities and dietary/supplementation habits to a larger extent than adults within a family for example outdoor stay, sun protection, multivitamin use and lunch in school.

The serum 25(OH)D concentrations found in the present study were higher and the prevalence of 25(OH)D <50 nmol/l was lower than the values reported in previous studies among similar age and ethnicity groups in Denmark [10, 20-23], other European countries [11, 23, 29-33], USA [34, 35] and Canada [36]. Most of these studies measured vitamin D status across different seasons or in the winter as opposed to the late summer measurement in the present study, however, the prevalence of 25(OH)D <50 nmol/l found in the present study was also lower than the rates found during summer. Most of our participants were of normal weight which could favourable impact the serum 25(OH)D concentration compared with for example the high rate of obesity in the USA [37]. Nevertheless, the studies should be compared with caution as differences may also depend on the laboratory and method used for serum 25(OH)D measurement [17]. In the present study we used the LC-MS/MS method that might be considered the golden standard [38, 39], and our method was standardized and calibrated against the International reference material by NIST [18]. The chromatographic methods are more specific compared with the frequently used immunoassays that are limited in their ability to detect vitamin D_2 [38, 40]. In DEQAS (The Vitamin D External Quality Assessment Scheme) the LC-MS method is positively biased for the All-Laboratory Trimmed Mean (ALTM) whereas the

immunoassays are mostly negatively biased [17]. Several studies have also found the LC-MS method to yield higher results than some other 25(OH)D assays [15, 16, 39, 40, 41]. In a study of the German population standardization to the LC-MS/MS method reduced the prevalence of vitamin D deficiency (<30 nmol/l) from approximately 48 % to 16 % [42]. This might partly explain the higher serum 25(OH)D concentrations found in the present study compared with previous studies in similar population groups.

Similarly to the common finding of an inverse relation between 25(OH)D and PTH concentration [43], we observed a negative trend between PTH and 25(OH)D group in adults and the children aged 4-10 years. In our study relatively few subjects had a low serum 25(OH)D concentration (<25 nmol/l), but the PTH concentration was markedly higher in this group than in the other 25(OH)D groups. Elevated PTH concentrations may result in increased bone resorption in adults whereas the meaning for bone health in children is unclear [44].

For all subjects outdoor stay in light clothes and sun vacation were major determinants for serum 25(OH)D concentration in late summer. In the adults sun preference, solarium use and BMI were also strong determinants. We expected vitamin D status to be related to sun exposure as cutaneous vitamin D synthesis is considered the major source of vitamin D during the summer [45]. It is interesting though that several expressions of the sun exposure were related to serum 25(OH)D concentrations at the same time. Most suggestive may be that vitamin D status was associated with sun vacation (abroad) despite that the hours of sunshine the preceding summer (2010) in Denmark was only slightly below (3 %) the average hours of sunshine for the preceding ten years [46]. To our knowledge, this association between vitamin D status and sun vacation during the summer season has not been shown previously. The dietary vitamin D intake was also associated with serum 25(OH)D concentration in this study despite the median intake (2·5 µg/d) being much lower than the new recommended intake (RI) by the Nordic Nutrition Recommendation. The RI was recently increased from 7·5 µg/d to 10 µg/d for 2-60 year olds [47]. This makes room for an even greater improvement in vitamin D intake, and our finding suggests that dietary vitamin D is also important during summer even in a group of children and adults that frequently stay outside.

Vitamin D status was not associated with age in this study. Some previous studies have shown an association between vitamin D status and age in both children [35] and adults [11], whereas others did not find an association between 25(OH)D and age [10, 31, 32]. The observed higher vitamin D status in boys compared with girls has been reported previously among similar age groups [48]. In our study

this gender difference was not attributable to differences in dietary vitamin D intakes or multivitamin use. An explanation might be the higher level of physical activity in the boys compared to the girls (56 % of the boys compared to 35 % of the girls reported to do sport and physical active play in their leisure-time, results not shown) assuming that these activities were primarily outdoors. Serum 25(OH)D concentration was strongly inversely related to BMI in the adults, whereas there was no association in the children. This might be due to the fact that no children were categorised as obese and only a few were overweight. Another study with healthy weight children with a broad age span did not find an association between 25(OH)D and BMI or fat mass either [35]. The association between 25(OH)D concentration and obesity is a common finding [11, 34, 49] and one explanation could be sequestration of vitamin D in fat tissue [50].

In the adults serum 25(OH)D concentration was not related to lifestyle when assessed together with the influence of vitamin D source related factors except for a borderline relation with leisure-time physical activity. It might be that physical activity acts like a surrogate marker for sun exposure assuming that the activities are mainly outdoors. Previous studies have found an association between 25(OH)D and various lifestyle factors [10, 11]. One study used an overall lifestyle index and found that vitamin D concentrations were substantially higher in those with the healthiest lifestyle compared with the less healthy lifestyle and that this difference was substantially higher than the differences between the single components of the lifestyle index [11]. This suggests that high vitamin D concentrations may serve as an indicator of a generally healthy lifestyle.

The strength of the present study was the random and population-based inclusion of families which made it possible to compare vitamin D status across age and gender groups. This has not been done previously in studies of the Danish population. Another strength was the use of detailed information on vitamin D sources including several variables for sun exposure, dietary vitamin D intake and supplement use. A limitation of the present study was that it was conducted at a single site in Denmark. However, our study population was large and randomly selected with few exclusion criteria and we believe that the results are likely to be generalizable.

We presented vitamin D status in a representative sample of Danish families measured by a standardised and calibrated method and thus the results are useful for future comparisons of vitamin D status between populations. In conclusion, the majority of children and adults among families had serum 25(OH)D concentrations >50 nmol/l in late summer in Denmark. Vitamin D status was associated with BMI, dietary vitamin D, multivitamin use, solarium use, outdoor stay in light

clothes, sun preference and sun vacation, but not with lifestyle factors in the adults when these were assessed together with the other determinants. The children within a family seemed to be more alike than the adults within a family with respect to vitamin D status.

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Tables

Table 1. Characteristics of the study population (*n* 755)

	All subjects		Children		Adults	
	n	%	*n*	%	*n*	%
Gender						
Female	386	51	178	52	208	50
Male	369	49	162	48	207	50
Age						
4-10 y	179	24	179	53	-	-
11-17 y	161	21	161	47	-	-
18-40 y	198	26	-	-	198	48
41-60 y	217	29	-	-	217	52
BMI						
Obese	54	7	1	<1	53	13
Overweight	156	21	20	6	136	33
Normal weight	545	72	319	94	226	55
Dietary vitamin D*						
Q1: <1·7 µg/d	186	25	85	25	101	24
Q2: 1·7-2·4 µg/d	193	25	83	24	110	27
Q3: 2·5-3·3 µg/d	186	25	93	27	93	22
Q4: >3·3 µg/d	190	25	79	23	111	27
Multivitamin use						
Yes	244	32	140	41	104	25
No	511	68	200	59	311	75
Outdoor transport to school/work						
<15 min/d	369	50	162	48	207	51
15-30 min/d	236	32	132	39	104	26
31-60 min/d	95	13	36	11	59	15
>60 min/d	43	6	10	3	33	8
Solarium use						
Yes	12	2	2	<1	10	2
No	743	98	338	99	405	98
Outdoor stay in light clothes						
Most of the time	408	54	199	59	209	50
Often	273	36	116	34	157	38
Sometimes	62	8	18	5	44	11
Seldom/never	12	2	7	2	5	1
Sun preference						
Prefer sun	246	33	103	30	143	35
Sometimes in sun	467	62	222	65	245	59
Avoid sun	42	6	15	4	27	7
Sun screen use						
Always	68	9	47	14	21	5
Most of the time	274	36	169	50	105	25
Sometimes	224	30	80	24	144	35
Seldom/never	189	25	44	13	145	35
Sun vacation						
Yes	363	48	168	49	195	47
No	392	52	172	51	220	53

*Quartiles for the whole study population.

Table 2. Lifestyle related characteristics of the adult study population (*n* 415)

	n	%
Education		
None/technical	115	28
<3 years higher education	59	14
3-4 years higher education	133	32
>4 years higher education	108	26
Smoking status		
Current	77	19
Former	106	26
Never	232	56
Alcohol consumption		
Never	19	5
<1 time per month	61	15
1-3 times per month	103	25
1 time per week	67	16
2-4 times per week	124	30
5-6 times per week	27	7
Daily	14	3
Leisure-time physical activity		
Mainly sedentary	44	11
Light to moderate activity	155	37
Regular sport and exercise	168	41
Athletic training	48	12
Self-rated physical shape		
Really good	29	7
Good	154	37
Fairly good	167	40
Bad	53	13
Really bad	12	3
Self-rated health		
Excellent	51	12
Really good	167	40
Good	170	41
Less good/bad	27	7
Effort to eat healthily		
Very often	125	30
Often	211	51
Sometimes	65	16
Seldom/never	14	3

Table 3. Serum 25(OH)D concentrations and prevalence by age and gender groups among families in late summer in Denmark (56 °N)

	Girls		Boys		Women		Men		All children	All adults	All subjects
	4-10 years	11-17 years	4-10 years	11-17 years	18-40 years	41-60 years	18-40 years	41-60 years			
n	94	84	85	77	102	106	96	111	340	415	755
Serum 25(OH)D (nmol/l)*											
Geometric mean	71·7	70·3	76·0	72·2	74·1	70·7	71·2	71·0	72·5	71·7	72·1
Interquartile range	64·1, 82·0	59·9, 82·0	65·1, 89·5	63·1, 84·6	60·7, 87·7	60·0, 88·5	61·0, 87·4	61·3, 87·2	62·7, 84·9	60·8, 88·0	61·5, 86·7
Prevalence (%)											
<25 nmol/l	0	0	0	0	0	1	2	1	0	1	1
25-50 nmol/l	5	8	5	8	7	15	6	9	7	9	8
51-74 nmol/l	55	55	46	48	45	35	40	42	51	41	45
75-125 nmol/l	39	35	49	44	44	47	51	48	42	48	45
>125 nmol/l	0	2	1	0	5	2	1	0	1	2	2

* No overall differences in serum 25(OH)D concentration between age (P=0·190), gender (P=0·332) and the combined age and gender groups (P=0·223) as analysed in the linear mixed model with family as a random variable, the following categorical variables: gender, age, BMI, dietary vitamin D, multivitamin use, solarium use, outdoor stay in light clothes, sun preference and sun vacation and the logarithm of the serum 25(OH)D concentration as the dependent variable.

Table 4. Associations between potential determinants and serum 25(OH)D concentrations*

	All subjects (*n* 755)			Children (*n* 340)			Adults (*n* 415)		
Variables	Ratio of means†	95 % CI	*P*	Ratio of means†	95 % CI	*P*	Ratio of means†	95 % CI	*P*
Gender			**0·332**			**0·034**			**0·792**
Female	0·98	0·95, 1·02		0·95	0·91, 1·00		1·01	0·96, 1·06	
Male	1	1		1	1		1	1	
Age			**0·190**			**0·823**			**0·937**
4-10 y	0·99	0·94, 1·05	0·778	1·01	0·96, 1·06		-	-	
11-17 y	0·95	0·91, 1·01	0·082	1	1		-	-	
18-40 y	1·01	0·96, 1·07	0·653	-	-		1·00	0·94, 1·06	
41-60 y	1	1		-	-		1	1	
BMI			**<0·001**			**0·348**			**0·001**
Obese	0·83	0·77, 0·90	<0·001	0·97	0·61, 1·54	0·908	0·84	0·77, 0·92	<0·001
Overweight	0·97	0·92, 1·02	0·173	0·93	0·85, 1·03	0·148	0·98	0·92, 1·04	0·519
Normal weight	1	1		1	1		1	1	
Dietary vitamin D‡			**0·008**			**0·065**			**0·034**
Q1: <1·7 µg/d	0·92	0·87, 0·97	0·002	0·94	0·88, 1·00	0·068	0·91	0·84, 0·98	0·015
Q2: 1·7-2·4 µg/d	0·97	0·92, 1·02	0·284	0·95	0·88, 1·01	0·104	1·00	0·93, 1·08	0·982
Q3: 2·5-3·3 µg/d	1.00	0·95, 1·05	0·927	1·01	0·95, 1·08	0·741	0·99	0·92, 1·08	0·899
Q4: >3·3 µg/d	1	1		1	1		1	1	
Multivitamin use			**0·019**			**0·066**			**0·045**
Yes	1·06	1·01, 1·10		1·05	1·00, 1·11		1·07	1·00, 1·14	
No	1	1		1	1		1	1	
Solarium use			**0·006**			**0·199**			**0·007**
Yes	1·2	1·06, 1·43		0·82	0·61, 1·11		1·29	1·07, 1·55	
No	1	1		1	1		1	1	
Outdoor stay in light clothes			**0·001**			**0·013**			**<0·001**
Most of the time	1·30	1·11, 1·51	0·001	1·20	1·01, 1·43	0·034	1·58	1·20, 2·08	0·001
Often	1·29	1·11, 1·50	0·001	1·25	1·05, 1·49	0·011	1·51	1·15, 2·00	0·003
Sometimes	1·16	0·99, 1·36	0·063	1·32	1·10, 1·59	0·004	1·29	0·97, 1·71	0·083
Never/seldom	1	1		1	1		1	1	
Sun preference			**0·002**			**0·621**			**0·001**
Prefer sun	1·14	1·04, 1·25	0·004	0·96	0·85, 1·09	0·570	1·24	1·09, 1·40	0·001
Sometimes in sun	1·07	0·98, 1·17	0·116	0·95	0·84, 1·07	0·393	1·14	1·01, 1·29	0·034
Avoid sun	1	1		1	1		1	1	
Sun vacation			**<0·001**			**<0·001**			**0·021**
Yes	1·09	1·04, 1·15		1·11	1·06, 1·17		1·07	1·01, 1·14	
No	1	1		1	1		1	1	

* Analyzed in linear mixed models with family as a random variable, the following categorical variables: gender, age, BMI, dietary vitamin D, multivitamin use, solarium use, outdoor stay in light clothes, sun preference and sun vacation and the logarithm of the serum 25(OH)D concentration as the dependent variable.

† The regression coefficients have been exponentially transformed (10^{β}) so that the figures represent the estimated ratio of means and the corresponding confidence limits.

‡ Quartiles for the whole study population.

Table 5. Standard deviations for within (σ) and between (ω) family effects and the intra-family correlation (ρ)*

Parameter	**All subjects (*n* 755)**	**Children (*n* 340)**	**Adults (*n* 415)**
ω	0·060	0·063	0·061
σ	0·098	0·074	0·11
ρ†	**0·27**	**0·42**	**0·24**

* Derived from linear mixed models with family as a random variable, the following categorical variables: gender, age, BMI, dietary vitamin D, multivitamin use, solarium use, outdoor stay in light clothes, sun preference and sun vacation and the logarithm of the serum 25(OH)D concentration as the dependent variable.
† Calculated as $\rho = \omega^2/(\omega^2+ \sigma^2)$.

Paper 3:

Relationship between winter vitamin D intake and status in Denmark.

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Relationship between winter vitamin D intake and status in Denmark

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Serum 25(OH)D, dose-response, children, adults

Abbreviations

25(OH)D, 25-hydroxyvitamin D; DEQAS, Vitamin D External Quality Assessment Scheme; FFQ, Food Frequency Questionnaire; LC-MS, liquid chromatography mass spectrometry; IQR, interquartile range; NNR, Nordic Nutrition Recommendation; RDA, Recommended Daily Allowance; RI, Recommended Intake.

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Trial registration

This trial was registered at clinicaltrials.gov as NCT01184716.

Abstract

Background: How much vitamin D is needed to maintain an optimal vitamin D status year-round is unknown. Objective: The present study investigated the relationship between actual vitamin D intake and status, taking into account effect modifiers and the estimated required winter vitamin D intake in healthy Danish people aged 4-60 years.

Design: Blood samples (n = 692) were collected in September-October and March-April, and serum 25-hydroxyvitamin D (25(OH)D) concentration was measured by LC-MS/MS. Dietary intake was determined by a self-administered FFQ.

Results: The median (IQR) total vitamin D intake was 6.2 (2.8-11.1) µg/d and the geometric mean 25(OH)D was 52.2 (39.1-73.1) nmol/L. The vitamin D intake-status relationship was best fitted using a log-transformation. The effect of vitamin D intake on serum 25(OH)D was 4% higher in men compared with women and 10% higher in the group with the lowest initial 25(OH)D (<61.5 nmol/L) compared with the group with the highest initial 25(OH)D (>86.9 nmol/L). An estimated intake of 5, 11, 23 and 39 µg/d was required for 50, 75, 90 and 95% of the population to maintain a vitamin D status >50 nmol/L during winter. It was higher for the group with the lowest initial 25(OH)D (11, 18, 34 and >34 µg/d) and lower for the group with the highest initial 25(OH)D (<1, 3, 8 and 17 µg/d).

Conclusion: The vitamin D intake-status relationship in a population of Danish families was non-linear and modified by gender and initial vitamin D status.

Introduction

Vitamin D is provided by endogen synthesis in the skin after UVB exposure and by intake through the diet and dietary supplements. Exposure to sunlight is considered the main source of vitamin D in the general population (1). However, when sun exposure is restricted, for example during winter at higher latitudes (2), vitamin D from intake becomes essential (3). Moreover, limited sun exposure as a consequence of changes in life style, such as spending more time indoors and increased awareness about skin cancer (4), means that vitamin D intake may play a more important role than previously assumed (5).

It is unclear how much vitamin D is needed to maintain an optimal vitamin D status all year round. Furthermore, while there is consensus about serum 25-hydroxyvitamin D (25(OH)D) being the best marker for vitamin D status (6), there is less agreement on the concentration defining the optimal status. The traditional approach was to determine sufficiency as the level at which the short-latency diseases for vitamin D deficiency, rickets and osteomalacia, were avoided. However, the prevention of the clinical symptoms of these diseases is not the same as the optimal status, because less severe degrees of deficiency can produce longer-latency diseases such as osteoporosis (7). Current research focuses on defining the optimal vitamin D intake and status for health outcomes rather than the lack of explicit clinical disease (8). Fifty nmol/L is the target used in the Nordic Nutrition Recommendation (NNR) for its Recommended Intake (RI) of 10 µg/d (9) and by the US Dietary Committee for its Recommended Daily Allowance (RDA) of 15 µg/d to define the optimal intake for bone health (10). In the present study, we operated with the limits of 25 and 50 nmol/l that are commonly used in Denmark to define deficiency and sufficiency (11).

Most supplemental studies have found that vitamin D intake increases the concentration of serum 25(OH)D, but various dose-response approaches have been used (12). Some have looked at the change in serum 25(OH)D concentration, whereas others have looked at the absolute 25(OH)D concentration as a function of the vitamin D intake. Earlier studies used specified doses, often beyond the recommended range, and most were conducted with adults. It is well-known that supplemental effects may depend on many factors, so dose-response studies have been criticized for not paying attention to these factors, especially the initial vitamin D status (13).

The aim of the present study was to investigate the relationship between total vitamin D intake from natural foods, fortified foods and dietary supplements, and the vitamin D status in children and adults in late winter in Denmark, taking into account the initial vitamin D status, age, gender and BMI class. Furthermore, we estimated the vitamin D intake needed to maintain 25(OH)D concentrations >25 and >50 nmol/L in specified proportions of the population.

Participants and methods

Study population

The present study used data from the VitmaD intervention (14) conducted in Denmark (56°N). Apparently healthy children and adults aged 4-60 years were recruited as families randomly drawn from the Danish Civil Registration System. The families received either vitamin D fortified or non-fortified milk and bread for six months during the winter (September 2010–April 2011). They were requested to replace their usual consumption of bread and milk with the study products and in all other aspects to live their lives as normal. Participants were examined as previously described in detail (22) and categorized into BMI classes based on standards for children (15) and the WHO standard for adults (16). Out of the 782 participants recruited, 692 had serum 25(OH)D concentrations measured both at baseline and at the end of the study as well as complete questionnaire data. The present analyses were conducted with these participants. Written informed consent for participation was obtained from all adult participants and from the guardians of the children. The study protocol was approved by the Research Ethics Committee of the Capital Region of Denmark (record no. H-4-2010-020) and registered in ClinicalTrials.gov (NCT01184716).

Serum 25(OH)D concentration

Non-fasting venous blood samples were drawn in an authorized laboratory (the Copenhagen General Practitioners' Laboratory, Søborg, Denmark). The serum was collected and stored at -80 ˚C until analysis. All blood samples were analyzed as single determinations in a random order at the Clinical Biochemical Department, Holbæk Hospital, Denmark. Serum 25(OH)D concentrations were determined by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) using principles described elsewhere (17). The method was

77 calibrated with the standard reference material vitamin D in human serum (SRM 972) (18).
78 The interassay CVs for our method were 2.2% and 2.8% at 30 and 180 nmol/L, respectively,
79 for 25(OH)D_3 and 7.6% and 4.6% at 43 and 150 nmol/L, respectively, for 25(OH)D_2. The
80 analytical quality of our method was assured by participation in the Vitamin D External
81 Quality Assessment Scheme (DEQAS). In this validation scheme, the mean bias for our
82 method compared with the mean of the DEQAS LC-MS group during the period of the
83 present analyses was -3.2%.

84 *Vitamin D intake*

85 Information on vitamin D intake was obtained from detailed self-administered web-based
86 questionnaires. Dietary vitamin D intake was registered in a semi-quantitative food frequency
87 questionnaire (FFQ) adapted from an FFQ used in the European Union project *Towards a*
88 *strategy for Optimal Vitamin D Fortification* (19). The vitamin D intake from natural foods
89 was calculated based on the reported consumption frequencies and the vitamin D
90 concentrations in the food items given in the Danish Food Composition Databank (20). The
91 vitamin D intake from the fortified foods was calculated based on the reported consumption
92 frequencies for milk and bread and the measured concentrations of vitamin D in these
93 fortified products (14). The contribution of vitamin D from dietary supplements was
94 calculated as the frequency of use multiplied by the self-reported content of vitamin D in the
95 supplement used. The total vitamin D intake used in the present analysis is the sum of vitamin
96 D from natural foods, fortified milk and bread, and dietary supplements.

97 *Statistics*

98 The data were analyzed using the STATA statistical software (StataCorp. 2011, *Stata*
99 *Statistical Software: Release 12*, College Station, TX: StataCorp LP) and their statistical
100 significance was evaluated at a level of $P<0.05$ (two-sided). Mixed models with family as a

random factor were applied in all analyses to take account of the non-independency of the participants. Before analysis, serum 25(OH)D concentrations were logarithmically transformed to meet the model requirements.

The dose-response relationship between the total vitamin D intake and the serum 25(OH)D concentration at the end of the winter was modeled as: linear, square, logarithm and restricted cubic spline with knots at 0.8, 4, 8 and 25. The fit of the various curves was compared using Akaike's Information Criterion (AIC) with small values indicating the best fit. Using the best fitted model, gender, age group (4-10, 11-17, 18-40, 41-60 y), BMI class (normal, overweight, obese), and initial serum 25(OH)D concentrations in four equal-sized groups were included as explanatory variables in the initial model. In this joint model, interactions with total vitamin D intake were tested, one at a time, to see whether any of these factors modified the effect of total vitamin D intake on serum 25(OH)D concentration. The vitamin D intake needed to maintain serum 25(OH)D above 25 and 50 nmol/L in 50, 75, 90, 95 and 97.5% of the population were determined by the lower prediction limits calculated using the method described by Jiang and Zhang (21).

Results

Individual serum 25(OH)D concentrations at the end of the winter ranged from 8.2 to 192.9 nmol/L with an overall geometric mean (IQR) of 52.2 (39.1, 73.1) nmol/L. The initial serum 25(OH)D concentration was 72.0 (61.4, 86.8) nmol/L. Individual total vitamin D intake ranged from 0.2 to 39.1 µg/d with a median (IQR) of 6.2 (2.8, 11.1) µg/d.

Table 1 shows the contributions from natural foods, fortified foods and dietary supplements to the total vitamin D intake. Approximately half of the participants received fortified products and 39% of the children and 26% of the adults (overall 32%) used dietary supplements with vitamin D. The majority used multivitamins. Total vitamin D intake decreased with the decreasing number of sources for vitamin D, and the serum 25(OH)D concentrations followed the same pattern. Thus, both vitamin D intake and status were highest in the participants receiving vitamin D from all three sources (Table 1). In the group receiving vitamin D from all three sources, 78.6% had a total vitamin D intake of ≥10 µg/d, whereas less than 1% reached this level in the group receiving vitamin D from natural foods only.

The intake-response relationship between the total vitamin D intake and the serum 25(OH)D concentration was best fitted in a log-model yielding a non-linear association (**Figure 1**). From this model, a doubling in the vitamin D intake was estimated to result in an 18% (95% CI: 15, 21%) increase in serum 25(OH)D ($P < 0.0001$). An intake of 10 µg/d predicted a serum 25(OH)D concentration (95% CI) of 60.2 (57.5, 63.0) nmol/L (**Table 2**).

This effect of the total vitamin D intake on serum 25(OH)D concentration was not modified by age group ($P = 0.132$) or BMI class ($P = 0.884$), but was modified by gender ($P = 0.014$) and initial 25(OH)D group ($P < 0.001$). For each doubling of vitamin D intake, 25(OH)D in men increased by 4% more than in women, and vitamin D intake has a 10% higher effect on

participants in the lowest starting 25(OH)D group than on participants in the highest starting 25(OH)D group (**Table 3**).

Based on the initial simple mixed model, total vitamin D intakes of 39, 23, 11 and 5 μg/d were required to maintain a winter vitamin D status above 50 nmol/L in 95, 90, 75 and 50% of the population (**Figure 2**). Similarly 95, 90, 75 and 50% would be above 25 nmol/L with daily intakes of 4, 3, 1 and <1 μg/d. Because the effect of vitamin D intake was dependent on the starting 25(OH)D level, lower vitamin D intakes were needed for the highest starting 25(OH)D group to have the same effect on vitamin D status as the lowest starting 25(OH)D group (**Table 4**). Thus, for 90% of the population to maintain a vitamin D status above 50 nmol/L during winter, 8 μg/d were required in the highest starting 25(OH)D group compared with an intake of 34 μg/d required in the lowest starting 25(OH)D group.

Discussion

We found that the relationship between the total vitamin D intake and status was best fitted by a non-linear curve and that the relationship was modified by gender and initial vitamin D status but not by age and BMI. This non-linear relationship is similar to previous observations (12, 22-27), whereas others have reported response estimates based on linear analyses (6, 12, 22, 28-35). These response curves have been found to deflect at an intake of 25 μg/d (27) or 35 μg/d (22). In the relatively narrow vitamin D intake interval in the present study, the dose-response curve deflected ~6 μg/d.

Studies of vitamin D supplementation in various doses and durations have yielded rate constants from 0.4 to 5.0 $nmol \cdot L^{-1} \cdot \mu g^{-1}$ with a mean of 2.1 $nmol \cdot L^{-1} \cdot \mu g^{-1}$ (36). The value of 2.0 $nmol \cdot L^{-1} \cdot \mu g^{-1}$ has been estimated to be a risk-averse approach to use when setting dietary recommendations (36). Studies using higher doses have found lower constants in the range 0.66-1.15 (22, 26, 28, 37). Meta-regression analyses using linear modeling have found slopes of 0.4-0.8 $nmol/L \cdot \mu g^{-1}$ (6, 29). However, the figures can be calculated by different means; some have used the change in vitamin D status, while others have used the final vitamin D status as a function of vitamin D dose, as we did in the present study. Our study was different in that we used the actual total vitamin D intake instead of controlled doses. Systematic reviews have also concluded that studies have been heterogeneous in their design, population and 25(OH)D assay (12, 27, 31), which may well explain the observed differences between studies.

In the present study, the response to vitamin D intake in serum 25(OH)D concentration was not modified by age, but the response in men was higher than in women. This has also been found in older people (≥64 y) (34), but most other studies have not found an effect of gender

171 (22, 26) or age (12, 22, 27, 38). While obese people have been found to respond less to
172 vitamin D supplementation than thin people (39, 40), BMI class was not an effect modifier in
173 the present study. Others have not found the response in 25(OH)D to vitamin D intake to be
174 affected by BMI either (22, 25, 32, 37, 38). On the other hand, the BMI interval in our study
175 was relatively narrow and most participants were normal weight, which might explain the
176 lack of effect of BMI class on the response to vitamin D intake. The response in serum
177 25(OH)D concentration to vitamin D intake was larger for the group with the lowest initial
178 25(OH)D than for the group with the highest initial 25(OH)D, which is a common finding
179 (22, 23, 26, 27, 31, 38, 41). It has been suggested that this is to be explained by the
180 acceleration of the hydroxylation of 25(OH)D into inactive metabolites with increases in
181 25(OH)D creating a threshold effect (41).

182 The estimated vitamin D intake required to maintain a specific serum 25(OH)D concentration
183 during winter varied depending on the 25(OH)D goal and the selected proportion of the
184 population. For example 39 µg/d was needed to sustain a vitamin D status >50 nmol/L in 95%
185 of the population. The intake required was reduced to 23 µg/d if the proportion was set to
186 90% and again to 11 µg/d for 75% and 5 µg/d for 50%. The estimated intake to maintain
187 25(OH)D in 95% of the population was higher for the 50 nmol/L goal and lower for the 25
188 nmol/L goal compared with previous estimates in children, adults and the elderly (24, 32, 34).
189 The intake required for the median of the three population groups to be >50 nmol/L was 10.4,
190 10.2 and 7.1 µg/d, respectively, which are all higher than our estimate of 5 µg/d. Interestingly,
191 the meta-analysis by Cashman et al. estimated that a vitamin D intake of only 9-12 µg/d was
192 needed to sustain 97.5% of the population above 50 nmol/L during winter (12). These
193 differences may be largely due to the different methods used for analysis of 25(OH)D (42-
194 44).

Similarly, the initial vitamin D status had a considerable influence on the vitamin D intake required to maintain a specified vitamin D status. For example, for 90% to be above 50 nmol/L, the required intakes were 34, 22, 19 and 8 µg/d for the four initial vitamin D status groups, respectively, despite the fact that the range between the four groups was relatively small (<61.5 to >86.9 nmol/L) and that the lowest group was not critically low. Similarly, others have found that lower vitamin D intakes were needed in groups with higher initial 25(OH)D than in groups with lower initial 25(OH)D (22, 32). Sun exposure may be a surrogate marker for summer vitamin D status or vice versa. Studies estimating required vitamin D intake based on sun exposure groups found less differences in the required intake between high (7.9-8.8 µg/d) and low sun exposure groups (11.4-12.3 µg/d) (24, 34) than we did between the corresponding high and low initial 25(OH)D groups (<1 and 11 µg/d for 97.5% >25 nmol/L). This supports the view that initial vitamin D status and/or cutaneous synthesis during the summer probably offsets the dietary requirement for vitamin D during winter (24, 32). In Danish girls (11-13 y) and women (70-75 y) with a typical dietary vitamin D intake (2.3-3.3 µg/d), usual summer sun exposure and no supplement use, a summer vitamin D status of 100 nmol/L was estimated to be needed to maintain vitamin D status above 50 nmol/L during winter (45). Our study supports that an intake of ~3 µg/d is sufficient to maintain 75% of the population >50 nmol/L during winter if the summer vitamin D status is >86.9 nmol/L. Intake-sun modeling has shown that 28 µg/d was needed to maintain the average vitamin D status above 50 nmol/L with usual outdoor behaviors in British adults (1 h on week days and 1.5 h on weekend days) (3). This intake is five times as much as our estimated required intake and more than the double the intake estimated by Cashman et al. (24, 32, 34).

What the relative contributions from oral intake and sunlight exposure should be to maintain an adequate vitamin D status is still not clear (3). Furthermore, it is not known how much impact the seasonal fluctuations in vitamin D status have on health (32). It might be that people who live in northern latitudes have adapted to these circumstances (1), so that some decline in serum 25(OH)D concentration during winter is acceptable and that a chronically low concentration is the condition more critical for long-term health. Nevertheless, vitamin D from the diet or dietary supplements is a requirement in most people during winter (24) and as the present and previous studies have demonstrated, the required vitamin D intake depends on several factors. One novelty in our study is that we estimated the required vitamin D intake based on the initial vitamin D status. The strengths of the present study are the broad span in age for both genders and the use of actual total vitamin D intake instead of predetermined doses to model the dose-response. However, this also poses an uncertainty factor in the estimated intake, because they are based on the subjects' self-reported usual dietary intake.

In conclusion, the dose-response between the total vitamin D intake from natural foods, fortified foods and dietary supplements and serum 25(OH)D concentrations in a sample of Danish children and adults was best modeled by a non-linear relation. This relationship was modified by gender and initial vitamin D status, but not by age and BMI.

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Tables

Table 1. Vitamin D intake by source and serum 25(OH)D concentration at the end of the winter in the four groups of the study population: participants receiving fortified milk and bread and using dietary supplements, participants receiving fortified milk and bread and not using dietary supplements, participants not receiving fortified milk and bread and using dietary supplements and participants not receiving fortified milk and bread and not using dietary supplements (n = 692)

	Fortified foods		Non-fortified foods	
	Dietary supplement users	Non-users	Dietary supplement users	Non-users
n	103	230	118	241
Vitamin D intake (μg/d) *				
Natural foods	2.6 (1.9, 3.5)	2.3 (1.6, 3.2)	2.0 (1.2, 2.6)	2.3 (1.6, 3.1)
Fortified foods	6.6 (4.2, 8.9)	7.0 (4.2, 9.7)	-	-
Dietary supplements	3.6 (2.0, 7.1)	-	3.6 (2.1, 7.1)	-
Total	14.7 (10.6, 18.2)	9.6 (6.3, 12.9)	6.2 (4.1, 9.0)	2.3 (1.6, 3.1)
Serum 25(OH)D (nmol/L)†				
Initial‡	77.4 (65.2, 92.3)	71.3 (60.5, 85.3)	75.2 (64.5, 87.0)	69.2 (60.3, 84.6)
Final	72.2 (62.2, 83.1)	65.5 (55.0, 78.5)	53.2 (40.1, 72.9)	36.4 (27.1, 47.5)

* Median (IQR)
† Geometric mean (IQR)
‡ n = 688

Table 2. Predicted late winter serum 25(OH)D concentration (95% CI) for specified total vitamin D intake in the initial simple log mixed model with family as random factor (n = 692)

Vitamin D intake (µg/d)	Predicted serum 25(OH)D (nmol/L)
2	41.1 (38.9, 43.3)
5	51.1 (49.0, 53.2)
10	60.2 (57.5, 63.0)
15	66.3 (62.8, 69.9)
20	71.0 (66.7, 75.3)
25	74.9 (69.9, 79.9)
30	78.2 (72.6, 83.8)
35	81.1 (75.0, 87.3)
40	83.8 (77.1, 90.4)

Table 3. Estimated effect of total vitamin D intake for gender and for the four groups of initial vitamin D status (n = 688)

	n	Estimated fold increase in 25(OH)D per doubling in total vitamin D intake (95% CI)*	p
Gender			
Females	354	1.13 (1.10, 1.16)	<0.0001
Males	334	1.17 (1.15, 1.20)	<0.0001
Initial serum 25(OH)D†			
<61.5 nmol/L	173	1.20 (1.16, 1.24)	<0.0001
61.5-73.4 nmol/L	171	1.18 (1.14, 1.22)	<0.0001
73.4-86.9 nmol/L	172	1.13 (1.09, 1.17)	<0.0001
>86.9 nmol/L	172	1.10 (1.06, 1.14)	<0.0001

*All estimates are adjusted for gender, age group, BMI class and initial vitamin D status in a log mixed model with random family effect.

† Groups according to quartiles for the initial serum 25(OH)D concentration.

Table 4. Estimated vitamin D intake to maintain vitamin D status above 25 or 50 nmol/L in specified proportions of the population during winter for the four initial vitamin D status groups (n = 688)*

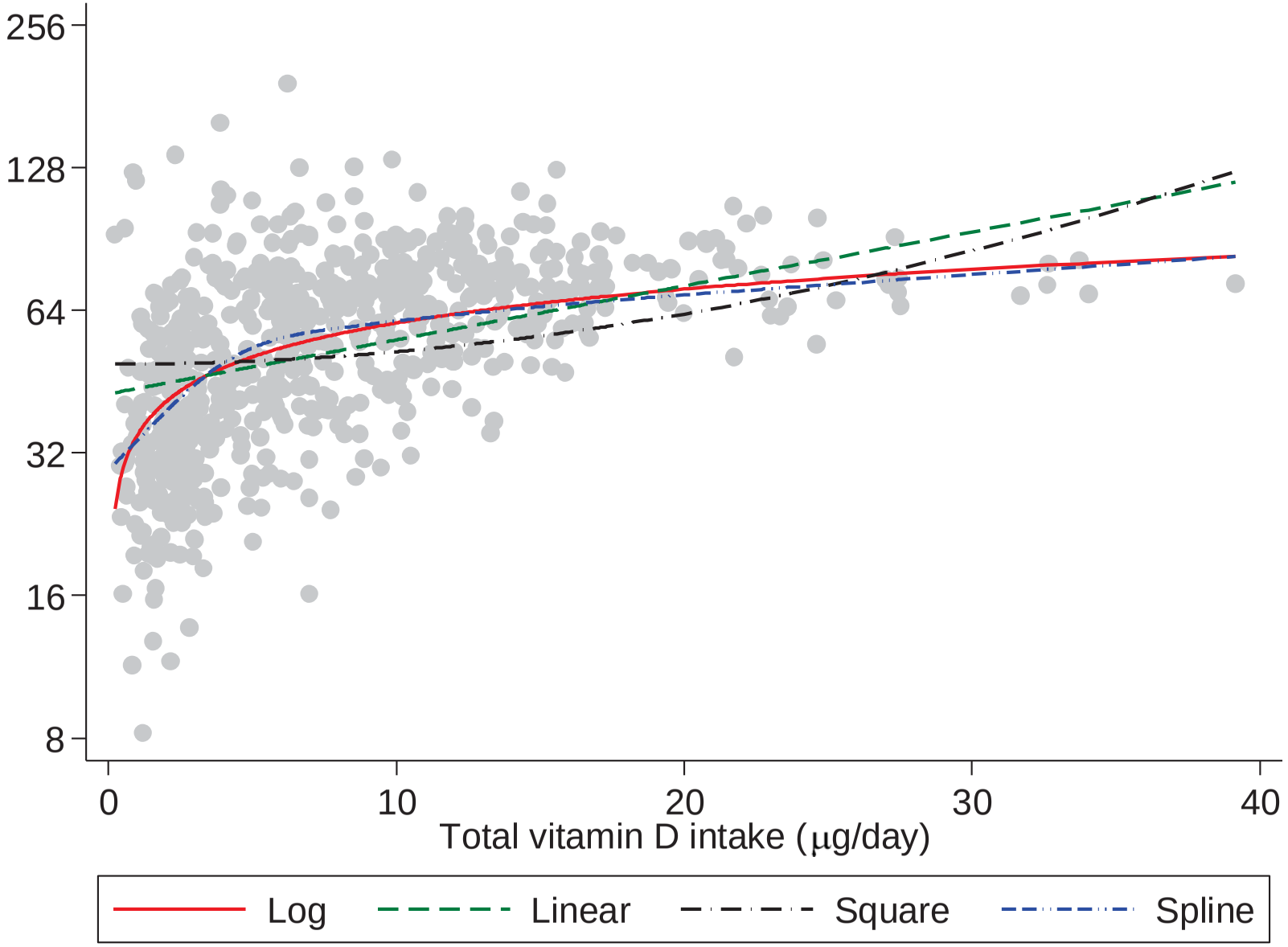
Vitamin D intake (µg/d)	97.5% limit	95% limit	90% limit	75% limit	50% limit
Aim >50 nmol/L					
Overall		39	23	11	5
Initial <61.5 nmol/L	-	-	34	18	11
Initial 61.5-73.4 nmol/L	-	37	22	1	6
Initial 73.4-86.9 nmol/L	-	37	19	7	4
Initial >86.9 nmol/L	39	17	8	3	<1
Aim >25 nmol/L					
Overall	6	4	3	1	<1
Initial <61.5 nmol/L	11	7	5	3	2
Initial 61.5-73.4 nmol/L	7	4	3	2	<1
Initial 73.4-86.9 nmol/L	4	2	<1	<1	<1
Initial >86.9 nmol/L	<1	<1	<1	<1	<1

* Prediction limits are based on a simple log mixed model with random family effect.

Figure legends

Figure 1. Four different models for the relationship between total vitamin D intake and serum 25(OH)D concentrations in Danish people aged 4-60 years at the end of the winter. Models are simple mixed models with family as a random factor (n = 692)

Figure 2. Prediction limits based on simple log2 mixed models with random family effect (n = 692). Horizontal lines mark the points where serum 25(OH)D concentrations of 25 and 50 nmol/L are reached for the specified prediction limits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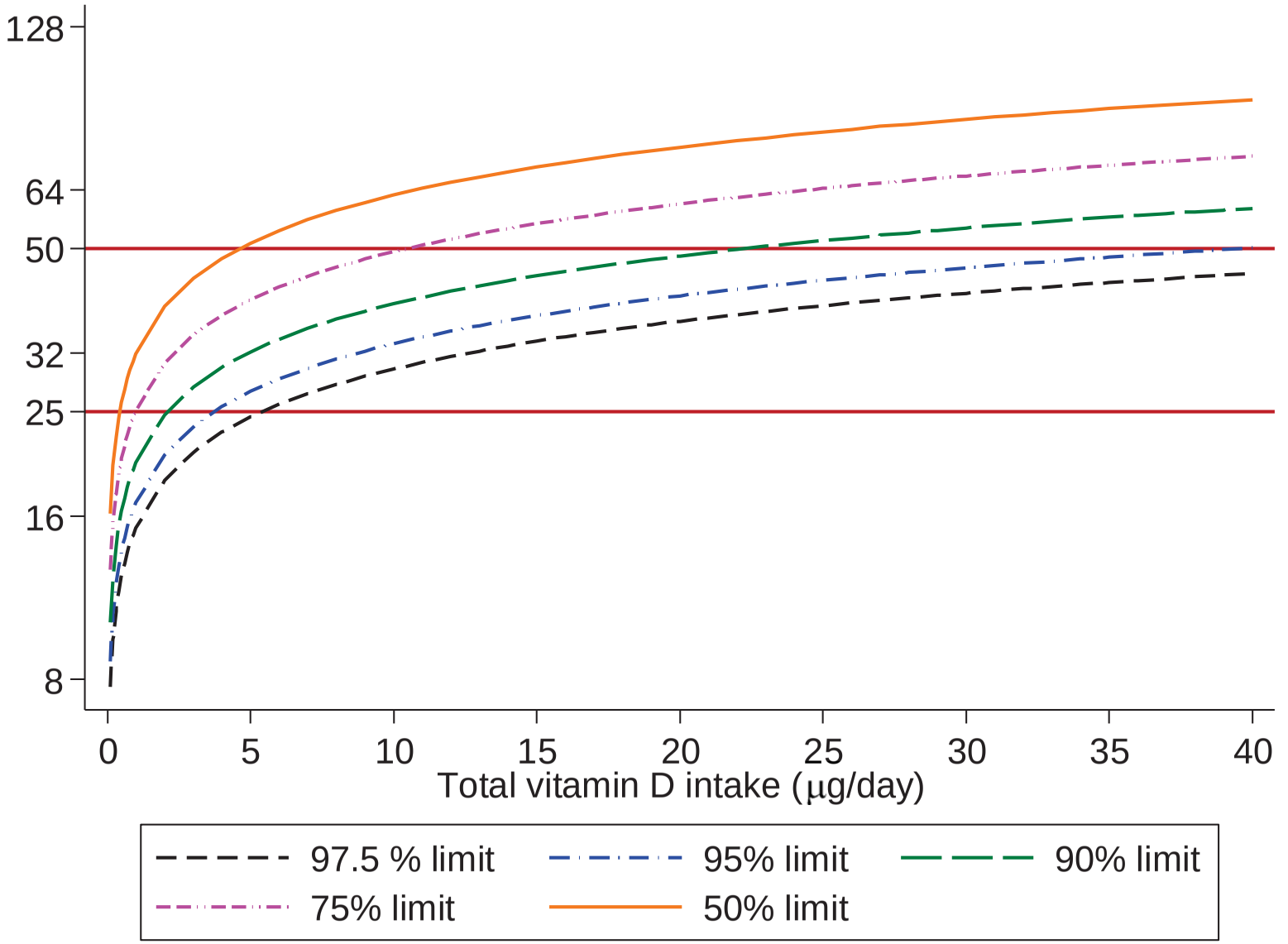
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Total vitamin D intake (µg/day)
97.5 % limit
95% limit
90% limit
75% limit
50% limit

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