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Division for Diet, Disease Prevention and Toxicology
Vitamin D and food fortification

Food based solutions for optimal vitamin D nutrition and health among women of Danish and Pakistani origin

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Preface

This PhD thesis is based on the randomised controlled trial ODIN FOOD, conducted at the Division for Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark. The work was initiated in September 2014 and has lasted 4 years including one year of maternity leave.

ODIN (Food-based solutions for optimal vitamin D nutrition and health through the life cycle) is a European Commission-funded large-scale collaborative project aiming at decreasing the prevalence of vitamin D deficiency in Europe as well as improving public health at all stages of the life cycle. This project has received funding from the European Union’s Seventh Framework Program (FP7/2007-2013) and the National Food Institute, Technical University of Denmark.

The Danish work package assessing women of Danish and Pakistani origin, ODIN-FOOD, was a three-month randomised double-blinded, placebo-controlled, intervention trial carried out between January and April 2016. My main responsibilities in the project have been the detailed planning of the intervention, recruitment of the participants as well as the collection of data together with the ODIN FOOD team. Subsequent analyses and interpretations of data have formed the basis for the three papers included in this PhD thesis.

Ida Marie Grønborg, Lyngby, August 2018
List of papers

I) **Ida M. Grønborg, Inge Tetens, Majken Ege, Tue Christensen, Elisabeth Wreford Andersen, Rikke Andersen** (2018). Modelling of adequate and safe vitamin D intake in Danish women using different fortification and supplementation scenarios to inform fortification policies. [Eu J Nutr]

II) **Ida M Grønborg, Inge Tetens, Tue Christensen, Elisabeth W Andersen, Jette Jakobsen, Mairead Kiely, Kevin D Cashman, Rikke Andersen.** Vitamin D fortified foods improve wintertime vitamin D status in women of Danish and Pakistani origin living in Denmark: A randomised controlled trial. [Submitted]

III) **Ida M Grønborg, Inge Tetens, Elisabeth Wreford Andersen, Michael Kristensen, Rikke E K Larsen, Thanh L L Tran, Rikke Andersen.** Effect of vitamin D fortified foods on bone markers and muscle strength during winter in women of Pakistani and Danish origin living in Denmark: a randomised controlled trial [Manuscript in preparation]
Summary

**Background:** Low vitamin D status is especially prevalent in countries of northern latitudes. Notably, many immigrants living in Denmark, and other Nordic countries, are at a higher risk of vitamin D deficiency compared to the native residents. Low vitamin D status has been linked with adverse bone health outcomes and decreased muscle strength. Food-based strategies aiming at improving vitamin D intake and status across the population, such as vitamin D food fortification may be a suitable solution. Data on the effects and safety of a food-based approach, especially in at-risk population subgroups, is lacking.

**Aim:** The overall aim of this project was to study the effect and safety of food based solutions to prevent vitamin D deficiency in high risk populations of women of Caucasian and south Asian origin living in Denmark. Additionally, we assessed the effect of the intervention on the bone markers osteocalcin (OC), Bone specific Alkaline Phosphatase (BALP), Procollagen type 1 amino-terminal propeptide (P1NP), C-terminal crosslinked telopeptide of type 1 collagen (CTX), and on muscle strength measured by hand grip strength, knee extension strength and a 30-second chair stand test.

**Design and methods:** A three-month randomised double-blinded, placebo-controlled trial. The subjects in the intervention groups were given four different fortified foods (cheese, yoghurt, eggs, crisp-bread) contributing with ≈ 30 µg/day of vitamin D3, while the placebo groups received equivalent foods not fortified with vitamin D. The main endpoint was the change in serum 25(OH)D concentration (**Paper II**). Secondary endpoints were muscle strength, markers of bone turnover (**Paper III**). Prior to the recruitment of study participants a fortification model was prepared (**Paper I**).

**Results:** In the fortification modelling we found that adequate and safe levels of intake were present in all the Danish women (n = 855) in the scenarios in which fortified foods (20 µg/dag) and a daily supplement of vitamin D up to 40 µg/day were added to the habitual diet (**Paper I**).

A total of 143 women of Danish and Pakistani origin were enrolled in the ODIN FOOD study. The baseline serum 25(OH)D was 49.6 (18) nmol/L and 46.9 (22) nmol/L among women of Danish and Pakistani origin, respectively. The prevalence of serum 25(OH)D < 30 nmol/L and < 50 nmol/L was 9 and 50 % of the women of Danish origin and 24 and 32 % of the women of Pakistani origin, respectively. Following the intervention mean (standard deviation) endpoint serum 25(OH)D
concentration in the fortified food groups were 77.8 (15) nmol/L and 54.7 (18) nmol/L among the women of Danish origin and Pakistani origin, respectively. At endpoint the prevalence of 25(OH)D concentration < 30 nmol/L in the Danish fortified group and Pakistani fortified group were 0 % and 3 %, respectively, compared with 7 % and 11 % in their respective control groups. The increase in serum 25(OH)D concentration following the intervention, among the of the participants receiving fortified foods, was dependent on the, baseline serum 25(OH)D concentration, ethnicity and to a lesser degree BMI. Compliance with the fortified foods was significantly higher among the women of Danish origin compared to women of Pakistani origin (Paper II).

The bone formation marker osteocalcin (OC) were significantly higher among the women of Danish origin at baseline, compared to women of Pakistani origin. The intervention did not result in significant changes of the bone turnover markers OC, Bone specific Alkaline Phosphatase (BALP), Procollagen type 1 amino-terminal propeptide (P1NP), C-terminal crosslinked telopeptide of type 1 collagen (CTX). Baseline handgrip and knee extension strength were 4.8 and 6.3 kg higher among participants of Danish origin compared to participants of Pakistani origin. Fortification with vitamin D did not have an effect on the muscle strength measured as hand grip strength in a linear model. Analysis of the knee extension strength showed an approximately 2 kg higher increase among the women of Danish origin compared to women of Pakistani origin, when adjusting for intervention group, BMI and baseline knee strength (Paper III).

**Conclusion:** By performing modelling of vitamin D fortification in the population of Danish women 18–50 years, we showed that adequate and safe levels of intake were present in scenarios including low dose fortification of several foods and vitamin D supplement intake below 40 μg/day.

In the ODIN FOOD intervention study we found that vitamin D fortification of 30 μg/day, provided in four different foods, for 12 weeks during winter was effective in increasing vitamin D status and reducing the prevalence of vitamin D deficiency among women of Danish and Pakistani origin. Compliance to the fortified foods was higher among the women of Danish origin compared to women of Pakistani origin. Following the intervention no significant changes of the bone turnover markers osteocalcin, BALP, P1NP and CTX were found. Muscle strength measured as hand grip strength and chair-stand test did not change significantly following the intervention. However, the change in knee extension strength following intervention showed a tendency to be higher among the participants of Danish origin compared to participants of Pakistani origin.
**Resumé (Danish summary)**


**Formål:** Det overordnede formål med denne Ph.d. afhandling var at undersøge effekten af fødevare-baserede løsninger på forebyggelse af D-vitaminmangel hos voksne kvinder af dansk og pakistansk oprindelse; en risikogruppe for lav D-vitamin status. Desuden undersøgte vi effekten af interventionen på knoglemarkørerne osteocalcin (OC), knogle specifik basisk fosfatase (BALP), Prokollagen 1 aminoterminal propeptid (P1NP), collagen 1 krydsbundne C-terminal telopeptid (CTX), og effekten på muskelstyrke målt som håndgrebs styrke, knæ ekstension og en 30-sekunders stol-til-stående test.

**Design og metode:** Et 3-måneders randomiseret dobbeltblindet, placebo-kontrolleret forsøg. Deltagere i interventionsgruppen fik fire forskellige berigede fødevarer (ost, yoghurt, æg og knækbrød) bidragende med $\approx 30 \mu g/dag$ D3 vitamin, hvorimod placebo grupperne modtog lignende fødevarer, uden D-vitamin. Hovedeffektparameteren var ændringen i serum 25-hydroxyvitamin D (25(OH)D) koncentrationen (**Paper II**). Sekundær parametre var knoglemarkører og muskelstyrke (**Paper III**). En berigelses-modellering blev udført forud før rekrutteringen af deltagere til interventionen (**Paper I**).

**Resultater:** Vi fandt at alle kvinder undersøgt i berigelsesmodelleringen havde et tilstrækkeligt og samtidigt ikke for højt indtag af D-vitamin ($n = 855$) i de scenarier hvor de indtog berigede fødevarer (20 $\mu g/dag$) og et dagligt D-vitamin tilskud på op til 40 $\mu g/dag$ udover den normale kost (**Paper I**).

I alt blev 143 kvinder af dansk og pakistansk oprindelse tilmeldt ODIN FOOD forsøget. Før forsøget fandt vi at serum 25(OH)D var 49.6 (18) nmol/L og 46.9 (22) nmol/L for henholdsvis kvinder af dansk og pakistansk oprindelse. Prævalensen af serum 25(OH)D < 30 nmol/L og < 50 nmol/L var henholdsvis 9 og 50 % for kvinder af dansk oprindelse samt henholdsvis 24 og 32 % af kvinder med pakistansk oprindelse. Efter interventionen var middelværdien (standardafvigelse) for...
serum 25(OH)D koncentrationen blandt kvinder af dansk og pakistansk oprindelse i den berigede gruppe, henholdsvis 77.8 (15) nmol/L og 54.7 (18) nmol/L. Efter interventionen var prævalensen af 25(OH)D koncentration < 30 nmol/L 0 % and 3 %, i henholdsvis den danske og den pakistanske berigede gruppe, sammenlignet med 7 % og 11 % i de respektive placebogrupper. Ændringen i serum 25(OH)D koncentrationen efter interventionen i de berigede grupper var afhængig af start serum 25(OH)D koncentrationen, etnicitet og i mindre grad BMI. Det daglige indtag af D-vitamin fra de berigede fødevarer var signifikant højere blandt de kvinder med dansk oprindelse, sammenlignet med kvinder af pakistansk oprindelse (Paper II).

Markøren for knogledannelse osteocalcin (OC) var signifikant højere blandt kvinder af dansk oprindelse ved starten af forsøget, sammenlignet med kvinder af pakistansk oprindelse. Der blev ikke fundet signifikante ændringer i knoglemarkørerne OC, BALP, P1NP, CTX efter interventionen. Håndgrebsstyrken ved starten af forsøget og knæekstension var henholdsvis 4.8 og 6.3 kg højere hos kvinder af dansk oprindelse, sammenlignet med kvinder af pakistansk oprindelse. D-vitamin berigelse havde ikke nogen effekt på muskelstyrke målt som håndgrebsstyrke og stol-til-stående test i en lineær model. Ændringen i knæekstensionen var omkring 2 kg højere blandt kvinder af dansk oprindelse, sammenlignet med kvinder af pakistansk oprindelse, efter justering for interventionsgruppe, BMI og knæekstension målt ved forsøgets start (Paper III).

Konklusion: I berigelsesmodelleringen med data fra danske kvinder mellem 18 og 50 år viste vi, at alle kvinder havde et tilstrækkeligt og ikke for højt indtag af D-vitamin (n = 855) i scenarierne med berigede fødevarer og et dagligt D-vitamin tilskud på op til 40 µg/dag udeover den normale kost. I ODIN FOOD interventionen fandt vi at D-vitamin berigelse med 30 µg/dag, fordelt i fire forskellige fødevarer, i 12 uger i vinterperioden var effektivt til at øge D-vitamin status og reducere prævalensen af D-vitaminmangel blandt kvinder af dansk og pakistansk oprindelse. Indtaget af de berigede fødevarer var højere blandt kvinder af dansk oprindelse, sammenlignet med kvinder af pakistansk oprindelse. Der blev ikke fundet signifikante ændringer knoglemarkørerne OC, BALP, P1NP, CTX efter interventionen. D-vitamin berigelse havde ikke nogen effekt på muskelstyrke målt som håndgrebsstyrke og stol-til-stående test. Dog var der en tendens til en højere stigning i knæekstensions styrke blandt kvinder af dansk oprindelse, sammenlignet med kvinder af pakistansk oprindelse.
Abbreviations:

DANSDA – Danish National Survey of Dietary Habits and Physical Activity (2011-13)

FFQ – Food Frequency Questionnaire

DBP – Vitamin D binding protein

D2 – Ergocalciferol

D3 - Cholecalciferol

25(OH)D – Serum 25-hydroxyvitamin D

1,25(OH)2D – 1,25-dihydroxyvitamin D

PTH – Parathyroid Hormone

IOM – Institute Of Medicine

NNR – Nordic Nutrition Recommendations

BMD – Bone Mineral Density

P1NP – Procollagen type 1 amino-terminal propeptide

BALP – Bone specific Alkaline Phosphatase

OC – Osteocalcin

CTX – C-terminal crosslinked telopeptide of type 1 collagen

CVD – Cardiovascular Disease

VDSP – Vitamin D Standardization Program

DEQAS – International Vitamin D Quality Assessment Scheme

DRV’s – Dietary Reference Values

RDA – Recommended Dietary Allowance

EAR/AR – Estimated Average Requirement

RI – Recommended Intake

AI – Adequate Intake

SNP – Single Nucleotide Polymorphism

GWAS – Genome Wide Association Study

UL – Upper Level of Intake

EFSA – European Food Safety Authority

DTU – Technical University of Denmark

ODIN – Food-based solutions for optimal vitamin D nutrition and health through the life cycle

UCC – University College Cork

LC-MS/MS – Liquid chromatography-tandem mass spectrometry

CV – Coefficient of variance ((SD/mean)*100)
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1. INTRODUCTION

1.1 Rationale

Low vitamin D status is a prevalent worldwide problem\(^1\). This is especially relevant in countries in which the absence of vitamin D from UVB sources during winter increases the risk of low vitamin D status\(^2, 3\). The dietary intake of vitamin D in Denmark is generally low and fat-rich fish is the main dietary source\(^4\).

Immigrants living in Denmark are considered at risk of low vitamin D status due to factors such as skin colour, cultural clothing and dietary habits\(^5-7\). This risk group extends to ethnic Danish women with low UVB exposure and a low dietary intake of vitamin D\(^3, 4\). Food fortification with vitamin D may be a safe and effective solution\(^8, 9\). Moreover, food fortification has the ability to target entire populations rather than individuals, in a safe manner\(^10\).

Few studies have been conducted aiming at assessing the effect of food based solutions to vitamin D deficiency in different ethnic groups living in Denmark.

1.2 Aim

The overall aim of this project was to study the effect and safety of food based solutions to prevent vitamin D deficiency in high risk populations of women of Caucasian and south Asian origin living in Denmark.

1.3 Specific objectives

- To create a fortification model assessing the effect of vitamin D fortification on vitamin D status in a population of Danish women at risk of vitamin D deficiency, based on low-dose vitamin D-fortified foods and using data from 18-50-year-old female participants from a representative national dietary survey (DANSDA) (Paper I).

- To tailor a vitamin D-specific Food Frequency Questionnaire (FFQ) to be applied among women of Danish origin as well as women of Pakistani origin (Thesis results).

- To describe the prevalence of vitamin D deficiency and insufficiency among women of Danish and Pakistani origin (immigrants and descendants) living in Denmark (Paper II).
- To estimate the habitual dietary intake of vitamin D among women of Danish and Pakistani origin (immigrants and descendants) living in Denmark (Paper II).

- To investigate the effects of vitamin D fortification of four different foods on vitamin D status among women of Danish and Pakistani origin (immigrants and descendants) 18-50 years of age (Paper II).

- To investigate whether there are any ethnic specific differences between women of Danish and Pakistani origin (immigrants and descendants) in the effects of vitamin D fortified foods on vitamin D status (Paper II).

- To examine the effects of vitamin D fortification on muscle strength in women of Danish and Pakistani origin (immigrants and descendants) (Paper III).

- To examine the effects of vitamin D fortification on specific markers of bone turnover in women of Danish and Pakistani origin (immigrants and descendants) (Paper III).
2. BACKGROUND

2.1 Vitamin D

2.1.1 Biochemistry, sources and metabolism

Vitamin D is a fat soluble vitamin and it is considered a pro-hormone\textsuperscript{(11)}. Vitamin D exists in two forms; D2 and D3, Figure 1. Vitamin D2 is found in plant sources (e.g. mushrooms) and vitamin D3 is derived from animal sources (e.g. fish). Few foods in our diet contain large amounts of vitamin D, mainly fat-rich fish such as salmon, herring and mackerel, meat and eggs\textsuperscript{(12)}. Vitamin D intake from oral sources may only be essential if UVB exposure is limited\textsuperscript{(13)}. UVB exposure is the predominant source of vitamin D, however countries such as the Scandinavian are subjective to seasonal variations resulting in a “Vitamin D Winter”, during which the UVB exposure is limited\textsuperscript{(3, 14)}. Besides sun-exposure and food sources, vitamin D can also be supplied via dietary supplements and fortified foods.

![Figure 1. Chemical structure of vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol).](image)

When the skin is exposed to UVB radiation (290-315 nm), the photo production of vitamin D is initiated in the skin. The epidermis and dermis of the skin contain high concentrations of 7-dehydrocholesterol which is transformed to pre-vitamin D3 by the action of the UVB light. The heat in the skin acts on the thermodynamically unstable pre-vitamin D3 to form vitamin D3.
Excessive amounts of sunlight and a high degree of skin pigmentation are both regulators of vitamin D production in the skin. Pigments absorb the UVB light and thereby decrease the production of pre-vitamin D3, and in a situation of prolonged exposure to sunlight, pre-vitamin D3 will be transformed to biologically inactive bi-products to avoid overproduction and toxicity. Vitamin D3 formed in the skin is released into circulation and transported by vitamin D binding protein (DBP) to the liver where it is activated, or it may be stored in adipose tissue\(^\text{11, 15}\), Figure 2.

**Figure 2.** Vitamin D metabolism, adapted from Holick 2006\(^\text{16}\).
Following absorption in the intestines, vitamin D from oral sources (food and supplements) is also transported to the liver in the form of vitamin D3 or D2 through a different pathway, namely by chylomicron transportation in the lymphatic system to the blood circulation. In the liver, vitamin D3 is hydroxylated (in the C25 of the side-chain) by the enzyme 25-hydroxylase to form 25-hydroxyvitamin D (25(OH)D).

Subsequently, newly formed 25(OH)D is transported, bound to DBP, to the kidneys. 25(OH)D is transported into the renal cell mitochondria, the major production site of 1,25-dihydroxyvitamin D (1,25(OH)2D), where the enzyme 1-25-hydroxylase converts (by an additional hydroxylation of the A-ring) 25(OH)D to 1,25(OH)2D; the biologically active form of vitamin D, Figure 2. The metabolite 1,25(OH)2D has a shorter half-life (∼4 hours) than 25(OH)D and the actions of 1,25(OH)2D are under strict endocrine regulation\(^\text{(16, 17)}\). The active metabolite 1,25(OH)2D has both genomic and non-genomic functions given that it interacts with nuclear as well as membrane receptors and act as a hormone, depending on the tissue or the situation\(^\text{(17)}\).

### 2.1.2 Musculoskeletal functions

The most well-established mechanistic function of vitamin D is the ability to ensure adequate intestinal calcium and phosphorus absorption and thereby support a tightly regulated calcium and phosphorus balance in the blood to the benefit of bone health\(^\text{(15, 17)}\). Taking place by the binding of 1,25(OH)2D to the vitamin D receptor (VDR) in intestinal, bone and kidney cells\(^\text{(17)}\). The metabolite 1,25(OH)2D may also act through non-genomic pathways like a steroid hormone, these actions are rapid (seconds to minutes) compared to the genomic actions that can unfold over hours to days\(^\text{(17, 18)}\). A continuous inadequate exposure (limited UVB exposure or oral intake) to vitamin D increases the risk of developing insufficiency and deficiency defined by the Institute Of Medicine (IOM) and Nordic Nutrition recommendations (NNR) as a serum 25(OH)D concentration below 50 or 30 nmol/L, respectively\(^\text{(19, 20)}\). A state of insufficiency or deficiency may have consequences for the absorption of calcium and phosphorus and increase the risk of long-term negative effects on the bone health\(^\text{(19, 20)}\).

When calcium concentrations in circulation is low, increased parathyroid hormone (PTH) secretion enable an endocrine feedback pathway to ensure adequate blood calcium\(^\text{(13)}\). The mechanism involves limiting the calcium excretion by enhancing the renal reabsorption and mobilisation of bone calcium as well as increasing the production of 1,25(OH)2D\(^\text{(11)}\). The tightly regulated serum calcium will thus be maintained at normal concentration despite vitamin D insufficiency or low
calcium intake, except in severe cases, Figure 2. Need and colleagues demonstrated that the
decrease in calcium absorption may not exist with a vitamin D status > 20 nmol/L, however,
decreased calcium absorption was evident at serum 25(OH)D concentrations < 10 nmol/L\(^{\text{(13)}}\). At
this stage, the production of 1,25(OH)\(_2\)D is also affected, suggesting that calcium absorption is
dependent on the 1,25(OH)\(_2\)D concentration\(^{\text{(13)}}\).

The IOM conclude that no consistent evidence has been found that a vitamin D status > 50 nmol/L
enhances the calcium absorption\(^{\text{(19)}}\). The bone-related consequences of vitamin D deficiency
involves decreased mineralisation leading to low bone mineral density (BMD), growth retardation
in children and bone pain. Long-term consequences of vitamin D deficiency are rickets in children,
osteomalacia and osteoporosis in adults resulting from a vitamin D-deficiency-induced chronically
low intestinal calcium absorption\(^{\text{(15, 16)}}\).

The bone turnover can be monitored by bone biomarkers in the blood (different enzymes and
peptides), this is a potential cost-effective method to complement BMD measurements and may be
used in situations where BMD is not a feasible endpoint e.g. studies of shorter duration\(^{\text{(21, 22)}}\). There
are several available markers of bone formation and they are categorized as either by-products of
collagen synthesis e.g. Procollagen type 1 amino-terminal propeptide (P1NP), osteoblast enzymes
e.g. Bone specific Alkaline Phosphatase (BALP) or matrix proteins e.g. Osteocalcin (OC). The
markers of bone resorption can also be categorized as collagen degradation products e.g. C-terminal
crosslinked telopeptid of type 1 collagen (CTX), non-collagenous proteins, osteoclastic enzymes
or osteocyte activity markers\(^{\text{(21)}}\).

Closely related to bone health, vitamin D has long been associated with muscle strength and
function\(^{\text{(1)}}\) and it has been found that changes in the muscle function due to vitamin D deficiency
may be some of the first physical signs of the effects of vitamin D deficiency on body functions that
subsequently may affect the bone health\(^{\text{(23)}}\). Associations of vitamin D status < 30 nmol/L with
decreased muscle strength, muscle pain and difficulty rising from a chair have been found in elderly
adults\(^{\text{(24)}}\). Most studies of vitamin D and muscle strength have been performed in elderly adults and
there is thus a lack of data on young adults. Meta-analyses from 2011 and 2014 show mixed results,
one concluding that vitamin D supplementation had no effect on muscle strength and the latter
finding a small positive effect of supplementation, it should be noted that Stockton et al. found a
larger effect in participants with a mean 25(OH)D concentration < 25 nmol/L\(^{\text{(25, 26)}}\).
The underlying mechanism of vitamin D in muscle function has not been agreed upon, however, it has been hypothesised that 1,25(OH)\(_2\)D concentrations through direct and indirect pathways influence the calcium influx of the muscle cells and thereby affect the contractility\(^{(24, 27)}\). The direct (genomic) pathway involves 1,25(OH)\(_2\)D and its binding to the VDR and subsequent effects on intracellular calcium concentration as described in relation to skeletal effects of vitamin D. The indirect (non-genomic) pathway involves the classic vitamin D-related serum calcium regulation in the kidneys and intestines primarily, the result increase or decrease the blood and intracellular calcium concentration likewise affecting muscle contractility\(^{(24, 28)}\).

### 2.1.3 Non-skeletal functions

There is a large amount of observational data assessing vitamin D and non-skeletal endpoints such as cancer, cardio-vascular diseases and diseases of the immune system\(^{(29)}\). Although several observational studies have shown associations between vitamin D and non-skeletal diseases, very few randomised controlled trials (RCT’s) have been designed to study these specific endpoints. Expression of the enzyme 1-\(\alpha\)-hydroxylase and the vitamin D receptor (VDR) in cells other than those traditionally related to the vitamin D metabolism (intestinal, renal and bone) such as cells of the immune system, pancreas, heart, brain and reproductive system has fuelled a large increase in studies focusing on these cells as targets of vitamin D, although the discovery of both the enzyme and receptor in numerous cell types has not been fully agreed upon\(^{(28, 30, 31)}\). A recent umbrella-systematic review assessing 54 meta analyses (including data from 210 RCT’s) including the endpoints cardiovascular diseases, blood pressure, type 2 diabetes, body weight, birth weight, malignant diseases, respiratory tract infections (excluding tuberculosis), depression, and mortality provides a comprehensive overview of the evidence-base and quality of the data\(^{(32)}\). Results show that as little as a quarter of the included RCT’s were designed for the purpose of assessing vitamin D and the specific non-skeletal outcome in focus. Sixteen of the included meta-analyses showed a beneficial effect of vitamin D supplementation e.g. respiratory tract infections and total mortality. None of the meta-analyses reported an effect on cardiovascular diseases, body weight, cancer or type 2 diabetes. In half of the studies, participants were reported having a baseline serum 25(OH)D concentration \(< 50\ \text{nmol/L}\), for this reason we cannot reject the hypothesis that vitamin D supplementation may be beneficial to individuals with a low vitamin D status in relation to specific disease endpoint\(^{(32)}\).
2.1.4 Measurement of vitamin D status

Vitamin D status is measured by determining the serum concentration of the metabolite 25(OH)D. This marker of vitamin D status is considered the gold standard and is the accepted marker of total exposure\(^{(19)}\), although several others such as PTH, BMD and free 25(OH)D concentrations have been considered\(^{(33, 34)}\). The metabolite 25(OH)D is not tightly regulated and with a half-life of \(\approx 3\) weeks it reflects both cutaneous production and oral intake\(^{(16, 35)}\). An oral dose of vitamin D3 has a delayed serum 25(OH)D3 response with the blood concentration rising slowly with peaks around seven days after a single large oral dose of vitamin D3 and 6-8 weeks is required to reach a steady state after the start of vitamin D supplementation\(^{(33, 36)}\).

High degrees of between-laboratory variability for serum 25(OH)D concentration analyses are seen, even when the assay is the same type\(^{(37)}\). There is a high need for eliminating bias in the determination of serum 25(OH)D concentration, thus enabling a comparison between studies and countries. Recent advances aiming at creating a protocol for comparable analytical methods have been done in the vitamin D standardization program (VDSP) as an addition to the international vitamin D Quality Assessment Scheme (DEQAS)\(^{(38)}\). The VDSP is based on a re-analysis of subsamples from older studies to create reference values for standardizing all the previously analysed samples within each study. For future studies, the protocol recommends following the methodological and analytic guidelines to enable comparison of the results with other standardized data\(^{(38)}\). A Nordic standardization study, used the VDSP protocol on existing data from a Danish, Finnish and a Norwegian survey as well as a Danish RCT, results showed highly variable effects of the standardization procedure on the serum 25(OH)D concentrations, highlighting the importance of standardizing data prior to comparison. The overall changes following standardization was increased comparability of the Nordic studies\(^{(39)}\).
2.1.5 Cut-off values and dietary reference values for vitamin D status

In terms of cut-off values there is no agreement as to what concentration of serum 25(OH)D concentration is the optimal for human health\(^{40,41}\). In the USA, The IOM based Dietary Reference values (DRV’s) on endpoints related to bone health in a comprehensive evaluation. A number of studies were evaluated in order to derive an Estimated Average Requirement-type reference value (EAR) covering 50% of the population needs and a Recommended Dietary Allowance-type reference value (RDA) covering 97.5% of the population needs (not to be used for population DRV), for infants an Adequate Intake (AI) was defined instead of RDA due to inadequacy of the evidence. The IOM state that a serum 25(OH)D concentration < 30 nmol/L was associated with decreased calcium absorption and risk of rickets, osteomalacia and low BMD and thus define a state of vitamin D insufficiency\(^{19}\). A cut-off value of vitamin D status > 50 nmol/L was defined as sufficient vitamin D since above this cut-off no additional benefits in terms of calcium absorption, BMD, incidence of rickets and osteomalacia were seen\(^{19}\). In line with the IOM, the European Food Safety Authority (EFSA) conclude that a vitamin D status > 50 nmol/L is suitable for all age- and gender groups\(^{41}\). The NNR, a regional collaboration between countries of the North (Denmark, Norway, Sweden, Iceland, Finland, Faroe Islands) use the same cut-off value as defined by the IOM and EFSA, a serum 25(OH)D concentration > 50 nmol/L for sufficient vitamin D status. In Denmark the National health Board has also defined the cut-off for sufficiency to be > 50 nmol/L, however they use < 25 nmol/L as the cut-off for deficiency and additionally define a cut-off for severe deficiency of < 12 nmol/L. They also define an optimal concentration for patients suffering from osteoporosis or kidney diseases of 75-150 nmol/L\(^{42}\), Table 1.
Table 1
Cut-off values for vitamin D deficiency and insufficiency

<table>
<thead>
<tr>
<th>Cut-off value (nmol/L)</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;12</td>
<td>Severe deficiency</td>
</tr>
<tr>
<td>12-25</td>
<td>Deficiency</td>
</tr>
<tr>
<td>25-50</td>
<td>Insufficiency</td>
</tr>
<tr>
<td>&gt;50</td>
<td>Sufficiency</td>
</tr>
<tr>
<td>75-150</td>
<td>Optimal for osteoporosis or kidney patients</td>
</tr>
</tbody>
</table>

Institute of medicine (IOM) 2011\(^{(19)}\)

<table>
<thead>
<tr>
<th>Cut-off value (nmol/L)</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 30</td>
<td>Deficiency</td>
</tr>
<tr>
<td>30-50</td>
<td>Insufficiency</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>Sufficiency</td>
</tr>
</tbody>
</table>

Nordic Nutrition Recommendations (NNR) 2014\(^{(20)}\)

<table>
<thead>
<tr>
<th>Cut-off value (nmol/L)</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 30</td>
<td>Deficiency</td>
</tr>
<tr>
<td>30-50</td>
<td>Insufficiency</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>Sufficiency</td>
</tr>
</tbody>
</table>

European Food Safety Authority (EFSA) 2016\(^{(41)}\)

<table>
<thead>
<tr>
<th>Cut-off value (nmol/L)</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 50</td>
<td>Sufficiency</td>
</tr>
</tbody>
</table>

In our RCT and throughout this thesis, the NNR and IOM cut-off values will be used to define deficiency, insufficiency and sufficiency of vitamin D in accordance with Table 1.

There is no consensus in Europe on the specific vitamin D intake recommendations given and most countries have defined their own ranging from 2.5 µg/day recommended in the Netherlands to 10 µg/day recommended for adults between 25 and 50 years in several countries including Albania, Iceland and Italy\(^{(43)}\). Specifically for the agencies IOM, NNR, EFSA the DRV’s are listed in Table 2, IOM state that the cut-off of 50 nmol/L corresponds with the RDA of 15 µg/d for adults < 70 years\(^{(19)}\). EFSA evaluated the evidence for vitamin D in 2016 and concluded that the evidence was insufficient to derive an Average Requirement and thus recommend 15 µg/day (AI) for all adults based on a meta-regression analysis of 83 trials assessing the relationship of vitamin D intake and serum 25(OH)D concentration\(^{(41)}\).
The NNR based their recommendations on systematic reviews and dose-response studies assessing bone-related end-points. The NNR set their Average Requirement (AR) of vitamin D to be 7.5 µg/day and the Recommended Intake (RI) for adults to be 10 µg/day\(^{(20)}\) (see Table 2).

### Table 2
Dietary reference values (DRV’s) for vitamin D in µg/day.

<table>
<thead>
<tr>
<th>Age group</th>
<th>EAR</th>
<th>RDA/RI</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Institute of Medicine (IOM) 2011(^{(19)})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - &gt;70</td>
<td>10</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>1 - 70</td>
<td>10</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>&gt;70</td>
<td>10</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><strong>Nordic Nutrition Recommendations (NNR) 2014(^{(20)})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 - 74 years</td>
<td>7.5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>≥ 75 years</td>
<td>7.5</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><strong>European Food Safety Authority (EFSA) 2016(^{(41)})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - &gt;70</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
</tbody>
</table>

EAR: Estimated Average Requirement and AR: Average Requirement, values estimated to cover 50% of population needs. RDA: Recommended Dietary Allowance, a value intended to cover the majority of the population (97.5%). RI: Recommended Intake, the Average Requirement + 2SD. Recommendations for infants are not included in the table. AI: Adequate Intake, defined when data is insufficient to determine RDA.

IOM does not give specific recommendations for sun-exposure, but state that when sun-exposure is limited it is important to increase dietary intake by e.g. taking supplements. The IOM DRV’s are defined using studies performed during times of minimal UVB exposure\(^{(19)}\). EFSA specify that sun-exposure may be a source of large variation in vitamin D status and the recommendations are created using data from trials performed during minimal sun-exposure. This also means that their recommendations are targeted low UVB exposed populations\(^{(41)}\). The NNR considers some vitamin D from UV-sources in their recommendations for dietary vitamin D and thus have higher recommendations for elderly (≥ 75 years)\(^{(20)}\). The differences in the methods when creating the DRV’s, i.e., evaluation of the included studies and performing the regression analyses as well as how UVB exposure is considered result in the varying recommendations given by e.g. the NNR and EFSA\(^{(20,41)}\), Table 2.
2.1.6 Genetic variation and vitamin D status

A Single Nucleotide Polymorphisms (SNP) is defined as an inherited DNA sequence variation of a single nucleotide (A, G, C, T) in which one nucleotide is substituted with another and the occurrence of this SNP is >1% of the population. SNP’s can be located in different regions of the gene, the promotor region, protein coding region or even between genes (intronic regions). The location of the SNP dictates the consequences of the specific SNP on the metabolism of e.g. vitamin D. A specific SNP may change the regulation of the gene, alter production of proteins or properties of the enzymes.

Several genes modulating vitamin D, more specifically vitamin D status, have been identified in Genome Wide Association Studies (GWAS)\(^{(44, 45)}\). Studies have shown that by determining the carriers of certain SNP’s or risk-alleles it may be possible to predict the vitamin D status on a population scale\(^{(46, 47)}\).

A recent Danish study found that an accumulation of risk-alleles affect the vitamin D status in a dose-response manner. Consequently, the group with the most risk alleles had a higher risk of vitamin D deficiency compared to the group having few risk alleles\(^{(48)}\). Most studies on genes and vitamin D has to this date been performed in Caucasian populations. However one study assessing healthy African Americans and Caucasian participants (n = 379 and n = 379, respectively) find similar trends as the Danish study, namely that an accumulation of risk alleles predict the vitamin D status and risk of vitamin D deficiency, although only among the African Americans, however not the Caucasians. The highest risk allele score of 5 vs. a score of 1 was associated with a 7.5 nmol/L reduction in serum 25(OH)D concentration. The study had a low power, which may explain that they do not find associations among the Caucasian participants.

Thus, genotypes may give insights into yet unknown risk groups of vitamin D deficiency though the extensiveness of these potential classifications and the impact on health outcomes are still unknown.
2.2 Vitamin D - the Danish perspective

2.2.1 Status, intake and sources

In Denmark, studies have found that 50-90% of the population have a vitamin D status below the cut-off for sufficiency of 50 nmol/L during winter\textsuperscript{(20)}. A study performed at the Danish Cancer Society found that 11% of the Danish adults enrolled in the study (n $\approx$ 300) had a serum 25(OH)D concentration < 25 nmol/L in either spring or autumn\textsuperscript{(49)}.

The incidence of rickets in Danish children is very low and has been recorded as 2.9 per 100,000 children (0-14y) per year in the years 1995-2005 among ethnic Danish children; however, the incidence among immigrants in the same period was 60 per 100,000/year\textsuperscript{(50)}. Individuals with dark skin, sun-avoiding behaviour or all-year covered clothing are recommended to take a supplement containing 10 µg/day\textsuperscript{(42)}.

According to the most recent Danish National Survey of Dietary Habits and Physical Activity (DANSDA) 2011-13\textsuperscript{(4)} the median dietary intake for women (18-75 y) in Denmark is 3.0 µg/day (10th and 90th percentiles (1.3 ; 9.0)), and for men the intake is 3.7 µg/day (1.8 ; 11.0). This current intake is considerably lower than the AR of 7.5 µg/day. Fish contribute to the majority (61%) of the dietary vitamin D, but meat, milk and eggs are also important sources (see Figure 3).

\textbf{Figure 3.} Dietary sources of vitamin D in Denmark (n=3946) (created using data from Pedersen \textit{et al.} 2015 \textsuperscript{(4)})
Compared with other European countries, we generally see a high consumption of dietary supplements in Denmark\(^{(51)}\). The intake of supplements containing only vitamin D is similarly high, with around 42-63 % subjects self-reporting an intake of multivitamin or vitamin D supplements\(^{(4, 52)}\), a routine which is particularly widespread in certain population groups such as elderly women\(^{(49, 52, 53)}\). Supplementation as a public health strategy to increase the intake of a specific micronutrient, holds risks of deficiency in non-consumers and toxicity in individuals with a ‘more is better’-approach. Considering food-based population strategies, vitamin D fortification may thus be a relevant future solution in Denmark, aimed at increasing the intake of vitamin D of the entire population.

### 2.2.2 Seasonal variation in vitamin D status

In the northern European countries situated at high latitudes (≥ 45°N) there is a large seasonal variation in vitamin D status, evidently increasing with latitudes, originating from the decrease in vitamin D from UVB sources seen between October/November and April\(^{(2, 3, 54)}\). The highest concentrations of serum 25(OH)D is seen in the autumn and the lowest in late winter, depending on the sun-exposure of the given year, see Figure 4.

*Figure 4.* Seasonal variation in hours of sunshine registered in Denmark by the Danish Institute of Meteorology in year 2000 and corresponding serum 25(OH)D concentrations. Figure reprinted from Thuesen et al.\(^{(3)}\) with permission.
In winter the solar zenith angle is very large as the sun is lower in the sky (see Figure 5). This affects the distance the photons must travel and overall this has a negative effect on the solar energy. The energy of the solar radiation in winter is therefore too low to initiate the cutaneous production of vitamin D in the skin\(^{(14)}\). During the months of summer the sun rays contain higher energy and the body is able to produce adequate vitamin D to sustain biological functions of vitamin D and the need for oral intake is diminished.

![Figure 5. The solar zenith angle.](image)

### 2.2.3 Risk groups of vitamin D deficiency

Immigrants from non-western countries living in Denmark or other Nordic countries are identified as a risk group of vitamin D deficiency, mainly due to a higher degree of skin pigmentation, cultural clothing habits and little time spent outdoors, but also a low intake of vitamin D\(^{(3, 5, 55, 56)}\). Several studies conducted in the Scandinavian countries have shown that immigrants from southern countries such as Pakistan, the Middle Eastern and African countries have a low dietary intake of vitamin D and generally a low vitamin D status, especially women and girls had extremely low vitamin D status\(^{(5, 6, 56)}\). Pakistani immigrants started coming to Denmark for work or for joining family already living in Denmark in the 1960’s and today almost 11,000 immigrants and approx. 10,000 descendants with Pakistani origin live in Denmark of which the majority lives in the Greater Copenhagen area\(^{(57)}\). Immigrants from Pakistan represent one of the major ethnic groups in Denmark.
Specifically moving to Denmark as opposed to another country such as Germany, Sweden or Norway may be considered a risk factor in itself because in Denmark we do not systematically fortify foods with vitamin D. In general, populations of special concern in terms of vitamin D deficiency are those who avoid the sun (e.g. seek the shade, wears covering clothing) and those who have a low intake of fish and supplements containing vitamin D. These risk groups include immigrants with dark skin and the elderly (>75 years), but may also apply to part of the adult population of ethnic Danish origin in a modern work life. Danish women who do not regularly eat fish have an extremely low intake of vitamin D of ~1.5 μg/day. This is considerably lower intake than the AR in the Nordic countries (7.5 μg/day). Generally women have a lower intake of vitamin D compared to men, which may primarily be due to a lower intake of energy and specifically meat, thus women of Danish as well as other ethnicities may be an important risk-group to study in relation to vitamin D.

### 2.3 Vitamin D fortification of foods

#### 2.3.1 Legislation, efficacy and safety

Denmark has traditionally been restrictive towards food fortification, mainly due to a lack of evidence on the population needs as well as benefits of fortification. A Danish survey (n =1263) concluded that the majority of the participants were positive to the idea of vitamin D fortification, but when it came down to buying either the fortified or the non-fortified product, they were more likely to choose the latter. The report additionally found that one third of Danes have a negative or somewhat negative attitude towards vitamin D fortification and the main criticism involved that fortified food was considered unnatural.

In Denmark, we follow the selective approach (in line with the Codex Alimentarius general principles) in which fortification is a public health issue governed by the authorities. In 2006 the European fortification legislations were harmonized with the introduction of Regulation (EC) No. 1925/2006, in order to accommodate the European free trade philosophy. Denmark is thereby part of the voluntary fortification practice already used in several European countries such as the UK and Ireland. In order to sell a fortified product in Denmark a permission given by the Danish Veterinary and Food Administration is required.
Fortification of foods with vitamin D has been found an effective and safe public health strategy when assessing populations. It is now generally accepted that low daily doses are more effective and safe in terms of maintaining optimal vitamin D status throughout the year compared with large bolus doses which in some cases have shown to increase fall and fracture incidences\(^{(63)}\). In finding a strategy to deliver daily vitamin D in adequate amounts, food fortification has been proposed as a possible solution. The effects of food fortification with vitamin D has shown as convincing results as supplementation in terms of increasing the serum 25(OH)D concentration on an individual level\(^{(8, 9)}\). Moreover, food fortification has the ability to target entire populations in a safe manner, rather than individuals\(^{(10)}\).

In terms of safety, an Upper safe level of intake (UL) has been proposed from several agencies defined as a safe intake that can be consumed daily over a lifetime without adverse health effects. EFSA set their UL for adults (\(>17\) years) and adolescents (11-17 years) at 100 µg/day\(^{(64)}\). The IOM also use an UL of 100 µg/day for adults from 19 years of age\(^{(19)}\). The NNR follow EFSA and IOM and use their proposed UL value\(^{(20)}\). In the 2016 assessment the British Scientific Advisory Committee on Nutrition (SACN) did not identify further studies on hypercalcemia than had been used in the definitions of IOM and EFSA and agreed on the 100 µg/day UL\(^{(65)}\). Sustained intakes above this limit may lead to hypercalcemia and hypercalciuria, nephrocalcinosis and kidney failure as well as calcification of other soft tissue\(^{(19, 20, 41)}\). Children are more vulnerable to toxic doses of vitamin D and we recently saw a serious case of accidental intoxication of children in Denmark caused by a 75 times too large dose (150 µg/day) of vitamin D added to vitamin D drops\(^{(66, 67)}\).

Nutrients and vitamins may have dual risk, meaning a risk of deficiency in the lower end of intakes and a risk of toxicity with high intakes. Vitamin D is a fat soluble vitamin and it is known to have a slow turnover and a long half-life in the body\(^{(64)}\). This means that a continuously high (>100 µg/day) intake with time may lead to toxicity. In terms of hazard characterisation, vitamin D is listed as a category A nutrient which is defined as a nutrient having a narrow range between a healthy intake and the UL\(^{(59)}\). Meltzer et al. argue that when dealing with food fortification of a category A nutrient it is necessary to respect the potential harmful effects of these micronutrients\(^{(59)}\).
At this stage the Regulation No. 1925/2006 introduced in the EU does not contain any maximum limits for addition of nutrients to foods. In Denmark the practice is therefore to follow the national authorization procedure in which a risk-assessment calculation based on UL for the specific nutrient and dietary intake data from national representative surveys are utilized every time a product is introduced to the Danish market\(^{68}\).

2.3.2 **Effect of vitamin D3 fortified foods on vitamin D status**

A collection of studies assessing the effect of food fortification with vitamin D3 on serum 25(OH)D concentration were compiled in a PubMed literature search (Table 3) (search terms and eligibility criteria are listed in appendix B).

Vitamin D fortification increased vitamin D status in 24 of the 27 studies listed in Table 3. Those not showing an effect of vitamin D fortification had either low doses (< 10 µg/day) or were short-term studies (≤ 8 weeks) (see Table 3). The results of the included studies resemble those found in meta-analyses on this subject\(^{(8, 69)}\). However, the authors of these meta-analyses reported that the heterogeneity was high and the ability to compare the studies was therefore low. Seventy percent of the studies used doses < 20 µg/day. The majority (63 %) of the studies had a duration of 12 weeks or longer duration. Most of the studies only used one fortified food, however five studies assessed the effect of two different fortified foods.
<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Population</th>
<th>n</th>
<th>Food</th>
<th>Dose (μg/day)</th>
<th>Duration Season</th>
<th>Assay</th>
<th>Baseline serum 25(OH)D</th>
<th>Effect compared with placebo</th>
<th>Ethnicity</th>
<th>Standardized analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keane et al. 1998&lt;sup&gt;(70)&lt;/sup&gt;</td>
<td>Ireland</td>
<td>66-91 y, Women, Men</td>
<td>51</td>
<td>Milk</td>
<td>5</td>
<td>12 months</td>
<td>CPBA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.0 (placebo) 24.7 (fortified)</td>
<td>↑</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>De Jong et al. 1999&lt;sup&gt;(71)&lt;/sup&gt;</td>
<td>Netherlands</td>
<td>78 y, women, men</td>
<td>145</td>
<td>Fruit product + Dairy product</td>
<td>10</td>
<td>17 weeks</td>
<td>CPBA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.1 (placebo) 24.3 (fortified)</td>
<td>↑</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Chee et al. 2003&lt;sup&gt;(72)&lt;/sup&gt;</td>
<td>China</td>
<td>55-65 y, women</td>
<td>200</td>
<td>Milk</td>
<td>10</td>
<td>1 year</td>
<td>RIA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.4 (placebo) 69.1 (fortified)</td>
<td>↑</td>
<td>Chinese Asian</td>
<td>NA</td>
</tr>
<tr>
<td>Tangpricha et al. 2003&lt;sup&gt;(73)&lt;/sup&gt;</td>
<td>USA</td>
<td>22-60 y</td>
<td>26</td>
<td>Orange juice</td>
<td>25</td>
<td>12 weeks Spring</td>
<td>CPBA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50.0 (placebo) 37.0 (fortified)</td>
<td>↑</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Johnson et al. 2005&lt;sup&gt;(74)&lt;/sup&gt;</td>
<td>USA</td>
<td>≥ 60 y, women, men</td>
<td>100</td>
<td>Cheese</td>
<td>15</td>
<td>8 weeks</td>
<td>RIA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57.4 (placebo) 49.9 (fortified)</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Daly et al. 2006&lt;sup&gt;(75)&lt;/sup&gt;</td>
<td>Australia</td>
<td>50-87 y, women, men</td>
<td>167</td>
<td>Milk</td>
<td>20</td>
<td>2 years</td>
<td>RIA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.1 (placebo) 77.2 (fortified)</td>
<td>↑</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Natri et al. 2006&lt;sup&gt;(76)&lt;/sup&gt;</td>
<td>Finland</td>
<td>25-45 y, women, men</td>
<td>41</td>
<td>Bread (wheat and rye)</td>
<td>10</td>
<td>3 weeks</td>
<td>HPLC&lt;sup&gt;f&lt;/sup&gt;</td>
<td>29.6 (placebo) 29 (fortified wheat)</td>
<td>↑</td>
<td>NA</td>
<td>DEQAS</td>
</tr>
<tr>
<td>Wagner et al. 2008&lt;sup&gt;(77)&lt;/sup&gt;</td>
<td>Canada</td>
<td>18-60 y, women, men</td>
<td>80</td>
<td>Cheese</td>
<td>100</td>
<td>8 weeks Winter</td>
<td>RIA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55 (placebo) 55 (fortified)</td>
<td>↑</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Manios et</td>
<td>Greece</td>
<td>55-65 y</td>
<td>101</td>
<td>Dairy</td>
<td>7.5</td>
<td>5 months</td>
<td>CIA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>63.4 (placebo)</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Study</td>
<td>Region</td>
<td>Age/Gender</td>
<td>Products</td>
<td>Season</td>
<td>Duration</td>
<td>Method</td>
<td>Baseline</td>
<td>Fortified</td>
<td>Type</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>al. 2009[78]</td>
<td>Women</td>
<td>18-84 y, women, men</td>
<td>Orange Juice</td>
<td>Spring</td>
<td>25</td>
<td>LC-MSa</td>
<td>49.2 (placebo)</td>
<td>44.8 (fortified)</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biancuzzo et al. 2010[79]</td>
<td>USA</td>
<td>18-47 y women</td>
<td>Milk powder</td>
<td>Winter</td>
<td>5</td>
<td>RIAb</td>
<td>74 (placebo)</td>
<td>76 (fortified)</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green et al. 2010[80]</td>
<td>New Zealand</td>
<td>&gt; 55 y Women</td>
<td>Milk</td>
<td>16 weeks</td>
<td>9.6</td>
<td>CIAc</td>
<td>43.3 (Placebo Indonesia)</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Houghton et al. 2011[82]</td>
<td>New Zealand</td>
<td>12-20 month toddlers</td>
<td>Milk</td>
<td>20 weeks</td>
<td>3.7 (0-10.4)</td>
<td>RIAb</td>
<td>48.8 (placebo)</td>
<td>52.8 (fortified)</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manios et al. 2011[83]</td>
<td>Greece</td>
<td>55-65 women</td>
<td>Dairy products</td>
<td>30 months</td>
<td>7.5 - 22.5</td>
<td>CIAc</td>
<td>59.0 (placebo at 0, 6, 12 and 30 mo)</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nikooyeh et al. 2011[84]</td>
<td>Iran</td>
<td>30-60 y (diabetics)</td>
<td>Yoghurt drink</td>
<td>12 weeks</td>
<td>25</td>
<td>HPLCf</td>
<td>41.6 (placebo)</td>
<td>44.4 (fortified)</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rich-Edwards et al. 2011[85]</td>
<td>Mongolia</td>
<td>9-11 y, girls, boys</td>
<td>Milk</td>
<td>7 weeks</td>
<td>7.5</td>
<td>LC-MSa</td>
<td>20 (placebo Mongolian milk)</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Country</td>
<td>Age Group</td>
<td>Gender</td>
<td>Intervention</td>
<td>Duration</td>
<td>Season</td>
<td>Method</td>
<td>PV (Placebo)</td>
<td>PV (Fortified)</td>
<td>Significance</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
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</tr>
<tr>
<td>Shab-Bidar et al. 2011</td>
<td>Iran</td>
<td>30-60 y (diabetics)</td>
<td>women, men</td>
<td>Yoghurt drink</td>
<td>12 weeks</td>
<td>Winter</td>
<td>HPLC f</td>
<td>38.0</td>
<td>38.5</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Bonjour et al. 2012</td>
<td>France</td>
<td>50-65 y</td>
<td>Women</td>
<td>Cheese</td>
<td>6 weeks</td>
<td>Fall</td>
<td>RIA b</td>
<td>57.3</td>
<td>58.8</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Fisk et al. 2012</td>
<td>UK</td>
<td>18-65 y</td>
<td>Women</td>
<td>Milk drinks</td>
<td>4 weeks</td>
<td>Winter</td>
<td>LC-MS a</td>
<td>33.5</td>
<td>31.3 (fortified 5)</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Kanelakis et al. 2012</td>
<td>Greece</td>
<td>55-65 y</td>
<td>Women</td>
<td>Dairy products</td>
<td>12 months</td>
<td></td>
<td>CIA e</td>
<td>58.3</td>
<td>57.0</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Kruger et al. 2012</td>
<td>China</td>
<td>&gt; 55 y</td>
<td>Women</td>
<td>Milk</td>
<td>12 weeks</td>
<td></td>
<td>CIA e</td>
<td>29.3</td>
<td>33.1</td>
<td>↑ Asian</td>
<td></td>
</tr>
<tr>
<td>Khadgawat et al. 2013</td>
<td>India</td>
<td>10-14 y, Girls, boys</td>
<td></td>
<td>Milk</td>
<td>12 weeks</td>
<td></td>
<td>CIA e</td>
<td>29.4</td>
<td>28.6 (fortified 15)</td>
<td>↑ South East Asian/Indian</td>
<td></td>
</tr>
<tr>
<td>Madsen et al. 2013</td>
<td>Denmark</td>
<td>4-6 y</td>
<td>Families</td>
<td>Milk and bread</td>
<td>6 months</td>
<td></td>
<td>LC-MS a</td>
<td>71.1</td>
<td>73.1</td>
<td>↑ Caucasian</td>
<td></td>
</tr>
<tr>
<td>Hayes et al. 2016</td>
<td>Ireland</td>
<td>45-70 y</td>
<td>Men, women</td>
<td>Eggs</td>
<td>8 weeks</td>
<td>Winter</td>
<td>LC-MS a</td>
<td>41.2</td>
<td>48.2 (fortified D3)</td>
<td>↑ Caucasian</td>
<td></td>
</tr>
<tr>
<td>Manios et al. 2017</td>
<td>Greece</td>
<td>55-75 y</td>
<td>Women</td>
<td>Cheese</td>
<td>8 weeks</td>
<td></td>
<td>LC-MS a</td>
<td>42.9</td>
<td>47.3</td>
<td>↑ DEQAS, VDSP</td>
<td></td>
</tr>
</tbody>
</table>

Notes:
- PV: Plasma Vitamin
- HPLC: High Performance Liquid Chromatography
- RIA: Radio Immuno Assay
- LC-MS: Liquid Chromatography Mass Spectrometry
- CIA: Cholesterol Immuno Assay
- DEQAS: Diet, Exercise, and Quality of Life Study
- VDSP: Vitamin and Dietary Supplement Project

Significance:
- ↑: Significant increase
- ↓: Significant decrease
- NS: Not significant
<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Age Range (y)</th>
<th>Gender</th>
<th>Frequency</th>
<th>Intervention</th>
<th>Duration</th>
<th>Analytical Method</th>
<th>Baseline</th>
<th>Treatment</th>
<th>Outcome</th>
<th><code>South Asian and white</code></th>
<th>Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guo et al. 2017&lt;sup&gt;95&lt;/sup&gt;</td>
<td>UK</td>
<td>30-65</td>
<td>Men</td>
<td>17</td>
<td>Dairy drink</td>
<td>1 single dose (acute test)</td>
<td>LC-MS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.5 (placebo) 31.0 (Fortified D3) 30.4 (fortified HyD3&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>↑</td>
<td>-</td>
<td>Validated</td>
<td></td>
</tr>
<tr>
<td>Tripkovic et al. 2017&lt;sup&gt;96&lt;/sup&gt;</td>
<td>UK</td>
<td>20-64</td>
<td>Women</td>
<td>335</td>
<td>Orange juice, biscuit</td>
<td>12 weeks</td>
<td>HPLC&lt;sup&gt;f&lt;/sup&gt;</td>
<td>58.8 (placebo white) 30.8 (placebo SA) 57.3 (fortified Juice, white) 27.3 (fortified juice, SA) 63.4 (fortified biscuit, white) 20.5 (fortified biscuit, SA)</td>
<td>↑</td>
<td>South Asian and white</td>
<td>DEQAS</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Some studies assessed the effect of D2 as well as D3, only the D3 arms are included in the table.  
<sup>b</sup>LC-MS, liquid chromatography mass spectrometry.  
<sup>c</sup>RIA, radioimmunoassay.  
<sup>d</sup>CPBA, competitive protein-binding assay.  
<sup>e</sup>biofortification.  
<sup>f</sup>CIA, Chemiluminescence immunoassay.  
<sup>g</sup>HPLC, high-performance liquid chromatography. NA: not available. NS: not significant.
2.3.3 Nordic fortification experiences

Since populations in the Nordic countries have considerable seasonal variations in regards to vitamin D status, several countries have taken actions to avoid the decrease of vitamin D status seen in winter. Voluntary fortification with vitamin D is common in Nordic countries such as Finland, Sweden, Norway and Denmark, however the extensiveness of the fortification practices varies between countries\(^{(97, 98)}\). In Finland specifically, the National Nutrition Council suggested the implementation of fortification to help increase the low intake recorded at the time (7.4 µg/day in 2002). All liquid dairy products and fat spreads were fortified with concentrations of 0.5 µg D3/100 ml and 1.0 µg D3/100 g, respectively. The intake of vitamin D improved following this fortification action, however not sufficiently. Consequently, in 2010 the amount of vitamin D added to dairy products and fat spreads were doubled\(^{(99)}\). An 11-year follow-up study focusing on vitamin D supplement non-users show how this large public health action has increased the serum 25(OH)D concentration of the follow-up group (\(n \approx 1300\)) with an average of 20 nmol/L. At follow-up 91% of supplement non-users had a serum 25(OH)D concentration > 50 nmol/L\(^{(98)}\).

Norway has had fortified margarine and butter on the market since 1950 and 1990, respectively. Also, a few types of fortified milk are sold, moreover the traditional source of vitamin D still consumed in Norway is the cod liver oil\(^{(100)}\). The Swedish National Food Administration recently (April 2018) expanded their mandatory requirements for foods fortified with vitamin D to also include sour milk, plant-based milk alternatives and cooking oil and the amount of vitamin D in margarines and the concentration of vitamin D in fat spreads was increased to 20 µg/100g\(^{(101)}\).

In Denmark, we have voluntary fortification with vitamin D, however very few fortified products are available on the market and the acceptance of new fortified products is low according to a market survey\(^{(101)}\). The increased focus on vitamin D in recent years has therefore resulted in an increase in the intake of dietary supplements with vitamin D among the Danish people, especially middle aged women\(^{(52, 102)}\).

2.3.4 Fortification vehicles

Vitamin D3 is currently the metabolite used for fortification of foods, previously both vitamin D3 and D2 were used. Vitamin D3 is quite stable during cooking, depending on the method. A study showed that boiling and frying of eggs gave the highest (82-88%) retention of vitamin D compared
to oven-baked (39-45%)\textsuperscript{(103)}. Foods suited for vitamin D fortification have previously been identified to include milk and milk products, margarines, bread and juice\textsuperscript{(104–106)}. However, vitamin D may be added to many types of foods and one of the more novel fortification practices are biofortification of eggs. Vitamin D biofortification of eggs can be achieved by both sun-exposure (free range chickens)\textsuperscript{(107)} and in a more controlled setting in which the animal feed is fortified with vitamin D3 and 25(OH)D and the vitamin D concentration of the eggs corresponds closely to the feed concentration\textsuperscript{(93,108)}.

An RCT conducted at the Danish National Food Institute (DTU) showed that fortifying two basic foods (milk and bread) prevented the decrease in vitamin D status generally seen in winter\textsuperscript{(104)}. Food fortification of a variety of foods has shown potential as a safe and effective solution to solve vitamin D deficiency problems in the Nordic countries\textsuperscript{(9,104)}.

Carefully planned fortification programs where several different foods are fortified with lower concentrations of vitamin D, rather than few foods fortified with higher concentrations are more effective on the population level because it limits the number of non-consumers, and it is safe in terms of toxic intakes, as high intakes are more difficult to achieve\textsuperscript{(104, 105, 109)}. It still needs to be investigated whether several vitamin D fortified foods can improve vitamin D status among highly vitamin D deficient risk groups, including immigrants and ethnic Danish populations at risk of vitamin D deficiency.

2.3.5 Fortification modelling

The goal of population-based food fortification is generally to increase the intake of the lower tails without bringing the top tails in danger of intoxication and this may be difficult to obtain with a micronutrient like vitamin D for which the span between a beneficial intake and UL is narrow\textsuperscript{(59, 110)}. Fortification modelling using habitual dietary intake of vitamin D may be a tool to demonstrate whether you can reach those in need of the specific nutrient or not\textsuperscript{(8)}. Prior to implementation of a national fortification program it is important to assess the current intakes of vitamin D and how the population intakes are distributed in order to avoid actions leading to toxic intakes. This can be done by using population-based survey data, representative for the country of focus e.g. the DANSDA\textsuperscript{(4)}. 

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In a simulation model it is possible to show how different concentrations in different fortification vehicles contribute with vitamin D in addition to the habitual dietary intake of vitamin D. This has previously been done in the UK by Allen and colleagues\(^{(10)}\). Using nationally representative data on the food consumption they identified best fortification vehicles as being wheat flour and milk. A range of different concentrations were used in their model aiming at the whole population and they found that wheat flour with a concentration of 10 µg vitamin D/100 g was effective in reaching those not meeting the dietary recommendations for vitamin D without any exceeding the UL\(^{(10)}\).
3. METHODS

3.1 Overview of the work included in this thesis

This thesis consists of a few additional parts other than the intervention study ODIN FOOD. Prior to the recruitment of the ODIN FOOD study population a fortification model was prepared using data from the DANSDA (2011–13)(4). The data and results from the modelling formed basis for Paper I. During the preparations for the intervention study, efforts were put into the tailoring of an FFQ aimed at women of Danish and women of Pakistani origin (presented in the thesis results). Subsequently, the recruitment for the ODIN FOOD intervention study began and the study was carried out between January and April 2016. Results from the ODIN FOOD intervention study forms basis for Paper II and III, Figure 6.

Figure 6. Timeline of the work included in this thesis.

3.2 Fortification model

A graded modelling of dietary vitamin D intake adding extra vitamin D from fortified foods and supplements to the habitual diet of 855 women was performed prior to the ODIN FOOD intervention study. We aimed at a contribution of 20 µg/day from the four different fortified foods included in our model. The choices of fortified foods for the model as well as the ODIN FOOD intervention trial were based on the Danish dietary habits with consideration of ethnic minority
The chosen fortified foods were yoghurt, cheese, eggs and crisp-bread. Some of the available foods for our study were produced for the German and Dutch markets and the concentration of vitamin D was therefore predetermined (yoghurt and cheese), see Table 4. The vitamin D content of the eggs were determined by the concentration of vitamin D3 and 25(OH)D allowed by EFSA in the chicken feed,. The crisp-bread contained the remaining vitamin D in order to meet the aim of 20 µg/day, see Table 4.

Table 4. Planned portion sizes and vitamin D concentrations of the vitamin D fortified foods used in the fortification model as well as the intervention ODIN FOOD.

<table>
<thead>
<tr>
<th>Food product</th>
<th>Daily portion size (g/day)</th>
<th>Planned vitamin D content of fortified product (µg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yoghurt</td>
<td>150</td>
<td>2.25</td>
</tr>
<tr>
<td>Cheese</td>
<td>60</td>
<td>7.0</td>
</tr>
<tr>
<td>Eggs</td>
<td>54</td>
<td>3.0</td>
</tr>
<tr>
<td>Crisp-bread</td>
<td>8.8</td>
<td>8.0</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>20.25</td>
</tr>
</tbody>
</table>

Individual intake data (dietary vitamin D) on 855 Danish (Caucasian) women aged 18–50 years were extracted from the DANSDA (2011–13)(4). The 855 women had all completed seven consecutive days of individual dietary recordings. Using the 7-day dietary recordings combined with the Danish food databank (Frida)(12), the median individual dietary intake of vitamin D was calculated (habitual intake). The percentiles were calculated and the distribution of the population plotted in order to illustrate the habitual intake and visualize the effects of subsequent addition of fortified foods and vitamin D supplements in the model scenarios.
3.3 Tailoring of a Food Frequency Questionnaire (FFQ) by Cognitive interviewing

A Food Frequency Questionnaire (FFQ) was used to capture the habitual daily intake of vitamin D. This type of questionnaire is known to be a useful tool, especially for vitamin D since it is distributed in few foods and not necessarily consumed daily\(^{(111)}\). The FFQ used in this study is a retrospective questionnaire asking about the participants’ habitual dietary patterns over the preceding three months, this method and duration of recall is applied because vitamin D-containing foods are affected by a highly variable consumption pattern and a shorter time period may therefore not be able to capture the general pattern of consumption\(^{(111)}\).

The FFQ used in the ODIN FOOD study were developed prior to the intervention start based on two existing questionnaires used in previous RCT’s (OPTIFORD and VitmaD) at the National Food Institute\(^{(104, 112)}\). The questionnaire tailoring, here defined by the addition of ethnic specific questions fitting both Danish and Pakistani persons regarding foods that contribute with vitamin D and calcium, testing the changes by cognitive interviewing in the target population. Expert knowledge was applied to create the ethnic specific food list fitting to Danish and Pakistani dietary habits and make it as comprehensive as possible. The questions and the structure of the questionnaire was then carefully updated using DANSDA\(^{(4)}\), the Food Composition database (version 7)\(^{(113)}\), information on product sales and knowledge from supermarket duties to check for any new fortified products. Four major supermarkets and four ethnic shops were checked for any new fortified foods, imported foods and specific foods eating by the Pakistani population that was not included in the previous questionnaires.

3.3.1 Conceptual framework and methods

The raw FFQ was tailored to the two target populations of Danish and Pakistani women by use of cognitive ‘read-think-aloud’ interviews. This method was chosen to ensure best possible understanding of the questions in a population of Danish origin and a population of Pakistani origin. The aim of the tailoring approach was an improved conceptualization as well as concept and content validity all of which lead to fewer misinterpretations by the participants and thus better data.

Cognitive interviews have a long history for being applied in survey development and validations to identify sources of error in the participant responses. Cognitive interviewing aims at elucidating these misconceptions in question understanding and interpretation in order to improve data quality.
In any questionnaire it is crucial that each question or concept is fully understood by the target population, especially when studying specific ethnic groups as in the present ODIN FOOD study. Looking towards the qualitative sciences including cognitive psychology, it has been shown that using ‘think-aloud’ cognitive interviewing in the development of the FFQ can improve the accuracy of the questionnaire\(^{(114, 115)}\). In this study population we expected the cognitive interviews to find ethnic-related differences in the relevance and understanding of questions related to the dietary intake of vitamin D and calcium.

The sub-group population recruited specifically for the FFQ development were enrolled 6 months in advance of the main study in order to target and improve the questionnaire before applying it to a larger study group. The participants were in total 13 individuals, 6 were of Pakistani origin and 7 were of Danish origin, i.e., similar ethnic groups as the main study population of ODIN FOOD. The subjects were recruited by advertisement at Universities, local schools, libraries and Pakistani shops. In addition we used social media and interactions with network in the recruitment. Subjects were mainly recruited from the Copenhagen area. The interviews were carried out at the participants’ homes, at local libraries or at the National Food Institute, DTU.

The interviews were semi-structured ‘think-aloud’ interviews in which the participants were asked to think out aloud when answering the questions in the FFQ. Probing questions were used by the interviewer to assist the participants to think out loud more easily (for example when the participant responded with ‘uhm’ or ‘ah’ or changed the answer), the probes were pre-scripted, but additional unscripted probes were applied when needed. The interviews were concurrent (participants gave their thought during the interview instead of summarizing their thought at the end of the interview) to minimise details lost due to reliance on long-term memory and to get an insight into the thought processes of the participants. The interviews were not recorded and transcribed due to limited resources, instead the interviewer noted all problems in a paper version of the questionnaire while the participant where working in the online version. Summary of problems noted during the interviews were analysed by verbal protocol analysis using a Problem Coding Frame which aids in classifying the verbal responses into themes\(^{(116)}\). The cognitive interviews were applied with the expectance of elucidating any ethnic differences in the understanding of questions and concepts and the results will facilitate a targeting of the FFQ in the specific study population.
3.4 The European collaborative project ODIN

ODIN (Food-based solutions for optimal vitamin D nutrition and health through the life cycle) is a European Commission-funded large-scale collaborative project aiming at decreasing the prevalence of vitamin D deficiency in Europe as well as improving public health at all stages of the life cycle. The project involves 31 partners from 19 countries and had duration of 4 years, starting November 2013. By assessing the efficacy and safety of biofortified and fortified food products among a population of immigrant and ethnic Danish women as well as the dose-response of vitamin D supplementation in RCT’s among children, teenagers and pregnant women the project aim to gain knowledge and fill gaps in the evidence regarding the vitamin D intake requirements\(^{117, 118}\).

3.5 Study design of ODIN FOOD

The Danish work package of the European ODIN project was a food-based RCT called ODIN FOOD, a three-month randomised double-blinded, placebo-controlled, intervention trial carried out between January and April 2016. The study population consisted of a total of 143 women of Danish and Pakistani origin, aged between 18 and 50 years. The trial was performed during the winter months in Denmark to minimize interference from UVB-induced cutaneous synthesis of vitamin D3. Participants were randomised into four groups stratified for ethnicity (two groups with women of Danish origin and two groups with women of Pakistani origin). The randomisation sequence was generated by a researcher not involved in the project using block randomisation with a block size of four within each ethnic group (Figure 7). The subjects in the intervention groups were given four different fortified foods aiming to contribute an additional 20 µg/day of vitamin D3, while the placebo groups received equivalent foods not fortified with vitamin D. Participants were seen before the start of intervention start and at the end of the 3-month intervention. The main endpoint was the change in serum 25(OH)D concentration. Secondary endpoints were muscle strength, markers of bone turnover and dietary vitamin D intake.

The study protocol was approved by the local ethical committee (protocol no. H-15008276) prior to recruitment and registered at ClinicalTrials.gov with identifier: NCT02631629. The study was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

The intervention was double-blinded, and the study foods were colour and letter coded. The blinding was managed by a researcher not involved in the project. Both the participants and the
researchers working within the project, during the intervention and subsequent analyses, were all blinded until the statistical analyses were completed.

The sample size calculation was based on 90% power (alpha = 5%). In total n=140 (35 in each group) were required to detect a change in serum 25(OH)D concentration of 20 nmol/L in the treatment group compared to the placebo group with a standard deviation (SD) of 23 nmol/L accounting for a drop-out rate of 20%.

3.6 Subjects

The subjects were recruited from mid-September 2015, from the Copenhagen area, city and suburbs (Denmark, 55°N). The recruitment of the participants with Pakistani origin was done by advertising, e-mail, networking and interactions with local community groups, media, and social and cultural initiatives in the Copenhagen area, as well as visiting local shops, libraries, mosques and women’s societies. We participated in events, parties, Pakistani radio-shows and TV-shows and visited private homes to talk about the project. Eligible women between 18 and 50 years of age were invited to information meetings in which the study procedures were explained in detail. Written consent was obtained from all participants. The participants of Danish origin were recruited by advertisements and posters in the Copenhagen area (libraries, schools, community centres and more), as well as e-mail recruitment among previous participants in a vitamin D fortification trial at the DTU\(^{(104)}\).

3.6.1 Inclusion and exclusion criteria

Inclusion criteria were a low consumption of fish and fish products (less than weekly), a low frequency of use of vitamin D-containing supplements (less than weekly), no use of tanning facilities, no planned sun-holiday (to a location more southerly than 47°N) between October 2015 and May 2016. The participants were asked to stop taking vitamin D containing supplements at the date of recruitment and during the whole study period. Exclusion criteria were pregnancy and breastfeeding, menopause, non-Danish speakers, serious diseases (cancer, server liver or kidney insufficiencies, sarcoidosis and other granulomatous diseases) and medication affecting vitamin D metabolism (steroids, antiepileptic, thyroid hormones, bisphosphonates, oestrogen).
Figure 7. Consort flow diagram of the number of participants enrolled, randomised, completed and analysed in the ODIN FOOD study. Consort, Consolidated standards of Reporting Trials. DK: Women of Danish origin, Pa: Women of Pakistani origin.
Participants were seen before the intervention start (January 2016) and at the end of intervention after 3 months (April 2016) (Figure 8), at the visits blood were drawn, anthropometrics (height and weight, waist-hip circumference and body composition), and muscle strength was measured. Two questionnaires were applied, a background questionnaire and an FFQ to estimate the habitual dietary vitamin D intake.

Throughout the study, the participants were encouraged to take contact to a researcher from the trial if they had questions, problems or other queries regarding the study. We had biweekly participant contact, primarily at the food pick-up, but additional communication was carried out by e-mail, text messages and phone calls to aid compliance. Foods given during the study period were free of charge for the participants and were made available for pick-up at the National Food Institute, DTU or at easy accessible local outlets; if participants were not able to pick up their designated food package it was delivered to their home address. The foods were handed out every two weeks during the 12 week intervention period. The first package was collected in connection with visit 1 (baseline) at the National Food Institute, DTU, see Figure 8.
3.7 Intervention foods

The study foods were chosen with the aim of increasing the total vitamin D status in an as effective and safe manner as possible. The intervention foods were low-fat Milner cheese (gouda) and yoghurt (plain) produced free of cost by FrieslandCampina in the Netherlands, eggs (Livskraft) produced free of cost by Hedegaard in Denmark, and whole grain biscuits produced by Smorum konditori (confectionary) using ingredients provided free of cost by Lantmännen Cerealia and vitamin D3 supplied by DSM nutritional products, Switzerland. The study foods were chosen because they are commonly consumed by both ethnic groups. Dietary calculations and pilot taste tests were carried out to ensure the acceptance of the products. The taste tests were performed among 12 women of Danish and Pakistani origin and based on this assessment we chose the final flavours and types of products. The majority of the foods were low in fat and they were considered nutritionally suitable substitutions for the participants’ habitual intake of these food products.

The participants were given either placebo foods or vitamin D-fortified foods (aimed at providing approximately 20 μg/day of vitamin D). A daily dose of 20 μg vitamin D3 in the intervention foods was expected, together with the remaining diet, to maintain a serum vitamin D concentration > 30 nmol/L among the vast majority of the individuals and it allows for a large margin of safety in relation to the UL of 100 μg/day. The fortified and non-fortified foods had the same fat percentage and a comparable content of nutrients, except for the content of vitamin D. The packaging of the food products was identical except for colour and letter coding added to distinguish the fortified from the placebo foods. Random samples from every batch were analysed for vitamin D content at the National Food Institute in Denmark.

The participants were given a daily dose consisting of 1 egg, 150 g (1 small container) yoghurt (plain), 60 g (2 slices) of low fat Milner cheese (gouda) and 1 whole wheat crisp-bread. The foods were provided every two weeks. The participants were allowed to freely plan how they distributed the foods over a week as long as they consume the designated 7 eggs, 7 portions of yoghurt, 7 x 60 g cheese and 7 crisp-bread per week. This approach towards the weekly dose of vitamin D has been chosen to mimic a real-life situation in order for the results to be used in a public health setting. By allowing people to consume for instance 4 eggs and 60 g cheese in one meal (e.g. omelette) we believed that the overall compliance would be higher and the end-result better than what would be achieved by attempting to restrict the participants to consume the same dose every day. All foods given to the participants were provided in portions that fit into a healthy diet, and in order to
maintain body weight the participants were instructed to substitute the study foods with their habitual intake of similar products. If a participant did not like one of the study foods they had the option of replacing it with another of the study foods. The weekly compliance was monitored by a user-friendly compliance questionnaire in which the participant would mark each food item once consumed, see appendix C.

3.8 Questionnaires

At baseline, the participants completed two questionnaires. Firstly, a general background questionnaire, assessing the health, lifestyle, physical activity and sun habits, and other factors affecting the vitamin D status. Secondly, a semi-quantitative FFQ estimating the average intake of vitamin D and calcium from the habitual diet. All questionnaires were self-administered, however to ensure that every participant completed the questionnaire and to avoid misunderstandings of cultural as well as language related character, we set up a questionnaire-room where all participants were introduced to the questionnaires. In this room computers with questionnaire access and an assisting staff member to help with any questions and difficulties were available.

3.8.1 Data management of the FFQ

All FFQ’s were given during the participant’s first visit in January 2016. The questionnaire contained the 8 food groups (fish, meat, milk and milk products, egg, cheese, bread, fats and pulses) contributing to the majority of dietary vitamin D (98%) and calcium (71%) found in a general Danish diet including the ethnic specific Pakistani foods(4). Further questions provided information on vitamin D-containing supplements prior to the study start, in order to estimate the prevalence of supplement users at baseline. The questionnaire took between 30 and 50 minutes to complete and all participants completed the questionnaire.

The data obtained from the FFQ’s were matched to specific foods and recipes along with the individually reported portion sizes. When questions contained groups of foods, such as fat-rich fish or lean fish as cold cuts these groups were defined by experts in consumption and recipe development. As an example, 100 g of fat-rich fish cold cuts was composed of 53 g of marinated herring, 25 g of gravad salmon, 17 g of smoked salmon and 5 g of smoked herring. The mentioned proportions was then combined to data from the Danish Food Composition database (version 7)(113). The choice of the components in case of fat-rich fish cold cuts reflects the most commonly eaten fat-rich fish in Denmark(4). The same procedure was completed for several compound foods like
lassi, i.e., a yoghurt drink, low fat cheese, high fat cheese, milk, flat-bread and organ meats etc. Other foods with known recipes could be connected directly to a similar food in the Danish Food Composition database. Fortified products were handled as a known product, e.g., margarine, and then extra vitamin D was added to fit the amount of that specific brand or product. The vitamin D concentration of eggs in the food composition database is regarded as high (3.2 µg/egg) and can be explained by a large conversion factor of 5, used when calculating the bioactivity of 25(OH)D in the eggs. Since these data were added to the database, new knowledge on the magnitude of the conversion factors have emerged and following consultancy with experts within this area we decided to lower the conversion factor to a conservative value of 2.5 based on new findings\textsuperscript{(120)}. The total vitamin D intake for each participant prior to the study start was estimated as the sum of dietary vitamin D and contribution from personal supplements, if consumed.

3.8.2 Assessment of habitual intake of supplements

The supplements that we assessed in the ODIN FOOD project were vitamin D, calcium, fish oil, multi vitamins and cod liver oil.

In the FFQ, all participants were asked about their habits in regards to the intake of vitamins and dietary supplements during the previous 6 months. A list of 30 supplement types and brands including pictures was shown together with the questions to help the participants remember the type/brand that they consumed. The participants were asked to estimate in how many of the preceding 6 months they had consumed the reported dose of supplements. The daily average dose was calculated based on the participant’s frequency and dose/product of consumption.

Calcium doses were very poorly reported by the participants and very few could remember the dose. This micro nutrient was therefore not used in the calculations. Only 2 participants were using cod-liver oil; 5 µg/serving were added to these participants total vitamin D intake in the calculations.

3.8.3 Assessment of compliance

The weekly compliance was monitored by a user-friendly (pictures of every food item) printed compliance questionnaire in which the participant would mark each food on a picture after consumption (see appendix C). The compliance questionnaires were collected every two weeks during the food delivery. The compliance questionnaire was a paper-based self-reported
questionnaire showing pictures of the intervention foods to be marked every day after eating it. Reasons for not eating the foods could be reported if necessary.

Compliance was estimated individually since the participants had individual start and finish dates and thereby number of days in the study. By dividing the amount of food delivered to each individual with the individual self-reported intake and the compliance is shown as the percentage consumed foods of delivered foods. Due to packaging sizes, an individual would sometimes get more foods than they needed and they were asked not to consume extra foods after fulfilling their weekly amount. However, if they did consume the extra foods this was accounted for in the compliance. The individual compliance could therefore in some cases result in more than 100 % (see appendix C for compliance questionnaire). The compliance was reported for all participants and separately for each ethnic group.

3.9 Clinical examinations

The participants were examined twice at the National Food Institute at the Technical University of Denmark. A baseline visit in January and a follow-up visit in April 2016.

Blood sampling
At each visit non-fasting blood samples were obtained between 9 am and 16 pm for the majority of the samples. Efforts were made to book the endpoint measurement on the same time as baseline visit for each participant. Forty mL blood were taken from each participant, the venepuncture was performed by an experienced phlebotomist or a nurse. The serum tubes were left to coagulate for 30 minutes prior to centrifuging. The plasma tubes were centrifuged immediately. Blood samples were collected with the aim of analysing serum 25(OH)D and the bone turnover markers OC, BALP, P1NP and CTX. Serum for assessing 25(OH)D had highest priority during the sampling.

Anthropometrics
Anthropometric measures were completed with participants wearing thin/light clothing, no shoes and after emptying their bladder. The measures were height (wall-mounted stadiometer) and weight, waist-hip circumference (standard tape measure) averaged of three repeated measurements and body composition (Tanita BC 418 MA, Tokyo, Japan). The majority of the measurements were handled by the same person in order to minimize systematic error.
**Methods**

*Muscle strength*

Both upper and lower limb muscle strength were measured using different physical tests. Upper limb muscle strength was measured as isometric hand grip strength (measured in kg) using a digital hand dynamometer (SAEHAN Corporation, South Korea)\(^{(121)}\). Participants were seated on a chair with a 90° elbow flexion, and the forearm in mid-prone neutral position. The handle of the dynamometer was placed in position 2. All participants were allowed two test contractions prior to the maximal contraction test. A minimum of 3 maximal isometric contractions were conducted using the dominant hand with a 90-second break between each measurement. If the last contraction was the highest, the participants had up to two additional contractions. During each contraction test the instructor gently supported below the instrument to minimized an effect of gravity. Muscle strength was calculated as the mean of the three highest values obtained (averaged peak grip strength). The intra-assay coefficient of variation for hand grip strength was 4.0%.

Lower limb muscle strength was measured as isometric knee extension strength (measured in kg) using a digital handheld muscle tester (MicroFET2, Hoggan Scientific, USA). The participants were seated on a table fitted with a non-skid material with a 90° hip flexion and arms placed on the lap. The lower leg was hanging down without touching the floor. The MicroFET2 dynamometer was placed on the front of the ankle, 4 cm above the lateral malleoli. The participants were asked to stretch their leg as much as they could for approximately 5 seconds, without using their arms. The MicroFET2 dynamometer was belt-stabilized to the table, so that the instructor would only have to concentrate on holding the dynamometer in the correct place position on the ankle\(^{(122)}\). The participants had two familiarization trials followed by 3-5 maximal contractions with the dominant leg with a 90-second break between each measurement and muscle strength was calculated as the mean of the three highest values obtained. The intra-assay coefficient of variation for knee extension strength was 4.2%.

Lower limb muscle function was also measured by a 30-second chair stand test, as a measurement of anaerobic muscle function. Participants were seated on a 43 cm high chair (without arm rest). Arms were folded across the chest, and the number of full stands achieved in 30 seconds were recorded.
3.10 Biochemical analyses

Serum concentrations of total 25(OH)D (i.e., 25(OH)D2 plus 25(OH)D3) of all serum samples (baseline and endpoint visits) were measured at the Cork Centre for Vitamin D and Nutrition Research, University College Cork (UCC), using the ODIN core LC-MS/MS analytical platform for serum 25OHD, described in detail elsewhere\textsuperscript{(123)}. The intra-assay coefficient of variance (CV) of the method was < 5\% for all 25(OH)D metabolites, while the inter-assay CV was < 6\%. UCC is a member of the Vitamin D Standardization Program and their method is certified by the Center for Disease Control and Prevention’s VDSP.

The biochemical analyses of serum calcium, PTH, creatinine as well as four different markers of bone turnover were performed at the University Hospital Aarhus. Serum calcium had an analysis precision of ± 0.032 mmol/L SD at a concentration of 2.161 mmol/L and ± 0.047 mmol/L (SD) at a concentration of 3.134 mmol/L. Serum PTH had a precision of ± 0.2 pmol/L (SD) at a concentration of 2 pmol/L and ± 1.0 pmol/L (SD) at a concentration of 10 pmol/L. Lastly, serum creatinine had the following precisions at specific concentrations: At 81 µmol/L ± 7.4\% (95\% CI) and at 567.0 µmol/L ± 5.0\% (95\% CI) and biological variation of ± 11.9\% (95\% CI).

The markers of bone turnover were OC, BALP, P1NP, CTX. OC had a precision of ± 0.55 µg/L (SD) at a concentration of 19 µg/L and ± 2.75 µg/L (SD) at a concentration of 92 µg/L. BALP had a precision of ± 0.45 µg/L (SD) at a concentration of 4.5 µg/L and ± 4.6 µg/L (SD) at a concentration of 45.9 µg/L. P1NP had a precision of ± 1.1 µg/L (SD) at a concentration of 30 µg/L and ± 7.6 µg/L (SD) at a concentration of 205 µg/L. CTX had a precision of ± 0.015 µg/L (SD) at a concentration of 0.26 µg/L and ± 0.03 µg/L (SD) at a concentration of 0.59 µg/L.
3.11 Statistics

Descriptive statistics were done for the two ethnic groups at baseline, and the ethnic groups were compared using a two-sample t test when the data could be assumed to be normal; otherwise using a non-parametric Kruskal Wallis test. Categorical variables were compared using a Pearson’s chi-square test.

Comparison of the daily vitamin D intake, serum 25(OH)D concentration and serum PTH at baseline and endpoint across the intervention groups were done by simple one way analysis of variance (ANOVA) and if significant differences were observed, a Tukey HSD test was performed in order to assess the between group differences. Analysis of covariance (ANCOVA) was used to assess the effect of the intervention on the primary outcome variable, i.e., change in vitamin D status (endpoint - baseline 25(OH)D) as well as secondary outcomes, i.e., muscle strength and markers of bone turnover. For each endpoint, two models were created: a minimal adequate model and a maximal model, which controlled for specific covariates that may influence the outcome. The minimal adequate model included baseline 25(OH)D concentration as a covariate and the factors intervention group, ethnicity and their interaction allowing the effect of intervention to differ between the two ethnic groups of women. The interaction was tested for statistical significance to see whether the model could be simplified. In the maximal model, the covariates age and BMI at baseline were added, as they are likely to have a strong association with the outcome (serum 25(OH)D concentration).

Similar models were constructed for the secondary outcomes – muscle strength and markers of bone turnover, i.e., OC, BALP, P1NP and CTX which also included covariates found to be relevant for the muscle or bone-specific endpoint, e.g., age, ethnicity and BMI. The variables included in the models as covariates were chosen based on factors known to affect the change in vitamin D status following fortification and the variables that were significantly different between the two ethnic groups at baseline.

To check the model assumptions the standardized residuals of the final models were assessed for normality, variance homogeneity, and linearity.

Statistical analyses were performed using RStudio for Windows\(^{(124)}\) (Version 1.1.414 – © 2009-2018 RStudio, Inc.) with a significance level of \(\alpha = 0.05\).
4. RESULTS

4.1 Modelling of adequate and safe vitamin D intake in Danish women using different fortification and supplementation scenarios to inform fortification policies

4.1.1 Results

The following results are presented in Paper I (appendix A). Illustrated on Figures 9 and 10 are the distribution of the habitual dietary vitamin D intake of the 855 women assessed in the DANSDA (2011–13)\(^4\). The habitual dietary vitamin D intake, excluding and including the vitamin D contribution from fish is shown in scenario 1 and 2, respectively, Figure 9. We subsequently added the four fortified foods (yoghurt, cheese, eggs and crisp-bread) contributing with approx. 20 μg/day to the habitual diet excluding fish (scenario 3). In the next scenario, a daily supplement of 10 μg was added (scenario 4). Fish was then again added to the diet (scenario 5). Scenarios 6 and 7 represent increasing daily amounts of vitamin D supplements of 40 and 80 μg/day, see Figures 9 and 10 and Table 5.

![Figure 9](image_url)

**Figure 9.** Distribution of habitual vitamin D intake excluding and including vitamin D from fish, and addition of foods fortified with vitamin D contributing with 20 μg/day in a population of Danish women (n = 855).
Figure 10. Distribution of vitamin D intake in four scenarios adding foods fortified with vitamin D (≈ 20 µg/day) and doses of vitamin D supplements ranging from 10-80 µg in a population of Danish women (n = 855).

A prevalence of 88 % of the women had a dietary vitamin D intake < AR of 7.5 µg/day, when assessing the habitual diet including fish, i.e., scenario 2. We saw a large variation in the daily intake of vitamin D when fish was a part of the habitual diet ranging from 1.0-17.7 µg/day, Table 5. Safe levels of intake < the UL (100 µg/day) were seen in all scenarios except scenario 7 in which a high-dose supplement of 80 µg/day were added.
### Table 5
Fortification scenarios 1-7 based on the habitual dietary intake (HD) of vitamin D among 855 Danish women.

<table>
<thead>
<tr>
<th>Scenarios (µg/day)</th>
<th>Percentiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>1 Vitamin D from HD including fish</td>
<td>1.0</td>
</tr>
<tr>
<td>2 Vitamin D from HD without fish</td>
<td>0.7</td>
</tr>
<tr>
<td>3 Scenario 2 + 4 fortified foods¹</td>
<td>21</td>
</tr>
<tr>
<td>4 Scenario 2 + 4 fortified foods + 10 µg/day supplement</td>
<td>31</td>
</tr>
<tr>
<td>5 Scenario 1 + 4 fortified foods + 10 µg/day supplement</td>
<td>31.3</td>
</tr>
<tr>
<td>6 Scenario 1 + 4 fortified foods + 40 µg/day supplement</td>
<td>61.3</td>
</tr>
<tr>
<td>7 Scenario 1 + 4 fortified foods + 80 µg/day supplement</td>
<td>109.3</td>
</tr>
</tbody>
</table>

¹Fortified foods contribute with 20 µg/day vitamin D₃ distributed in yoghurt, cheese, eggs and crisp-bread.

All values in the table are represented in µg/day unless otherwise specified.

### 4.1.2 Summary

The current vitamin D intake was below the AR of 7.5 µg/day among 88% of the 855 Danish women. By performing modelling of vitamin D fortification in the population of Danish women 18–50 years, we showed that adequate and safe levels of intake were present in all women in the scenarios in which fortified foods and a daily supplement of vitamin D up to 40 µg/day. A daily vitamin D supplement of 80 µg/day or more, however, would increase the risk of exceeding the UL value. The fortification modelling was successful in predicting the impact of low-dose fortification in a population of Danish women at risk of vitamin D deficiency.
4.2 FFQ tailored by cognitive interviews

4.2.1 Results

The results of the FFQ tailoring assessed in a population of 13 women recruited prior to the ODIN FOOD main study population. The 13 participants represent a similar, however smaller study population of women of Danish and Pakistani origin recruited for the tailoring of the FFQ with the aim of improving the validity of the FFQ. The verbal responses were analysed by a Problem Coding Frame which aids in classifying the verbal responses\textsuperscript{(116)}. The issues found by this method were mainly confusing formulations and structural issues (problem code [2] and [3] in Table 6) and the solutions to these were rephrasing and elaboration of difficult concepts as well as structural changes as suggested in Table 6.
### Table 6
Problem definitions, problem codes, prevalence of problems among women of Danish and Pakistani origin and overall solutions resulting from the cognitive interviews.

<table>
<thead>
<tr>
<th>Problem definitions</th>
<th>Problem code [1-4]</th>
<th>Number of problematic questions (Danish n=7)</th>
<th>Number of problematic questions (Pakistani n=6)</th>
<th>Overall solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confusing question structure/construction in LimeSurvey¹</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>Change question structure and design</td>
</tr>
<tr>
<td>Vague/confusing/difficult question formulation/wording, need for elaboration or examples</td>
<td>2</td>
<td>28</td>
<td>22</td>
<td>Formulate questions clearly, elaborate where needed, give more examples</td>
</tr>
<tr>
<td>Missing/no suitable answer category</td>
<td>3</td>
<td>19</td>
<td>14</td>
<td>Add the missing categories</td>
</tr>
<tr>
<td>Culturally selective question</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>Make question more general</td>
</tr>
</tbody>
</table>

¹LimeSurvey is the online questionnaire program used in the ODIN-FOOD intervention

Following the problem coding and suggestion of overall changes, the specific problems were solved and the online questionnaire updated accordingly. Examples of problems and specific solutions regarding questions in four different food groups are listed in Table 7. The problems were mainly language and structurally related, however one cultural issue became visible. We asked the participants about their consumption of some specific Pakistani food items (chapatti and roti) and the Danish participants were confused about this and did not know the foods. All participants were asked the same questions, regardless of ethnicity, thus the more ethnic specific questions were changed to include more general terms and we added e.g. flatbread to describe the specific bread types as most Danish and Pakistani are familiar with this term.
Table 7
Examples of problems found by cognitive interviews, proportion of problems among the participants and specific solutions.

<table>
<thead>
<tr>
<th>Food group</th>
<th>Problematic questions – examples</th>
<th>Problem</th>
<th>Problem code [1-4]</th>
<th>Proportion 1</th>
<th>Proportion 1</th>
<th>Specific changes of problematic question examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish/meat</td>
<td>Choose what kind of fish you eat on bread and how often. Portion sizes are chosen on the right side (A-F), see pictures 7,8,9</td>
<td>Participants forget to fill out the second part of the question – portion sizes</td>
<td>1</td>
<td>5/7</td>
<td>4/6</td>
<td>Divide the question into two separate questions instead of asking two questions in one</td>
</tr>
<tr>
<td>Milk</td>
<td>Have you been drinking milk (hot and cold) in the past 3 months?</td>
<td>Participants were confused by what milk this covers. All? Also in tea and coffee?</td>
<td>2</td>
<td>5/7</td>
<td>3/6</td>
<td>Add a help text explaining that this does not cover milk in tea/coffee or chocolate milk</td>
</tr>
<tr>
<td>Fats</td>
<td>Which of the following products do you use most regularly when cooking?</td>
<td>Many participants use oil, this option is within answer category ‘other fats’ because it contains no vitamin D</td>
<td>3</td>
<td>5/7</td>
<td>6/6</td>
<td>Add oil as an answer possibility even if it does not contain any vitamin D, so that people feel they can deliver their answer</td>
</tr>
<tr>
<td>Bread</td>
<td>Did you consume chapatti (roti) or paratha during the past 3 months?</td>
<td>I don’t know what this is, why are you asking this?</td>
<td>4</td>
<td>3/7</td>
<td>0/6</td>
<td>Make question more general by adding more familiar types such as flat bread</td>
</tr>
</tbody>
</table>

1Proportion of participants having problems with the specific question
4.2.2 Summary

By use of cognitive interviews; a method from the field of social sciences, we identified several problems in the online questionnaire of language as well as structural character. The questionnaire were updated based on the results from the cognitive interviews in order to tailor it to the two specific ethnic groups. The data quality of the FFQ was improved considerably by use of methods from the social sciences, although not validated against other dietary assessment methods or the original questionnaire.
4.3 **Vitamin D fortified foods improve wintertime vitamin D status in women of Danish and Pakistani origin living in Denmark: A randomised controlled trial.**

4.3.1 **Results**

The following results are presented in Paper II (appendix A). The dietary intake of vitamin D was low among women of Danish as well as Pakistani origin, 1.5 (1.0 ; 2.0) µg/day and 1.1 (1.0 ; 2.0) µg/day, respectively. The baseline serum 25(OH)D concentration was 49.6 (18) nmol/L and 46.9 (22) nmol/L among women of Danish and Pakistani origin, respectively, **Table 8**. The mean baseline serum 25(OH)D concentration was similar \( (P = 0.43) \) among both ethnic groups. At baseline, none of the participants, irrespective of ethnicity, had serum 25(OH)D concentrations < 10 nmol/L. The prevalence of serum 25(OH)D concentrations < 30 nmol/L and < 50 nmol/L was 9 and 50 % of the women of Danish origin and 24 and 32 % of the women of Pakistani origin, respectively.
Table 8  
Baseline characteristics of participants by ethnicity

<table>
<thead>
<tr>
<th></th>
<th>Danish Total (n=66)</th>
<th>Pakistani Total (n=70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Born in Denmark (%)</td>
<td>99</td>
<td>33</td>
</tr>
<tr>
<td>Mean Age (y)</td>
<td>33 (11)</td>
<td>36 (9)</td>
</tr>
<tr>
<td>Mean weight in kg (SD)</td>
<td>68 (13)</td>
<td>70 (12)</td>
</tr>
<tr>
<td>Mean BMI (kg/m²)</td>
<td>24 (5)</td>
<td>27 (5)*</td>
</tr>
<tr>
<td>Mean fat percentage (%)</td>
<td>31 (8)</td>
<td>37 (6)*</td>
</tr>
<tr>
<td>Mean baseline serum 25(OH)D</td>
<td>49.6 (18)</td>
<td>46.9 (22)</td>
</tr>
<tr>
<td>&lt; 9.9 nmol/L, n (%)</td>
<td>0 (0)</td>
<td>0 (0) *</td>
</tr>
<tr>
<td>≥ 10 - &lt; 29.9 nmol/L, n (%)</td>
<td>6 (9)</td>
<td>17 (24)</td>
</tr>
<tr>
<td>≥ 30 - &lt; 49.9 nmol/L, n (%)</td>
<td>33 (50)</td>
<td>22 (32)</td>
</tr>
<tr>
<td>≥ 50 nmol/L, n (%)</td>
<td>27 (41)</td>
<td>31 (44)</td>
</tr>
<tr>
<td>Mean PTH at baseline (pmol/L)</td>
<td>5.0 (1.7)</td>
<td>4.4 (1.8)*</td>
</tr>
<tr>
<td>Median vitamin D intake from the diet (µg/day)</td>
<td>1.5 (1.0 ; 2.0)</td>
<td>1.1 (1.0 ; 2.0)*</td>
</tr>
<tr>
<td>Total median vitamin D intake from vitamin D suppl. and multivitamin suppl. (µg/day)</td>
<td>2.9 (1.8; 9.0)</td>
<td>13 (6.8; 29.3)*</td>
</tr>
<tr>
<td>Median Calcium intake from the diet (mg/day)</td>
<td>465 (324 ; 688)</td>
<td>441 (260 ; 589)</td>
</tr>
<tr>
<td>Vitamin D supplements at baseline (%)</td>
<td>15</td>
<td>61*</td>
</tr>
<tr>
<td>Multivitamin supplements at baseline (%)</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Alcohol drinkers (%)</td>
<td>86</td>
<td>1.4*</td>
</tr>
<tr>
<td>Wearing hijab (%)</td>
<td>0</td>
<td>43*</td>
</tr>
</tbody>
</table>

\*Means and SD unless otherwise specified. If non-normally distributed, medians and 25\textsuperscript{th} and 75\textsuperscript{th} percentiles. No variables were transformed for analysis.

\*Means significantly different from women of Danish origin; Unpaired t test, P <0.05

\*Percentage significantly different from women of Danish origin; Pearson’s chi² test, P <0.05

The analysis of the crisp-bread showed a slightly higher vitamin D concentrations than expected due to a calculation error in the production of the flour mix, whereas the eggs contained less vitamin D than expected due to lower vitamin D (D3 and 25(OH)D3) doses in the chicken feed than expected, and this affected the total daily dose of the fortified foods which was thus ≈ 30 µg/day.
The participants of Danish origin in the fortified group consumed a significantly higher amount of vitamin D (µg/day) from the provided fortified foods (P < 0.001), compared to the participants of Pakistani origin reflecting the compliance of 92% among participants of Danish origin and 73% among participants of Pakistani origin (P < 0.05). The consumed study foods contributed to a median (25th, 75th percentiles) daily intake of vitamin D of 32.0 (27.0, 34.4) µg/day in the participants of Danish origin randomised to the fortified food study group, and 24.2 (19.2, 30.8) µg/day in the participants of Pakistani origin randomised to the fortified food study group. The study foods used in the ODIN FOOD trial were yoghurt, cheese, eggs and crisp-bread. The distribution of vitamin D intakes of the fortified study groups are shown in Figure 11 in which the participants of Pakistani origin (blue dots) are grouped to the left.

Figure 11. Daily dose of vitamin D (µg/day) of the fortified groups (Danish and Pakistani) plotted against serum 25(OH)D concentration (nmol/L).
**Effect of intervention**

The mean (SD) endpoint serum 25(OH)D concentration among the women of Danish origin in the fortified food group was 77.8 (15) nmol/L, whereas among the women of Pakistani origin in the fortified food group, it was significantly ($P < 0.01$) lower at 54.7 (18) nmol/L (Table 9). Following the intervention, none of the women of Danish origin in the fortified group had a serum 25(OH)D concentration < 30 nmol/L. Among the women of Pakistani origin, 3 and 41% of the fortified food group had an endpoint serum 25(OH)D concentration < 30 and < 50 nmol/L, respectively.

**Table 9**

Total vitamin D intake, and serum 25(OH)D concentration and PTH concentration at baseline and endpoint in each of the four study groups

<table>
<thead>
<tr>
<th>Intervention groups</th>
<th>DK Placebo ($n=35$)</th>
<th>DK Fortified ($n=31$)</th>
<th>PA Placebo ($n=37$)</th>
<th>PA Fortified ($n=33$)</th>
<th>$P^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total vitamin D Intake</strong>, $\mu$g/d</td>
<td>1.5 (1.0, 2.0)$^{a}$</td>
<td>32.0 (27.0, 34.4)$^{b}$</td>
<td>1.1 (0.8, 1.4)$^{a}$</td>
<td>24.2 (19.2, 30.8)$^{c}$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Serum 25(OH)D, nmol/L:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>46.2 (19)$^{d}$</td>
<td>53.3 (17)</td>
<td>49.0 (23)</td>
<td>44.5 (21)</td>
<td>0.31</td>
</tr>
<tr>
<td>Endpoint</td>
<td>44.0 (17)$^{a}$</td>
<td>77.8 (14)$^{b}$</td>
<td>36.5 (16)$^{a}$</td>
<td>54.7 (18)$^{c}$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Change</td>
<td>-2.8 (9)$^{a}$</td>
<td>26.4 (16)$^{b}$</td>
<td>-11.2 (12)$^{a}$</td>
<td>10.5 (18)$^{c}$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Serum PTH, pmol/L:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.3 (1.9)</td>
<td>4.9 (1.6)</td>
<td>4.2 (1.8)</td>
<td>4.5 (1.8)</td>
<td>0.07</td>
</tr>
<tr>
<td>Endpoint</td>
<td>4.7 (1.4)</td>
<td>4.4 (1.8)</td>
<td>4.3 (1.8)</td>
<td>4.3 (1.5)</td>
<td>0.97</td>
</tr>
<tr>
<td>Change</td>
<td>-0.54 (9.0)</td>
<td>-0.42 (16)</td>
<td>-0.002 (12)</td>
<td>-0.27 (18)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

$^a$DK = Danish, PA = Pakistani; 25(OH)D = 25-hydroxyvitamin D; PTH, parathyroid hormone.

$^b$Total vitamin D intake during study; placebo groups only had dietary intake, fortified groups had diet plus study fortified foods.

$^c$Median (25th, 75th percentiles).

$^d$Means (SD), all such values.

$^e$P values for baseline comparisons by intervention group were determined with the use of a simple one-way ANOVA, followed by a Tukey HSD test.

$^{a,b,c}$Different superscript letters represent significant ($P<0.01$) differences in group means for endpoint total vitamin D intake, serum 25(OH)D concentration and change in serum 25(OH)D concentration.
The mean increase in serum 25(OH)D concentration from baseline to endpoint among the fortified food group was higher ($P < 0.01$) in the women of Danish origin ($\Delta 26.4 (16) \text{ nmol/L}$) compared to that in the women of Pakistani origin ($\Delta 10.5 (18) \text{ nmol/L}$), $P < 0.0001$. Serum 25(OH)D concentration decreased by 2.8 (9) nmol/L in the Danish placebo group and by 11.2 (12) nmol/L in the Pakistani placebo group over the 12 weeks of winter ($P = 0.02$), Figure 12 and Table 9.

![Figure 12. The unadjusted change in serum 25(OH)D concentrations (nmol/L) following intervention in the four study groups.](image)

To assess the adjusted intervention effect we performed an analysis of covariance (ANCOVA) including factors influencing the change in serum 25(OH)D concentration following the intervention. The ANCOVA was done in two steps, firstly a minimal adequate model (Model 1) and secondly, a maximal model (Model 2) including covariates known to affect the change in vitamin D status following fortification or supplementation (Table 10).
### Table 10
Analysis of covariance models exploring the intervention effects on serum 25(OH)D response.

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Effect (2.5;97.5) % CI</th>
<th>( P )</th>
<th>Effect (2.5;97.5) % CI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intercept</strong>³</td>
<td>16.5 (9.76; 23.2)</td>
<td>&lt;0.0001 (***)</td>
<td>33.8 (19.6; 48.1)</td>
<td>&lt;0.0001 (***)</td>
</tr>
<tr>
<td><strong>Baseline 25(OH)D</strong></td>
<td>-0.41 (-0.52; -0.30)</td>
<td>&lt;0.0001 (***)</td>
<td>-0.42 (-0.53; -0.30)</td>
<td>&lt;0.0001 (***)</td>
</tr>
<tr>
<td><strong>Ethnicity:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Danish</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Pakistani</strong></td>
<td>-8.41 (-13.9; -2.14)</td>
<td>0.008 (***)</td>
<td>-6.12 (-12.14; -0.07)</td>
<td>0.05 (*)</td>
</tr>
<tr>
<td><strong>Danish</strong>*Fortified**</td>
<td>31.1 (25.0; 37.2)</td>
<td>&lt;0.0001 (***)</td>
<td>33.8 (19.6; 48.1)</td>
<td>&lt;0.0001 (***)</td>
</tr>
<tr>
<td><strong>Pakistani</strong>*Fortified**</td>
<td>20.3 (14.3; 26.3)</td>
<td>&lt;0.0001 (***)</td>
<td>27.7 (12.2; 43.3)</td>
<td>&lt;0.0001 (***)</td>
</tr>
<tr>
<td><strong>BMI at baseline</strong></td>
<td>-0.64 (-1.16; -0.12)</td>
<td>0.02 (*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>-0.05 (0.006; 0.20)</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

³Model 1 (minimal): change in vitamin D status ~ Baseline status + intervention group + (ethnicity*intervention group).

²Model 2 (maximal): change in vitamin D status ~ Baseline status + intervention group + (ethnicity*intervention group) + BMI + Age.

³The reference group is included in the intercept: Danish ethnicity and placebo study group.

Based on the output of Model 1, 12 weeks of intervention with vitamin D fortified foods, compared to that of an equivalent woman in the non-fortified group, resulted in an adjusted change of endpoint serum 25(OH)D concentration of 31.1 nmol/L (25.0; 37.2) and 20.3 nmol/L (14.3; 26.3) in the women of Danish and Pakistani origin, respectively (Table 10). The intervention effect was significantly higher in the Danish group compared to the Pakistani group (\( P = 0.008 \)).

In terms of other factors which affected the response of serum 25(OH)D to intervention, the ANCOVA models showed that baseline 25(OH)D status and BMI had a negative effect on the change in serum 25(OH)D concentration following the intervention, however the effect of BMI was very small. According to the model, participants with e.g. a higher baseline serum 25(OH)D concentration had an expected lower increase in serum 25(OH)D concentration following the intervention (Table 10 and Figure 13).
Results

Figure 13. The change in vitamin D status following the intervention plotted against baseline serum 25(OH)D concentration (nmol/L).

4.3.2 Summary

The dietary intake of vitamin D was below 2 µg/day among both ethnicities. Vitamin D fortification of 30 µg/day, provided in four different foods, for 12 weeks during winter was effective in increasing vitamin D status and reducing the prevalence of vitamin D deficiency among women of Danish and Pakistani origin living in Denmark. Women of Pakistani origin had a lower response to the intervention than did women of Danish origin. Compliance to the fortified foods was significantly higher among the women of Danish origin compared to women of Pakistani origin.
4.4 Effect of vitamin D fortified foods on bone markers and muscle strength during winter in women of Pakistani and Danish origin living in Denmark: A randomised controlled trial.

4.4.1 Results

The following results are presented in Paper III (appendix A). Baseline characteristics including, anthropometrics, dietary intake of vitamin D and calcium, vitamin D status, self-reported physical activity and health are listed in Table 11. Assessment of the baseline markers of bone turnover revealed significant ethnic differences of OC, a marker of bone formation, with higher concentrations among the women of Danish origin (t test, $P < 0.001$, data not shown). Baseline handgrip and knee extension strength were 4.8 and 6.3 kg higher among participants of Danish origin compared to participants of Pakistani origin (both $P < 0.001$, data not shown). The women of Pakistani origin had a slightly higher BMI and fat percentage compared to the women of Danish origin (both $P < 0.001$).

Among the participants of Danish origin 24% reported their health as “very good”; this was only the case with 7% among the participants of Pakistani origin. Accordingly, 33% of the women of Pakistani origin reported “somewhat bad”, whereas only 4% of the women of Danish origin did. When asked to compare the overall health with others of the same age, 6% and 26% of the women of Danish and Pakistani origin, respectively, reported “worse”.
Table 11
Baseline characteristics by ethnicity and study group

<table>
<thead>
<tr>
<th></th>
<th>Women of Danish origin</th>
<th>Women of Pakistani origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (n=66)</td>
<td>Total (n=70)</td>
</tr>
<tr>
<td>Born in Denmark (%)</td>
<td>99</td>
<td>32</td>
</tr>
<tr>
<td>Mean Age in years (SD)</td>
<td>33 (11)</td>
<td>36 (9)</td>
</tr>
<tr>
<td>Mean weight in kg (SD)</td>
<td>68 (13)</td>
<td>69 (12)</td>
</tr>
<tr>
<td>Mean BMI (kg/m²)</td>
<td>24 (5)</td>
<td>27 (5)</td>
</tr>
<tr>
<td>Mean fat percentage (%)</td>
<td>31 (8)</td>
<td>37 (6)</td>
</tr>
<tr>
<td>Serum 25(OH)D nmol/L</td>
<td>50 (18)</td>
<td>47 (22)</td>
</tr>
<tr>
<td>Mean plasma creatinine (µmol/l)</td>
<td>61.5 (9.7)</td>
<td>58.0 (8.8)</td>
</tr>
<tr>
<td>Median dietary vitamin D intake µg/d*</td>
<td>1.5 (0.8)</td>
<td>1.1 (0.7)</td>
</tr>
<tr>
<td>Median dietary Calcium intake mg/d</td>
<td>464 (375)</td>
<td>441 (362)</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>Alcohol drinkers (%)¥</td>
<td>86</td>
<td>1.4</td>
</tr>
<tr>
<td>Wearing hijab (%)¥</td>
<td>0</td>
<td>43</td>
</tr>
</tbody>
</table>

Total physical activity

<table>
<thead>
<tr>
<th>Physical activity score, no job, n (%)</th>
<th>n total DK = 3</th>
<th>n total Pa = 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Sedentary</td>
<td>0 (0%)</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>2: Light</td>
<td>1 (33%)</td>
<td>15 (68%)</td>
</tr>
<tr>
<td>3: Medium</td>
<td>2 (67%)</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>4: Heavy</td>
<td>0 (0%)</td>
<td>1 (4%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Physical activity score, job and leisure, n (%)</th>
<th>n total DK = 63</th>
<th>n total Pa = 48</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Sedentary</td>
<td>5 (8%)</td>
<td>10 (21%)</td>
</tr>
<tr>
<td>2: Light</td>
<td>31 (49%)</td>
<td>28 (58%)</td>
</tr>
<tr>
<td>3: Medium</td>
<td>22 (35%)</td>
<td>9 (19%)</td>
</tr>
<tr>
<td>4: Heavy</td>
<td>5 (8%)</td>
<td>1 (2%)</td>
</tr>
</tbody>
</table>
### Results

<table>
<thead>
<tr>
<th>Self-rated health, n (%)</th>
<th>n total DK = 66</th>
<th>n total Pa = 70</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Very good</td>
<td>24 (36%)</td>
<td>5 (7%)</td>
</tr>
<tr>
<td>2: Somewhat good</td>
<td>37 (56%)</td>
<td>34 (49%)</td>
</tr>
<tr>
<td>3: Somewhat bad</td>
<td>4 (6%)</td>
<td>23 (33%)</td>
</tr>
<tr>
<td>4: Very bad</td>
<td>1 (2%)</td>
<td>8 (11%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Self-rated health compared to others of same age, n (%)</th>
<th>n total DK = 66</th>
<th>n total Pa = 70</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: A lot better</td>
<td>6 (9%)</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>2: Somewhat better</td>
<td>16 (24%)</td>
<td>21 (30%)</td>
</tr>
<tr>
<td>3: The same</td>
<td>40 (61%)</td>
<td>27 (38%)</td>
</tr>
<tr>
<td>4: Worse</td>
<td>4 (6%)</td>
<td>18 (26%)</td>
</tr>
</tbody>
</table>

*Means and SD unless otherwise specified. If non-normally distributed, medians and 25th and 75th percentiles.

*Significant difference between Danish (all) and Pakistani (all) women in an unpaired t test ($P < 0.05$).

$\dagger$Significant difference between Danish (all) and Pakistani (all) women in a Pearson’s chi² test.

Although the intervention was effective in increasing the mean serum 25(OH)D concentration after 12 weeks of fortification during winter (the change in serum 25(OH)D concentration among Danish and Pakistani were $\Delta$ 26.4 (16) nmol/L and $\Delta$ 10.5 (18) nmol/L, respectively, among the women randomised to the fortified foods) the markers of bone formation OC, BALP, P1NP and the marker of bone resorption, CTX, did not respond significantly to the intervention. Only the baseline concentration of each marker of bone turnover had a significant influence on the endpoint concentration of the specific bone marker as assessed in linear models of Table 12.
Table 12
The effect of intervention with vitamin D fortified foods on the markers of bone turnover OC, BALP, P1NP and CTX, analysed in an ANCOVA including the baseline covariates that may influence the outcome (age, ethnicity, intervention group and BMI)

### Δ OC

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept(^1)</td>
<td>6.13</td>
<td>(1.16; 11.1)</td>
<td>0.016 (*)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.001</td>
<td>(-0.07; 0.07)</td>
<td>0.98</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>-0.53</td>
<td>(-0.88; 1.94)</td>
<td>0.46</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>0.75</td>
<td>(-2.05; 0.54)</td>
<td>0.25</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.10</td>
<td>(-0.26; 0.06)</td>
<td>0.22</td>
</tr>
<tr>
<td>Baseline OC</td>
<td>-0.20</td>
<td>(-0.28; -0.12)</td>
<td>&lt;0.0001 (***)</td>
</tr>
</tbody>
</table>

### Δ BALP

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept(^1)</td>
<td>0.50</td>
<td>(-2.6; 3.56)</td>
<td>0.75</td>
</tr>
<tr>
<td>Age</td>
<td>0.02</td>
<td>(-0.03; 0.07)</td>
<td>0.42</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>0.08</td>
<td>(-0.91; 1.07)</td>
<td>0.88</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>0.19</td>
<td>(-0.75; 1.13)</td>
<td>0.70</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.05</td>
<td>(-0.07; 0.16)</td>
<td>0.42</td>
</tr>
<tr>
<td>Baseline BALP</td>
<td>-0.25</td>
<td>(-0.33; -0.17)</td>
<td>&lt;0.0001 (***)</td>
</tr>
</tbody>
</table>

### Δ P1NP

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept(^1)</td>
<td>13.0</td>
<td>(0.66; 25.4)</td>
<td>0.04 (*)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.16</td>
<td>(-0.35; 0.03)</td>
<td>0.10</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>1.55</td>
<td>(-2.00; 5.10)</td>
<td>0.39</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>-1.83</td>
<td>(-5.21; 1.56)</td>
<td>0.29</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.04</td>
<td>(-0.37; 0.45)</td>
<td>0.85</td>
</tr>
<tr>
<td>Baseline P1NP</td>
<td>-0.19</td>
<td>(-0.28; -0.10)</td>
<td>&lt;0.0001 (***)</td>
</tr>
</tbody>
</table>
Fortification with vitamin D did not have an effect on the muscle strength measured as hand grip strength in a linear model, only the baseline hand grip strength had a significant influence on the endpoint strength (Table 13). Analysis of the knee extension strength showed a negative influence of Pakistani ethnicity compared to Danish ethnicity. Thus, following the intervention, we saw an approximately 2 kg difference in the change in knee extension strength between the participants of Danish and Pakistani origin, when adjusting for intervention group, BMI and baseline knee strength.
Table 13
The change in muscle strength measured as handgrip strength and knee extension strength and a 30 second chair-standing test, analysed in an ANCOVA including the baseline covariates that may influence the outcome (age, ethnicity, intervention group and BMI)

### Δ Handgrip strength

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept¹</td>
<td>6.88</td>
<td>(3.34;10.4)</td>
<td>&lt;0.0001 (***)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.03</td>
<td>(-0.05;0.05)</td>
<td>0.99</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>-0.52</td>
<td>(-1.64;0.60)</td>
<td>0.36</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>0.40</td>
<td>(-0.60;1.40)</td>
<td>0.43</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.01</td>
<td>(-0.10;0.13)</td>
<td>0.88</td>
</tr>
<tr>
<td>Baseline handgrip strength</td>
<td>-0.22</td>
<td>(-0.31;-0.12)</td>
<td>&lt;0.0001 (***)</td>
</tr>
</tbody>
</table>

### Δ Knee extension strength

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept¹</td>
<td>9.93</td>
<td>(4.26;15.6)</td>
<td>&lt;0.0001 (***)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.03</td>
<td>(-0.11;0.05)</td>
<td>0.46</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>-1.95</td>
<td>(-3.67;-0.22)</td>
<td>0.03 (*)</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>1.21</td>
<td>(-0.35;2.78)</td>
<td>0.13</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.02</td>
<td>(-0.20;0.17)</td>
<td>0.87</td>
</tr>
<tr>
<td>Baseline knee extension strength</td>
<td>-0.25</td>
<td>(-0.37;-0.12)</td>
<td>&lt;0.0001 (***)</td>
</tr>
</tbody>
</table>

### Δ Chair-standing test

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept¹</td>
<td>5.99</td>
<td>(0.90;11.1)</td>
<td>0.02 (*)</td>
</tr>
<tr>
<td>Age</td>
<td>0.02</td>
<td>(-0.05;0.09)</td>
<td>0.58</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>-0.08</td>
<td>(-1.42;1.25)</td>
<td>0.90</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>0.95</td>
<td>(-0.30;2.21)</td>
<td>0.14</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.16</td>
<td>(-0.31;-0.01)</td>
<td>0.04 (*)</td>
</tr>
<tr>
<td>Baseline chair-standing</td>
<td>-0.06</td>
<td>(-0.17;0.05)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

¹The reference group is included in the intercept: Danish ethnicity and placebo study group
4.4.2 Summary

Consumption of vitamin D fortified foods for 12 weeks did not result in significant changes of the bone turnover markers OC, BALP, P1NP and CTX. Muscle strength measured as hand grip strength and chair-stand test did not change significantly following the intervention. However, the change in knee extension strength following the intervention showed a tendency to be higher among the participants of Danish origin compared to participants of Pakistani origin.
5. DISCUSSION

5.1 Discussion of main results

In a condensed summary of the main results, the present ODIN FOOD study demonstrated that carefully planned vitamin D fortification of four different foods given for 12 weeks during winter (January through to April) was effective in increasing serum 25(OH)D concentration among Danish and Pakistani women and reducing the prevalence of serum 25(OH)D concentrations < 30 and 50 nmol/L during the winter months. The mean baseline serum 25(OH)D concentration was 49.6 (18) and 46.9 (22) nmol/L in the Danish and the Pakistani group, respectively.

The Danish fortified group increased more than the fortified Pakistani group resulting in an endpoint serum 25(OH)D concentration of 77.8 (14) nmol/L among Danish women and 54.7 (18) nmol/L among women of Pakistani origin. Compliance among the women of Pakistani origin was lower compared to the women of Danish origin. The increase in serum 25(OH)D concentration following the intervention, among the participants receiving fortified foods, was dependent on baseline serum 25(OH)D concentration, ethnicity and to a lesser degree BMI. The results show a successful preventive effect of vitamin D fortified foods in a in a population of women at risk of deficiency.

5.1.1 Effects of vitamin D fortification on vitamin D status

In the present ODIN FOOD study, the increase in serum 25(OH)D concentration seen among the participants receiving vitamin D fortified foods was dependent on baseline serum 25(OH)D concentration and ethnicity. The dose consumed by the women of Danish and Pakistani origin in the groups receiving fortified foods were thus 32.0 (27.0; 34.4) and 24.2 (19.2; 30.8) µg/day and this was sufficient to ensure that none of the women of Danish origin were < 50 nmol/L however, 41% of the women of Pakistani origin were < 50 nmol/L at endpoint, in the groups receiving the fortified foods (Paper II).

The cut-off value often used in Denmark to define vitamin D insufficiency is serum 25(OH)D concentrations < 50 nmol/L, however, the evidence that forms basis for this cut-off may not be adequate. The question is still left unanswered; how much vitamin D is the optimal for health at the different life stages? This remains one of the key challenges in vitamin D research. There are several modifiable factors blocking the way to more clear and meaningful cut-off values for vitamin D status e.g. analytical variability, study heterogeneity, UVB exposure and methods by which the
regression analysis of the dose-response are performed. Cashman et al. have highlighted the lack of individual participant data in the regression analyses behind the DRV’s presented by the IOM and argue that by use of combined data from each RCT we are missing important information on the between-participant variability that seem to be the most important, given the goal of the DRV’s is to give recommendations to the individual\(^{(119)}\).

This being said, the results from the present ODIN FOOD study are in line with the findings of a number of the fortification studies, summarized in Table 3 of the background section. The effect of vitamin D fortification in the majority of the studies included in Table 3 is convincingly positive in terms of maintaining or increasing vitamin D status, depending on the concentration of the fortified foods, baseline serum 25(OH)D concentration, season and duration of study\(^{(81, 88, 96, 104)}\). A large Danish study assessed the effects of vitamin D fortified foods (milk and bread) among ethnic Danish families (total n = 782) during winter\(^{(104)}\). The daily vitamin D contribution from the fortified foods was 9.4 µg and the results indicated a successful prevention of winter vitamin D deficiency among the fortified individuals since < 1% and 16 % of the participants in the fortified study group had winter vitamin D status < 30 and 50 nmol/L, respectively\(^{(104)}\).

Results from the Finnish fortification program recently assessed in an 11-year follow-up study likewise showed a positive effect of fortification, here tested in a large scale implementation of a national voluntary, but comprehensive, vitamin D fortification policy. The study revealed an average increase in vitamin D status of 20 nmol/L (95% CI: 19; 21) among supplement non-users, no records of ethnic background was obtained\(^{(98)}\). There seem little doubt about the positive effect of vitamin D fortification on vitamin D status, however the magnitude of the effect depends on the rate of compliance with the fortified foods as well as several factors of which baseline vitamin D status may be the primary.

5.1.2 Baseline serum 25(OH)D concentration

The ODIN FOOD study aimed at studying the effects of vitamin D fortification in a risk group of vitamin D deficiency. In order to recruit women at risk of vitamin D deficiency we used restrictive inclusion criteria allowing only women with a low intake of fish and supplements as well as a low UVB exposure to be enrolled in the study. Despite vitamin D exposure-specific inclusion criteria, the mean baseline vitamin D status of the participants in this trial were 49.6 (18) and 46.9 (22) nmol/L, measured among participants of Danish and Pakistani origin, respectively (Paper II). This baseline vitamin D status was higher than expected, especially among the women of Pakistani
origin, when compared with data from a previous Danish study which included only participants of Pakistani origin living in Denmark. In that study, conducted in 2002 (all seasons), a low vitamin D status was reported for both girls and women of Pakistani origin (median serum 25(OH)D concentration was 10.9 and 12.0 nmol/L, respectively)\(^5\). It should be noted, however, that the earlier study by Andersen et al.\(^5\) was performed prior to the initiation of the VDSP and this may to some degree affect the comparability of serum 25(OH)D data. More importantly, several factors may have affected the change in vitamin D status over the course of the last 15+ years. For example, it may partly be a result of the strong focus on vitamin D in the general public as well as public health actions initiated by the Danish health authorities between 2005 and 2010, targeting the general population, ethnic minorities as well as health professionals\(^{125, 126}\). An additional result of the mentioned public health actions may be an increased intake of dietary supplements containing vitamin D. In the present ODIN FOOD study we found that 61 % of the Pakistani women reported a baseline use of vitamin D containing supplements (not including multivitamins), this was only 15 % for the Danish women (\textbf{Paper II}), importantly, participants taking supplements prior to the study start were asked to stop their supplement routine during the study.

\textbf{5.1.3 Effect of vitamin D on markers of bone turnover}

The ODIN FOOD intervention with vitamin D fortified foods had no effect on the markers of bone turnover OC, CTX, BALP and P1NP (\textbf{Paper III}). Observational studies have reported increased bone resorption with vitamin D deficiency\(^{127, 128}\). However, when studied in RCT’s the evidence appears mixed for markers of bone turnover\(^{112, 129-132}\) as well as muscle strength\(^{32}\).

A Danish study assessed the effects of one year of vitamin D supplementation (10 or 20 µg/day) on bone markers among immigrant families in Denmark (ages 13-53 years)\(^{112}\). The baseline serum 25(OH)D concentrations were 10.9, 12.0 and 20.7 nmol/l for girls, women and men, respectively, however, no effects on the markers of bone turnover OC and urinary pyridinoline and deoxypyridinoline were seen\(^5, 112\). Likewise an RCT from Norway assessed an immigrant population with a baseline serum 25(OH)D concentration of 28.9 (17.6) nmol/L, after 16 weeks of vitamin D supplementation (10 and 25 µg/day) they found no changes in serum P1NP or CTX\(^{129}\). A more recent Austrian study by Schwetz and colleagues studied hypertensive adults (mean age 62 y) with a mean baseline serum 25(OH)D concentration of 55 nmol/L and found no effect of 8 weeks of vitamin D supplementation (70 µg) on BALP, CTX, P1NP or OC\(^{131}\). On the contrary, a Brazilian study assessing menopausal women (mean age 59 y) with a normal BMD, recently found
a significant decrease in the bone resorption marker CTX\(^{(132)}\). However, the study also found a decrease of the bone formation marker P1NP following 9 months of vitamin D supplementation with 25 µg/day in the supplemented group compared to baseline, however no group differences were found (compared to placebo). The baseline status of the participants in the vitamin D supplemented group were 37.4 (18.7) nmol/L, increasing to 68.6 (26.0) nmol/L following the intervention, the placebo group decreased from 42.2 (16.7) nmol/L to 34.4 (15.0) nmol/L and no changes were seen in the bone turnover\(^{(132)}\).

The relatively high baseline serum 25(OH)D concentration seen among the women of Danish and Pakistani origin of 49.6 (18) and 46.9 (22) nmol/L, respectively, may be a reason for not finding an effect of vitamin D supplementation on markers of bone turnover. When vitamin D status is above the suggested cut-off optimal concentration (> 50 nmol/L), PTH concentration will be within the normal range and calcium will not be released from the bone matrix to support the calcium balance, thus the bones are neither resorbed added upon in any detectable degree\(^{(128)}\).

### 5.1.4 Effect of vitamin D on muscle strength

In the present ODIN FOOD study we saw that the baseline handgrip and knee extension strength were 4.8 and 6.3 kg higher among participants of Danish origin compared to participants of Pakistani origin. After the intervention, we saw an approximately 2 kg difference in the change of knee extension strength between the participants of Danish and Pakistani origin, when adjusting for intervention group, BMI and baseline knee strength (\textit{Paper III}). Though the intervention effect on muscle strength in our study was not convincing, we cannot exclude the possibility that effect could have been found in participants with a baseline serum 25(OH)D concentration < 30 nmol/L as suggested by some studies.

The majority of the studies assessing vitamin D and muscle strength (observational as well as RCT’s) have been performed in older adults (> 50 years)\(^{(25,26)}\). Two larger systematic review and meta-analyses by Stockton \textit{et al.} and Beaudart \textit{et al.} including 17 and 30 RCT’s, respectively, found that the effect of vitamin D supplementation may only be present among vitamin D deficient people (Vitamin D concentrations < 25-30 nmol/L)\(^{(25,26)}\). A smaller systematic review and meta-analysis assessing healthy young adults (18-40 y) included six RCT’s and one controlled study assessing upper and lower limb strength and vitamin D. They conclude that vitamin D supplementation had a significant positive effect on muscle strength, however they argue that the lack of studies in the young adult population calls for further studies\(^{(133)}\).
A Danish study by Glerup et al. assessed the effects of high-dose (2500 µg/week for 1 month followed by 2500 µg/month for 5 months) vitamin D supplementation in women of Arabic origin (n = 55) with a Danish control group (n = 22). The women of Arabic origin had a baseline serum 25(OH)D concentration of 6.7 (0.6) nmol/L compared to the women of Danish origin having a serum 25(OH)D concentration of 47.1 (4.6) nmol/L. They observed a very low baseline knee extension strength (maximum voluntary), among the women of Arabic origin of 26.4 kg, which was 34% lower than the 32.7 kg among the women of Danish origin. After high-dose treatment, the ethnic difference disappeared. In addition, they observed a significant reduction in reports of muscle and bone pain among the women of Arabic origin (23). Results from our present ODIN FOOD study showed an ethnic difference of 2 kg in the change of knee extension strength following intervention, which is smaller compared to the study by Glerup et al. described above.

Studies have demonstrated that vitamin D may primarily affect the lower limb muscle strength following supplementation or fortification with vitamin D (23, 134, 135), therefore knee extension strength may be a more suitable measure in future studies as compared to hand grip strength. Adding to this, vitamin D supplementation has been found to mainly have effects on type II muscle fibres (fast twitch) (23). The type II fibres may therefore not be activated during the relatively slow movements of the hand grip strength or knee extension strength measure used in the ODIN FOOD study (Paper III).

5.1.5 Ethnic differences in vitamin D response

Ethnic differences in vitamin D status have been shown in several cases comparing indigenous populations with immigrants in Norway, Finland, Sweden and Denmark (45, 6, 136, 137). A higher Bone Mineral Density and a lower incidence of fractures have been shown among black Americans despite a lower vitamin D status compared to white Americans (138, 139). Moreover, the study by Powe et al. further showed that the concentration of DBP was lower among African Americans when compared to white Americans which may help explain the lower serum 25(OH)D concentrations in black Americans (139). However, the effect of supplementation on the change in vitamin D status seems to be less clear and most studies do not find any ethnic-specific response to vitamin D supplementation or fortification. In the present ODIN FOOD study the participants were randomised to either fortified foods or placebo foods within each ethnic group to enable subsequent assessment of ethnic differences.
A Finnish study published in 2018 was designed to assess the dose-response in different ethnic groups and thus compared women of Finnish origin with women of East African (Somali) origin (n=147). They showed an equal response to vitamin D supplementation in the two ethnic groups after adjusting for baseline serum 25(OH)D\(^{(137)}\). A Norwegian study from 2007 giving multivitamin tablets and fish oil tablet with the same amount of vitamin D also enrolled participants of immigrant background\(^{(136)}\). The participants of immigrant background represented 32% and 37% of the two study groups (n = 9 and 10, respectively). Following supplementation with vitamin D through the two different sources, serum 25(OH)D concentration increased equally in both ethnic groups, therefore the change did not depend on ethnicity\(^{(136)}\). A recent RCT from the UK including women of white Europeans and South Asian descent found no ethnic difference in the response to vitamin D supplementation, however the south Asian women had a higher increase in vitamin D status, but this was hypothesised to stem from their lower baseline vitamin D status that was \(\approx 30 \text{ nmol/L}\) lower than the white European women\(^{(96)}\).

A study mentioned in the above discussion on markers of bone turnover studied the bone health of Pakistani immigrants living in Denmark\(^{(112)}\). The vitamin D status of the immigrant participant were extremely low, however one year of vitamin D supplementation with 10 or 20 \(\mu\text{g/day}\) did not result in any clinically relevant changes of BMD, Bone Area (BA), Bone Mineral Content (BMC) or the markers of bone turnover mentioned in the previous discussion section, despite four-fold and two-fold increases of serum 25(OH)D concentrations in the men and women, respectively\(^{(112)}\). Results from this study and other similar\(^{(129)}\) raises questions as to whether some population groups have a lower target for optimal vitamin D status to maintain healthy bones.

In the present ODIN FOOD study the effect of intervention with vitamin D fortified foods on vitamin D status was different in the two ethnic groups, however this ethnic difference may in part be explained by the differences in compliance with the study foods, as compliance while high (73%), was significantly lower in the Pakistani women compared to the compliance of the Danish women (92%). Assessing the distribution of baseline serum 25(OH)D concentration it was shown that despite a similar mean vitamin D status, the IQR revealed a much longer lower tail among the women of Pakistani origin which was evident as per the higher percentage \(< 30 \text{ nmol/L}\) of 24 % compared to 9 % among the women of Danish and among Pakistani origin, respectively (Paper II).

To summarise, poor vitamin D status have been found in immigrant groups of in countries with low winter UVB exposure and this may be a result of sun-habits, a high degree of skin pigmentation or
dietary habits. Based on the available evidence regarding ethnic differences in response to vitamin D supplementation we cannot exclude the possibility that differences exist and the question ‘how much vitamin D is the optimal for health at the different life stages?’ may extend to different ethnic groups and remain a key research goal.

5.1.5.1 Genetic variation in vitamin D modulating genes

Knowledge on genotypes and genetic variation in vitamin D modulating genes may in the future answer some of the questions regarding what concentrations of vitamin D are optimal for whom and aid in defining risk groups of vitamin D deficiency based on risk alleles. This may assist us in finding and comparing risk groups of vitamin D deficiency across different ethnic groups, assess disease endpoints and optimize the recommendations as well as treatments. Data from trials investigating the genetic predispositions (SNP’s in vitamin D modulating genes) to low vitamin D status and the effect of supplementation and fortification with vitamin D have been able to predict and group participants based on their genetic profile\(^\text{140, 141}\). Though most of the published studies have investigated Caucasian populations a few studies link ethnic differences in vitamin D status to genetic predictors\(^\text{142, 143}\).

Evidence from twin studies has found the heritability of serum 25(OH)D concentration to be as high as > 80 %\(^\text{144}\). However, results from a recent (2018) large-scale GWAS, studying genotypes and serum 25(OH)D concentrations of nearly 80,000 Europeans, challenge this by their findings of a considerably lower heritability (SNP heritability) of 7.4 % of a person’s vitamin D status is explained by the genetic variation of vitamin D modulating genes and they argue that environmental factors may be more important in the general population than SNP’s in terms of determining a person’s vitamin D status\(^\text{145}\).

In the present ODIN FOOD study, we have collected whole blood samples, extracted the DNA and commenced the analyses of SNP. The data are not yet ready for statistical analysis, but we aim to assess this in the near future to investigate whether differences in the increase of vitamin D status may explained by the genotype. However, since our study has quite few participants for assessing genetic variants, we aim to merge our data with data from a Finnish trial assessing serum 25(OH)D responses to vitamin D supplementation in two different ethnic groups\(^\text{137}\).
5.1.6 Public health strategies and safety

The dietary intake of vitamin D among women in Denmark is 3.0 µg/day and if we want the population to fulfil the proposed AR of 7.5 covering the need 50% of the population, action must be taken. Inadequate dietary intake of vitamin D can be tackled in different ways. E.g. public health campaigns may encourage people to increase the intake of fish. We have seen several of these in Denmark in the last decade\(^\text{(125, 126)}\), and still the dietary intake is below the AR. In the DANSDA the non-consumers of fish in the recording period were recorded as 10% of the included individuals (n=3016)\(^4\).

In Denmark, fish consumption contribute to more than 60% of the dietary vitamin D intake and we cannot reject the fact that it is an important source, however, only among consumers\(^4\). In Paper I, we saw a large variation in the daily intake of vitamin D from the habitual diet including fish, ranging from 1.0-17.7 µg/day among Danish women, Table 5. Similarly, a meta-analysis including 9 studies showed that only fatty fish were effective in maintaining vitamin D status, this was not the case with lean fish. The review conclude that in consumers, fish may be important in maintaining vitamin D status, however it may not be possible to increase vitamin D status without the use of supplements or food fortification\(^{146}\).

The variation in the content of vitamin D in different fish species is large and it is hypothesised to depend on the feed and the season among other factors\(^{146}\), e.g. farmed salmon has been found to contain only 25% of the vitamin D compared to wild\(^{147}\). In addition, the vitamin D content has been shown to vary from 2.5 to 6.9 µg/100 g, depending on the food composition database used\(^{146}\). The variation of the vitamin D content of fish in the Danish food composition database range from 1 µg/100 g in the cod (lean fish), to 30 µg/100g in wild Atlantic salmon. The farmed salmon has a content of 8.6 μg/100 g according to the Danish data\(^{12}\).

Supplementation with vitamin D may currently be the best source of vitamin D in Denmark during the winter months since the vitamin D-rich sources in the diet are scarce and the availability of fortified foods even scarcer\(^{12}\). Supplementation as a strategy to increase vitamin D intake is very effective at an individual level in consumers with a regular intake, however, high numbers of non-consumers, low adherence and the risk of high-dose intakes are ever present\(^{9, 53, 148}\).

The intake of dietary supplements in Denmark is high, 60% of female participants and 51% of male participants enrolled in a nationally representative survey (n = 4649) reported taking supplements of
some kind, participants were classified as supplement users regardless of their reported frequency of consumption\textsuperscript{(53)}. Though the intake of dietary supplements containing vitamin D is relatively high in Denmark, this is not the case for all population groups. Tetens et al. reported that individuals between 50 and 75 had significantly higher intakes compared to the 18-49 year old, similar results was shown by Knudsen et al. who found that the intake of supplements was strongly associated with age and was shown to be most prevalent among elderly women\textsuperscript{(148)}. A large American study using data from National Health and Nutrition Examination Survey (NHANES 2007-2010) found that, as we see in Denmark, more than half of Americans use dietary supplements, however large differences were found between users and non-users of supplements. Supplement users reported better health status, performed more exercise and had higher educational levels and socioeconomic status\textsuperscript{(149)}. If we want to capture the whole population by one strategy and increase the overall intake of vitamin D, supplementation may therefore not be the best solution given that it might only be effective among a selective health conscious and highly motivated group.

An alternative strategy suggested to avoid non-consumers and the low adherence seen with supplements, has been given as a large monthly or even bi-annually bolus doses of vitamin D. One review assessing 30 RCT's using large single doses of vitamin D supplementation conclude that the use of large bolus doses had a similar effect on vitamin D status as daily low-dose supplementation or even better, specifically due to low adherence to daily supplementation\textsuperscript{(150)}. However, today it is generally accepted that large bolus doses are not as safe and effective as the daily, smaller doses. Specifically, a recent high-dose vitamin D supplementation trial assessing falls and fracture risk showed increases of fall and fracture risk at very high doses after 3-5 years of supplementation duration (500 000 IU/12.5 mg) (n = 2256 women, ≥ 70 y)\textsuperscript{(63)}. A recent study by Bislev et al. found that much lower doses may also cause problems since three months of vitamin D supplementation with 70 µg/day found small, though significant negative effect of treatment on muscle strength measured as hand grip and knee flexion strength in postmenopausal women with a baseline serum 25(OH)D concentration of 31 (10) and 35 (9) in the two study groups, respectively \textsuperscript{(151)}.

In Table 3 of the background section of this thesis, a collection of studies assessing the effects of vitamin D fortification on vitamin D status was summarized. Since 2010, 18 new RCT's have been published. This not only reflects the high focus on vitamin D in recent years, but also the need for public health solutions to reach those in need of increased intakes of vitamin D, not only in Denmark, but throughout Europe.
Discussion

Well planned and continuously monitored low-dose fortification of foods with vitamin D, though not free of all pitfalls, may be a solution contributing to reaching those in need of increased vitamin D in a safe and effective manner\(^9\). Naturally, the effect of fortification is dependent on the intake of the fortified foods and non-consumers of e.g. dairy products must be considered by distributing the vitamin D into a wide range of different food products in order for a fortification program to have an impact on the entire population\(^9\).

Several studies have investigated fortification of a single food\(^{72, 73, 75, 90}\), while fewer studies have been conducted in which fortification of several foods was used\(^{9, 92, 96}\), and from a population perspective the latter could be a useful strategy. For the ODIN FOOD study we decided on an approach in which the daily dose of vitamin D was spread out into four foods in order to achieve increases of vitamin D status in as many participants as possible in an as safe manner as possible in accordance with previous studies from Finland and Denmark\(^{104, 109}\).

### 5.1.7 Fortification vehicles

Since our study participants were of both Danish and Pakistani origin, we made special considerations when choosing the fortification vehicles since acceptance of the foods in both groups was of high importance. As an example of our considerations we did not use milk as a fortification vehicle despite its suitability in previous Nordic studies\(^{98, 104, 109}\), since the prevalence of lactose intolerance among individuals of Pakistani origin is > 60 %\(^{152}\). Instead we chose plain yoghurt. Yoghurt has a lower content of lactose and is suitable for most lactose intolerant people, it can be used in breakfast, snacks and sweet or savoury cooking. Several studies have used cheese and bread or other types of flour based biscuits with good results\(^{76, 77, 94, 104}\), based on this knowledge from previous studies we used a low fat cheese and a small crisp-bread. All the study foods were provided with no cost for the participants in portions that fit into a healthy diet. The foods were all low in fat and energy. We instructed the participants to substitute their normal diet with food from the study, so that they would remain approximately isocaloric throughout the study. This was successful and we saw no changes in the body composition (BMI) of the participants following the intervention.
5.1.7.1 Biofortification and novel sources of vitamin D

Since vitamin D-rich dietary sources are rare and the acceptance of traditionally fortified foods are low in Denmark\(^{(61)}\), it may be essential to look for alternative sources for e.g. non-consumers of fish and individuals with very low intakes of fish. The general Danish population may also benefit from this since we found in Paper I that 88% of the assessed Danish women had a vitamin D intake below the Average Requirement of 7.5 \(\mu\)g/day, including consumers of fish\(^{(153)}\). Thus, there is a need for more reliable and available sources of vitamin D in the general diet. Novel sources of vitamin D may include biofortified eggs produced with the use of 25(OH)D in animal diets and UVB irradiated yeast and mushrooms.

The natural concentration of 25(OH)D in foods such as milk, eggs and meat are very low compared to concentrations of vitamin D\(_3\)\(^{(12, 154)}\). It has been hypothesised that the activity of 25(OH)D is markedly higher (up to 5 times) than the activity of D\(_3\) and this conversion factor is used in food composition databases in several countries including Denmark\(^{(12, 155)}\). However, it should be noted that a recent Danish study challenge the high conversion factor of 5 and claim that it may only be 2 or 1.5\(^{(120)}\). It is currently not allowed to add 25(OH)D directly to human foods, so the only way to make use of the possible higher activity of 25(OH)D today is to add it to animal diets.

Eggs have shown to respond effectively and in a linear manner to biofortification in terms of the increase of 25(OH)D concentration of the yolk\(^{(147)}\). However, the present ODIN FOOD study failed to replicate this linear dose-response in the eggs used in the intervention, due to a lower concentration of vitamin D and 25(OH)D in the animal diet than expected. One might speculate that a biofortified food e.g. eggs may be a novel source of vitamin D that are more easily accepted by the consumers in Denmark due to its more natural way of production compared to the traditional fortified foods, however this has not yet been tested.
5.2 Strengths and limitations

One of the greatest strengths of the present ODIN FOOD study lies in the study design, this was a real-life based design in which the participants would incorporate the study foods by substitution of their habitual intake of similar foods. We encouraged the participants to consume the same amount of the study foods every day to create a habit and incorporate the foods into their normal diet, however, we allowed for the participants to include foods from several days in one meal, allowing people to consume e.g. 4 eggs and 60 g cheese in one meal (e.g. omelette). This approach was chosen to mimic a real-life situation in order for the results to be generalized to a public health setting as well as to strengthen the study by increasing compliance and decreasing drop-out rate. The study achieved a good compliance and a low drop-out rate for this study population.

A limitation of the study was the inclusion of only one family member as opposed to the entire family, this might have affected the participant’s motivation and decreased the compliance of the study foods since the participants could not share them with family. This was very hard for some of our participants, especially the older generation of women of Pakistani origin, they normally cooked for the entire family and found it harder to incorporate the study foods into their habitual meal-routine.

As a strength, the ODIN FOOD trial was carried out in winter time when there is no cutaneous production of vitamin D, which lowers the risk of contamination of vitamin D from UVB sources. The fact that the study had to finish before the sun became stronger in April put a large time-pressure on the whole process and we had to finalise all baseline measurements within 2-3 weeks. Due to this very tight scheduled study visits we did not attempt to collect fasting blood samples as this would not have allowed us as many participants completing the study visits per day.

The intake of dietary vitamin D was very low, compared to the DANSDA (2011-13) that showed a vitamin D intake of 3 µg/day among women, however, the participants were recruited based on their low intake of fish, so this was not surprising. The dietary calcium intake recorded in the FFQ was also very low, and part of this can be explained by the questionnaire, while aiming at capturing the majority of dietary vitamin D is was not designed to capture more than 70% of dietary calcium.

A clear strength of the FFQ was the inclusion of fortified foods presently on the market in the questions and subsequent calculations. Although the prevalence of fortified foods in Danish shops is low it has a high impact on the individual dietary intake of vitamin D if consumed.
Despite having several advantages, an FFQ is affected by recall bias and participant reporting bias when asked to report their average/habitual their intake of a certain food or food group. But for the estimation of dietary vitamin D and calcium an FFQ is the best known tool. The main results from the use of cognitive interviewing process was identification of several problems in the questionnaire of language as well as structural character based on the found issues the questionnaire was changed in order to target the two specific ethnic groups. The use of qualitative methods in life science has its benefits, but also limitations. It gives the opportunity to shape the FFQ and tailor it to a very specific population.

On the limitation side, as is the case of some qualitative studies there is no existing framework for data analysis and data processing\textsuperscript{(116, 156)}. The subjectivity of the data analysis is a weakness of the method. However, in an attempt to overcome this flaw, we have applied tools such as verbal analysis protocol and Problem Coding Frame that has been previously described and used in analyses of data with qualitative characteristics\textsuperscript{(116)}. This being said, using a qualitative approach is the only method to discover perceptions, thought processes and interpretations of the participants and thereby problems in the understanding of questions and concepts that stem from poorly designed phrases and inadequately elaborated concepts in the FFQ. This method lead to improved data quality in this specific study population consisting of two different ethnic groups and we managed to discover errors and misunderstandings of concepts that would otherwise not have been found, not even in a subsequent assessment of the data. However, as a limitation, the changes were not validated against the original FFQ or a traditional dietary recording method. Also, the cognitive interviews were not recorded. Instead the researcher manually recorded the main issues during the interview.

In the ODIN FOOD study we saw a high self-reported intake of vitamin D from supplements, and although participants taking supplements prior to the study start were asked to stop their supplement routine during the study, this relatively high prevalence of supplement users among our study population was unexpected. Therefore not enough time was allocated for wash-out. The lack of a wash-out period is a limitation of the study and should be included in any future studies assessing the effects of fortified foods.

The chemical analysis of the crisp-bread showed a slightly higher concentration than expected due to a calculation error in the production of the flour mix, whereas the eggs contained less vitamin D than expected due to lower vitamin D (D3 and 25(OH)D3) doses in the chicken feed than expected,
and this affected the total daily dose from fortified foods of approximately 30 μg/day. This was an error during the production and it did not increase the risk of toxicity of vitamin D since the concentrations were still quite low. The fortified foods and biofortification is still a relatively new area in Denmark and there is still a lot to learn in order to make successful products ready for the consumers.

5.2.1 Study design and measurements

The power calculation was based on the detection of a change in serum 25(OH)D concentration of 20 nmol/L in the fortified study groups compared to the placebo groups, with a SD of 23 nmol/L. With the planned sample size the study proved to have enough power to detect the intended change in serum 25(OH)D concentration in the Danish fortified group, however not in the Pakistani fortified group, likely due to a lower compliance among the women of Pakistani origin compared to the women of Danish origin. The significantly lower compliance in the Pakistani study group affects the power to detect significant changes, however for our main outcome this was not a problem. For the secondary outcomes a lower power is more often seen and the risks of false negatives (type II error) are thus increased. This is also the case in the present ODIN FOOD study.

Recruitment bias may affect all studies since the participants responding to the recruitment may in general be more interested in their own health, which might affect the generalizability of the results. We applied a number of eligibility criteria to narrow down the general population to a well-defined risk group of vitamin D deficiency. Although this population group may be prevalent in Denmark, having many eligibility criteria naturally limits the pool of eligible participants and subsequently lowers the generalizability of the results to other types of populations. The pool of eligible and interested participants contained more women of Danish origin compared to Pakistani. This inequality reflects the number of women with Pakistani and Danish ethnicity living in the Copenhagen area. Drop-outs were equally distributed between the ethnic groups.

A training day was arranged for all the ODIN FOOD investigators prior to the study start. On this day the time plan, measurements, procedures and standard operating protocols (SOPs) were tested on test subjects (colleagues not involved in the project). Effort was put into standardizing the measurements as much as possible. For this purpose we used the same investigator in the collection of data for anthropometrics, blood samples, questionnaires and muscle strength in the vast majority of the cases. In case of two or more investigators sharing the measurement duty, careful planning
followed to book the participant to have their endpoint measurement done by the same investigator as did the baseline.

As mentioned, we saw a systematic difference in the compliance of the two ethnic groups and this may introduce bias. We included all participants in the analyses to comply with intention-to-treat, however we explored the compliance of the two ethnic groups in further detail through plots and IQR. Compliance was self-reported in a questionnaire and participants would bring the questionnaire back every two weeks at food delivery and finally at the endpoint measurements. One may argue that this method is not as reliable as compared to methods such as biomarker based compliance. However, we estimated that a bi-weekly user-friendly questionnaire would be effective, put minimal burden on the participants while at the same time be applied using limited resources.

Finally, the statistical analyses were performed by an investigator blind to the treatment allocation and the codes were only revealed after the main analyses had been carried out. This was again an effort made to minimize bias by removing the preconceptions of the investigator from the analysis process.
6. CONCLUSION

Vitamin D fortification of 30 µg/day provided in four different foods for 12 weeks during winter was effective in increasing vitamin D status and reducing the prevalence of vitamin D deficiency among women of Danish and Pakistani origin living in Denmark. The baseline serum 25(OH)D concentration was higher than expected in the Danish and the Pakistani group, with a mean (SD) baseline 25(OH)D concentrations of 49.6 (18) and 46.9 (22) nmol/L, respectively. The Danish fortified group increased more than the fortified Pakistani group resulting in an endpoint serum 25(OH)D concentration of 77.8 (14) nmol/L among Danish women and 54.7 (18) nmol/L among women of Pakistani origin. Compliance among the women of Pakistani origin was lower compared to the women of Danish origin. At endpoint the prevalence of 25(OH)D concentration < 30 nmol/L in the Danish fortified group and Pakistani fortified group were 0 % and 3 %, respectively, compared with 7 % and 11 % in their respective control groups. Fortification of four foods with a total of 30 µg/day was safe and none of the participants had too high serum 25(OH)D concentration as a result of the fortification. The increase in serum 25(OH)D concentration following the intervention, among the of the participants receiving fortified foods, was dependent on the, baseline serum 25(OH)D concentration, ethnicity and to a lesser degree BMI.

Overall the fortification modelling was successful in predicting the impact of low-dose fortification in a population of Danish women at risk of vitamin D deficiency. Adequate and safe levels of intake were present in all women in the scenarios in which fortified foods and a daily supplement of vitamin D up to 40 µg/day. A daily vitamin D supplement of 80 µg/day or more, however, would increase the risk of exceeding the UL value.

Consumption of vitamin D fortified foods for 12 weeks did not result in significant changes of the bone turnover markers OC, BALP, P1NP and CTX. Muscle strength measured as hand grip strength and chair-stand test did not change significantly following the intervention. However, the change in knee extension strength following the intervention showed a tendency to be higher among the participants of Danish origin compared to participants of Pakistani origin.
FUTURE PERSPECTIVES

Results of the present ODIN FOOD study may be applicable for designing fortification programs and shaping policies prior to implementation.

In Denmark, we have an increasingly multi-ethnic society. Research studies involving two or more ethnicities are therefore highly needed in order to give suitable health advice and ensure equality in health of all citizens in Denmark.

Large dose-response studies should be conducted to create an evidence base on how the prevalence of certain vitamin D related SNP’s link to health outcomes.

Analyses protocols and analytical standards for measuring serum 25(OH)D concentration should be harmonized and future studies should aim at performing the serum 25(OH)D analyses in standardized laboratories to enable future comparisons of studies.
8. ACKNOWLEDGEMENTS

I consider myself very fortunate to have participated on this 3-year journey as part of a European collaborative project. It has not only been a scientific journey, but a personal one as well. I could not have come this far without the invaluable support of my family, friends, supervisors, and perhaps the best colleagues in the world.

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10. APPENDICES
Appendix A: Paper I, II and III

**Paper I:** Modelling of adequate and safe vitamin D intake in Danish women using different fortification and supplementation scenarios to inform fortification policies.

**Paper II:** Vitamin D fortified foods improve wintertime vitamin D status in women of Danish and Pakistani origin living in Denmark: A randomised controlled trial.

**Paper III:** Effect of vitamin D fortified foods on bone markers and muscle strength in women of Pakistani and Danish origin living in Denmark: a randomised controlled trial.
Paper I

Modelling of adequate and safe vitamin D intake in Danish women using different fortification and supplementation scenarios to inform fortification policies
Modelling of adequate and safe vitamin D intake in Danish women using different fortification and supplementation scenarios to inform fortification policies

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Abstract

Purpose Fortification of foods with vitamin D may be a population-based solution to low vitamin D intake. We performed modelling of vitamin D from diet, fortified foods and supplements in a population of Danish women 18–50 years, a risk group of vitamin D deficiency, to inform fortification policies on safe and adequate levels.

Methods Based on individual habitual dietary vitamin D intake of female participants from the Danish National Survey of Dietary Habits and Physical Activity (DANSDA) (n = 855), we performed graded intake modelling to predict the intake in six scenarios increasing the vitamin D intake from a habitual diet without fish to habitual diet including fish, fortified foods and supplements (40/80 µg). Four different foods were used as potential foods to fortify with vitamin D.

Results The vitamin D intake was below the Average Requirement (AR) of 7.5 µg/day for 88% of the assessed women. Safe levels of intake (< 100 µg/day) were observed after adding four different fortified foods (plain yoghurt, cheese, eggs and crisp-bread) contributing with a total of 20 µg/day and a vitamin D supplement of 40 µg/day to the habitual diet. Consumption of fish, fortified foods and a vitamin D supplement of 80 µg resulted in intakes above the Tolerable Upper Intake Level (UL) < 100 µg/day.

Conclusions In a Danish female population with a low vitamin D intake, low-dose fortification of different foods with vitamin D may be an effective and safe population-based approach.

Keywords Vitamin D · Fortification · Intake modelling · Danish women

Introduction

Deficiency and insufficiency of vitamin D, defined as a serum 25-hydroxy vitamin D (25(OH)D) below 30 and 50 nmol/L, is a prevalent public health problem that applies for the Nordic countries mainly due to a 4- to 6-month-long winter period without sufficient sun exposure to initiate the cutaneous vitamin D production [1–3]. In Denmark, the prevalence of vitamin D status below 50 nmol/L has recently been estimated to be approximately 23% in a study of 3904 adults and an RCT with 420 adults; both studies were part of a recent Vitamin D Standardization Program (VDSP) publication [4]. A recent Danish survey showed that 11% of the adult population (n = 2625) had a vitamin D status below 25 nmol/L [5]. Sustained vitamin D deficiency (25(OH)D below 30 nmol/L) is known to be associated with risk of poor bone health, muscle pain and weakness [6–8]. Vitamin D status, measured as serum 25(OH)D, is affected by sun exposure, intake from the habitual diet and the consumption of vitamin D-containing supplements. The contribution from the habitual diet, however, is generally low. According to the most recently conducted Danish National Survey of Dietary Habits and Physical Activity (DANSDA) 2011–13 [9], the median dietary intake for women (18–75 years) in Denmark is 3.0 µg/day [10th and 90th percentiles (1.3; 9.0)], and for men the intake is 3.7 µg/day (1.8; 11.0). This current intake is considerably
lower than the Average Requirement (AR) of 7.5 µg/day stated by the Nordic Nutrition Recommendations (NNR) [10]. In general, women have a lower dietary intake of vitamin D than men and are therefore at greater risk of deficiency [11].

The consumption of vitamin D supplements has proven to be effective in increasing vitamin D status, although this strategy is naturally only effective in those who consume the supplements and the risk of too high intakes is ever present [12]. Compared with other European countries, the consumption of dietary supplements in Denmark is high [13] and also the intake of vitamin D-containing supplements is high, and 57% of the women between 18 and 50 years have an intake of vitamin D supplements, self-reported in DANSDA [9]. Supplementation as a strategy holds risks of deficiency in non-consumers and toxicity in individuals with a ‘more is better’ approach, whereas food-based population strategies such as vitamin D fortification may be a potential future goal for countries like Denmark.

Vitamin D fortification has not yet been widely implemented and accepted in Denmark although voluntary fortification with vitamin D in selected products has been allowed since 2005 [14]. At present, only few products in categories such as fat spreads, sports drinks and lactose-free milk products are fortified with vitamin D. Low-dose fortification may be a strategy to increase the intake of those individuals in the lower end of the intake distribution range without increasing the risk of the upper end reaching toxic intake levels [12]. Previous studies from Denmark and Finland have shown that fortifying several foods with a low dose is a safer and more effective approach than fortifying a single food [15, 16]. Foods suited for vitamin D fortification have previously been identified to include milk and milk products, margarines, bread and juice because these foods are consumed by the majority of the assessed populations [15, 17, 18]. The chosen foods for fortification with vitamin D in our model were plain yoghurt, cheese, eggs and crisp-bread, all foods found in a habitual Danish diet and eaten by the majority of the population, but not yet available as fortified foods [9].

The objective of this study is to create an intake model focusing on Danish women at risk of vitamin D deficiency based on low-dose vitamin D-fortified foods using data from 18- to 50-year-old female participants from a representative national dietary survey (DANSDA). Dietary vitamin D intake from habitual diet, four different fortified foods and supplements were used in the model in order to inform fortification policies on safe and adequate levels in a Danish setting.

**Methods**

We performed a graded modelling of dietary vitamin D intake adding extra vitamin D from fortified foods and supplements to the habitual diet of 855 women. Individual intake data (dietary vitamin D) on 855 Danish (Caucasian) women aged 18–50 years were extracted from the Danish National Survey of Dietary Habits and Physical Activity (DANSDA) 2011–13 [9]. The 855 women had all completed seven consecutive days of individual dietary recordings. Using the 7-day dietary recordings combined with the Danish food databank [19], the median individual intake of dietary vitamin D was calculated, assuming that this was the habitual intake of the individuals. We calculated the percentiles and plotted the distribution of the population to illustrate the habitual intake and visualize the subsequent addition of fortified foods and vitamin D supplements in the model scenarios listed in Table 1 and shown in Fig. 1a, b.

The distribution of the habitual dietary intake of vitamin D excluding the intake from fish (scenario 1) calculated from the dietary recordings of the 855 individuals is shown in Fig. 1a. From this level, we added the intake of vitamin D from fish in order to get the habitual vitamin D intake of women that consume fish (Scenario 2) (Fig. 1a). We then added the four fortified foods (plain yoghurt, cheese, eggs and crisp-bread) contributing with approx. 20 µg/day (Scenario 3) (Fig. 1a). In the next scenario, a daily supplement of 10 µg was added (scenario 4) (Fig. 1b). Fish was then again added to the diet to explore whether some women eating

<table>
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<tr>
<th>Scenarios</th>
<th>Description of the scenarios</th>
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<tr>
<td>1</td>
<td>Vitamin D from habitual diet including fish</td>
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<tr>
<td>2</td>
<td>Vitamin D from habitual diet without fish</td>
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<td>3</td>
<td>Scenario 2 + fortified foods* (plain yoghurt, cheese, eggs and crisp-bread)</td>
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<td>4</td>
<td>Scenario 2 + fortified foods* (plain yoghurt, cheese, eggs and crisp-bread) + 10 µg/day supplement</td>
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<td>5</td>
<td>Scenario 1 + fortified foods* (plain yoghurt, cheese, eggs and crisp-bread) + 10 µg/day supplement</td>
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<tr>
<td>6</td>
<td>Scenario 1 + fortified foods* (plain yoghurt, cheese, eggs and crisp-bread) + 40 µg/day supplement</td>
</tr>
<tr>
<td>7</td>
<td>Scenario 1 + fortified foods* (plain yoghurt, cheese, eggs and crisp-bread) + 80 µg/day supplement</td>
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*The four fortified foods contribute with 20 µg/day vitamin D₃ distributed in plain yoghurt, cheese, eggs and crisp-bread
fish and a daily supplement would be at risk of too high an intake (Scenario 5) (Fig. 1b). Finally, we added two different vitamin D supplements with doses of 40 and 80 µg/day as these supplements are sold and consumed in Denmark (Scenario 6–7) (Fig. 1b).

The choice of fortified foods and portion sizes (150 g plain yoghurt, 60 g cheese, 1 egg of 60 g egg and 10 g crisp-bread) were based on previous intervention studies and the Danish National Survey of Dietary Habits and Physical Activity (DANSDA) 2011–13 [9, 15] in order to fit the Danish dietary habits. In the modelling exercise, the assumption was that every person in the population would eat all of the fortified food portions assigned per day and consume 100% of the supplements given. This approach is a choice to ensure the safety of the entire population. The dose of vitamin D from the four fortified foods was approx. 20 µg/day which is the level recommended for persons at risk of osteoporosis in Denmark, and we found this level appropriate since our model was aimed at women with a very low intake of vitamin D and non-consumers of fish [20]. The daily dose of 20 µg/day of vitamin D3 was distributed in plain yoghurt, cheese, eggs and crisp-bread.

Fig. 1 a Distribution of habitual vitamin D intake with and without fish, and addition of vitamin D-fortified foods contributing with 20 µg/day in a population of Danish women (n = 855). b Distribution of vitamin D intake in four scenarios adding foods fortified with vitamin D (20 µg/day) and different doses of vitamin D supplements in a population of Danish women (n = 855)
Different concentrations of vitamin D supplements were used in our model and these were chosen from a study of the Danes use of supplements [21], and the high doses represent doses that can legally be sold in Denmark as well as the internationally accepted Tolerable Upper Intake Level (UL) (< 100 µg/day).

The modelling was performed in Microsoft Excel, and vitamin D intake is represented as µg/day. The percentage of women below the Average Requirement (AR) of 7.5 µg/day was calculated based on the habitual intake of vitamin D obtained in a 7-day food diary from each of the 855 women (µg/day).

Results

Vitamin D intake scenarios

By use of individual habitual dietary vitamin D intake data from the 855 women in the nationally representative DANSDA survey, the initial habitual diet distribution and the six scenarios were created [9] (Fig. 1a, b). The specific vitamin D intakes at the percentiles 5–99 are listed in Table 2.

The women of the DANSDA survey have a wide distribution of vitamin D intake ranging between 0.7 µg/day and 17.7 in the 1st and 99th percentiles (Fig. 1a; Table 2) (DANSDA 2011–13). The percentage of women below the AR of 7.5 µg/day given by the Nordic Nutrition Recommendations (NNR) was 88%, when looking at the habitual dietary intake (including fish), emphasizing the extensiveness of the problem affecting this population of women [8]. The intake of fish is responsible for the difference between the two first curves from the left (the black and the grey non-dashed curves) (Fig. 1a) and is a result of the large variation in the daily fish intakes and the fact that fish potentially contribute with a high concentration of vitamin D, depending on the type of fish. Among the women between 18 and 50 years, 57% reported consuming vitamin D-containing supplements and these women had a median intake of vitamin D of 9.5 µg/day from supplements. In our model, we follow a risk-averse approach and assume that 100% of the women consume the designated portions of fortified foods as well as supplements.

Scenarios 1 and 2 (habitual vitamin D intake not including fish and including fish) show the low intake levels between 0.7 and 4.5 µg/day at the 5th, 25th, 50th and 75th percentiles. Safe levels of intake below 100 µg/day are observed in Scenarios 3–5. Also in Scenario 6 the total vitamin D intake of the 99th percentile is considered safe and does not exceed 80 µg/day, which is below the tolerable Upper intake Level (UL) of 100 µg/day [20, 22]. Scenario 7 depicts a situation of a high vitamin D supplement intake (80 µg/day) and a diet that includes fortified foods, which results in all of the 855 women having a total intake above the UL of 100 µg/day. The maximum intake in scenario 7 was 132 µg/day.

Discussion

The main findings of this paper are that in a setting of a low habitual dietary intake of vitamin D where 88% of the assessed female population had a habitual intake below the AR of 7.5 µg/day, the addition of four vitamin D-fortified foods, contributing with a total daily dose of 20 µg/day, was safe in a population of Danish women. Only those consuming an additional daily vitamin D supplement of 80 µg/day or more may be at risk of exceeding the UL of intake.

In Denmark, we observe an extremely low dietary intake of vitamin D and a relatively high intake of vitamin D from supplements, especially in certain population groups such as elderly women [9, 21]. This pattern is a result of the dietary preferences of the Danish people, current guidelines for intake of supplements and a lack of mandatory vitamin D fortification. Prior to implementing a national fortification

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<td>1 Vitamin D from HD including fish</td>
<td>1.0</td>
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<tr>
<td>2 Vitamin D from HD without fish</td>
<td>0.7</td>
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<tr>
<td>3 Scenario 2 +4 fortified foods</td>
<td>21</td>
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<tr>
<td>4 Scenario 2 +4 fortified foods +10 µg/day supplement</td>
<td>31</td>
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<tr>
<td>5 Scenario 1 +4 fortified foods +10 µg/day supplement</td>
<td>31.3</td>
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<tr>
<td>6 Scenario 1 +4 fortified foods +40 µg/day supplement</td>
<td>61.3</td>
</tr>
<tr>
<td>7 Scenario 1 +4 fortified foods +80 µg/day supplement</td>
<td>109.3</td>
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Fortified foods contribute with 20 µg/day vitamin D₃ distributed in plain yoghurt, cheese, eggs and crispbread

All values in the table are represented in µg/day unless otherwise specified
programme, it is relevant to look at the possible effects and safety of such a programme in a model situation to predict the consequences on a population level.

In the present study, we chose to focus on Danish women in the modelling of vitamin D intake levels because women are known to have a lower intake of vitamin D than men, despite similar intake recommendations for the daily intake [9, 10]. Previous intervention and cohort studies have reported extremely low vitamin D status (defined as 25(OH) D < 12 nmol/L) in women [23, 24]. Some of the reasons for women having a lower intake may be due to a lower intake of calories and meat compared to men [9].

After reviewing the literature for foods well suited for vitamin D fortification, we identified the most commonly fortified foods as being milk and milk products, margarines, bread and juice [15, 17, 18]. Eggs appeared as an interesting novel food and a good bio-fortified (vitamin D added to the animal diet) source of vitamin D [25]. We chose the four foods based on relevancy in a Danish setting. The fortified foods were yoghurt, cheese, eggs and crisp-bread, all foods included in a habitual Danish diet and eaten by the majority of the population [9]. Low-dose fortification was chosen to create a realistic model for a Danish fortification situation. The use of several foods in the fortification model and lower concentrations of vitamin D in each food makes it more safe and efficient, whereas using a single food such as milk may be problematic because non-consumers skewed the intake across a population [16, 26].

In the modelling, each individual in the population (DANSDA) was assumed to eat the same daily portion of the four fortified foods. This assumption makes the curve shift equally in every percentile as shown in Fig. 1a, b. Adding a vitamin D supplement of 10 μg to the diet again shifts the entire population under the assumption that everyone has an equal intake, except for the individual habitual dietary intake. These assumptions ensure the safety of the fortification levels when each individual has a daily consumption of the foods of plain yoghurt (150 g), cheese (60 g), one egg (60 g) and one crisp-bread (10 g). In a real-life situation, we will inevitably see varying consumption patterns of the fortified foods, meaning that not all will eat the proposed amount used in our model. However, we chose to perform the modelling with a risk-averse approach and the above assumptions to ensure the safety of the entire population in case of the introduction of a national fortification scheme in Denmark. In this way, we have a margin of safety, which in our view is ideal when dealing with a fat-soluble vitamin such as vitamin D.

The NNR has stated an AR of vitamin D for the age group of 7.5 μg/day and The Danish National Health Authorities recommend a vitamin D intake of 10 μg/day for children and adults and 20 μg/day for elderly (> 70 y) and risk groups of osteoporosis (Danish National Health Authority 2010).

Therefore, our goal was to increase the intake of the Danish women to 20 μg/day from fortified foods, to ensure that this risk group of a low vitamin D intake will have their vitamin D status lifted and maintained throughout the year. The scenarios including extra vitamin D supplements simulate the real-life situation in which the intake of supplements in Denmark is high [5, 21, 27].

Strengths and limitations

A clear strength of this paper was the inclusion of individual consumption data from a nationally representative survey which provides results applicable to a Danish setting. These data allow for the use of these results in a Danish setting by policy makers and public health agencies. We did not assess all levels of supplementation, only the chosen three levels being 10, 40 and 80 μg/day as well as the UL level of 100 μg/day.

Conclusion

The current vitamin D intake was below the AR of 7.5 μg/day in 88% of the 855 Danish women assessed in this paper. By performing modelling of vitamin D fortification in the population of Danish women 18–50 years, we showed that adequate and safe levels of intake were present in all women consuming the fortified foods and a daily supplement of vitamin D as high as 40 μg/day. When consuming a daily vitamin D supplement of 80 μg/day or more, all of the women were at risk of reaching the UL of intake (100 μg/day).

Acknowledgements We thank Karin Hess Ygil for assisting with dietary intake calculations and the project group behind the Danish National Survey of Dietary Habits and Physical Activity (DANSDA) 2011-13.

Compliance with ethical standards

Author Contributions ME and TC managed the intake data. IMG undertook the statistical analyses and wrote the paper. EWA assisted with the statistical analyses. IMG, RA and IT designed the study. All the authors contributed to the manuscript.

Conflict of interest None of the authors have any conflicts of interest.

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References

Paper II

Vitamin D fortified foods improve wintertime vitamin D status in women of Danish and Pakistani origin living in Denmark: A randomised controlled trial
Vitamin D fortified foods improve wintertime vitamin D status in women of Danish and Pakistani origin living in Denmark: A randomised controlled trial.

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Abbreviations:
25(OH)D – Serum 25-hydroxyvitamin D
PTH – Parathyroid Hormone
RCT – Randomised Controlled trial
ODIN – Food-based solutions for optimal vitamin D nutrition and health through the life cycle
SD – Standard Deviation
CV – Coefficient of variance ((SD/mean)*100)
UL – Tolerable Upper Intake Level
FFQ – Food Frequency Questionnaire
UCC – University College Cork
LC-MS/MS – Liquid chromatography-tandem mass spectrometry
VDSP – Vitamin D Standardization Program
ANOVA – Analysis of variance
ANCOVA – Analysis of co-variance
CI – Confidence Interval
ABSTRACT

**Purpose:** Low vitamin D status is prevalent worldwide. We aim to investigate the effect of vitamin D fortification on serum 25-hydroxyvitamin D (25(OH)D) concentration in women of Danish and Pakistani origin at risk of vitamin D deficiency.

**Methods:** A 12-week randomised, double-blinded, placebo-controlled intervention trial during winter time, designed to provide 20 μg vitamin D3/day through fortified yoghurt, cheese, eggs and crisp-bread and assess the change in serum 25(OH)D concentration. Participants were 143 women of Danish and Pakistani origin, living in Denmark, randomised into four groups, stratified by ethnicity.

**Results:** Mean (SD) baseline 25(OH)D concentrations among women of Danish and Pakistani origin were 49.6 (18) and 46.9 (22) nmol/L, respectively ($P = 0.4$). While 9% of Danish women had a 25(OH)D concentration < 30 nmol/L, the prevalence among women of Pakistani origin was 24%. Median (IQR) vitamin D intake among Danish and Pakistani women at endpoint were 32.0 (27.0, 34.4) μg/d and 24.2 (19.2, 30.8) μg/d, respectively. Endpoint serum 25(OH)D concentration increased in fortified groups to 77.8 (14) nmol/L among Danish women and 54.7 (18) nmol/L among women of Pakistani origin ($P < 0.01$). At endpoint, 0% in the Danish fortified group and 3% in the Pakistani fortified group had 25(OH)D < 30 nmol/L, compared with 7% and 11% in their respective control groups.

**Conclusions:** Vitamin D fortification of four different foods for 12-weeks during winter was effective in increasing serum 25(OH)D concentration and reducing the prevalence of very low vitamin D status among women of Danish and Pakistani origin.

**Keywords:** Vitamin D; Food-based RCT; ODIN; women of Pakistani origin; women of Danish origin; fortified foods.

INTRODUCTION

Very low and low vitamin D status (reflected by 25(OH)D concentrations < 30 and < 50 nmol/L, respectively [1, 2]) are prevalent amongst the general population in countries of northern latitudes [3], as well as being a more worldwide concern [4]. Notably, many immigrants (first generation immigrants and descendants) living in Denmark, and other Nordic countries, are at a higher risk of vitamin D deficiency compared to the native
residents [3, 5–8]. Low vitamin D status has been linked with risk of various chronic diseases, and in particular, with adverse bone health outcomes [1, 2, 9–11].

Denmark, located at 55° North, experiences a five-month long ‘vitamin D winter’, during which there is increased emphasis on dietary supply of vitamin D [12]. However, food sources of vitamin D are sparse (fish, meat, eggs, cheese) and their consumption patterns irregular [13]. Data from the most recent Danish National Survey of Dietary Habits and Physical Activity (DANSDA 2011-13) shows that median intakes of vitamin D are 3-4 µg/day (without supplement contribution) [13], considerably lower than the Average Requirement of 7.5 µg/day for the Nordic region [10]. In Denmark, while vitamin D supplement use is only recommended for populations at risk of deficiency; infants, pregnant women, elderly and individuals with dark skin [14], the proportion in the general population taking a vitamin D supplement is relatively high, e.g., 50-60% of the adult females [15, 16]. However, while vitamin D supplementation is an effective approach for increasing vitamin D status in those individuals who consume them regularly, it has limitations as a strategy at a population level due to a high proportion of non-users as well as concerns about potential risk of excessive intake amongst high users [17].

Food-based strategies aiming at improving vitamin D intake and status across the population, such as vitamin D food fortification, have been highlighted as being of high potential as a public health measure in Europe [17–20]. Furthermore, while many of the environmental factors that contribute to the elevated risk of vitamin D deficiency in populations, such as latitude, skin color, and cultural clothing practices are not modifiable, in contrast, dietary supply of vitamin D is an important modifiable factor, again emphasizing the need for food-based strategies to offset low intakes [21].

Vitamin D fortification of food has not yet been widely implemented and accepted in Denmark, despite voluntary fortification of certain foods being permissible for over a decade [20, 22]. Low-dose fortification of several foods rather than higher dose fortification in few foods has been proposed as an effective and safe strategy on the population level [18, 23]. Yet, randomised controlled trial (RCT) data on the effectiveness and safety of this approach, especially in at-risk population subgroups, is lacking despite the importance of such data
in informing food fortification policy. Thus, we aimed to investigate the effect of low-dose, vitamin D₃ fortification of a number of commonly consumed foods (yoghurt, cheese, eggs and crisp-bread) on vitamin D status in a population of adult women of Danish and Pakistani origin at increased risk of vitamin D deficiency, using a 12-week, winter-based RCT.

**SUBJECTS AND METHODS**

*Study design*

The ODIN-FOOD study was part of the European Commission-funded large scale collaborative ODIN (Food-based solutions for optimal vitamin D nutrition and health through the life cycle) Project. The ODIN-FOOD study was a three-month randomised double-blinded, placebo-controlled, intervention trial carried out during the winter months (January-March) of 2016, and enrolled a total of 143 women of Danish and Pakistani origin, aged between 18 and 50 years. Immigrant women in Denmark are considered an at risk group for vitamin D deficiency [5, 7]. The risk extends to the ethnic Danish female population in a modern work-life setting with a sedentary lifestyle, low UVB exposure and a low habitual vitamin D intake, particularly if they avoid or limit fish intake [13, 24]. The trial was performed during the winter months to minimize interference from UVB-induced cutaneous synthesis of vitamin D₃. Participants were randomised into four groups, stratified for ethnicity (two groups with women of Danish origin and two groups with women of Pakistani origin). The randomization sequence was generated by a researcher not involved in the project using block randomization with a block size of four within each ethnic group (Fig. 1). The subjects were given four different fortified foods aiming to contribute an additional 20 µg/day of vitamin D₃ or equivalent non-fortified foods (placebo). Participants were seen before the start of intervention and at the end of 3-month intervention, and at both visits non-fasting blood were drawn, anthropometrics, dietary vitamin D intake and muscle strength was measured. The main endpoint was the change in serum 25(OH)D concentration. Secondary endpoints included anthropometric measures and dietary intake of vitamin D.

The intervention was double-blinded, and the study foods were color and letter coded. The blinding was managed by a researcher not involved in the project. Both the participants and the researchers working within
the project, during the intervention and subsequent analyses, were all blinded until the statistical analyses were completed.

Sample size of 143 women of two ethnicities was based on 90% power (alpha = 5%) to detect a change in serum 25(OH)D concentration of 20 nmol/L in the treatment groups with a standard deviation (SD) of 23 nmol/L, and included a drop-out rate of 20%.

Subjects

We recruited women of Pakistani origin (first generation immigrants or descendants) and women of Danish origin from the Copenhagen area, city and suburbs (Denmark, 55°N). The recruitment was done by e-mail, advertising, networking and interactions with local community groups, media, and social and cultural initiatives in the Copenhagen area, as well as visiting local shops, libraries, mosques and women’s societies. Eligible women were invited to information meetings in which the study procedures were explained by a researcher from the project group. Written informed consent was obtained from all participants on enrolment. Inclusion criteria were a low consumption of fish and fish products (less than weekly), a low frequency of use of vitamin D-containing supplements (less than weekly), no use of tanning facilities, no planned sun-holiday (to a location more southerly than 47°N) between October 2015 and May 2016. There was no upper limit on the vitamin D dose of the supplements as long as participants discontinued them during the study period. Exclusion criteria were pregnancy and breastfeeding, menopause, non-Danish speakers, serious diseases (cancer, server liver or kidney insufficiencies, sarcoidosis and other granulomatous diseases) and medicines affecting vitamin D metabolism (steroids, antiepileptic, thyroid hormones, bisphosphonates, estrogen).

Fortification vehicles

The intervention foods of choice were low-fat Milner cheese (gouda) and yoghurt (plain) produced and provided free of cost by FrieslandCampina in the Netherlands, eggs (Livskraft) produced and provided free of cost by Hedegaard in Denmark, and whole grain crisp-bread produced by Smørum Konditori (confectionary) using
ingredients provided free of cost by Lantmännen Cerealia, Denmark, the vitamin D₃ for the crisp-bread was supplied by DSM nutritional products, Switzerland. The study foods were chosen because they are commonly consumed by both ethnic groups. Dietary calculations and pilot taste tests were carried out to ensure the acceptance of the products. The taste tests were performed among 12 women of Danish and Pakistani origin. The majority of the foods were low in fat and they are considered suitable substitutions for the participants’ habitual intake of these food products (Table 1). The participants were given either placebo foods or vitamin D-fortified foods (aimed at providing approximately 20 μg/day of additional vitamin D₃) [20] at no cost for the participants.

A daily dose of 20 μg, together with the contribution from habitual intake (~3-4 μg/d [6]), would be expected to maintain winter serum 25(OH)D concentration above of 30 nmol/L in the vast majority of individuals [25] and it allows for a large margin of safety in relation to the Tolerable Upper Intake Level (UL) of 100 μg/day [20, 26]. The fortified and non-fortified foods have the same fat content and comparable content of nutrients, except for the vitamin D concentration (Table 1). The packaging of the food products was identical, except for color and letter coding added to distinguish the fortified from the placebo foods.

Laboratory analysis of food samples

The vitamin D₃ and 25-hydroxyvitamin D₃ content of food were analysed at the National Food Institute at the Technical University of Denmark using modifications of a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described elsewhere [27]. The method was performed in a laboratory accredited according to ISO17025, and has a limit of quantification at 0.01 μg/100 g, and a precision <10% (CV). For crisp-bread and eggs, eight and six batches, respectively, were produced and all were analysed. Only one batch was produced for yoghurt and cheese and for this the stability was controlled. Analysis confirmed there was no decrease of vitamin D during the 12 weeks of intervention. Total vitamin D activity of each food was calculated as the vitamin D₃ content of the food plus the 25-hydroxyvitamin D₃ content × 2.5. The conversion factor of 2.5 was used as a conservative estimate based on factor of 5 as currently used [28] and a factor of 1.5 recently obtained in an intervention study in Denmark [29].
**Intervention**

After the baseline visit, participants were each given food for two weeks (1 egg per day, 150 g (1 small pot) yoghurt, 60 g (2 slices) of low fat Gouda cheese and 1 crisp-bread per day). The participants were allowed to freely plan how they distributed the provided foods over a week as long as they consumed the designated 7 eggs, 7 portions of yoghurt, 7 x 60 g cheese and 7 crisp-breads per week. To maintain body weight, participants were advised to substitute some of their regularly consumed foods with the study foods. Fresh foods were distributed every two weeks. Compliance was monitored on a weekly basis by a user-friendly printed compliance questionnaire in which the participant would mark each food item on a picture once consumed. Compliance was estimated individually by dividing the individual amount of food received by self-reported compliance, expressed as a percentage. Due to packaging, an individual would sometimes receive more than they needed for that period, and were asked not to consume the surplus after fulfilling their weekly amount, however, if they did consume the extra foods, this was accounted for. Thus, individual compliance could in some cases be > 100 %.

**Measurements**

Participants were examined twice at the National Food Institute at the Technical University of Denmark, including a baseline visit in January and an endpoint visit in April 2016. At each visit, a 40 mL non-fasting blood sample was obtained from each participant by an experienced phlebotomist or nurse. Anthropometric measures were completed with participants wearing thin/light clothing, no shoes and after emptying their bladder. The measures were height (wall mounted stadiometer, seca, Hamburg, Germany) and weight, waist-hip circumference (standard tape measure) and body composition (Tanita BC 418 MA, Tokyo, Japan).

At baseline, participants completed two questionnaires, a general background questionnaire, assessing the health, lifestyle and sun habits, and other factors affecting the vitamin D status and a semi-quantitative Food Frequency Questionnaire (FFQ) estimating the average intake of vitamin D and calcium. The FFQ was a retrospective questionnaire asking about the participants’ habitual dietary intake of vitamin D-rich foods over
the 3 preceding months. The FFQ used in the ODIN-FOOD study was developed from existing questionnaires used in previous RCT’s (OPTIFORD and VitmaD) at the National Food Institute [5, 22].

All questionnaires were self-administered, however to ensure that all participants completed the questionnaire and to avoid misunderstandings, cultural as well as language-related, we set up a questionnaire room, where all participants were introduced to the questionnaires and offered help when needed. The questionnaires contained ethnic-specific questions regarding foods that contribute vitamin D and calcium. All FFQ’s were administered during the participant’s first visit in January 2016. The questionnaire contained the 8 food groups (Fish, meat, milk and milk products, egg, cheese, bread, fats and pulses) contributing to the majority of dietary vitamin D (98%) and calcium (71%) [13]. Further questions were asked to estimate intake of vitamin D-containing supplements prior to the study start. The questionnaire took between 30 and 50 minutes to complete and all participants completed the questionnaire.

Estimates of vitamin D intake were made from the data obtained from the FFQ matched to specific foods and recipes made from the Danish food composition database (version 7) using the individually reported portion sizes [30]. The total vitamin D intake for each participant prior to the study start was estimated as the sum of dietary vitamin D and contribution from personal supplements, if consumed.

Laboratory analysis of blood samples

Serum concentrations of total 25(OH)D (i.e., 25(OH)D$_2$ plus 25(OH)D$_3$) of all serum samples (baseline and endpoint visits) were measured at the Cork Centre for Vitamin D and Nutrition Research, University College Cork (UCC), using the ODIN core LC-MS/MS analytical platform for serum 25(OH)D, described in detail elsewhere [31]. The intra-assay CV of the method was < 5 % for all 25(OH)D metabolites, whilst the inter-assay CV was < 6 %. The LC-MS/MS method at UCC is certified by the Center for Disease Control and Prevention’s Vitamin D Standardization Certification Program.

The biochemical analyses of serum calcium and Parathyroid Hormone (PTH) were performed at the University Hospital Aarhus. Total serum calcium had an analysis precision of ± 0.032 mmol/L (standard deviation [SD]) at a concentration of 2.161 mmol/L and ± 0.047 mmol/L (SD) at a concentration of 3.134 mmol/l. Serum PTH
had a precision of ± 0.2 pmol/L (SD) at a concentration of 2 pmol/L and ± 1.0 pmol/L (SD) at a concentration of 10 pmol/l.

**Statistical analysis**

Results are shown as means and SDs unless otherwise specified. Descriptive statistics were generated for the two ethnic groups at baseline, and the groups were compared using a two-sample *t* test when the data could be assumed to be normal; otherwise using a non-parametric Kruskal Wallis test. Categorical variables were compared using a Pearson’s chi-square test.

Comparison of the daily vitamin D intake, serum 25(OH)D concentration and serum PTH at baseline and endpoint across the intervention groups were done by simple one way ANOVA and if significant differences were observed a Tukey HSD test were performed in order to assess differences between groups. Analysis of covariance (ANCOVA) was used to analyse the effect of the intervention on the outcome variable *change in vitamin D status* (endpoint - baseline 25(OH)D). Two models were run – a minimal adequate model (Model 1) and a maximal model (Model 2), which controlled for specific covariates that may influence the outcome.

Model 1 included *baseline 25(OH)D* as a covariate and the factors *intervention group, ethnicity* and their interaction allowing the effect of intervention to differ between the two groups of women. The interaction was tested for statistical significance to see whether the model could be simplified. In Model 2, the covariates *Age and BMI* at baseline were added to Model 1 construct as they are likely to have a strong association with the outcome (serum 25(OH)D concentration). The variables were chosen based on factors known to affect the change in vitamin D status following fortification and the variables that were significantly different between the two ethnic groups at baseline.

To check the model assumptions the standardized residuals of the final models were assessed for normality, variance homogeneity and linearity.

Statistical analyses were performed using RStudio for Windows [32] (Version 1.1.414 – © 2009-2018 RStudio, Inc.) with a significance level of $\alpha = 0.05$. 
RESULTS

A total of 143 women of Danish and Pakistani origin were randomly allocated to either vitamin D-fortified foods or placebo (similar non-fortified foods), forming the four study groups. The completion rate of the study was 89% with the number of drop-outs at 16 in total for reasons as described within the Consort flow diagram in Fig. 1. Drop-outs were equally distributed across the two ethnic groups ($P = 0.67$). In total, six participants who finished the intervention were excluded from the analyses due to unplanned travels to countries more southerly than 47° N during the study period and one was excluded as an outlier due to a baseline serum 25(OH)D concentration > 125 nmol/L, which the Institute of Medicine (IOM) suggest is potentially of concern, if sustained [1].

Compliance to consumption of the study foods was 92% among participants of Danish origin and 73% among participants of Pakistani origin ($P < 0.05$). The study foods and the habitual dietary intake contributed to a median (25th, 75th percentiles) daily intake of vitamin D of 32.0 (27.0, 34.4) µg/day in the participants of Danish origin randomised to the fortified food study group, and 24.2 (19.2, 30.8) µg/day in the participants of Pakistani origin randomised to the fortified food study group (Table 3). The chemical analysis of the crisp-bread showed a slightly higher concentration of vitamin D than expected due to a calculation error in the production of the flour mix, whereas the eggs contained less vitamin D than expected due to lower vitamin D (D$_3$ and 25(OH)D$_3$) doses in the chicken feed than expected, and this led to a slightly higher total daily contribution of vitamin D from fortified foods (Table 1). The margin of safety was still sufficient (UL:100 µg/day) [20].

Baseline characteristics of the participants

There were no differences in baseline characteristics between the groups receiving vitamin D-fortified foods or placebo foods within either ethnicity (data not shown); however, there were significant differences in some baseline anthropometric characteristics between the two ethnic groups (Table 2). Women of Pakistani origin had a significantly higher mean BMI and fat percentage compared to the women of Danish origin ($P = 0.004$ and $P < 0.001$, respectively). The intake of supplements containing only vitamin D was significantly higher among the women of Pakistani origin compared to the women of Danish origin ($P < 0.05$). More than 60% of the women
of Pakistani origin reported taking a supplement with vitamin D before study start. The proportion of the participants using multivitamins was statistically similar in the two ethnic groups \((P > 0.30)\) (Table 2). No significant differences \((P > 0.05)\) were found in mean age between the two ethnic groups (Table 2). The intake of supplements containing only vitamin D was significantly higher among the women of Pakistani origin, compared to Danish \((P = 0.001)\). The intake of vitamin D from dietary sources were significantly higher among the women of Danish origin \((P < 0.05)\), however both groups had very low intake.

At baseline, none of the participants, irrespective of ethnicity, had serum 25(OH)D concentration < 10 nmol/L. The prevalence of 25(OH)D concentration < 30 nmol/L and < 50 nmol/L was 9 and 50 % of the women of Danish origin and 24 and 32 % of the women of Pakistani origin, respectively. The mean baseline serum 25(OH)D concentration was similar \((P = 0.43)\) among both ethnic groups (Table 2).

Baseline serum PTH was significantly higher among the women of Danish origin than of Pakistani origin \((P < 0.02)\), but all group values were within the reference range for normal PTH (Table 2). While serum PTH was positively associated with serum 25(OH)D concentration at baseline among the women of Pakistani origin \((P = 0.02)\), there was no significant \((P > 0.90)\) association between these two variables in women of Danish origin (data not shown).

**Effect of intervention**

The following analyses included 136 individuals following an intention to treat approach. There were no significant changes in body weight (between baseline and endpoint) in either ethnic group \((P = 0.70 \text{ and } P = 0.66 \text{ for Danish and Pakistani women, respectively})\) (data not shown).

The total vitamin D intake from the fortified foods during the intervention was higher among the Danish women, compared to the women of Pakistani origin \((P = 0.004)\), reflecting the lower compliance of the women of Pakistani origin. The mean (SD) endpoint serum 25(OH)D concentration among the women of Danish origin in the fortified food group was 77.8 (15) nmol/L, whereas among the women of Pakistani origin in the fortified food group, it was significantly \((P < 0.01)\) lower at 54.7 (18) nmol/L (Table 3). The mean increase in serum 25(OH)D concentration from baseline to endpoint among the fortified food group
was higher ($P < 0.01$) in the women of Danish origin ($\Delta 26.4 (16)$ nmol/L) compared to that in the women of Pakistani origin ($\Delta 10.5 (18)$ nmol/L). Serum 25(OH)D concentration decreased by 2.8 (9) nmol/L in the Danish placebo group and by 11.2 (12) nmol/L in the Pakistani placebo group over the 12 weeks of winter ($P = 0.02$).

Following the intervention, none of the women of Danish origin in the fortified group had a serum 25(OH)D concentration < 30 nmol/L. Among the women of Pakistani origin, 3 and 41 % of the fortified food group had an endpoint serum 25(OH)D concentration < 30 and < 50 nmol/L, respectively (Table 3). In contrast, vitamin D deficiency (serum 25(OHD < 30) was evident in about a quarter of the Danish and a third of the Pakistani women in the placebo groups at endpoint. Likewise, vitamin D insufficiency (serum 25(OH)D < 50) was evident in about two-thirds and three-quarters of the Danish and Pakistani women in the placebo groups, respectively, at endpoint (Table 4).

There was no significant increase in PTH concentration of the placebo groups (Danish and Pakistani) following the intervention (0.21 and $P = 0.85$, respectively, $t$ test). Likewise, there was no significant decrease in PTH concentration of the fortified food groups (Danish and Pakistani) following the intervention ($P = 0.40$ and 0.58, respectively, $t$ test).
Analysis of covariance including factors influencing the change in serum 25(OH)D concentration following the intervention

Based on the output of Model 1, compared to that of an equivalent woman in the non-fortified group, 12 weeks of intervention with vitamin D fortified foods (together with habitual diet, collectively supplying a total of 30 µg vitamin D/day), resulted in an improvement of endpoint serum 25(OH)D concentration of 31.1 nmol/L (25.0; 37.2) and 20.3 nmol/L (14.3; 26.3) in the women of Danish and Pakistani origin, respectively (Table 5). The intervention effect is significantly higher in the Danish group compared to the Pakistani group ($P = 0.008$).

In terms of other factors which affected the response of serum 25(OH)D concentration to intervention, the ANCOVA models showed that baseline 25(OH)D concentration status and BMI had a negative effect on the change in serum 25(OH)D concentration following the intervention, however the effect of BMI was very small. According to the model, participants with e.g. a higher baseline serum 25(OH)D concentration had an expected lower increase in serum 25(OH)D concentration following the intervention (Table 5).
The present study demonstrated that fortification of four different foods for 12 weeks during winter (January through to April) was effective in increasing serum 25(OH)D concentration among Danish and Pakistani women and reduced the prevalence of low and very low vitamin D status during the winter months. The Danish fortified group increased significantly more than the fortified Pakistani group. To our knowledge few previous studies have assessed the effects of vitamin D food fortification with two different ethnic groups \[33\] and none in Denmark, the results of our study may be of importance for the planning of fortification policies in countries with multi-ethnic populations and no vitamin D fortification program.

The increase in serum 25(OH)D concentration of the fortified participants was mainly the baseline serum 25(OH)D concentration and ethnicity in accordance with the linear models. However, we saw significant difference in the intake of vitamin D from the fortified foods provided in the study due to a lower compliance in the women of Pakistani origin compared to women of Danish origin. Generally, the results show a successful preventative effect of an intervention with vitamin D-fortified foods in a country without mandatory fortification in a population of women at risk of deficiency, as well as an important effect in terms of maintaining serum 25(OH)D concentration > 50 nmol/L (reflective of sufficiency) in all participants of Danish origin and in 59% of the participants of Pakistani origin.

These results are in line with the findings of an 11-year Finnish follow-up study which showed a positive effect of an implementation of a national voluntary vitamin D fortification policy. The study revealed an average increase in vitamin D status of 20 nmol/L (95% CI: 19, 21) among supplement non-users, although no records of ethnic background was obtained \[19\].

Several studies have investigated fortification of a single food, but only few have experience from low dose fortification of several foods \[17, 22\]. We decided on a low-dose approach in which the daily dose of vitamin D was spread out into several (four) foods in order to ensure the effectiveness and safety in a population approach, based on previous experience \[22, 23\]. Our study participants were of Danish and Pakistani origin, therefore we made special considerations when choosing the fortification vehicles since acceptance of the foods in both groups was of high importance. As an example of our considerations we did not use milk as a
fortification vehicle despite its use in previous Nordic studies [19, 22, 23], since the prevalence of lactose intolerance among individuals of Pakistani origin is > 60% [34]. Instead we chose plain yoghurt. Yoghurt has a lower level of lactose and is suitable for most lactose intolerant people, it can be used in breakfast, snacks and sweet or savoury cooking.

All the study foods were provided with no cost for the participants in portions that fit into a healthy diet. The foods were all low in fat and energy. We instructed the participants to substitute their normal diet with food from the study, so that they would remain approximately isocaloric throughout the study. This was successful and we saw no changes in the body composition (BMI) of the participants following the intervention.

The effect of intervention with fortified foods on vitamin D status was significantly different in the two ethnic groups. This ethnic difference may in part be explained by the differences in compliance with the study foods as compliance while high (73%) was significantly lower in the Pakistani women. Assessing the distribution of baseline serum 25(OH)D concentration it was evident that despite a similar mean vitamin D status, the IQR revealed a much longer tail among the women of Pakistani origin which was evident as per the higher percentage < 30 nmol/L. In the statistical models ethnicity was significant and there could be some ethnic-specific differences in the serum 25(OH)D response that stem from genetic variation of vitamin D modulating genes. Analyses of Single Nucleotide Polymorphism (SNP) data collected in this study, but not yet studied, may explain parts of the found ethnic specific intervention effect.

We found that the baseline serum 25(OH)D concentration among the women of Pakistani origin was higher than expected (mean ≈ 50 nmol/L), when compared with data from a previous Danish study which included only participants of Pakistani origin living in Denmark. In that study, conducted in 2002, a low vitamin D status was reported for both girls and women of Pakistani origin (median serum 25(OH)D concentration was 10.9 and 12.0 nmol/L, respectively) [5]. It should be noted, however, that the earlier study by Andersen et al. [5] was performed prior to the initiation of the Vitamin D Standardization Program (VDSP) and this may
affect the comparability of serum 25(OH)D concentration data. More importantly, several factors may have
decided the change in vitamin D status over the course of the last 15+ years. For example, it may partly be a
result of the strong focus on vitamin D in the general public as well as public health actions initiated by the
Danish health authorities between 2005 and 2010, targeting the general population, ethnic minorities as well
as health professionals [35, 36].

An additional result of the mentioned public health actions may be an increased intake of dietary
supplements containing vitamin D. In our study we saw a relatively high self-reported intake of vitamin D
from supplements, importantly, participants taking supplements prior to the study start were asked to stop
their supplement routine during the study. The prior intake of vitamin D-containing supplements was
adjusted for by ensuring that baseline serum 25(OH)D concentration was an input in all of the linear models.
We did not intend to perform a screening serum 25(OH)D concentration test prior to study start due to
practical reasons as well as the time constraint present in this vitamin D RCT’s due to the relatively short
study period of a winter only study.

**Strengths and limitations**

This study was a real-life based design in which the participants would incorporate the study foods by
substitution of their habitual intake of similar foods. We encouraged the participants to consume the same
amount of the study foods every day to create a habit and incorporate the foods into their normal diet,
however, we allowed for the participants to include foods from several days in one meal, permitting people
to consume e.g. 4 eggs and 60 g cheese in one meal (e.g. omelette). This approach was chosen to mimic a
real-life situation in order for the results to be used in a public health setting as well as to strengthen the
study by increasing compliance and decreasing drop-out rate. The study achieved a good power and a low
drop-out rate for this study population.

In Denmark we have an increasingly multi-ethnic society. Research studies involving two or more ethnicities
are therefore highly needed in order to give suitable health advice and ensure equality in health of all citizens
in Denmark. The trial was carried out in winter time when there is no cutaneous production of vitamin D,
this lowers the risk of contamination of vitamin D from UV sources.
The intake of dietary vitamin D was very low, compared to the Danish National Survey of Dietary Habits and Physical Activity (DANSDA 2011-13) [13], however, the participants were recruited based on their low intake of fish, so this was not surprising. The dietary calcium intake recorded in the FFQ was also very low, and part of this can be explained by the questionnaire, while aiming at capturing the majority of dietary vitamin D is was not designed to capture more than 70% of dietary calcium.

The chemical analysis of the crisp-bread showed a slightly higher concentration than expected due to a calculation error in the production of the flour mix, whereas the eggs contained less vitamin D than expected due to lower vitamin D (D₃ and 25(OH)D₃) doses in the chicken feed than expected, and this affected the total daily dose from fortified foods of approximately 30 μg/day (Table 1).

**Perspectives**

Results may be applicable for designing fortification policies prior to implementing them in modern multi-ethnic population groups. Future studies may go further into the topic of vitamin D fortification at Northern latitude and effects of genetic variation in different ethnic populations complying with the VDSP when analysing the serum 25(OH)D concentration.
CONCLUSION

Vitamin D fortification of 30 µg/day, provided in four different foods, for 12 weeks during winter was effective in increasing vitamin D status and preventing vitamin D deficiency in women of Danish and Pakistani origin living in Denmark. Women of Pakistani origin had a lower response to the intervention that did women of Danish origin. Compliance was lower among the women of Pakistani origin compared to the women of Danish origin.
Acknowledgements

We thank the following individuals for their help and assistance with this trial: Majken Ege, Karin Hess Ygil, Dorte L Korsbech, Erika Baig for assisting with the measurements, dietary intake calculations and FFQ work, Bashy Quraishy, Raza Mustafa, Saima Raza, Shahnaz Qureshi and Shais Anais for introducing us to the Pakistani societies in Copenhagen, Denmark. We would also like to thank Dr. George Hull and Ms. Kirsten Dowling at University College Cork for their contribution to the analysis of serum 25(OH)D concentration. We thank our industrial partners FrieslandCampina, Hedegaard, Lantmännen Cerealia and DSM nutritional products for delivering the foods used in the trial.

Contributors: IMG and RA collected the data, TC managed intake data, JJ analysed the vitamin D content of the food, KC analysed the serum 25(OH)D, IMG undertook the statistical analyses and wrote this paper. EWA assisted with the statistical analyses. RA, IT, KC and MK designed the study. All contributed to the manuscript.

Ethical standards:

Written informed consent was obtained from all participants on enrolment. The study protocol was approved by the local ethical committee (protocol no. H-15008276) and registered at ClinicalTrials.gov with identifier: NCT02631629. The study was carried out in accordance with the Declaration of Helsinki.

Conflicts of interest:

None of the authors had conflicts of interest. The industry partners had no influence on the design of the study, the interpretation of the results or the writing of this manuscript.
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Fig. 1
Consort flow diagram of the number of participants enrolled, randomised, completed and analysed in the *ODIN-FOOD* study. Consort, Consolidated standards of Reporting Trials. 
DK: Women of Danish origin, Pa: Women of Pakistani origin
Table 1
Nutritional composition, portion size, energy and mean total vitamin D contribution from the fortified and placebo foods

<table>
<thead>
<tr>
<th>Food product</th>
<th>Macro nutrients (g/100g)¹</th>
<th>Kcal/100g²</th>
<th>Daily portion size (g/day)</th>
<th>Kcal/day</th>
<th>Total vitamin D contribution from fortified product (µg/day)³</th>
<th>Total vitamin D contribution from placebo product (µg/day)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yoghurt</td>
<td></td>
<td>70</td>
<td>150</td>
<td>105</td>
<td>2.00 (0.03)³</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>3.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHO</td>
<td>6.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>2.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese</td>
<td></td>
<td>272</td>
<td>60</td>
<td>163</td>
<td>8.37 (0.27)</td>
<td>0.1 (0.01)</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHO</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eggs</td>
<td></td>
<td>141</td>
<td>54</td>
<td>76</td>
<td>0.86 (0.52)</td>
<td>1.4 (0.1)</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>9.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHO</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>12.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crisp-bread</td>
<td></td>
<td>460</td>
<td>8.8</td>
<td>40.5</td>
<td>18.8 (0.11)</td>
<td>&lt; 0.025</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHO</td>
<td>58</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>943</td>
<td>-</td>
<td>384.5</td>
<td>30.0 (8.20)</td>
<td>1.5 (0.05)</td>
</tr>
</tbody>
</table>

¹Macro nutrient composition for the specific products given by the suppliers.
²Fortified and placebo foods had similar energy content, only shown once.
³Analysed total vitamin D concentrations of fortified and placebo foods (D₃ + 2.5 x 25(OH)D).
⁴Mean (SD) for all such values.
### Table 2
Baseline characteristics of participants by ethnicity

<table>
<thead>
<tr>
<th></th>
<th>Danish Total (n=66)</th>
<th>Pakistani Total (n=70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Born in Denmark (%)</td>
<td>99</td>
<td>33</td>
</tr>
<tr>
<td>Mean Age (y)</td>
<td>33 (11)</td>
<td>36 (9)</td>
</tr>
<tr>
<td>Mean weight in kg (SD)</td>
<td>68 (13)</td>
<td>70 (12)</td>
</tr>
<tr>
<td>Mean BMI (kg/m²)</td>
<td>24 (5)</td>
<td>27 (5)*</td>
</tr>
<tr>
<td>Mean fat percentage (%)</td>
<td>31 (8)</td>
<td>37 (6)*</td>
</tr>
<tr>
<td>Mean baseline serum 25(OH)D</td>
<td>49.6 (18)</td>
<td>46.9 (22)</td>
</tr>
<tr>
<td>&lt; 9.9 nmol/L, n (%</td>
<td>0 (0)</td>
<td>0 (0) b</td>
</tr>
<tr>
<td>≥ 10 - &lt; 29.9 nmol/L, n (%)</td>
<td>6 (9)</td>
<td>17 (24)</td>
</tr>
<tr>
<td>≥ 30 - &lt; 49.9 nmol/L, n (%)</td>
<td>33 (50)</td>
<td>22 (32)</td>
</tr>
<tr>
<td>≥ 50 nmol/L, n (%)</td>
<td>27 (41)</td>
<td>31 (44)</td>
</tr>
<tr>
<td>Mean PTH at baseline (pmol/L)</td>
<td>5.0 (1.7)</td>
<td>4.4 (1.8)*</td>
</tr>
<tr>
<td>Median vitamin D intake from the diet (µg/day)</td>
<td>1.5 (1.0 ; 2.0)</td>
<td>1.1 (1.0 ; 2.0)*</td>
</tr>
<tr>
<td>Total median vitamin D intake from vitamin D suppl. and multivitamin suppl. (µg/day)</td>
<td>2.9 (1.8 ; 9.0)</td>
<td>13 (6.8 ; 29.3)*</td>
</tr>
<tr>
<td>Median Calcium intake from the diet (mg/day)</td>
<td>465 (324 ; 688)</td>
<td>441 (260 ; 589)</td>
</tr>
<tr>
<td>Vitamin D supplements at baseline (%)</td>
<td>15</td>
<td>61*</td>
</tr>
<tr>
<td>Multivitamin supplements at baseline (%)</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Alcohol drinkers (%)</td>
<td>86</td>
<td>1.4*</td>
</tr>
<tr>
<td>Wearing hijab (%)</td>
<td>0</td>
<td>43*</td>
</tr>
</tbody>
</table>

*Means and SD unless otherwise specified. If non-normally distributed, medians and 25th and 75th percentiles. No variables were transformed for analysis.

*Means significantly different from women of Danish origin; Unpaired t test, P <0.05

¶Percentage significantly different from women of Danish origin; Pearson’s chi² test, P <0.05
Table 3
Total vitamin D intake, and serum 25(OH)D concentration and PTH concentration at baseline and endpoint in each of the four study groups

<table>
<thead>
<tr>
<th>Intervention groups</th>
<th>DK Placebo (n = 35)</th>
<th>DK Fortified (n = 31)</th>
<th>PA Placebo (n = 37)</th>
<th>PA Fortified (n = 33)</th>
<th>P^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total vitamin D Intake^2, µg/d</td>
<td>1.5 (1.0, 2.0)^a</td>
<td>32.0 (27.0, 34.4)^b</td>
<td>1.1 (0.8, 1.4)^a</td>
<td>24.2 (19.2, 30.8)^c</td>
<td>0.0001</td>
</tr>
<tr>
<td>Serum 25(OH)D, nmol/L:</td>
<td>46.2 (19)^a</td>
<td>53.3 (17)</td>
<td>49.0 (23)</td>
<td>44.5 (21)</td>
<td>0.31</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endpoint</td>
<td>44.0 (17)^a</td>
<td>77.8 (14)^b</td>
<td>36.5 (16)^a</td>
<td>54.7 (18)^c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Change</td>
<td>-2.8 (9)^a</td>
<td>26.4 (16)^b</td>
<td>-11.2 (12)^a</td>
<td>10.5 (18)^c</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum PTH, pmol/L</td>
<td>5.3 (1.9)</td>
<td>4.9 (1.6)</td>
<td>4.2 (1.8)</td>
<td>4.5 (1.8)</td>
<td>0.07</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endpoint</td>
<td>4.7 (1.4)</td>
<td>4.4 (1.8)</td>
<td>4.3 (1.8)</td>
<td>4.3 (1.5)</td>
<td>0.97</td>
</tr>
<tr>
<td>Change</td>
<td>-0.54 (9.0)</td>
<td>-0.42 (16)</td>
<td>-0.002 (12)</td>
<td>-0.27 (18)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

^DK = Danish, PA = Pakistani; 25(OH)D = 25-hydroxyvitamin D; PTH, parathyroid hormone.

^2Total vitamin D intake during study; placebo groups only had dietary intake, fortified groups had diet plus study fortified foods.

^3Median (25th, 75th percentiles).

^4Means (SD), all such values.

^5P values for baseline comparisons by intervention group were determined with the use of a simple one-way ANOVA, followed by a Tukey HSD test.

^a,b,cDifferent superscript letters represent significant (P<0.01) differences in group means for endpoint total vitamin D intake, serum 25(OH)D concentration and change in serum 25(OH)D concentration.
Table 4  
Number and percentage of women with serum 25(OH)D concentration below 30 and 50 at baseline and endpoint in each of the four study groups

<table>
<thead>
<tr>
<th></th>
<th>Danish placebo</th>
<th>Danish fortified</th>
<th>Pakistani placebo</th>
<th>Pakistani fortified</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 30 baseline, n (%)</td>
<td>5 (14)</td>
<td>1 (3)</td>
<td>8 (22)</td>
<td>9 (27)</td>
</tr>
<tr>
<td>&lt; 30 endpoint, n (%)</td>
<td>7 (23)</td>
<td>0 (0)</td>
<td>11 (34)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>&lt; 50 baseline, n (%)</td>
<td>25 (71)</td>
<td>14 (45)</td>
<td>19 (51)</td>
<td>20 (60)</td>
</tr>
<tr>
<td>&lt; 50 endpoint, n (%)</td>
<td>20 (65)</td>
<td>0 (0)</td>
<td>25 (78)</td>
<td>11 (41)</td>
</tr>
</tbody>
</table>
Table 5
Analysis of covariance models exploring the intervention effects on serum 25(OH)D concentration response

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Effect</th>
<th>95 % CI</th>
<th>( P )</th>
<th>Effect</th>
<th>95 % CI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept(^3)</td>
<td>16.5</td>
<td>(9.76; 23.2)</td>
<td>(&lt;0.0001) (***)</td>
<td>33.8</td>
<td>(19.6; 48.1)</td>
<td>(&lt;0.0001) (***)</td>
</tr>
<tr>
<td>Baseline 25(OH)D</td>
<td>-0.41</td>
<td>(-0.52; -0.30)</td>
<td>(&lt;0.0001) (***)</td>
<td>-0.42</td>
<td>(-0.53; -0.30)</td>
<td>(&lt;0.0001) (***)</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pakistani</td>
<td>-8.41</td>
<td>(-13.9; -2.14)</td>
<td>0.008 (**)</td>
<td>-6.12</td>
<td>(-12.14; -0.07)</td>
<td>0.05 (*)</td>
</tr>
<tr>
<td>Danish*Fortified</td>
<td>31.1</td>
<td>(25.0; 37.2)</td>
<td>(&lt;0.0001) (***)</td>
<td>33.8</td>
<td>(19.6; 48.1)</td>
<td>(&lt;0.0001) (***)</td>
</tr>
<tr>
<td>Pakistani*Fortified</td>
<td>20.3</td>
<td>(14.3; 26.3)</td>
<td>(&lt;0.0001) (***)</td>
<td>27.7</td>
<td>(12.2; 43.3)</td>
<td>(&lt;0.0001) (***)</td>
</tr>
<tr>
<td>BMI at baseline</td>
<td>-0.64</td>
<td>(-1.16; -0.12)</td>
<td>0.02 (*)</td>
<td>-0.64</td>
<td>(-1.16; -0.12)</td>
<td>0.02 (*)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.05</td>
<td>(0.006; 0.20)</td>
<td>0.69</td>
<td>-0.05</td>
<td>(0.006; 0.20)</td>
<td>0.69</td>
</tr>
</tbody>
</table>

\(^1\)Model 1 (minimal): change in vitamin D status \(\sim\) Baseline status + intervention group + (ethnicity*intervention group), adjusted \(R^2=65\%\).
\(^2\)Model 2 (maximal): change in vitamin D status \(\sim\) Baseline status + intervention group + (ethnicity*intervention group) + BMI + Age, adjusted \(R^2=67\%\).
\(^3\)The reference group is included in the intercept: Danish ethnicity and placebo study group.
Paper III

Effect of vitamin D fortified foods on bone markers and muscle strength during winter in women of Pakistani and Danish origin living in Denmark: a randomised controlled trial
EFFECT OF VITAMIN D FORTIFIED FOODS ON BONE MARKERS AND MUSCLE STRENGTH IN WOMEN OF PAKISTANI AND DANISH ORIGIN LIVING IN DENMARK: A RANDOMISED CONTROLLED TRIAL.

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Orcid ID: 0000-0001-7316-1073

Running title: Effect of vitamin D fortified foods on muscle strength and bone markers

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Contributors: RA and IT designed the study; MK, REKL, TLLT, IMG and RA collected the data; MK, REKL and TLLT designed the muscle strength tests and managed the muscle strength data; IMG undertook the statistical analyses and wrote this paper. All contributed to the manuscript.

ABBREVIATIONS

DTU – Technical University of Denmark
ODIN – Food-based solutions for optimal vitamin D nutrition and health through the life cycle
25(OH)D – Serum 25-hydroxyvitamin D
OC – Osteocalcin
BALP – Bone specific Alkaline Phosphatase
P1NP – Procollagen type 1 amino-terminal propeptide
CTX – C-terminal crosslinked telopeptide of type 1 collagen
BMD – Bone Mineral Density
IOF – International Osteoporosis Foundation
VDR – Vitamin D receptor
RCT – Randomised Controlled trial
SD – Standard Deviation
FFQ – Food Frequency Questionnaire
UCC – University College Cork
LC-MS/MS – Liquid Chromatography – Liquid chromatography-tandem mass spectrometry
VDSP – Vitamin D Standardization Program
ANOVA – Analysis of variance
ANCOVA – Analysis of co-variance
ABSTRACT

Background: Low vitamin D status is prevalent worldwide and associated with decreased muscle strength and poor bone health. We investigated the effect of vitamin D fortification on muscle strength and bone markers in women of Danish and Pakistani origin, at risk of vitamin D deficiency.

Methods: A 12-week randomised double-blinded placebo-controlled winter intervention trial, providing 30 µg vitamin D$_3$/day through fortified yoghurt, cheese, eggs and crisp-bread. Participants were 143 women of Danish and Pakistani origin 18-50 years of age, living in Denmark, randomised into four groups stratified by ethnicity. Serum 25-hydroxyvitamin D (25(OH)D) and the endpoints: muscle strength (handgrip, knee extension strength, chair-standing), and four specific bone markers (osteocalcin (OC), Bone specific Alkaline Phosphatase (BALP), Procollagen type 1 amino-terminal propeptide (P1NP), C-terminal crosslinked telopeptide of type 1 collagen (CTX)) were assessed using one-way ANOVA, ANCOVA, Tukey HSD and subsequent linear models adjusted for relevant covariates.

Results: Serum 25(OH)D concentration increased significantly from 53.3 (17) to 77.8 (14) nmol/L and from 44.5 (21) to 54.7 (18) nmol/L in the Danish and Pakistani women in the groups receiving fortified foods, respectively (P <0.05). Baseline handgrip and knee extension strength were 4.8 and 6.3 kg higher among Danish participants compared to Pakistani (P < 0.001). Following the intervention, the bone markers OC, BALP, P1NP and CTX did not change significantly. The change in knee extension strength were approximately 2 kg higher among the Danish women compared to the Pakistani, when adjusting for intervention group, BMI and baseline knee strength, however, muscle strength by handgrip and chair-standing test did not change significantly following the intervention.
**Conclusions:** Consumption of vitamin D fortified foods for 12 weeks did not result in significant changes of the bone turnover markers OC, BALP, P1NP and CTX. Muscle strength measured as hand grip strength and chair-standing did not change significantly following the intervention. However, the adjusted change in knee extension strength was approximately 2 kg higher among the women of Danish origin compared to the women of origin Pakistani.

**Keywords:** Vitamin D, Food-based RCT, ODIN, women of Pakistani origin, women of Danish origin, fortified foods, muscle strength, bone markers, markers of bone turnover.

**INTRODUCTION**

Vitamin D has a well-established role in the calcium and phosphorus homeostasis and is essential for bone health [1, 2]. Studies have shown that the consequences of vitamin D deficiency and insufficiency defined as serum 25(OH)D concentrations < 30 and 50 nmol/L, respectively [3, 4], may include non-skeletal adverse effects such as muscular atrophy, muscle pain and decreased muscle strength [5–7]. Vitamin D deficiency and insufficiency is a prevalent worldwide problem [8]. Especially in northerly countries such as Denmark in which the absence of vitamin D from UVB sources during winter increases the risk of deficiency and insufficiency [9, 10]. Immigrants (first generation and descendants) living in Denmark as well as other Nordic countries are considered at risk of vitamin D deficiency due to factors such as skin colour, cultural clothing and dietary habits [11–13]. Bone tissue is a dynamic structure that undergoes constant remodelling, and optimal bone health is a balance between bone resorption and bone formation. In a case of chronic bone resorption, the bone mineral density (BMD) will decrease with time [14]. The bone turnover can be monitored by bone biomarkers in the blood (different enzymes and peptides), and is therefore a potential cost-
effective method to complement BMD measurements and may be used in situations where BMD is not a feasible endpoint e.g. studies of shorter duration [14, 15]. Because markers of bone resorption, such as bone specific Alkaline Phosphatase (BALP), osteocalcin (OC) and C-terminal crosslinked telopeptide of type 1 collagen (CTX) have been shown to respond in situations of vitamin D deficiency and insufficiency, they are utilised as indicators of bone resorption ([16, 17]. CTX has been suggested as a reference marker of bone resorption and Procollagen type 1 amino-terminal propeptide (P1NP) as a reference marker of bone formation by the International Osteoporosis Foundation (IOF) and International Federation of Clinical Chemistry and Laboratory Medicine [14, 18].

Recent advances have located vitamin D receptors (VDR) within muscle tissue, supporting the assumption that vitamin D has an effect on muscle function, have initiated a current debate on the mechanism regarding vitamin D and muscle strength [19, 20]. Some studies have found an effect on muscle strength [21, 22] and some effect in the number of falls [23], while other studies were unable to see an effect [24], the majority of the participants were elderly > 60 y. These inconsistent trends may be related to the baseline serum 25(OH)D concentration and the effect of vitamin D supplementation is more pronounced on vitamin D deficient people (25(OH)D concentrations < 25-30 nmol/L) [21, 22]. Additionally, assessment of the effect of vitamin D on muscle strength has primarily been studied among elderly subjects (mainly community dwelling), and there is a lack of data on the effects of vitamin D on muscle strength in young adults deficient in vitamin D [25]. The mechanism of vitamin D and muscle function has not been agreed upon, however, it has been hypothesized that the active metabolite of vitamin D (1,25(OH)2D) vitamin D is involved in the regulation of the calcium flow within the muscle through genomic and non-genomic pathways and is thus important for the contraction of muscle fibres [5, 20]. The genomic pathway involves 1,25(OH)2D and its binding to the VDR and subsequent effects on intracellular calcium
concentration as described in relation to skeletal effects of vitamin D. The non-genomic pathway involves the classic vitamin D-related serum calcium regulation in the kidneys and intestines primarily, the result increase or decrease the blood and intracellular calcium concentration likewise affecting muscle contractility [5, 20].

We investigated the effect of 12 weeks of intervention with vitamin D fortified foods on markers of bone turnover and muscle strength in a population of women of Danish and Pakistani origin between 18 and 50 years of age, at risk of vitamin D deficiency. The parameters investigated were; the bone markers OC, BALP, P1NP and CTX as well as muscle strength measured as handgrip strength, knee extension strength and a 30 second chair-standing test.

METHODS

Study design

This paper is based on intervention data from a food-based randomised controlled trial (RCT) ODIN-FOOD. The intervention was designed to assess the change in serum 25-hydroxyvitamin D₃ following fortification, while measurements of muscle strength and bone turnover markers were secondary outcomes.

The present study was a three month double-blinded RCT. A total of 143 women of Danish (n = 71) and Pakistani (n = 72) origin between 18 and 50 years were enrolled in the study. Details of the study design, randomization and blinding has been described previously [26]. The subjects were given four different vitamin D fortified foods contributing with 30 µg/day (intervention group) or non-fortified foods (placebo group). The intervention was performed during the winter months (Jan. - Apr.) to minimize interference from cutaneous produced vitamin D. Participants were seen before the intervention start (January 2016) and at the end of intervention after 3 months (April 2016). At the visits blood was drawn, anthropometrics, dietary vitamin D intake and muscle strength were
measured. The endpoints were hand grip strength, knee extension strength, number of chair-stands, and concentrations of the different bone turn over markers (OC, BALP, P1NP, CTX).

Sample size was based on 90 % power (alpha = 5 %) to detect a change in 25(OH)D concentration of 20 nmol/L in the treatment group with a standard deviation (SD) of 23, and a drop-out rate of 20 %.

Participants

The participants were recruited from the Copenhagen area, city and suburbs (Denmark, 55°N). The recruitment was carried out in the autumn of 2016 by e-mail, advertisements and networking, as described elsewhere [26]. Inclusion criteria were a low consumption of fish and fish products (less than weekly), a low intake of vitamin D-containing supplements (less than weekly), no use of tanning facilities, no planned sun-holiday (more southerly than 47°N) between October 2015 and May 2016. No upper limit of the concentration of vitamin D in the supplement was specified as long as the participants stopped taking them at the time of enrolment in the study. Exclusion criteria were pregnancy and breastfeeding, menopause, non-Danish speakers, serious diseases (cancer, severe liver or kidney insufficiencies, sarcoidosis and other granulomatous diseases) and medicines affecting the vitamin D metabolism (steroids, antiepileptic, thyroid hormones, bisphosphonates, oestrogen).

Study foods

The intervention foods were low-fat Milner cheese (gouda) and yoghurt (plain) produced and provided free of cost by FrieslandCampina in the Netherlands, eggs (Livskraft) produced and provided free of cost by Hedegaard in Denmark, and whole wheat biscuits produced by Smørum konditori (confectionary) with ingredients supplied free of cost by Lantmännen Cerealia, Denmark, with vitamin D$_3$ supplied by DSM nutritional products, Switzerland. The study foods were chosen
with the aim of increasing the total vitamin D status in an effective and safe manner in both ethnic
groups. Foods highly representative in both traditional Danish and Pakistani kitchen were chosen.
The fortified and non-fortified foods have the same fat percentage and comparable content of
nutrients, except for the content of vitamin D.
Random samples from every batch were analysed for vitamin D content at the National Food
Institute in Denmark (further details and methods described elsewhere, [26]).

**Measurements**

At each of the two study visits non-fasting blood samples were obtained between 9 am and 16 PM
for the majority of the samples. Efforts were made to book the endpoint measurement at the same
time as baseline visit for each participant. Anthropometric measures were height (wall mounted
stadiometer, seca, Hamburg, Germany), weight, waist-hip circumference (standard tape measure)
and body composition (Tanita BC 418 MA, Tokyo, Japan), as described elsewhere [26]. At baseline
the participants completed two questionnaires, a general background questionnaire containing
questions regarding general health, physical activity (during work and leisure time, if the
participants had a job) and lifestyle, and a semi-quantitative Food Frequency Questionnaire (FFQ)
estimating the average daily intake of vitamin D and calcium. The FFQ was a retrospective
questionnaire asking about the participants’ habitual dietary intake of vitamin D-rich foods over the
3 preceding months. The questionnaire contained the 8 food groups (fish, meat, milk and milk
products, egg, cheese, bread, fats and pulses) contributing to the majority of dietary vitamin D (98
%) and calcium (71 %) found in a general diet [27]. The weekly compliance was monitored by a
user-friendly compliance questionnaire as described in details elsewhere [26].
Markers of bone turnover

In this paper we used the bone markers OC, BALP and P1NP for the assessment of the bone formation and CTX as a marker of bone resorption, based on previous studies carried out in a similar population group [28, 29].

The biochemical analyses of the bone turnover markers OC, BALP, P1NP, and CTX were performed at the University Hospital Aarhus. OC had a precision of ± 0.55 µg/L (SD) at a concentration of 19 µg/L and ± 2.75 µg/L (SD) at a concentration of 92 µg/L. BALP had a precision of ± 0.45 µg/L (SD) at a concentration of 4.5 µg/L and ± 4.6 µg/L (SD) at a concentration of 45.9 µg/L. P1NP had a precision of ± 1.1 µg/L (SD) at a concentration of 30 µg/L and ± 7.6 µg/L (SD) at a concentration of 205 µg/L. CTX had a precision of ± 0.015 µg/L (SD) at a concentration of 0.26 µg/L and ± 0.03 µg/L (SD) at a concentration of 0.59 µg/L.

Muscle function

Both upper and lower limb muscle function were measured using different physical tests. Upper limb muscle strength was measured as isometric hand grip strength (measured in kg) using a digital hand dynamometer (SAEHAN Corporation, South Korea) which have previously been demonstrated to have high inter-rate and test-retest reliability [30]. Participants were seated on a chair with the arm in a 90º elbow flexion, and the forearm in mid-prone neutral position. The handle of the dynamometer was placed in position 2. All participants had two familiarization trials prior to the maximal contraction test. A minimum of three maximal isometric contractions were conducted with the dominant hand with a 90-second break between each measurement. If the last contraction was the highest, the participants had up to two additional contractions. The instructor gently supported below the instrument to minimized an effect of gravity. Muscle strength was calculated as the mean of the three highest values obtained (averaged peak grip strength). The intra-assay coefficient of variation (CV) for hand grip strength was 4.0%.
Lower limb muscle strength was measured as isometric knee extension strength (measured in kg) using a digital handheld muscle tester (MicroFET2, Hoggan Scientific, USA). The MicroFET2 dynamometer was belt-stabilized to the table, so that the instructor would only have to concentrate on holding the dynamometer in the correct position on the ankle [31]. The participants were seated on a table fitted with a non-skid material in an upright position with a 90° hip flexion and arms placed on the lap. The lower leg was hanging down about 1 centimetre from the edge of the table without touching the floor. The MicroFET2 dynamometer was placed on the front of the ankle, on the dominant leg, approximately 4 cm above the lateral malleoli. The participants were asked to stretch their leg as much as they could for approximately 5 seconds. The participants had two familiarization trials where they were asked to stretch their leg using ≈ half of maximal power. This was followed by 3-5 maximal contractions lasting approximately 5 seconds with a 90-second break between each measurement. Muscle strength was calculated as the mean of the three highest values obtained. The intra-assay CV for knee extension strength was 4.2%.

Lower limb muscle function was also measured by a 30-second chair-stand test, as a measurement of anaerobic muscle function. Participants were seated on a chair (43 cm high, without arm rest). Arms were folded across the chest, and the number of full stands achieved in 30 seconds were recorded.

25(OH)D-measurements and other biochemical measurements

Serum concentrations of total 25(OH)D (i.e., 25(OH)D$_2$ plus 25(OH)D$_3$) of all serum samples (baseline and endpoint visits) were measured at the Cork Centre for Vitamin D and Nutrition Research, University College Cork (UCC), using the ODIN core LC-MS/MS analytical platform for serum 25(OH)D, described in detail elsewhere [32]. The intra-assay CV of the method was < 5 % for all 25(OH)D metabolites, whilst the inter-assay CV was < 6 %. The LC-MS/MS method at UCC...
is certified by the Centre for Disease Control and Prevention’s Vitamin D Standardization Certification Program (VDSP).

Serum creatinine a marker of muscle mass [33] was measured at the University Hospital Aarhus. Creatinine had the following precisions at specific concentrations: At 81 µmol/L ± 7.4% (95% CI) and at 567.0 µmol/L ± 5.0% (95% CI) and biological variation of ± 11.9% (95% CI).

**Statistical analysis**

Results are shown as means (SD) unless otherwise specified. Descriptive statistics were calculated using a two-sample t test or the non-parametric Kruskal Wallis test for comparing the two ethnic groups. Categorical variables were compared using a Pearson’s chi-square test. Baseline comparisons by the four level factor (intervention group times ethnic group) were determined with the use of a one-way ANOVA followed by Tukey’s test. ANCOVA analysis of covariance was used to analyse the effect of the intervention on the respective outcome variables (muscle strength and markers of bone turnover). We adjusted for age, ethnicity, BMI and study group as well as the respective baseline variables. Finally, to check the model assumptions the standardized residuals of the models were assessed visually for normality, variance homogeneity and linearity. Compliance was estimated individually by dividing the individual amount of food with the individual self-reported compliance the final compliance is shown as the percentage consumed foods of delivered foods.

Statistical analyses were performed using RStudio for Windows [34] (Version 1.1.414 – © 2009-2018 Rstudio, Inc.) with a significance level of \( \alpha = 0.05 \).
RESULTS

Baseline characteristics relating to anthropometrics, dietary intake of vitamin D and calcium, vitamin D status, self-reported physical activity and health are listed in Table 1. The mean baseline vitamin D status among the women of Danish and Pakistani origin were 49.6 (18) and 46.9 (22) nmol/L, respectively (no ethnic difference $P = 0.4$). At baseline 9 % and 24.3 % of the participants of Danish and Pakistani origin, respectively, had a vitamin D status below 30 nmol/L (distribution data shown elsewhere [26]). The women of Pakistani origin had a slightly higher BMI and fat percentage compared to the women of Danish origin (t test, both $P < 0.001$, data not shown) and lower lean mass (t test, $P < 0.001$, data not shown).

Among the participants of Danish origin 24% reported their health as “very good”, this was only the case with 7% among the participants of Pakistani origin. Accordingly, 33% the women of Pakistani origin reported “somewhat bad”, whereas only 4% of the women of Danish origin reported this. When asked to compare the overall health with others of the same age, 6% and 26% of the women of Danish and Pakistani origin, respectively, reported “worse”.

Assessment of the baseline markers of bone turnover revealed significant ethnic differences of OC, a marker of bone formation, with higher concentrations among the women of Danish origin (t test, $P <0.001$, data not shown). Simple one-way ANOVA were used to test for significant baseline comparison across the four study groups (DK placebo, DK fortified, PA placebo, Pa fortified) and a subsequent Tukey HSD test showed which groups were responsible for the difference, Table 2. At endpoint we saw a persistent ethnic difference in OC significant difference between the Danish and the Pakistani fortified groups ($P = 0.03$), however when assessing the change in bone turnover markers following the intervention, we found no significant differences in the change in any of the markers were seen following intervention, unadjusted ANOVA ($P > 0.05$, Table 2).
Baseline handgrip and knee extension strength were 4.8 and 6.3 kg higher among participants of Danish origin compared to participants of Pakistani origin (both $P < 0.001$, data not shown). Results from the chair-standing test showed a significance difference of 1.7 rep/min more among the participants of Danish origin compared to participants of Pakistani origin (t test 0.03, data not shown). Again a simple ANOVA and subsequent Tukey HSD test were applied to show which comparisons carry the significance, Table 3. Generally, the differences seen at baseline were also observed at endpoint, thus we saw no significant differences in the changes in muscle strength following using an unadjusted ANOVA, despite that the intervention was effective in increasing the mean serum 25(OH)D concentration from 53.3 (17) to 77.8 (14) nmol/L and from 44.5 (21) to 54.7 (18) nmol/L among the fortified Danish and Pakistani women, respectively ($P < 0.05$) (data shown in full in previous publication [26]).

**Linear models**

The effects of 12 weeks of intervention with vitamin D fortified foods on markers of bone turnover as well as muscle strength were explored in linear ANCOVA models controlled for relevant covariates that may influence the outcome (Table 4 and 5).

The intervention with vitamin D fortified foods showed no effect on the markers of bone turnover OC, CTX, BALP and P1NP, analysed in an ANCOVA including the baseline covariates age, ethnicity, intervention group and BMI. Only the baseline concentration of each marker of bone turnover had a significant influence on the endpoint concentration of the specific bone marker (Table 4).

Considering the muscle strength tests, fortification with vitamin D did not have a significant effect on the muscle strength measured as hand grip strength, only the baseline hand grip strength had a significant influence on the endpoint hand grip strength (Table 5). Analysis of the knee extension
strength showed a significant negative effect of Pakistani ethnicity compared to Danish ethnicity. We saw an approximately 2 kg higher change of knee extension strength following the intervention among the participants of Danish, compared to participants of Pakistani origin, when adjusting for intervention group, BMI and baseline knee strength. In the chair-standing test the change following the intervention was negatively affected by BMI, and not baseline chair-standing as were the case with the rest of the parameters analysed (Table 5).
DISCUSSION

Following the 12-week intervention with vitamin D fortification no significant increase in muscle strength in the two fortified groups and no significant decrease in muscle strength of the placebo groups were measured. However, the women of Pakistani origin had significantly lower baseline muscle strength compared to the women of Danish origin. No significant changes in the concentration of the markers of bone turnover OC, BALP, P1NP and CTX following the intervention.

We expected a low baseline vitamin D status among our study population (< 30 nmol/L) as previous studies including women of Pakistani origin have found a very low baseline vitamin D status (< 15 nmol/L) [28]. In order to recruit only women at risk of vitamin D deficiency we used restrictive inclusion criteria allowing only women with a low intake of fish and supplements as well as a low UVB exposure to be enrolled in the study (described elsewhere in detail, [26]). Despite vitamin D exposure-specific inclusion criteria, the mean baseline vitamin D status of the participants in this trial were 49.6 (18) and 46.9 (22) nmol/L, measured among participants of Danish and Pakistani origin, respectively. The distribution of vitamin D deficiency (< 30 nmol/L) in our study population showed that 9% of the participants of Danish origin and 24.3% of participants of Pakistani origin had a serum 25(OH)D concentration below 30 nmol/L at baseline (data shown in full length elsewhere [26]). In an earlier study by Andersen et al. the distribution of low vitamin D status among the women of Pakistani origin were reported as serum 25(OH)D concentration ≤ 10 or >10- ≤ 25 nmol/L, and the prevalence was 40 and 44%, respectively at baseline [11]. Thus, vitamin D status among women of Pakistani origin living in Denmark may have improved significantly during the last 10 years, for the specific populations.
Observational studies report increased bone resorption with vitamin D deficiency [16, 35]. Sahota et al. reported higher concentrations of OC and BALP in women living in the UK with vitamin D deficiency compared to women with sufficient concentrations of vitamin D [35]. Despite observations of increased bone turnover, RCT’s assessing the effect of vitamin D supplementation on markers of bone turnover have shown inconsistent results [28, 29, 36–38]. A Danish study assessed the effects of one year of vitamin D supplementation (10 or 20 µg/day) on bone markers among immigrant families in Denmark (ages 13-53 y) [28]. The baseline serum 25(OH)D concentrations were 10.9, 12.0 and 20.7 nmol/l for girls, women and men, respectively, however, no effects on the bone markers OC and urinary pyridinoline and deoxypyridinoline were seen [11, 28]. Likewise an RCT from Norway assessed an immigrant population with a baseline serum 25(OH)D concentration of 28.9 (17.6) nmol/L, after 16 weeks of vitamin D supplementation (10 and 25 µg/day) they found no changes in serum P1NP or CTX [29]. A more recent Austrian study by Schwetz and colleagues studied hypertensive adults (mean age 62 y) with a mean baseline serum 25(OH)D concentration of 55 nmol/L and found no effect of 8 weeks of vitamin D supplementation (70 µg) on BALP, CTX, P1NP or OC [37]. On the contrary, a Brazilian study assessing menopausal women (mean age 59 y) with a normal BMD, recently found a significant decrease in the bone resorption marker CTX, however they also found a decrease of the bone formation marker P1NP following 9 months of vitamin D supplementation with 25 µg/day in the supplemented group compared to baseline, however no group differences were found (compared to placebo). The baseline status of the participants in the vitamin D supplemented group were 37.4 (18.7) nmol/L, increasing to 68.6 (26.0) nmol/L following the intervention, the placebo group decreased from 42.2 (16.7) nmol/L to 34.4 (15.0) nmol/L and no changes were seen in the bone turnover [38].

Our study differs from these previous RCT’s by including both women of Pakistani origin and Danish origin and we used four different vitamin D fortified foods instead of a vitamin D
supplements. The relatively high baseline serum 25(OH)D concentration seen among the women of Danish and Pakistani origin of 49.6 (18) and 46.9 (22) nmol/L, respectively, may be a reason for not finding an effect of vitamin D supplementation on markers of bone turnover. When vitamin D status is above the suggested cut-off optimal concentration (> 50 nmol/L), PTH concentration will be within the normal range and calcium will not be released from the bone matrix to support the calcium balance [16].

Observational data show correlations between vitamin D status and muscle strength [39], however, when studied in RCT’s the evidence appears mixed [40]. The majority of the studies (observational as well as RCT’s) have been performed in older adults (> 50 years). Few studies however, investigate the effect of vitamin D in younger adults, adolescents and children. E.g. one study assess the effects of one year of high dose vitamin D supplementation (35 or 350 µg/week ≈ 5 or 50 µg/day) among children and adolescents (10-17 years) with a mean baseline serum 25(OH)D concentration of 35 (20) [41]. They found no effect on hand grip strength in any of the groups. However, they found effects of vitamin D supplementation on lean mass and BMD of the postmenarcheal girls in the high-dose group, but not among the premenarcheal girls [41]. Although interesting and highly needed, a study like this assessing a younger population group may be difficult to interpret since they include both pre- and postmenarcheal girls at different stages in terms of peak bone mass. A study performed in young adults (31 years, n = 40) gave 1500 µg/week for 8 weeks followed by 1500 µg vitamin D3/month for 4 months with 1 g of calcium or placebo. The baseline serum 25(OH)D concentration was 25.4 (9.9) and 21.1 (9.4) in the two study groups, respectively and the results showed a significant increase in hand grip strength (≈ 2 kg) among those supplemented with vitamin D, compared to the placebo group [42]. Two larger systematic review and meta-analyses by Stockton et al. and Beaudart et al. including 17 and 30 RCT’s, respectively, found that the effect of vitamin D supplementation may only be present among
vitamin D deficient people (Vitamin D concentrations < 25-30 nmol/L) [21, 22]. A smaller systematic review and meta-analysis assessing healthy young adults (18-40 y) included six RCT’s and one controlled study assessing upper and lower limb strength and vitamin D. They conclude that vitamin D supplementation had a significant positive effect on muscle strength, however a lack of studies in the young adult population calls for further studies [43].

A Danish study assessed the effects of high dose (2500 µg/week for 1 month followed by 2500 µg/month for 5 months) vitamin D supplementation in women of Arabic origin (n = 55) with a Danish control group (n = 22). The women of Arabic origin had a baseline serum 25(OH)D concentration of 6.7 (0.6) nmol/L compared to the women of Danish origin having a serum 25(OH)D concentration of 47.1 (4.6) nmol/L. They observed a very low baseline knee extension strength (maximum voluntary), among the women of Arabic origin of 26.4 kg, which was 34% lower than the 32.7 kg among the women of Danish origin. After high dose treatment, the ethnic difference disappeared. In addition, they observed a significant reduction in reports of muscle and bone pain among the women of Arabic origin [44]. Our results showed an ethnic difference of 2 kg in knee extension strength, which is smaller compared to the study by Glerup et al. described above.

In the present study the serum 25(OH)D concentration in the placebo groups decreased by 2.8 (9) nmol/L in the Danish placebo group and by 11.2 (12) nmol/L in the Pakistani placebo group, however we did not see a significant decrease in muscle strength and bone formation markers or increase of the bone resorption marker (CTX) following the intervention. Based on the above, one could argue that a vitamin D status of ≈ 50 nmol/L measured at the height of winter (start of January) would be sufficient to maintain healthy muscle and bone during the following winter months.
The women of Danish origin reported a more active lifestyle compared to the women of Pakistani origin in the background questionnaire. In line with the self-reported physical activity, we saw that baseline muscle strength was significantly higher among the women of Danish origin, compared to the women of Pakistani origin in all of the three muscle strength tests. After correcting for baseline knee extension, BMI and intervention group in a linear model, participants of Pakistani origin had a significantly lower change in knee extension strength compared to the women of Danish origin.

**Strengths and limitations**

The ODIN FOOD study was carried out in winter which is a strength in relation to avoiding confounding from vitamin D originating from UVB sources. An additional strength was the real-life setting of the study and thereby the applicability of the results in a public health setting.

The study was designed to detect the effect of vitamin D fortification on serum 25(OH)D concentration and thus, the power calculation was made with vitamin D status as the response variable. This may affect the results of secondary endpoint analyses such as muscle strength and bone markers due to a too low power to detect relevant changes in the mentioned endpoints.

Several limitations are present when using markers of bone remodelling. Large pre-analytical and analytical variability are seen and markers of bone turnover are affected by modifiable factors such as circadian rhythm, season, ethnicity, fasting state, physical activity and menstrual cycle [14]. Additionally, there are no standardized assays for bone markers. We were not able to obtain fasting blood samples from the participants due logistical reasons. We attempted to book the participant at the same hour of the day at the two different measurements, however some of the bookings were cancelled and rescheduled and this may affect the bone markers especially sensitive to diurnal changes (such as CTX) [14].
With regard to the study duration, 12 weeks seems to be the minimum time used in previous studies and may be too short a period to detect any changes in muscle strength and bone markers [22, 29] especially if the participants are in a vitamin D replete state of vitamin D, little may change following an intervention with vitamin D [22].

At baseline, the participants would perform the muscle test for the first time, whereas at the endpoint measurement there would be some familiarity of the muscle tests. This is a limitation of the muscle strength measurements (hand grip and knee extension) and may influence the score because of inexperience and reluctance to exert full capacity of power at baseline, compared to the endpoint. However, we performed familiarization trails before the tests, and the participants were allowed up to two additional muscle tests trials if the last one were the highest.

Although successfully developed for older adults [45], the 30 second chair-stand test has also been used on people within our target group [46]. However, this test may not be completely optimal for this target group because the participants may have memorized their own baseline test results and some may even have tried to improve their score at the endpoint test. Also, participants with good physical fitness performed the test so fast that some had problems with the balance as a result of this.

**CONCLUSION**

Consumption of vitamin D fortified foods for 12 weeks did not result in significant changes of the bone turnover markers OC, BALP, P1NP and CTX. The change in endpoint knee extension strength were approximately 2 kg higher among the women of Danish origin compared to the women of origin Pakistani, when adjusting for intervention group, BMI and baseline knee strength. However, muscle strength measured as hand grip strength and chair-standing did not change significantly following the intervention.
ACKNOWLEDGEMENTS

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ETHICAL STANDARDS

Written informed consent was obtained from all participants on enrolment. The study protocol was approved by the local ethical committee (protocol no. H-15008276) and registered at ClinicalTrials.gov with identifier: NCT02631629. The study was carried out in accordance with the Declaration of Helsinki.

CONFLICTS OF INTEREST

None of the authors had conflicts of interest. The industry partners had no influence on the design of the study, or the interpretation of the results.
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### Table 1: Baseline characteristics by ethnicity and study group

<table>
<thead>
<tr>
<th></th>
<th>Women of Danish origin</th>
<th>Women of Pakistani origin</th>
</tr>
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<tbody>
<tr>
<td><strong>Total n = 136</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Born in Denmark (%)</td>
<td>99 (n=66)</td>
<td>32 (n=70)</td>
</tr>
<tr>
<td>Mean age in years (SD)</td>
<td>33 (11)</td>
<td>36 (9)</td>
</tr>
<tr>
<td>Mean weight in kg (SD)</td>
<td>68 (13)</td>
<td>69 (12)</td>
</tr>
<tr>
<td>Serum 25(OH)D nmol/L</td>
<td>49.6 (18)</td>
<td>46.9 (22)</td>
</tr>
<tr>
<td>Mean plasma creatinine (µmol/l)</td>
<td>61.5 (10)</td>
<td>58.0 (9)</td>
</tr>
<tr>
<td>Median dietary vitamin D intake µg/d</td>
<td>1.5 (0.8)</td>
<td>1.1 (0.7)</td>
</tr>
<tr>
<td>Median dietary calcium intake mg/d</td>
<td>464 (375)</td>
<td>441 (362)</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>Alcohol drinkers (%)¥</td>
<td>86</td>
<td>1.4</td>
</tr>
<tr>
<td>Wearing hijab (%)¥</td>
<td>0</td>
<td>43</td>
</tr>
</tbody>
</table>

#### Total physical activity

<table>
<thead>
<tr>
<th>Physical activity score, no job, n (%)</th>
<th>Women of Danish origin</th>
<th>Women of Pakistani origin</th>
</tr>
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<tbody>
<tr>
<td>n total DK = 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: Sedentary</td>
<td>0 (0%)</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>2: Light</td>
<td>1 (33%)</td>
<td>15 (68%)</td>
</tr>
<tr>
<td>3: Medium</td>
<td>2 (67%)</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>4: Heavy</td>
<td>0 (0%)</td>
<td>1 (4%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Physical activity score, job and leisure, n (%)</th>
<th>Women of Danish origin</th>
<th>Women of Pakistani origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>n total DK = 63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: Sedentary</td>
<td>5 (8%)</td>
<td>10 (21%)</td>
</tr>
<tr>
<td>2: Light</td>
<td>31 (49%)</td>
<td>28 (58%)</td>
</tr>
<tr>
<td>3: Medium</td>
<td>22 (35%)</td>
<td>9 (19%)</td>
</tr>
<tr>
<td>4: Heavy</td>
<td>5 (8%)</td>
<td>1 (2%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Self-rated health, n (%)</th>
<th>Women of Danish origin</th>
<th>Women of Pakistani origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>n total DK = 66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: Very good</td>
<td>24 (36%)</td>
<td>5 (7%)</td>
</tr>
<tr>
<td>2: Somewhat good</td>
<td>37 (56%)</td>
<td>34 (49%)</td>
</tr>
<tr>
<td>3: Somewhat bad</td>
<td>4 (6%)</td>
<td>23 (33%)</td>
</tr>
<tr>
<td>4: Very bad</td>
<td>1 (2%)</td>
<td>8 (11%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Self-rated health compared to others of same age, n (%)</th>
<th>Women of Danish origin</th>
<th>Women of Pakistani origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>n total DK = 66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: A lot better</td>
<td>6 (9%)</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>2: Somewhat better</td>
<td>16 (24%)</td>
<td>21 (30%)</td>
</tr>
<tr>
<td>3: The same</td>
<td>40 (61%)</td>
<td>27 (38%)</td>
</tr>
<tr>
<td>4: Worse</td>
<td>4 (6%)</td>
<td>18 (26%)</td>
</tr>
</tbody>
</table>

Means and SD unless otherwise specified. If non-normally distributed, medians and 25th and 75th percentiles.

¥Significant difference between Danish (all) and Pakistani (all) women in a Pearson’s chi² test.
Table 2: Markers of bone turnover: OC, CTX, BALP, P1NP at baseline, endpoint and the change following intervention

<table>
<thead>
<tr>
<th>Intervention groups</th>
<th>DK Placebo (n = 35)</th>
<th>DK Fortified (n = 31)</th>
<th>PA Placebo (n = 37)</th>
<th>PA Fortified (n = 33)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OC (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>23.9 (9.6)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25.2 (8.7)&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>19.6 (8.4)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.2 (5.6)&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>Endpoint</td>
<td>22.3 (9.0)</td>
<td>22.7 (6.8)&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>19.5 (8.9)</td>
<td>17.4 (5.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.03</td>
</tr>
<tr>
<td>Change</td>
<td>-1.2 (4.2)</td>
<td>-2.3 (4.6)</td>
<td>-0.20 (4.0)</td>
<td>-0.5 (2.9)</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>CTX (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.25 (0.14)</td>
<td>0.30 (0.15)</td>
<td>0.27 (0.19)</td>
<td>0.21 (0.1)</td>
<td>0.13</td>
</tr>
<tr>
<td>Endpoint</td>
<td>0.23 (0.14)</td>
<td>0.26 (0.12)</td>
<td>0.24 (0.16)</td>
<td>0.19 (0.1)</td>
<td>0.20</td>
</tr>
<tr>
<td>Change</td>
<td>-0.006 (0.1)</td>
<td>-0.05 (0.1)</td>
<td>-0.03 (0.1)</td>
<td>-0.02 (0.1)</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>BALP (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>15.5 (7.0)</td>
<td>16.6 (5.2)</td>
<td>15.9 (5.2)</td>
<td>16.9 (7.9)</td>
<td>0.79</td>
</tr>
<tr>
<td>Endpoint</td>
<td>13.4 (5.4)</td>
<td>14.0 (4.4)</td>
<td>14.6 (4.9)</td>
<td>14.6 (6.0)</td>
<td>0.74</td>
</tr>
<tr>
<td>Change</td>
<td>-1.5 (3.5)</td>
<td>-1.5 (3.6)</td>
<td>-1.4 (2.1)</td>
<td>-1.8 (4.5)</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>P1NP (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>50.7 (25.2)</td>
<td>54.6 (19.6)</td>
<td>46.6 (19.6)</td>
<td>48.1 (17.2)</td>
<td>0.42</td>
</tr>
<tr>
<td>Endpoint</td>
<td>46.4 (23.8)</td>
<td>51.1 (16.8)</td>
<td>48.5 (21.0)</td>
<td>45.7 (14.7)</td>
<td>0.72</td>
</tr>
<tr>
<td>Change</td>
<td>-2.0 (7.9)</td>
<td>-2.6 (11.0)</td>
<td>1.7 (10.3)</td>
<td>-1.7 (10.4)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means and SD unless otherwise specified.

<sup>2</sup>DK = Danish, PA = Pakistani.

<sup>3</sup>P values for comparisons over the four study groups were determined with the use of a one-way ANOVA followed by Tukey’s HSD test. Significant comparisons a: DK Placebo vs DK Fortified, b: PA Fortified vs DK Fortified, c: PA Placebo vs DK Fortified, d: PA Fortified vs DK Placebo, e: PA Placebo vs DK Placebo, f: PA Placebo vs PA Fortified.

OC: Osteocalcin, BALP: Bone specific Alkaline Phosphatase, P1NP: Procollagen type 1 amino-terminal propeptide, CTX: C-terminal crosslinked telopeptide of type 1 collagen.
Table 3: Muscle strength and body composition at baseline, endpoint and the change following intervention

<table>
<thead>
<tr>
<th>Intervention groups</th>
<th>DK Placebo (n = 35)</th>
<th>DK Fortified (n = 31)</th>
<th>PA Placebo (n = 37)</th>
<th>PA Fortified (n = 33)</th>
<th>p&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Handgrip strength (kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>34.5 (4.2)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>33.2 (4.2)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.9 (6.6)&lt;sup&gt;es&lt;/sup&gt;</td>
<td>30.3 (6.2)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endpoint</td>
<td>34.07 (3.8)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>33.3 (4.3)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.0 (6.1)&lt;sup&gt;es&lt;/sup&gt;</td>
<td>30.3 (5.5)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Change</td>
<td>-0.46 (2.3)</td>
<td>-0.1 (3.0)</td>
<td>0.2 (3.0)</td>
<td>0.5 (3.5)</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>Knee extension strength (kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>34.0 (5.7)&lt;sup&gt;s&lt;/sup&gt;</td>
<td>36.5 (5.9)&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>27.7 (6.5)&lt;sup&gt;es&lt;/sup&gt;</td>
<td>31.1 (7.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endpoint</td>
<td>35.0 (5.9)&lt;sup&gt;s&lt;/sup&gt;</td>
<td>37.5 (5.4)&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>27.5 (6.7)&lt;sup&gt;es&lt;/sup&gt;</td>
<td>31.2 (7.8)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Change</td>
<td>0.3 (4.5)</td>
<td>1.0 (3.9)</td>
<td>-0.15 (5.0)</td>
<td>0.6 (4.3)</td>
<td>0.79</td>
</tr>
<tr>
<td><strong>Chair-stand test (Mean rep/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>23.4 (5.9)</td>
<td>24.0 (5.0)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.9 (5.5)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.6 (7.1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Endpoint</td>
<td>24.7 (6.6)</td>
<td>26.8 (6.5)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.9 (5.9)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.9 (7.2)</td>
<td>0.045</td>
</tr>
<tr>
<td>Change</td>
<td>1.2 (3.0)</td>
<td>2.7 (3.6)</td>
<td>1.3 (3.4)</td>
<td>1.6 (3.6)</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Mean BMI (kg/m²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>24.6 (5.1)</td>
<td>24.1 (4.0)&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>27.1 (24.8)&lt;sup&gt;s&lt;/sup&gt;</td>
<td>27.4 (4.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.007</td>
</tr>
<tr>
<td>Endpoint</td>
<td>24.4 (4.3)</td>
<td>24.1 (3.9)&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>27.2 (5.1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.2 (4.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.005</td>
</tr>
<tr>
<td>Change</td>
<td>0.1 (0.5)</td>
<td>0.2 (0.5)</td>
<td>0.2 (0.5)</td>
<td>0.1 (0.5)</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Mean fat %</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>32.6 (7.6)&lt;sup&gt;de&lt;/sup&gt;</td>
<td>30.1 (7.3)&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>37.1 (6.1)&lt;sup&gt;es&lt;/sup&gt;</td>
<td>37.6 (5.7)&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endpoint</td>
<td>32.0 (7.4)&lt;sup&gt;de&lt;/sup&gt;</td>
<td>30.7 (7.1)&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>36.6 (6.4)&lt;sup&gt;es&lt;/sup&gt;</td>
<td>37.4 (5.6)&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Change</td>
<td>-0.11 (1.9)</td>
<td>0.7 (2.3)</td>
<td>-0.3 (1.4)</td>
<td>0.16 (1.7)</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Mean lean mass (kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>46.5 (4.7)&lt;sup&gt;s&lt;/sup&gt;</td>
<td>47.1 (3.0)&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>42.8 (4.7)&lt;sup&gt;es&lt;/sup&gt;</td>
<td>43.8 (4.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endpoint</td>
<td>46.5 (4.3)&lt;sup&gt;s&lt;/sup&gt;</td>
<td>46.8 (3.0)&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>43.2 (4.8)&lt;sup&gt;es&lt;/sup&gt;</td>
<td>43.16 (4.1)&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Change</td>
<td>0.22 (1.3)</td>
<td>-0.05 (1.3)</td>
<td>0.43 (1.0)</td>
<td>0.06 (0.87)</td>
<td>0.37</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means and SD unless otherwise specified.
<sup>2</sup>DK = Danish, PA = Pakistani.
<sup>3</sup>P values for comparisons over the four study group were determined with the use of a one-way ANOVA followed by Tukey’s test. Significant comparisons a: DK Placebo vs DK Fortified, b: PA Fortified vs DK Fortified, c: PA Placebo vs DK Fortified, d: PA Fortified vs DK Placebo, e: PA Placebo vs DK Placebo, f: PA Placebo vs PA Fortified.
Table 4: The effect of intervention with vitamin D fortified foods on the markers of bone turnover OC, BALP, P1NP and CTX, analysed in an ANCOVA including the baseline covariates that may influence the outcome (age, ethnicity, intervention group and BMI)

Δ OC

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept¹</td>
<td>6.13</td>
<td>(1.16; 11.1)</td>
<td>0.016 (*)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.001</td>
<td>(-0.07; 0.07)</td>
<td>0.98</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>-0.53</td>
<td>(-0.88; 1.94)</td>
<td>0.46</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>0.75</td>
<td>(-2.05; 0.54)</td>
<td>0.25</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.10</td>
<td>(-0.26; 0.06)</td>
<td>0.22</td>
</tr>
</tbody>
</table>
| Baseline OC  | -0.20    | (-0.28; -0.12)| <0.0001 (***)

Δ BALP

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept¹</td>
<td>0.50</td>
<td>(-2.6; 3.56)</td>
<td>0.75</td>
</tr>
<tr>
<td>Age</td>
<td>0.02</td>
<td>(-0.03; 0.07)</td>
<td>0.42</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>0.08</td>
<td>(-0.91; 1.07)</td>
<td>0.88</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>0.19</td>
<td>(-0.75; 1.13)</td>
<td>0.70</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.05</td>
<td>(-0.07; 0.16)</td>
<td>0.42</td>
</tr>
</tbody>
</table>
| Baseline BALP| -0.25    | (-0.33; -0.17)| <0.0001 (***)

Δ P1NP

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept¹</td>
<td>13.0</td>
<td>(0.66; 25.4)</td>
<td>0.04 (*)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.16</td>
<td>(-0.35; 0.03)</td>
<td>0.10</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>1.55</td>
<td>(-2.00; 5.10)</td>
<td>0.39</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>-1.83</td>
<td>(-5.21; 1.56)</td>
<td>0.29</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.04</td>
<td>(-0.37; 0.45)</td>
<td>0.85</td>
</tr>
</tbody>
</table>
| Baseline P1NP| -0.19    | (-0.28; -0.10)| <0.0001 (***)

29
<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept¹</td>
<td>0.08</td>
<td>(-0.03; 0.18)</td>
<td>0.15</td>
</tr>
<tr>
<td>Age</td>
<td>0.0003</td>
<td>(-0.001; 0.002)</td>
<td>0.75</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pakistani</td>
<td>-0.0009</td>
<td>(-0.04; 0.02)</td>
<td>0.57</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>-0.02</td>
<td>(-0.05; 0.01)</td>
<td>0.25</td>
</tr>
<tr>
<td>Placebo</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.003</td>
<td>(-0.004; 0.003)</td>
<td>0.87</td>
</tr>
<tr>
<td>Baseline CTX</td>
<td>-0.34</td>
<td>(-0.45; -0.24)</td>
<td>&lt;0.0001 (***)</td>
</tr>
</tbody>
</table>

¹The reference group is included in the intercept: Danish ethnicity and placebo study group.
Table 5: The change in muscle strength measured as handgrip strength and knee extension strength and a 30 second chair-standing test, analysed in an ANCOVA including the baseline covariates that may influence the outcome (age, ethnicity, intervention group and BMI)

### Δ Handgrip strength

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>6.88</td>
<td>(3.34; 10.4)</td>
<td>&lt;0.0001 (***)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.03</td>
<td>(-0.05; 0.05)</td>
<td>0.99</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pakistani</td>
<td>-0.52</td>
<td>(-1.64; 0.60)</td>
<td>0.36</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>0.40</td>
<td>(-0.60; 1.40)</td>
<td>0.43</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.01</td>
<td>(-0.10; 0.13)</td>
<td>0.88</td>
</tr>
<tr>
<td>Baseline handgrip strength</td>
<td>-0.22</td>
<td>(-0.31; -0.12)</td>
<td>&lt;0.0001 (***)</td>
</tr>
</tbody>
</table>

### Δ Knee extension strength

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>9.93</td>
<td>(4.26;15.6)</td>
<td>&lt;0.0001 (***)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.03</td>
<td>(-0.11;0.05)</td>
<td>0.46</td>
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<td></td>
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<tr>
<td>Danish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pakistani</td>
<td>-1.95</td>
<td>(-3.67;-0.22)</td>
<td>0.03 (*)</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>1.21</td>
<td>(-0.35;2.78)</td>
<td>0.13</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>-0.02</td>
<td>(-0.20; 0.17)</td>
<td>0.87</td>
</tr>
<tr>
<td>Baseline knee extension strength</td>
<td>-0.25</td>
<td>(-0.37;-0.12)</td>
<td>&lt;0.0001 (***)</td>
</tr>
</tbody>
</table>

### Δ Chair-standing test

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>5.99</td>
<td>(0.90; 11.1)</td>
<td>0.02 (*)</td>
</tr>
<tr>
<td>Age</td>
<td>0.02</td>
<td>(-0.05; 0.09)</td>
<td>0.58</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pakistani</td>
<td>-0.08</td>
<td>(-1.42; 1.25)</td>
<td>0.90</td>
</tr>
<tr>
<td>Intervention group:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified</td>
<td>0.95</td>
<td>(-0.30; 2.21)</td>
<td>0.14</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>-0.16</td>
<td>(-0.31; -0.01)</td>
<td>0.04 (*)</td>
</tr>
<tr>
<td>Baseline chair-standing</td>
<td>-0.06</td>
<td>(-0.17; 0.05)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

1The reference group is included in the intercept: Danish ethnicity and placebo study group
Appendix B: Literature search strategy

Search terms

From a PubMed literature search, results listed in Table xx:

(("vitamin d"[MeSH Terms] OR "vitamin d"[All Fields] OR "ergocalciferols"[MeSH Terms] OR "ergocalciferols"[All Fields]) OR ("cholecalciferol"[MeSH Terms] OR "cholecalciferol"[All Fields]))


AND (Clinical Study[ptyp] OR Clinical Trial[ptyp] OR Controlled Clinical Trial[ptyp] OR Randomized Controlled Trial[ptyp])
Eligibility criteria

Eligible studies were RCT’s assessing the effect of vitamin D fortification compared with no treatment, unfortified foods or regular diet conducted in all populations regardless of age. Fifty English language publications were found in the search. Twenty-three studies were selected based on their content. Subsequently, an additional eight studies were found by assessing citations of the eligible 23 papers (snowballing). Four studies were subsequently excluded due to single-arm design, cross sectional design or use of only vitamin D2. The table contains information from the selected 27 vitamin D fortification studies.
Appendix C: Compliance questionnaire

1. Hvor mange af de udleverede yoghurt har du spist? (Sæt kryds/streg henover yoghurten)

UGE 1

UGE 2

UGE 3

UGE 4

2. Hvis du ikke har spist al yoghurten – sæt kryds ved årsagen

Havde ikke mere yoghurt ........................................................... 

Havde ikke lyst til yoghurt ...........................................................
Kunne ikke lide yoghurten .......................................................
Spiste ikke hjemme ...................................................................
Var på ferie/rejse eller lignende................................................
Var syg.......................................................................................
Andet........................................................................................

Hvor mange af de udleverede pakker ost har du spist? (Sæt kryds/streg henover ostene)

UGE 1

UGE 2

UGE 3

UGE 4
3. **Hvis du ikke har spist alosten – sæt kryds ved årsagen**

- Havde ikke mere ost......................................................... ☐
- Havde ikke lyst til ost....................................................... ☐
- Kunne ikke lide osten...................................................... ☐
- Spiste ikke hjemme......................................................... ☐
- Var på ferie/rejse eller lignende...................................... ☐
- Var syg........................................................................... ☐
- Andet.............................................................................. ☐
Hvor mange af de udleverede æg har du spist? (Sæt kryds/streg henover æggene)

UGE 1

UGE 2

UGE 3

UGE 4

4. Hvis du ikke har spist alle æg – sæt kryds ved årsagen

Havde ikke flere æg .................................................................
Havde ikke lyst til æg .............................................................
Kunne ikke lide æggene.........................................................
Spiste ikke hjemme .............................................................
Var på ferie/rejse eller lignende ............................................
Var syg..................................................................................
Andet.....................................................................................
5. Hvor mange af de udleverede pakker kiks har du spist? (Sæt kryds henover kiksene)

UGE 1

UGE 2

UGE 3

UGE 4

6. Hvis du ikke har spist alle pakker kiks – sæt kryds ved årsagen

Havde ikke flere kiks ................................................................. □
Havde ikke lyst til kiks .............................................................. □
Kunne ikke lide kiksene ........................................................... □
Spiste ikke hjemme ................................................................. □
Var på ferie/rejse eller lignende ............................................. □
Var syg ................................................................. □
Andet ..................................................................................... □

Andre kommentarer __________________________