The efficacy of vitamin D supplementation and fish consumption to optimize the vitamin D status in healthy volunteers

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Abbreviations

$1,23,25(OH)_3D_3$	1,23,25-trihydroxyvitamin D_3
$1,24,25(OH)_3D_3$	1,24,25-trihydroxyvitamin D_3
$1,25(OH)_2D$	1,25-dihydroxyvitamin D (calcitriol)
$1,25(OH)_2D_2$	1,25-dihydroxyvitamin D_2
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
1,25(OH)2D3-26,23-lactone	1,25-dihydroxyvitamin D_3-26,23-lactone
7-DHC	7-dehydrocholesterol
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
25(OH)D	25-hydroxyvitamin D (calcidiol)
$25(OH)D_2$	25-hydroxyvitamin D_2
$25(OH)D_3$	25-hydroxyvitamin D_3
AI	Adequate Intake
BMI	Body mass Index
CYP2R1	Cytochrom P450 2R1 (25-hydroxylase)
CYP24A1	Cytochrom P450 24A1 (25-hydroxyvitamin D-24-hydroxylase)
CYP27B1	Cytochrom P450 27B1 (25-hydroxyvitamin D 1-alpha-hydroxylase)
DACH	Deutschland-Österreich-Schweiz
DBP	Vitamin D-binding Protein
DEGS1	German Health Interview and Examination Survey for Adults
DGE	German Society for Nutrition
DNA	Deoxyribonucleic acid
DRV	Dietary reference values
EDTA	Ethylenediaminetetraacetate
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FFQ	Food Frequency Questionnaire
FGF-23	Fibroblast growth factor 23

HDL	High Density Lipoprotein
HPLC	High performance liquid chromatography
HDM	Health and Medicine Division (*former IOM*)
IOM	Institute of Medicine
IU	International units
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LDL	Low Density Lipoprotein
µg	Microgram
mW/m^2	Milliwatts per square meter
nm	Nanometer
NHANES	The National Health and Nutrition Examination Survey
NNR	Nordic Nutrition Recommendations
NOAEL	No observed effect level
Npt2a/c	Sodium-dependent phosphate co-transporters
NVH II	German Nutrition Health Survey II
PKA	Protein Kinase A
PKC	Protein Kinase C
PTH	Parathyroid hormone
RCT	Randomized controlled trials
RDA	Recommended Daily Allowance
SACN	British Scientific Advisory Committee on Nutrition
SD	Standard deviation
UK	United Kingdom
UL	Tolerable upper intake level
US	United States
UV	Ultraviolet
UVB	Ultraviolet B
VDR	Vitamin D-receptor
VDRe	Vitamin D-receptor response elements
VDSP	Vitamin D standardization program
WHO	World Health Organization

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1. Introduction

As early as in the 18th century scientists recognized the positive effects of sun and cod liver oil for the prevention and treatment of rickets [Park, 1940; Stamp, 1975; Chick, 1976; DeLuca, 2014] and osteomalacia [Compston et al., 1979] which were widespread then in northern European countries [Holick, 2010]. However, it was not until a century later that US-scientist McCollum identified the active substance responsible for this medical effect [McCollum et al., 1922]: Vitamin D – a compound which can be found in high amounts in cod liver oil and is further produced in the human skin upon exposure to UVB-radiation. Research into the effects and the optimal dose of vitamin D for human health is still ongoing and has received attention in the 21st century.

1.1. Absorption, degradation, metabolism and excretion

1.1.1. Sun exposure and skin synthesis

Vitamin D is formed when the skin is exposed to UVB-radiation, which comprises not more than 2 – 12% of all UV-radiation and consists of intermediate wavelengths (UVB) between 290 and 315 nm [Holick et al., 1981]. Furthermore, UVB light does not reach the earth's surface at areas beyond 40 degrees latitude - which correspond an imaginary line drawn through Boston – from November to February [Webb et al., 1988].

UVB wavelengths cause the photoconversion of 7-dehydrocholesterol (7-DHC) into vitamin D_3 mainly in the stratum basale and stratum spinosum of the epidermis [Havinga, 1973; Holick et al., 2007]. The double-bonds of 7-DHC absorb UVB-rays leading to the opening of the B-ring at C9 as well as C10 and, ultimately, the conversion to previtamin D_3 (also named precalciol) [Holick et al., 1981]. Previtamin D_3 is an unstable compound that is thermally isomerized, i.e. the three double bonds are re-arranged, to vitamin D_3 (cholecalciferol) [Havinga, 1973]. It consists of its characteristic structure of four combined rings (ring A-D) [Holick, 2011] with an open B-ring at C9-C10 [Jäpelt et al., 2013]. The vitamin is transported from the skin into the blood circulation where it is majorly bound to vitamin D-binding protein (DBP) [Haddad et al.,

1993] and to albumin to a lesser extent [Haddad, 1995]. With the blood stream, it is primarily carried to liver and kidney for hydroxylation.

1.1.2. Absorption and transport of dietary Vitamin D

It is estimated that the main source of vitamin D in humans is the cutaneous synthesis of cholecalciferol which accounts up to 90% of all vitamin D supply [Lehmann and Meurer, 2010], but is dependent on UVB radiation which is absent in long periods of the year in areas distant from the equator [Webb et al., 1988].

In these areas, humans depend on dietary vitamin D either from food or from supplements. Vitamin D is the overarching term for a number of substances differing in their side chains, and the most abundant forms are vitamin D_2 and vitamin D_3. Vitamin D_3 is derived from animal products and human skin [Holick et al., 2007; Schmid and Walther, 2013], while vitamin D_2 is derived from ergosterol in plants [Keegan et al., 2013]. There are little data on differences in absorption between vitamin D_2 and D_3, but it is assumed that both forms are absorbed to the same extent [Biancuzzo et al., 2010].

As with other fat soluble vitamins, dietary vitamin D is absorbed in the proximal small intestine [Dueland et al., 1983]. Dietary vitamin D reaches the enterocytes through passive diffusion. In the enterocytes, vitamin D is incorporated in chylomicrons that consist of triglycerides, cholesterol and other lipids which are released into the lymphatic system and then into the blood for transport to the liver and non-hepatic tissues [Holick, 2012]. Data on the amount of vitamin D absorbtion vary between 62-91% [Thompson et al., 1966]. Furthermore absorption can be affected in case of chronic malabsorption e.g. Crohn`s disease [Driscoll et al., 1982].

1.1.3. Metabolism

Vitamin D is taken up by the liver for hydroxylation or – if not needed – stored in adipose tissue [Heaney et al., 2009] and skeletal muscle [Chen et al., 2007]. After hepatic uptake both forms of vitamin D go through the same process of hydroxylation. In the liver, 25-hydroxylase (*CYP2R1*) hydroxylates vitamin D_3 to 25-hydroxyvitamin D_3 ($25(OH)D_3$), also referred to as calcidiol, at C25 [Henry, 2011]. The compound is then

transported to the kidney, where the second hydroxylation step is performed. Due its lipophilic characteristic it has to be bound to proteins like albumin, lipoproteins, and specific DBPs in order to be transported via blood stream [Bikle et al., 1985; Chun et al., 2014]. At the kidney, the 25(OH)D_3-DBP complex enters the organ via receptor-mediated endocytosis [Nykjaer et al., 2001]. The complex directly binds to megalin and cubilin, two endocytic receptors which are mainly expressed by the epithelial cells of the proximal tubulus [Rowling et al., 2006]. In the next step, the complex is transferred into the lysosome of the proximal tubular cells [Willnow and Nykjaer, 2002], where the 25(OH)D_3 is cleaved via peptidase from DBP and is released to the cytosol. The 25(OH)D_3 is hydroxylated at C1 to 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$), also known as calcitriol, via 25-hydroxyvitamin D 1-alpha-hydroxylase *(CYP27B1)* in the renal mitochondria [Prentice et al., 2008; Bikle, 2009] and bound at DBP within the intestinal fluid [Willnow and Nykjaer, 2002].

The synthesis of 1,25(OH)$_2D_3$ is precisely regulated by processes within the body and influenced by a variety of endocrine factors. The 1-alpha-hydroxylation depends on healthy proximal tubular cells – a prerequisite usually not given in case of kidney disease [Gallagher et al., 2007]. Under normal conditions, low concentrations of 1,25(OH)$_2D_3$, high parathyroid hormone (PTH) [Henry, 2011] or low phosphate, calcium and fibroblast growth factor 23 (FGF-23) concentrations [Shimada et al., 2004; Perwad et al., 2007] induce the activation of 1-alpha-hydroxylase.

Although the kidney is the main organ containing 1-alpha-hydroxylase, other tissues or cells such as macrophages [Adams and Gacad, 1985; Crowle et al., 1987], keratinocytes, monocytes, osteoblasts, breast and colon cells also express *CYP27B1* mRNA [Bikle et al., 1994; Lehmann et al., 1999; Norman et al., 2002; Holick, 2007b]. 1,25(OH)$_2D_3$ mediates its effects as the active form via the vitamin D receptor (VDR) [McDonnell et al., 1987]. Vitamin D receptors are present in many tissues in the organism and mediate various physiological effects [Bikle, 2009]. The main target tissues include those involved in mineral homeostasis as bones, intestine, kidney and parathyroidea [DeLuca, 2004]. Besides, VDR is also expressed in e.g. the skin [Reichrath et al., 1994], immune cells [Provvedini et al., 1983] or placenta [Pike et al., 1980].

1.1.4. Degradation and Excretion

While degradation of vitamin D metabolites, 25(OH)D_3 and 1,25(OH)$_2D_3$, can be mediated through the C24 oxidation pathway [Ohnuma and Norman, 1982; Napoli and Horst, 1983]. Additionally, 1,25(OH)$_2D_3$ can be catabolized via the C23 lactone pathway [Ohnuma and Norman, 1982].
The 24-alpha-hydroxylase (*CYP24A1*), a mitochondrial inner membrane cytochrome P-450 enzyme, limits body concentrations of 25(OH)D_3 and 1,25(OH)$_2D_3$ by formation of inactive vitamin D metabolites, either 24,25-dihydroxyvitamin D_3 (24,25(OH)$_2D_3$) or 1,24,25-trihydroxyvitamin D_3 (1,24,25(OH)$_3D_3$) [Norman, 2008]. In the kidney, 25(OH)D_3 can be converted by CYP24A1 at C24 to 24,25(OH)$_2D_3$. Additionally, high 1,25(OH)$_2D_3$-concentrations and low PTH concentrations activate the C24 oxidation pathway [Kleiner-Bossaller and DeLuca, 1974; Tanaka et al., 1977] which catalyses the conversion into 1,24,25(OH)$_3D_3$. Within the C24 oxidation pathway, the enzyme further catalyses the formation of calcitroic acid which is water-soluble and mainly secreted into bile [Jones, 1999]. Following it is excreted with faeces and, to a lesser extent, excreted with urine [Avioli et al., 1967].

1.2. Effects of Vitamin D

1.2.1. Biochemical functions

Vitamin D is primarily responsible for the regulation of bone metabolism and mineralization which, in turn, is regulated by the skeletons main components: calcium and phosphate [Holick, 1996]. Actions related to calcium homeostasis, i.e. bone metabolism and mineralization, are called *calcaemic effects* of vitamin D. They primarily help to ensure stabilisation of plasma calcium and phosphate concentrations and provide sufficient calcium for bone mineralization [Holick, 1996]. The active metabolite, 1,25(OH)$_2D_3$, has four so-called *traditional target tissues*: bone, kidney, intestine as well as parathyroid gland. One of the main functions is the homeostasis of the calcium concentration in blood within narrow bounds. The active metabolite, 1,25(OH)$_2D_3$ directly suppresses the synthesis of PTH in the parathyroid gland [Delmez et al., 1989]. Concentrations of PTH are largely determined by levels of 1,25(OH)$_2D_3$, calcium and phosphate [Lombardi et al., 2020]: A sufficient concentration of 1,25(OH)$_2D_3$ promotes the provision of calcium and phosphate (e.g. either through

intestinal absorption from food or re-absorption from urine in the kidney) and reduces the production of PTH. If there is an insufficient amount of $1,25(OH)_2D_3$ available, PTH is produced to a larger extent. This will, if needed, trigger an increased release of calcium and phosphate from the skeleton. In the kidney, $1,25(OH)_2D_3$ affects phosphate balance via regulation of bone-derived FGF-23 and its co-receptor α-Klotho [Hu et al., 2013]. Fibroblast growth factor 23 represses the reabsorption of phosphate in the kidney via inhibition of sodium-dependent phosphate co-transporter (Npt2a/c) [Gattineni et al., 2009]. Additionally, FGF-23 and α-Klotho are regulators of vitamin D metabolism [Kurosu et al., 2006]. They inhibit the activity of *CYP271* which converts $25(OH)D_3$ into $1,25(OH)_2D_3$ [Hu et al., 2013].

1.2.2. Molecular mechanisms

The biological active form, $1,25(OH)_2D_3$, assumes important regulatory functions in cellular growth, differentiation and apoptosis throughout the body, e.g. bones, pancreatic tissue [Pike et al., 1980], immune system [Provvedini et al., 1983], vascular [Cardus et al., 2009] and coronary muscle cells [Wu-Wong et al., 2007], muscle [Simpson et al., 1985; Costa et al., 1986] and nerve tissues [Smolders et al., 2013]. In order to mediate its functions, $1,25(OH)_2D_3$ binds to its specific vitamin D receptor (VDR), a ligand-activated transcription factor belonging to the superfamily of nuclear steroid receptors [Owen and Zelent, 2000; Yang et al., 2012]. VDR has been discovered in almost all tissues and cells in the human organism [MacDonald, 1999; Holick, 2004; Norman, 2008]. The VDR gene is located on chromosome 12, consists of nine exons [Li et al., 2009].

Ligand activation of VDR elicits the genomic pathway: The Vitamin D-VDR-complex interacts with the retinoid X receptor to form a heterodimer. The heterodimer binds to specific VDR-response-elements (VDRe) of the promoter region of $1,25(OH)_2D_3$ regulated target genes and modulates transcriptions of mRNAs which encodes for different proteins. VDR can bind either co-activators or co-repressors which results in activation or repression of pathways for e.g. cell proliferation or differentiation [Evans, 1988].

It has been shown that vitamin D metabolites differ in their affinity for VDR: $1,25(OH)_2D_3$ binds with high affinity to the VDR [Brumbaugh and Haussler, 1975], whereas other forms of vitamin D are significantly less potent for the receptor. For instance, the affinity of $25(OH)D_3$ for VDR is 100 to 1000 times lower than of $1,25(OH)_2D_3$ [Wecksler et al., 1978].

$1,25(OH)_2D_3$ acts also through an additional pathway: The non-genomic pathway. This pathway is responsible for rapid (seconds to minutes) cellular response effects and are mediated by activation of signal transduction pathways [Huhtakangas et al., 2004; Deeb et al., 2007].
Despite great efforts, the biochemistry of non-genomic actions and their effects on target cells is not fully understood. However it has been demonstrated that $1,25(OH)_2D_3$ affected the ion-channel activity via enzymes of signal transduction as protein kinase A and C (PKA and PKC) [Duval et al., 1983]. As a result influx of calcium and phosphate may mediate vitamin D-related functions [Fleet, 2004].

1.3. Status assessment, recommendations and toxicity

1.3.1. Vitamin D status assessment

Vitamin D status is defined by serum concentrations of 25(OH)D [Dawson-Hughes et al., 2005; Holick, 2009] which are more stable and reflect body stores more tightly than the highly regulated active form $1,25(OH)_2D$. The 25(OH)D has a half-time of two to three weeks and a high binding affinity to the DBP [Cooke and Haddad, 1989]. There is no consensus on the definition of normal vitamin D status [Lips, 2004; Holick, 2009] which is usually defined as deficient, inadequate or insufficient, adequate or sufficient and excessive. However, different cut-offs have been applied to define these stages. An overview on the different limits used in adults is provided in Table 1.

Table 1: Differentiation limits of adult vitamin D status set by leading advisory organizations

25(OH)D-concentration [nmol/l]	DACH	NORDEN	IOM	EFSA	Endocrine Society	SACN
<25 / 30	Deficient	Deficient	Deficient	Deficient	Deficient	Deficient
25 - 50	Insufficient	Insufficient	Uncertain[a]	Deficient	Deficient	
50 - 75	Sufficient	Sufficient	Sufficient	Sufficient	Insufficient	
> 75					Sufficient	

modified from [Lips et al., 2019]; [a] 30-50 nmol/l were adequate

DACH = [German Nutrition Society, 2012], NORDEN = [Nordic Council of Ministers, 2012], IOM = [Ross et al., 2011], EFSA = [EFSA, 2016], Endocrine Society = [Holick et al., 2011] , SACN = [Scientific Advisory Committee on Nutrition, 2016]

Prolonged vitamin D deficiency promotes risk for low bone mineral density and consequently may lead to osteoporosis [Holick, 1996]. Severe vitamin D deficiency with high probability of development of clinical deficiency symptoms as rickets (in children) and osteomalacia (in adults) is considered usually at less than 12 nmol/l [Lips, 2004].

The reference used (50 or 75 nmol/l, Table 1) has huge implications on the prevalence of vitamin D deficiency, as the majority of European adults have 25(OH)D-concentrations in the range of 40 – 70 nmol/l [Burnand et al., 1992; Chapuy et al., 1997; Carnevale et al., 2001; Lamberg-Allardt et al., 2001; Gomez et al., 2004; Hintzpeter et al., 2008]. In Germany, the mean 25(OH)D-concentration was 45 nmol/l in 1998 [Hintzpeter et al., 2008] and 47 nmol/l in 2008-11 [Rabenberg et al., 2015]. Thus, prevalence of inadequacy of 57% (with 15% of the population having levels less than 25 nmol/l) have been reported in Germany when measured in 1998 [Hintzpeter et al., 2008] and about 60% when measured between 2008-2011 [Rabenberg et al., 2015]. In the latter study, only about 12% of the participants had serum 25(OH)D-concentrations exceeding 75 nmol/l.

However, there are several challenges related to the use of the limits shown. This refers first to documented differences among the assays for 25(OH)D determination [Brouwer-Brolsma et al., 2013; Cashman et al., 2013; EFSA, 2016]. The vitamin D standardization program (VDSP) surmounted a number of issues regarding these

differences [Cashman and Dowling et al., 2016], also among Liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods, but in clinical practice, notable differences still occur. Furthermore, the serum 25(OH)D-concentrations in Europe undergoes a distinct seasonal variation with usually highest concentrations in late summer and lowest concentrations in late winter [Hyppönen and Power, 2007]. This variation follows a cosinor model with an amplitude of almost 16 nmol/l at a mean concentration of 60 nmol/l, as shown in a Norwegian cohort [Degerud et al., 2016]. This is usually not taken into account when status limits are defined.

1.3.2. Dietary recommendations

Dietary recommendations for vitamin D have been changed in many countries during the past decade when widespread deficiency was discovered and after a systematically update of the available literature (Table 2). Firstly, the Institute of Medicine (IOM), now the Health and Medicine Division (HDM), published new and increased vitamin D recommendations in 2011. The new intake recommendations were calculated to ensure a sufficient vitamin D status (>50 nmol/l) in 97.5% of the population [Ross et al., 2011]. Following the IOM, several European countries recalculated the vitamin D requirements and published new vitamin D recommendations [German Nutrition Society, 2012; Nordic Council of Ministers, 2012]. European recommendations usually refer to sunlight as the major source of vitamin D and are usually given for periods with limited access to UVB-radiation, e.g. in Germany from October to March [German Nutrition Society, 2012]. Indeed, most Europeans live in areas with limited UVB-radiation during winter when vitamin D supply is dependent on diet or supplements.

Table 2: Overview of global vitamin D recommendations

	DACH	NORDEN	IOM	EFSA	SACN
sufficient 25(OH)D-concentration [nmol/l]	*50*	*50*	*50*	*50*	*25*
Age group	Vitamin D intake in µg per day				
Infants (1-12 months)	10	10	-	10[b]	8.5-10
Children (>2-18 years)	20	10	15	15	10
Females (>18-69 years)	20	10	15	15	10
Males (>18-69 years)	20	10	15	15	10
Elderly (>70 years)	20	10 / 20[a]	20	15	10
Pregnancy	20	10	15	15	10
Lactation	20	10	15	15	10

reproduced from [Pilz et al., 2018];

DACH = [German Nutrition Society, 2012], NORDEN = [Nordic Council of Ministers, 2012], IOM = [Ross et al., 2011], EFSA = [EFSA, 2016], Endocrine Society = [Holick et al., 2011], SACN = [Scientific Advisory Committee on Nutrition, 2016]

[a] 20 µg for elderly > 75 years, [b] 10 µg for infants > 7 months

In the German speaking countries, the recommendations for vitamin D intake were updated in 2012, following a review of “Vitamin D and prevention of selected chronic diseases” [Linseisen et al., 2011]. This review showed a convincing evidence that vitamin D supplementation could prevent falls and fractures in old adults, and that there is a possible association of vitamin D supplementation with mobility and mortality among old adults. For other diseases, like many types of cancer, diabetes or cardiovascular diseases, either the evidence was missing, non-convincing or the database was regarded insufficient. Following this analysis, the recommendations were updated in 2012 and increased to 20 µg per day (µg/d) for the adult population [German Nutrition Society, 2012].

The IOM recognizes that dietary vitamin D is only one source of vitamin D, and that the role of dietary intake for achieving serum concentrations of 50 nmol/l is not clear. The report states that there is a lack of data on the association of vitamin D intake and health outcomes, and rather research on vitamin D status (which is the sum of diet and sun exposure) and health outcomes (p 363, [Ross et al., 2011]. The lack of a clear dose-response association of dietary vitamin D and serum concentrations of 25(OH)D

has also been highlighted by other authors [Lamberg-Allardt et al., 2013]. Indeed, the dose-response is also affected by sunshine exposure and also by the baseline concentrations of 25(OH)D.

Following the IOM approach, sufficient vitamin D status was defined as 25(OH)D-concentrations exceeding 50 nmol/l in almost all individuals by DACH [German Nutrition Society, 2012]. It was stated that the usual dietary vitamin D intake in Germany is low and not sufficient, and that either sun exposure to enhance endogenous synthesis or the intake of supplements is required to achieve a serum 25(OH)D-concentration of 50 nmol/l in 97% of the population.

Additionally other European countries changed their recommendations on vitamin D intake. For example, the Nordic countries sharing common recommendations called the Nordic Nutrition recommendations (NNR), published vitamin D recommendations in 2012 [Nordic Council of Ministers, 2012] as shown in Table 2. They also follow the approach of achieving 25(OH)D-concentrations of 50 nmol/l in the majority of the population [Lamberg-Allardt et al., 2013].

In 2016, the British Scientific Advisory Committee on Nutrition (SACN) published new recommendations to ensure a mean 25(OH)D-concentration of 25 nmol/l throughout the year in 97.5% of the population, also in periods when UVB radiation is limited [Scientific Advisory Committee on Nutrition, 2016], which are 8.5-10 µg/d vitamin D. In addition, supplements are recommended for pregnant and breastfeeding women, children <4 years, people with no or limited sun exposure, ethnic minorities and adults during wintertime (October – March).

Furthermore, the European Food Safety Authority (EFSA) changed the dietary reference values (DRV) for vitamin D in 2016 and set the adequate intake (AI) to 10 µg vitamin D in children <12 months or to 15 µg in children >12 months and adults. These DRVs apply under conditions of minimal cutaneous vitamin D synthesis, and it is concluded that in times of endogenous synthesis, less dietary vitamin D is recommended or even not required [EFSA, 2016].

The World Health Organisation (WHO) and the Food and Agriculture Organization of the United States (FAO) did not change their recommendations since 2004 [WHO & FAO, 2004].

In conclusion, discrepancies in both cut-offs of vitamin D status and for recommendations of vitamin D intake may be explained by the different health outcomes that are considered for sufficient vitamin D status. New recommendations may also take into account the association of low vitamin D status and chronic diseases [Linseisen et al., 2011] and provoke an adjustment of vitamin D recommendations in the USA. It has to be taken into account that the IOM recommendations are mainly based for achieving a sufficient vitamin D status in 50% of the population and on the importance of vitamin D status for bone health. However, even the outcome 'bone health' can be interpreted in different ways, e.g. maximising intestinal calcium absorption, bone mineral density, prevention of rickets and osteomalacia, or prevention of fractures and that these outcomes of bone health are associated with different limits and optimized 25(OH)D-concentrations [Ross et al., 2011].

Several other factors associated with 25(OH)D-status need to be considered. Among these, the most important are skin pigmentation [Holick, 2007a], age and BMI [Bischof et al., 2006; Lagunova et al., 2009]. Furthermore, lifestyle is important as it comes to time spend outside in the sun, use of sunscreens, veiling due to religious or other reasons, and smoking [Holick, 2007a]. In the German Health Interview and Examination Survey for Adults (DEGS1), as an example, obesity, low physical activity, non-use of vitamin D supplements, wintertime and media consumption were significantly associated with low 25(OH)D-concentrations in the German population [Rabenberg et al., 2015].

1.3.3. Non-traditional outcomes

In observational studies, low vitamin D status has been associated with increased risk for cardiovascular disease [Dobnig et al., 2008; Degerud et al., 2018], hypertension [Burgaz et al., 2011], diabetes [Pittas et al., 2007; Zhang et al., 2015], multiple sclerosis and other neurological [Evatt et al., 2008; Littlejohns et al., 2014] or autoimmune

diseases [Rossini et al., 2010; Wang et al., 2015] and various types of cancer [Garland et al., 1989; John et al., 1999; Ahonen et al., 2000] .

Randomized controlled trails (RCTs) with intermediate endpoints did not confirm the observational studies for cardiovascular risk factors [Jorde et al., 2010], insulin sensitivity in diabetic patients [Mousa et al., 2017]. In the meantime, several randomized clinical studies with clinical endpoints have been conducted and published, which, however, do not confirm the findings from observational studies for cardiovascular diseases [Manson et al., 2019], fractures and falls [Sanders et al., 2010; Khaw et al., 2017] and asthma [Castro et al., 2014].

1.3.4. Toxicity

High vitamin D intake may also exert toxic effects. The excess intake of vitamin D is described as “intoxication” or “hypervitaminosis D” and increases both calcium absorption in the intestines and calcium resorption from bone [Vieth, 1990; Selby et al., 1995]. This promotes both increased blood calcium levels (*hypercalcemia*) and increased calcium excretion in urine (*hypercalciuria*) [Rizzoli et al., 1994]. Furthermore, a deposition of calcium in soft tissues might cause kidney or cardiovascular damages [Zittermann and Koerfer, 2008].

In 2006, the EFSA Panel on Dietetic Products, Nutrition and Allergies evaluated the safety of vitamin D intake in all age groups and set hypercalcemia (serum calcium >2.75 nmol/l) as indicator of vitamin D toxicity [EFSA, 2016]. Taking into account studies with high vitamin D intake [Barger-Lux et al., 1998; Heaney et al., 2003], the ‘no observed effect level’ (NOAEL) was set at 250 µg/day [Hathcock et al., 2007]. When considering a safety factor of 2.5 for interindividual variation, the ‘tolerable upper intake level’ (UL) has been set to 100 µg/d for children >11 years and adults (EFSA 2012). This level is in line with the IOM that published in 2011 an UL of 100 µg/d for adults and children >9 years [Ross et al., 2011]. Indeed, it is discussed whether high serum levels of 25(OH)D exceeding 100 nmol/l, are associated with deleterious effects on the musculoskeletal system and probably on the cardiovascular system and mortality [Zittermann, 2017; Degerud et al., 2018].

1.4. Dietary Vitamin D

1.4.1. Dietary sources

What is commonly known as vitamin D is in fact a vitamer that has different chemical forms and consequently different sub-names. Therefore, various kinds of chemical vitamin D compounds with similar basic structures but different side chains exist. Vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) are the two kinds of vitamin D that are most relevant for human nutrition and vitamin D supply [Armas et al., 2004; Holick et al., 2008b; Biancuzzo et al., 2010]

In terms of chemical structure, vitamin D_2 and D_3, like all vitamin D forms, have a steroidal structure and are distinguished by different side chains (Figure 1) [Bikle, 2009]. Unlike vitamin D_3, vitamin D_2 contains one supplementary methyl-group at C24 and a double-bond at C22-C23 [Bikle, 2009; Keegan et al., 2013]. Vitamin D_2 and D_3 can be formed by UVB-radiation from their respective sterol precursors, ergosterol and 7-dehydrocholesterol .In the intestine, the absorption of vitamin D_2 and D_3 seems to be similar [Biancuzzo et al., 2010].

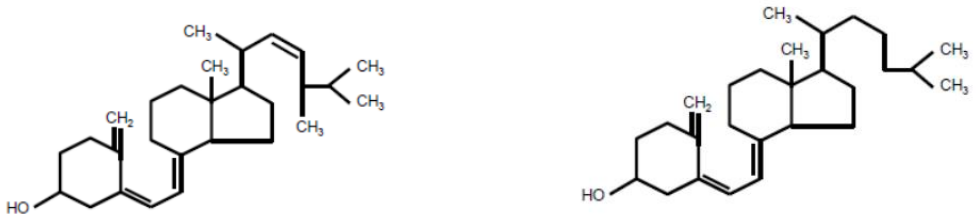

Figure 1: Chemical structure of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). (adopted from [Norman, 2008])

1.4.2. Vitamin D in foods

Vitamin D can be obtained from food in form of vitamin D_2 and D_3 or as the hydroxylated 25(OH)D_3 [Schmid and Walther, 2013]. Fish, fish liver, egg yolk, milk and milk products are good sources of vitamin D and contain mainly vitamin D_3 [Lehmann and Meurer, 2010]. The data on the vitamin D content in foods varies between different sources,

which may be explained by differences in housing, feeding [Chick and Roscoe, 1926], seasons [Mattila et al., 2011] but also analytical methods for the determination of vitamin D [Ovesen et al., 2003; Lamberg-Allardt et al., 2013]. An overview on vitamin D content of several foods in different food composition tables is provided in Table 3.

Eggs are a valuable source of vitamin D: They contain, mainly in the egg yolk, both vitamin D_3 and 25(OH)D_3 (which has a higher bioavailability). Additionally, meat contains only small amounts of vitamin D_3 and 25(OH)D_3 [Mattila et al., 1995].

Plant sources of vitamin D are mushrooms and yeasts, which contain vitamin D_2 [Mattila et al., 1994; Hohman et al., 2011]. However, the vitamin D_2 content of plant foods is low and in the order of less than 1 µg/100 g product [Souci et al., 2016]. The low availability of plant vitamin D-rich foods leads to low intake of vitamin D in vegetarians and vegans [Outila et al., 2000].

Table 3: Vitamin D content in foods in the German food composition database

	Souci-Fachmann-Kraut
Year	2008
	µg or ng/100g
Milk	74 ng
Milk *(1.5% fat)*	28 ng
Yoghurt *(3.5% fat)*	62 ng
Yoghurt *(1.5% fat)*	28 ng
Cheedar cheese *(50% fat)*	340 ng
Edam cheese *(40% fat)*	290 ng
Chicken egg *(total)*	2.9 µg
Egg yolk	5.6 µg
Egg white	0
Mushrooms	1.9 µg
Edible boletus	3.1 µg
Butter	1.2 µg
Margarine	2.5 µg
Margarine (*fat-reduced)*	2.5 µg

adopted from Souci-Fachmann-Kraut [Souci et al., 2016]

Fish is the one of the major food sources of vitamin D in several European countries including France [ANSES – French Agency for Food, Environmental and Occupa-tional Health & Safety, 2013], the United Kingdom [Henderson et al., 2003; Bates et al., 2014], Ireland [Irish Universities Nutrition Alliance, 2011], Norway [Calvo et al., 2005; Lamberg-Allardt et al., 2013], Spain [Serra-Majem et al., 2007] and Germany [Max Rubner-Institut, 2008]. Vitamin D is especially found in fatty fish like salmon, herring and mackerel [Mattila et al., 1995] and to a lower extent in lean fish like cod. Typical levels of vitamin D in fish are presented in Table 4. The data further indicate remarkable differences between food composition databases.

Table 4: Vitamin D content in fish in different food composition databases

Country	**Denmark**	**Germany**	**Netherlands**	**Norway**	**Sweden**	**United Kingdom**
	Fødevare-databanken	*Souci-Fachmann-Kraut*	*NEVO*	*MVT*	*Livsmedels-databasen*	*McCance and Widdowson's*
Year	(2009)	(2008)	(2013)	(2012)	(2014)	(2014)
				µg/100g		
Wild salmon *(Salmor salar L.)*	30.0	16.0	9.2	8.0	12.5	8.6
Farmed salmon				10.0	11.3	4.7
Atlantic herring *(Clupea harengus L.)*	10.7	25.0	19.0	11.5	8.19	19.0
Baltic herring *(Clupea harengus membras L.)*		7.8			5.78	
Farmed cod *(Gadus morhua L.)*	0.0	1.3	0.0	0.7	0.0	no data

Fødevare-databanken [Danish National Food Institute]; SFK – Souci-Fachmann-Kraut [Souci et al., 2016], NEVO [Dutch National Institute for Public Health and the Environment]; MVT – The Norwegian Food composition table [Norwegian Food Safety Authority], Livsmedels-datenbasen [Swedish National Food Agency]; McCance and Widdowson`s [McCance and Widdowson, 2014]

1.4.3. 25(OH)D_3-content of food

Data on the content of 25(OH)D_3 in foods are limited. It is known, for instance, that human milk and eggs contain substantial amounts of the total vitamin D_3 content in the 25-hydroxylated form [Hollis et al., 1986; Mattila et al., 2011; Kühn et al., 2014].

Usually, the total vitamin D content is calculated by factorisation of vitamin D_3 by 1 and 25(OH)D_3 by 4-5 [Cashman, 2012]. This is considered in several food composition databases, e.g. Switzerland, Denmark and United Kingdom [ETH Zurich and BAG; Saxholt et al., 2008; McCance and Widdowson, 2014]. The reason for the higher factorisation of 25(OH)D_3 is that this metabolite leads to higher serum 25(OH)D-concentrations than vitamin D_3 at equimolar amounts [Cashman et al., 2012].

1.5. Vitamin D-fortified foods

There is a long tradition of fortifying foods with vitamin D in several countries, e.g. milk in the United States [Park et al., 2001], or dairy products in Scandinavian [Burgaz et al., 2007; O'Mahony et al., 2011] countries. Indeed, milk, dairy products and spreads are regarded as suitable vehicles for fortification, as they are consumed by a large proportion of the population and are widely available [O'Mahony et al., 2011].

The efficacy of vitamin D fortified foods on the vitamin D status was investigated in >25 RCTs of various duration and doses of vitamin D which have been summarized in three meta-analyses [O'Donnell et al., 2008; Black et al., 2012; Tangestani et al., 2019]. These meta-analyses concluded, despite substantial heterogeneity among studies, that consumption of food items enriched with vitamin D is effective to increase the serum 25(OH)D-concentration. However, these meta-analyses did not report whether vitamin D_3 or D_2 were added to the food items.

In the US, where food fortification has a long tradition, numerous foods i.e. orange juice, breakfast cereals, bread, cheese and milk have been enriched with vitamin D [Holden and Lemar, 2008]. The daily intake of vitamin D from fortified food accounted in the National Health and Nutrition Examination Survey (NHANES) 1999-2000 for 65-86% of total dietary vitamin D intake [Moore et al., 2005]. In the overall population, 58% of men and 39% of women used vitamin D-enriched milk, making this item to the most important single contributor for vitamin D intake [O'Mahony et al., 2011]. Data from Finland, where fortification of fluid milk products and fat spreads was introduced in 2003, suggest that this measure was effective to increase the 25(OH)D-concentration in the Finish population [Jääskeläinen et al., 2017].

In Germany, only a few products, like margarine and other mixed fat products are allowed to be fortified with vitamin D in strictly limited amounts of 25 µg/kg in margarines and mixed fat products [LMvitV].

1.5.1. Bio-fortification

Increasing the vitamin D content of animal products through increased vitamin D intake of the animal is limited in the EU as there are strict regulations for the in-feed vitamin

D content of livestock [EFSA, 2016]. Another and relatively new option is the enrichment of vitamin D in food items by exposing the food to UVB-radiation and thus inducing the synthesis of vitamin D from either ergosterol or 7-DHC. This approach has been shown in 2011 already in mushrooms [Urbain et al., 2011], and since then was followed also for fish and eggs [Kühn et al., 2014]. It seems that the amount of vitamin D can be increased substantially by this approach. Wild living animals and mushrooms growing outside which are exposed to UVB-radiation, may have higher vitamin D contents than cultivated varieties [Mattila et al., 1994; Müller-Belecke A. et al., 2014]. In this field, further research is required.

1.6. Supplements

Vitamin D supplements were originally used to treat and prevent rickets [McCollum et al., 1922]. Both vitamin D forms, D_2 and D_3, have been shown to be effective to cure rickets [Jones et al., 1998; Jurutka et al., 2001].

Vitamin D supplementation can be administered using different regimes and routes. In addition to oral supplementation, intramuscular injections of high bolus doses have been used [Romagnoli et al., 2008; Leventis and Kiely, 2009]. Bolus supplementation can also given orally, and doses are up to 300 000 IU, although lower doses are regarded to be more physiological. The main advantage of bolus administration is to ensure compliance.

There are numerous vitamin D supplementation studies in the scientific literature, which have been summarized in several meta-analysis. For example, vitamin D_2 and D_3 supplementation was systematically elevated by Tripkovic et al. [2012], and low-to-moderate doses of vitamin D_3 were summarized by Whiting et al. [2015], while dose-response associations by vitamin D_3 were summarized by Shab-Bidar et al. [2014]. To date, there is no systematic evaluation (e.g. as a meta-analysis) available elucidating whether bolus or continuous supplementation is more effective to increase the 25(OH)D-concentrations. Results from available literature can be summarized as follows:

- Vitamin D supplements are effective in raising 25(OH)D-concentrations, and higher doses are associated with higher increases. However, other factors affecting the increase in 25(OH)D-concentrations are the baseline 25(OH)D-concentration, duration of the study and age of participants [Shab-Bidar et al., 2014].
- There is large variation in the increase of 25(OH)D-concentrations, which is especially evident at low to moderate doses of 400-1000 IU vitamin D per day [Cranney et al., 2007; Whiting et al., 2015].
- Tripkovic [Tripkovic et al., 2012] did not observe a difference between vitamin D_2 and D_3 in their ability to raise 25(OH)D-concentrations when given as oral, continuous supplements (5 studies), but vitamin D_3 was superior to vitamin D_2 when given as bolus (3 studies).

1.6.1. Supplements containing 25(OH)D_3

Recently, 25(OH)D_3 supplements became commercially available and have been tested in comparison to vitamin D_3 in healthy volunteers [Cashman et al., 2012; Jetter et al., 2014; Minisola et al., 2017; Vaes et al., 2018]. Although the evidence is limited (as these studies included in total about 250 participants), these three studies reported superiority of 25(OH)D_3 compared to vitamin D_3 in increasing the serum 25(OH)D-concentrations. Doses tested were in the range between 5 and 20 µg/d 25(OH)D_3, and the increase in serum 25(OH)D-concentrations was about 2-3 times [Jetter et al., 2014] or up to five times [Cashman et al., 2012] the increase after similar amounts of vitamin D_3. It is of interest that doses of 15 µg [Vaes et al., 2018] and 20 µg 25(OH)D_3 µg/d [Cashman et al., 2012] increased the 25(OH)D-concentrations on average to concentrations exceeding 100 nmol/l, Further studies are needed on this topic.

1.7. Habitual vitamin D intake *(Germany and Europe)*

Dietary intake of vitamin D is usually low and the average or median population intake is below the recommended dietary intake, even if older recommendations are used. In most countries, the mean vitamin D intake is about 2-5 µg/d. The Scandinavian countries report higher intakes. Integrating additionally the intake from supplements

into the considerations, the dietary recommendations are met exclusively in Norway and Finland [Elmadfa, 2009; Flynn et al., 2009].

Vitamin D intake in the German population was reported in 2008 (data obtained in the German Nutrition Health survey (NVS II) [Max Rubner-Institut, 2008], and were at median in men and women 2.9 and 2.2 µg/d. Thus, 82% of men and 91% of women did not meet the recommendations (that were 5 µg/d at that time) [Max Rubner-Institut, 2008]. In the DEGSI from 2008-2011, vitamin D intake was assessed by a semi-quantitative food-frequency questionnaire (FFQ) allowing the estimation of low intake (<1.65 µg/d), intermediate (1.65–2.81 µg/d) or high (>2.81 µg/d), which was measured in 40, 33 and 27% of the women and in 30, 31 and 38% of the men, respectively [Rabenberg et al., 2015]. Vitamin D supplements were taken by 6% of the women and 1% of men in the DEGSI survey [Rabenberg et al., 2015].

In the NVS II, the main sources of dietary vitamin D were fish and fish dishes (47%), butter and fats (11%), eggs and egg products (11%), and dairy (10%) [Max Rubner-Institut, 2008]. Other countries also report these food types as main sources. In Norway, even though the absolute intake of vitamin D is much higher, similar contributions of these foods groups to the vitamin D intake are reported [Totland et al., 2012].

1.7.1. Fish consumption

The average annual per capita fish consumption in Germany is 13.7 kg [BLE, 2018]. Most important fish species are pollock (19.2%), salmon (17.3%), tuna (12.4%) and herring (8.9%) [FIZ, 2019]. However, fish consumption is unevenly distributed with about 50% of the population who do not consume fish at all [Max Rubner-Institut, 2008].

With such a low dietary intake, it is not surprising that large parts of the population are regarded as vulnerable groups for vitamin D deficiency. Among these are:

- pregnant and breastfeeding women [Mulligan et al., 2010; Gellert et al., 2017]
- teenagers and young women [González-Gross et al., 2012]
- old adults over 65 years [Mosekilde, 2005]
- people who are affected by low exposure to sunlight [Webb et al., 1988]
- people who have high pigmentation of skin [Holick, 2006]
- people with bowel or kidney disease [Holick, 2007a]
- overweight or obese people [Alemzadeh et al., 2008]
- vegetarians or vegans [Lamberg-Allardt et al., 1993]

In conclusion, virtually all population groups are affected by vitamin D deficiency, which is also evident from the average 25(OH)D-concentrations which are in most population groups less than 50 nmol/l.

2. Aims

2.1. General aims of the thesis

This thesis aimed to investigate whether a sufficient vitamin D status can be achieved by supplements or fish consumption. It investigates the effect of different types and doses of vitamin D supplements and the effect of regular bio-fortified fish consumption on vitamin D status in apparently healthy volunteers in three separate studies. All studies were blinded, randomized and controlled. Further, the effect of regular fish consumption on 25(OH)D-concentrations was summarized in a meta-analysis of additional nine randomized controlled studies.

Specific aims of the individual studies:

Study 1 - Bioavailability of vitamin D_2 and vitamin D_3: As there was a debate on the efficacy of vitamin D_2 compared to vitamin D_3, it was the aim to compare the efficacy of oral 50 µg/d either vitamin D_2 or D_3 to increase the total 25(OH)D-, 25(OH)D_2- and 25(OH)D_3-concentrations in healthy volunteers over a period of 8 weeks during wintertime.

Study 2 - Effect of vitamin D_3 supplementation according to the new recommend-dations: In 2012, the dietary recommendations (RDA) for vitamin D in the German speaking countries have been increased from 5 to 20 µg per day [German Nutrition Society, 2012]. This amount should be able to increase the serum 25(OH)D_3-concentrations above the target level of >50 nmol/l (equivalent to 20 ng/ml) in the majority of the population. Therefore, it was the aim to study the efficacy of 20 µg/d vitamin D_3 to increase 25(OH)D_3-concentrations >50 nmol/l in healthy volunteers during wintertime when endogenous vitamin D production is absent. Furthermore, the study wanted to investigate the effect on relevant vitamin D metabolites and cardiovascular risk factors.

Study 3 - Efficacy of vitamin D-fortified rainbow trout on vitamin D status: The main source of vitamin D_3 in the diet is fish, especially fatty fish. However, it was unclear to which extent serum 25(OH)D_3-concentrations can be increased due to fish consumption. Therefore, the effect on 25(OH)D_3-concentrations after six times/week

consumption of 100 g rainbow trout that was fortified with vitamin D through UVB treatment was compared to ordinary rainbow trout in healthy volunteers over a period of four weeks.

Study 4 - Efficacy of fish consumption on vitamin D status: However, effects on the 25(OH)D-concentrations from other fish consumption studies were so far not systematically investigated. Therefore, it was the aim to conduct a systematic review and a meta-analysis on the effect on 25(OH)D-concentrations from randomized controlled fish consumption studies and derive an estimate of fish intake that ensures sufficient vitamin D status.

3. Studies

3.1. Study 1

Lehmann, U., Hirche, F., Stangl, G.I., Hinz, K., Westphal, S., & Dierkes, J. (2013). Bioavailability of vitamin D2 and D3 in healthy volunteers, a randomized placebo-controlled trial. *The Journal of Clinical Endocrinology & Metabolism*, *98*(11), 4339-4345.

Bioavailability of Vitamin D_2 and D_3 in Healthy Volunteers, a Randomized Placebo-Controlled Trial

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Background: The bioequivalence of the different forms of vitamin D, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), has been questioned. Earlier studies have suggested that vitamin D_2 is less biologically active than vitamin D_3.

Objective and Design: In a parallel study, we tested the effects of supplementation with 50-μg/d doses of vitamin D_2 or D_3 or a placebo over a period of 8 weeks on 25(OH)D_2, 25(OH)D_3, their sum 25(OH)D (primary outcome variables), and PTH in healthy volunteers applying a double-blind, randomized study design. The study was conducted during the winter of 2012 in Halle (Saale), Germany, at latitude 51°47N, when UVB irradiation is virtually absent. Blood samples for the determinations of vitamin D status and PTH were collected at baseline and after 4 and 8 weeks of supplementation.

Results: In the placebo group (n = 19), 25(OH)D_3 decreased from 39.4 ± 14.2 to 31.1 ± 12.4 nmol/L after 8 weeks ($P < .01$). In the vitamin D_3 group (n = 42), the concentrations of 25(OH)D_3 increased from 41.5 ± 22.8 nmol/L at baseline to 88.0 ± 22.1 nmol/L after 8 weeks ($P < .01$). In the group receiving vitamin D_2 (n = 46), the 25(OH)D_2 concentrations increased significantly, whereas the 25(OH)D_3 concentration fell from 36.4 ± 13.3 nmol/L at baseline to 16.6 ± 6.3 nmol/L after 8 weeks ($P < .01$). The total 25(OH)D was not different between the groups at baseline but differed significantly between the groups after 4 and 8 weeks ($P < .001$).

Conclusions: Vitamin D_3 increases the total 25(OH)D concentration more than vitamin D_2. Vitamin D_2 supplementation was associated with a decrease in 25(OH)D_3, which can explain the different effect on total 25(OH)D. **(*J Clin Endocrinol Metab* 98: 4339–4345, 2013)**

Vitamin D exists in two different forms: ergocalciferol (vitamin D_2), which occurs in plants, mainly in mushrooms; and cholecalciferol (vitamin D_3), which occurs in animals and is also produced in human skin. Vitamins D_2 and D_3 differ only in their side chains. The best dietary sources of vitamin D are fatty fish and products fortified with vitamin D (1, 2). It has been estimated that most of the vitamin D_3 in humans is derived from endogenous synthesis in the epidermis, which contains 7-dehydrocholesterol as a precursor for vitamin D_3, after irradiation with UVB light at wavelengths of 290–330 nm (3). Although vitamin D_2 is less frequently used in Europe, it is the standard form of fortification and supplementation outside Europe.

Thus, both forms can be found in human blood, as well as the hydroxylated forms 25(OH)D_2 and 25(OH)D_3.

It has been debated for many years whether the two forms are bioequivalent. A number of studies have shown that vitamin D_2 does not increase the serum total 25(OH)D concentrations to the same extent as vitamin D_3

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Abbreviation: BMI, body mass index.

(4–6), but this finding has also been questioned by other investigators (7, 8). Because fortification or supplementation with vitamin D is currently the subject of much discussion owing to the widespread occurrence of vitamin D deficiency in nearly all populations investigated (9–19), it is important to know which form is more effective in supplementation and fortification. Although some studies have already shown that serum 25(OH)D_3 is lowered after the administration of vitamin D_2, either these studies lack sufficient statistical power (5, 20) and a control group (21) and they measured only total 25(OH)D (6, 7), or they were conducted in specific population groups (eg, elderly) (21, 22). Furthermore, it seems that the route of administration (bolus vs daily) may affect the comparison of both vitamin D forms. A recent meta-analysis showed that there was no significant difference in total 25(OH)D after daily administration of either vitamin D_2 or vitamin D_3 (1). In this meta-analysis, studies using 1000–1600 IU of vitamin D_2 or vitamin D_3 were included, but it was also estimated that larger, more robust trials are required that further address this issue.

We therefore conducted a bioavailability study in healthy volunteers who received a placebo—50 μg/d of vitamin D_2 or 50 μg/d of vitamin D_3 (2000 IU/d). The aim was to investigate the effects of this high dose on the serum levels of the hydroxylated forms 25(OH)D_2 and 25(OH)D_3 and on their sum total 25(OH)D. In addition, we investigated PTH concentrations, which are regarded as a functional parameter of vitamin D status (23). The measurability of 25(OH)D_3 serum or plasma levels is superior to that of 1,25$(OH)_2D_3$, owing to the much lower concentrations of 1,25$(OH)_2D_3$ and its shorter half-life compared with 25(OH)D_3 (24). The PTH concentrations are higher in the presence of vitamin D deficiency and decline upon supplementation with vitamin D; they can therefore be used as a functional parameter of vitamin D metabolism.

Furthermore, due to the inclusion of a placebo group, we were able to monitor the decrease of 25(OH)D_3 and total 25(OH)D in healthy subjects during wintertime at latitude 51°North.

Subjects and Methods

Design

The trial was conducted as a double-blind, randomized study in parallel groups during January, February, and March 2012, when virtually no UVB irradiation is measurable in Halle and the surrounding region. Study visits were scheduled at baseline and after 4 and 8 weeks. The subjects were randomized (stratified for body mass index [BMI] as determined during the screening visit) to receive vitamin D_2 (50 μg/d; n = 46), vitamin D_3 (50 μg/d; n = 42), or placebo (n = 19).

The supplements were manufactured commercially (Zein-Pharma) and were outwardly indistinguishable from one another. The tablets were tested for their vitamin D content after the study by a liquid chromatography, tandem mass spectrometry method in four separate runs, and the content was found to be 54 ± 12 μg for vitamin D_2 and 48 ± 6 μg for vitamin D_3 per tablet.

The participants were issued containers of tablets at baseline and after 4 weeks and were instructed to take one tablet orally per day and to return any remaining tablets at 4 and 8 weeks. The containers were numbered by an investigator with no involvement in the trial. All investigators were unaware of the order of numbering. The participants were enrolled by the physician involved in the trial but were assigned to the intervention by another investigator. Compliance, which was checked by counting the returned tablets, was 97%. During each study visit, a venous blood sample was collected for determination of 25(OH)D_2, 25(OH)D_3, their sum 25(OH)D, PTH, and serum calcium. The samples were frozen at −80°C until the time of analysis. The study protocol had been evaluated and approved by the Ethics Committee of the Medical Faculty at the Martin-Luther-University Halle-Wittenberg, and each participant gave his or her written, informed consent before the start of the study. The study was registered at clinicaltrails.gov (NCT01503216).

Subjects

Participants were recruited through newspaper advertisements, personal contacts, and information in public institutions. During a screening in the autumn (about 2 mo before the start of the study), the participants answered a self-administered questionnaire on their medical history, weight, height, lifestyle (smoking, use of sun blocker-containing cosmetics), and dietary habits relating to food rich in vitamin D. The exclusion criteria were: use of vitamin D and calcium supplements, history of chronic illness and elevated serum creatinine (in females, ≥1.1 mg/dL; in males, ≥1.3 mg/dL), elevated serum calcium, pregnancy or lactation, and vacations in areas with abundant UVB irradiation in the course of the study.

A total of 119 subjects had been recruited for the intervention study (age range, 19–67 y), were finally included in the study, and were randomized by a computer-generated randomization list to the intervention groups with the BMI as the stratification criterion. Participants were randomized into three groups according to their BMI: normal weight (BMI below 25 kg/m^2), overweight (25–30 kg/m^2), and obese (above 30 kg/m^2). Before the start of the intervention, seven subjects (placebo group, n = 1; vitamin D_2 group, n = 3; vitamin D_3 group, n = 3) dropped out. During the study period, five subjects (vitamin D_2 group, n = 1; vitamin D_3 group, n = 4) dropped out for personal reasons. During each visit, the participants were interviewed about any adverse effect. In addition, the calcium concentration in serum was measured in serum obtained at each visit.

After completion of the study, all subjects, including those in the control group, were informed about their vitamin D status and offered vitamin D supplements.

Analytical methods

Serum concentrations of total 25(OH)D, 25(OH)D_3, and 25(OH)D_2 were determined by liquid chromatography coupled with mass spectrometry (MassChrom 25-OH Vitamin D_3/D_2 reagent kit for liquid chromatography, tandem mass spectrom-

Table 1. Characteristics of Study Participants at Baseline

	Vitamin D_2 Group	Vitamin D_3 Group	Placebo Group	*P* (ANOVA)
n	46	42	19	
Age, y	33.2 ± 12.4	35.6 ± 13.5	31.6 ± 9.3	.445
No. of males/females	15/31	16/26	8/11	.745
BMI, kg/m^2	23.7 ± 3.8	24.0 ± 4,2	23.7 ± 4.9	.928
Systolic blood pressure, mm Hg	121 ± 14	120 ± 15	115 ± 8	.201
Diastolic blood pressure, mm Hg	76 ± 8	76 ± 10	75 ± 6	.894
Creatinine at screening, mg/dL	0.80 ± 0.22	0.86 ± 0.23	0.88 ± 0.24	.298

Data are expressed as mean ± SD.

etry analysis; Chromsystems Instruments and Chemicals GmbH) on an API 2000 system (Applied Biosystems). The coefficient of variation for the determination of 25(OH)D_2 was 3.1% at a concentration of 44.8 nmol/L; for 25(OH)D_3, it was 5.3% at a concentration of 42.8 nmol/L. Total 25(OH)D was calculated as the sum of 25(OH)D_2 and 25(OH)D_3. The detection limit for both 25(OH)D_2 and 25(OH)D_3 was 2.5 nmol/L, and the limit of quantification was 7.5 nmol/L. However, the measured levels were used for the calculation of total 25(OH)D as the sum of 25(OH)D_2 and 25(OH)D_3, even in subjects with 25(OH)D_2 levels below the limit of quantification.

Intact PTH was measured in the serum by an ELISA (Biomerica Inc). Serum creatinine was determined spectrophotometrically (DiaSys Diagnostic Systems GmbH).

Statistical analyses

Statistical analyses were performed using PASW version 18.0 (SPSS Inc). All data are expressed in the form of means ± SD, with $P < .05$ as the significance threshold. The primary outcome variables were the 25(OH)D_2, 25(OH)D_3, and total 25(OH)D concentrations. These variables and PTH concentrations are presented in Table 2. Because changes in total 25(OH)D and PTH tend to depend on the baseline level, we used repeated measure analysis to analyze changes upon supplementation. We used the generalized linear models repeated measures procedure in PASW for this analysis. Total 25(OH)D and 25(OH)D_3 at baseline and at 4 and 8 weeks were used as the within-subjects factor, and the supplementation group was used as the between-subjects factor. In addition, post hoc analyses by Scheffé were used to detect differences between single groups. PTH was highly skewed and was therefore analyzed by the nonparametric Kruskal-Wallis test.

In addition, we calculated the absolute change and the percentage change in total 25(OH)D, 25(OH)D_3, and PTH (8 wk − baseline) and compared these changes among groups by ANOVA (Table 3).

According to a power calculation, 50 subjects per group would be required to show a difference of 10 nmol/L in the mean total 25(OH)D concentration after 8 weeks of supplementation between the vitamin D_2 and D_3 groups (at an assumed standard variation of 15 nmol/L for each group, at a power of 80%, and a significance level of 0.05). Because it was the main aim to compare vitamin D_2 with D_3, the size of the placebo group was only about half that of the vitamin D groups. Only subjects who finished the study according to protocol were included into the analyses.

Results

The characteristics of the subjects are presented in Table 1. The average total 25(OH)D concentration at baseline in January was 40.2 ± 18.0 nmol/L, indicating a high degree of suboptimal vitamin D status in these healthy, young volunteers, with no significant differences between the groups. The total 25(OH)D concentration increased significantly throughout the study in the groups supplemented with vitamin D_2 or vitamin D_3 and decreased significantly to 33.1 ± 13.9 nmol/L after 4 weeks and to 32.1 ± 12.8 nmol/L after 8 weeks in the placebo group. After 4 and 8 weeks, the 25(OH)D concentrations differed significantly between the groups (Table 2).

At baseline, the 25(OH)D_2 concentration was below the limit of quantification (7.5 nmol/L) in all but two participants. In neither the vitamin D_3 group nor the placebo group did the average 25(OH)D_2 rise above the limit of quantification in the course of the study. In the vitamin D_2 group, 25(OH)D_2 increased significantly to 39.6 ± 11.7 nmol/L at 4 weeks and to 51.2 ± 18.5 nmol/L at 8 weeks (Table 2).

At baseline, there was no difference in the 25(OH)D_3 concentration between the groups. Although in the vitamin D_3 group 25(OH)D_3 increased significantly after 4 and 8 weeks, it decreased significantly in the vitamin D_2 and placebo groups. The decrease was more pronounced in the vitamin D_2 group, and the difference from the placebo group was significant at both 4 and 8 weeks (Table 2).

The increases (4-wk baseline, 8-wk baseline) in the specific hydroxylated forms of vitamin D [either 25(OH)D_2 or 25(OH)D_3] were as follows: in the case of 25(OH)D_2 in the vitamin D_2 group, 38.4 ± 11.0 nmol/L after 4 weeks and 50.0 ± 18.0 nmol/L after 8 weeks; in the case of 25(OH)D_3 in the vitamin D_3 group, 34.2 ± 17.2 nmol/L after 4 weeks and 46.7 ± 21 nmol/L after 8 weeks. The increase was calculated from the baseline value in this group, without taking the decrease in 25(OH)D_3 in the placebo group into account. The increase was not significantly different at either 4 or 8 weeks.

Table 2. Vitamin D Metabolites in Healthy Volunteers Receiving Supplementation With Vitamin D_2, Vitamin D_3, or Placebo for 8 Weeks

	Vitamin D_2 Group	Vitamin D_3 Group	Placebo Group	*P* (ANOVA)
n	46	42	19	
Total 25(OH)D				
Baseline, nmol/L	37.6 ± 13.3	43.7 ± 23.3	40.7 ± 14.5	.292
4 wk, nmol/L	59.9 ± 15.2[a]	77.1 ± 23.5[b]	33.1 ± 13.9	.001
8 wk, nmol/L	67.8 ± 20.1[a]	89.2 ± 22.1[b]	32.1 ± 12.8	.001
Repeated measure analysis				<.001
25(OH)D_3				
Baseline, nmol/L	36.4 ± 13.3	41.5 ± 22.8	39.4 ± 14.2	.409
4 wk, nmol/L	20.3 ± 8.1[a]	75.7 ± 23.2[b]	31.1 ± 13.9	.001
8 wk, nmol/L	16.6 ± 6.3[a]	88.0 ± 22.1[b]	31.1 ± 12.4	.001
Repeated measure analysis				.001
25(OH)D_2				
Baseline, nmol/L	<7.5[c]	<7.5	<7.5	.110
4 wk, nmol/L	39.6 ± 11.7[a]	<7.5	<7.5	.001
8 wk, nmol/L	51.2 ± 18.5[a]	<7.5	<7.5	.001
Repeated measure analysis				.001
PTH				
Baseline, ng/mL	69.8 ± 45.2	59.3 ± 22.6	79.4 ± 49.2	.334
4 wk, ng/mL	63.0 ± 33.2	49.1 ± 19.5	65.0 ± 40.0	.086
8 wk, ng/mL	56.8 ± 26.5	40.3 ± 19.5	60.8 ± 38.1	.007
Repeated measure analysis				.651

Data are shown as mean ± SD. Differences between the groups at the various time points of the study were tested by one-way ANOVA with post hoc Scheffé comparison. The overall effect of supplementation was tested by an ANOVA with repeated measurement (PASW procedure GLM repeated measure). Due to the high degree of skewness, the Kruskal-Wallis test was used for testing differences in PTH between groups.

[a] Significantly different at $P < .01$ from vitamin D_3 group and placebo.

[b] Significantly different at $P < .01$ from vitamin D_2 group and placebo.

[c] Values for 25(OH)D_2 at baseline and in the vitamin D_3 and placebo groups in the course of the study are only provided for those levels exceeding the limit of detection (>2.5 nmol/L).

The PTH concentrations were not significantly different between the groups at baseline or after 4 and 8 weeks (Table 2). PTH concentrations decreased significantly during the course of the study in all groups.

The absolute and percentage differences in total 25(OH)D, 25(OH)D_3, and 25(OH)D_2 between baseline and 8 weeks were significant among the supplementation groups. Absolute or percentage differences in PTH concentrations were not significant among the groups (Table 3).

No adverse effects were reported by the participants. Serum calcium did not exceed the normal range in any of the participants (data not shown). The analysis for total 25(OH)D, the primary outcome variable, was repeated with all randomized subjects included (intention-to-treat analysis). This did not change the results (data not shown).

Table 3. Absolute and Percentage Changes in Total 25(OH)D, 25(OH)D_3, 25(OH)D_2 (Absolute Change Only), and PTH at 8 Weeks Compared to Baseline

	Vitamin D_2 Group	Vitamin D_3 Group	Placebo Group	*P* (ANOVA)
n	46	42	19	
Δ Total 25(OH)D at 8 wk (to baseline), nmol/L	+30.2 ± 20.1[c]	+45.5 ± 21.7[a]	−8.6 ± 7.3	.001
% Total 25(OH)D at 8 wk (of baseline)	200 ± 97%[a]	259 ± 149%[a]	79 ± 16%	.001
Δ 25(OH)D_3 at 8 wk (to baseline), nmol/L	−19.8 ± 9.6[c]	+46.5 ± 21.3[b]	−8.3 ± 6.1	.001
% 25(OH)D_3 at 8 wk (of baseline)	47 ± 14%	280 ± 183%[b]	79 ± 15%	.001
Δ 25(OH)D_2 at 8 wk (to baseline), nmol/L	+43.7 ± 18.5[d]	<7.5	<7.5	.001
Δ PTH at 8 wk (to baseline), ng/mL	−13.0 ± 35.4	−19.0 ± 29.4	−18.6 ± 35.1	.658
% PTH at 8 wk (of baseline)	95 ± 47%	80 ± 58%	82 ± 38%	.354

Data are shown as mean ± SD. Significance was tested by ANOVA, followed by a post hoc Scheffé comparison.

[a] Significantly different from placebo group.

[b] Significantly different from vitamin D_2 and placebo groups.

[c] Significantly different from vitamin D_3 and placebo groups.

[d] Significantly different from vitamin D_2 and vitamin D_3 groups.

Discussion

Our major finding is that vitamin D_3 increased 25(OH)D more effectively than vitamin D_2. By measuring the specific hydroxylated forms, we have been able to show that the underlying reason for this difference is a substantial decrease in 25(OH)D_3 in subjects receiving vitamin D_2. This had not been demonstrated earlier with sufficient statistical power. We have also been able to show that hydroxylation of vitamin D_2 was similar to hydroxylation of vitamin D_3 because the increase in the specific hydroxylated forms [25(OH)D_2 and 25(OH)D_3] was similar in the two groups (compare the absolute differences in Table 3).

Vitamins D_2 and D_3 have been compared earlier in a number of studies that differed in their design, supplement dosage, frequency of supplementation, use of the delivery method, and selection of participants and also in their conclusion regarding the bioequivalence of the two forms of the vitamin (4–8, 20–22, 25, 26). A recent meta-analysis that included seven of these studies (4–8, 21, 22) concluded that the change in 25(OH)D was significantly greater after supplementation with vitamin D_3 than after one with vitamin D_2, although the effect was largely due to the studies that used a bolus dose; it was not significant in studies with daily supplementation (1). However, for the latter analysis, only six studies (6–8, 21, 22) with a total number of 248 participants were available. Our study with 42 and 46 participants in the vitamin D_3 and D_2 groups, respectively, would have changed the result of this analysis, yielding a significant effect in favor of vitamin D_3 compared to vitamin D_2 also with daily supplementation (the analysis using present data in addition to those of Tripkovic et al [1] was made using Review Manager 5.2; data not shown).

The most interesting result of our study, however, is the decrease in 25(OH)D_3 after supplementation with vitamin D_2. This was already evident after 4 weeks, and the decrease was significantly different from the seasonal decrease observed in the placebo group. A decrease in 25(OH)D_3 after supplementation with vitamin D_2 was reported earlier by Glendenning et al (22) in elderly hip fracture patients receiving 1000 IU/d for a period of 3 months, and also by Armas et al (26), who studied single doses of 50 000 IU of D_2 and D_3 in healthy men with a follow-up period of 28 days. Interestingly, both groups of authors did not discuss these findings specifically. This was also observed by Binkley et al (21) after administration of 1600 IU daily for a period of 12 months. It is surprising that this effect was observed in only a few studies, although it should be pointed out that only studies using methods capable of distinguishing between 25(OH)D_2 and 25(OH)D_3 would be able to show this effect. The use of immunoassays will therefore not make it possible to observe the effect. The biological reason behind this finding remains to be elucidated.

It has been suggested that an increased catabolism of 25(OH)D takes place due to supplementation with vitamin D_2 (5). Heaney et al (5) studied, groups of 16 and 17 subjects who received 50 000 IU once weekly for 12 weeks, and a significantly higher $AUC_{25(OH)D}$ was observed after 84 days for vitamin D_3. Interestingly, vitamins D_3 and D_2 were also measured in the fat tissue of two participants, and a decrease in vitamin D_3 in fat tissue after supplementation with vitamin D_2 was observed. Because the authors measured vitamin D_2 in fat biopsies from only two participants, however, this finding did not reach statistical significance.

It has also been suggested that one reason for the lower increase in 25(OH)D after vitamin D_2 in comparison with supplementation with D_3 was due to impaired hydroxylation at C25 (atom of the vitamin D molecule) (27). We have shown that at least the increases in the specific hydroxylation products [either 25(OH)D_2 or 25(OH)D_3] were similar. However, we cannot exclude the possibility that vitamin D_2 impairs hydroxylation of vitamin D_3, which is also present in the circulation. Because the decrease in 25(OH)D_3 exceeded the observed decrease in the placebo group, this is a likely explanation. The problem should be investigated further.

Other explanations include an increased catabolism of the 25(OH)D_2 molecule due to a lower degree of binding to the vitamin D binding protein (28). Our data do not support an increased catabolism of 25(OH)D_2, although they cannot exclude it.

Because we did not measure any other metabolite [24,25$(OH)_2$D metabolites, 1,24,25$(OH)_3$D metabolites], we can only speculate about differences in the 24-hydroxylation step between 25(OH)D_2 and 25(OH)D_3. Further studies should include these metabolites to obtain a deeper insight into the competitive nature of the two forms of vitamin D.

Our study has several strengths and also some limitations. The strengths of the present study include its large sample size, which allowed us to detect small differences between vitamin D_2 and D_3 treatments that earlier studies had been unable to show. Another important strength is the measurement of both 25(OH)D_2 and 25(OH)D_3 in this study. Measurements of the specific hydroxylated forms of vitamin D enabled us to show the effect of vitamin D_2 on the 25(OH)D_3 levels. In addition, due to the inclusion of the placebo group, we were able to monitor the decrease in total 25(OH)D concentrations within healthy subjects living at the approximate latitude 51°North. We

observed a strong decrease from January to February and no further decrease from February to March.

One limitation of our study was that we did not measure the active forms, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$, or other metabolites. In addition, we did not obtain a dose-response curve after a single dose, and we did not determine the catabolic products $24,25(OH)_2D$, $24,25(OH)_2D_3$, or $24,25(OH)_2D_2$. Measurement of these metabolites would provide valuable insights into the metabolism of vitamin D_3 in the presence of vitamin D_2. We also studied only one dose, and the level of 50 μg/d is beyond current recommendations and fortification levels.

In future studies, the effect of lower doses of vitamin D that are closer to the recommended daily amounts should be investigated. In light of the decrease in $25(OH)D_3$ by vitamin D_2, the effect of vitamin D_2 supplementation on disease outcomes, eg, bone health and fractures, should be carefully analyzed. Indeed, the effect of vitamin D_2 on falls was found to be lower than that of vitamin D_3 in recent meta-analyses (29, 30).

PTH and vitamin D are both involved in bone metabolism (31) and show an inverse correlation. PTH secretion is directly modulated (23) and suppressed by 25(OH)D concentrations (31). Leventis and Kiely (32) demonstrated that vitamin D_3 affected PTH concentration more than vitamin D_2, a finding that is not supported by our data. However, our study was not designed to demonstrate an effect of vitamin D supplementation on PTH concentrations as the primary outcome. To demonstrate such an effect, we had to include even more subjects due to the large variation in PTH concentrations. Therefore, we may have missed an effect of vitamin D supplementation on PTH concentrations. This is in line with a number of other studies (8, 21, 22).

In conclusion, we have shown that vitamin D_3 is more effective in raising the vitamin D status than vitamin D_2 and that vitamin D_2 supplementation causes a decrease in $25(OH)D_3$. These findings question the usefulness of vitamin D_2 supplements. Instead, vitamin D_3 should be used for supplementation and fortification purposes.

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Author contributions: J.D. and G.I.S. designed the research. U.L., K.H., S.W., and F.H. conducted the study. J.D. and U.L. analyzed the data and wrote the paper. J.D. has primary responsibility for the final content. All authors read and approved the final manuscript. None of the authors declared any financial or personal relationships with other persons or organizations that could have an inappropriate influence on this work. The funding agency had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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3.2. Study 2

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Randomized control trials

Vitamin D_3 supplementation: Response and predictors of vitamin D_3 metabolites – A randomized controlled trial

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SUMMARY

Background & aims: Large parts of the population are insufficiently supplied with vitamin D, in particular when endogenous synthesis is absent. Therefore many health care providers recommend the use of vitamin D supplements. The current study aimed to investigate the efficacy of an once-daily oral dose of 20 μg vitamin D_3 to improve the vitamin D status and to evaluate predictors of response.
Methods: The study was conducted as a double-blind, randomized, placebo-controlled parallel trial from January till April 2013. In total, 105 subjects (20–71 years) were allocated to receive either a vitamin D_3 supplement (20 μg/d) or a placebo for 12 weeks. Circulating levels of vitamin D_3 metabolites such as the 25(OH)D_3 and the 24,25$(OH)_2D_3$, and biomarkers of calcium and phosphate metabolism were quantified.
Results: The 25(OH)D_3 serum concentrations in the placebo group decreased from 38 ± 15 nmol/L at baseline to 32 ± 14 nmol/L and 32 ± 13 nmol/L at weeks 8 and 12 of the study, respectively ($p < 0.01$). In the vitamin D_3 group, the serum 25(OH)D_3 concentration increased from 38 ± 14 nmol/L at baseline to 70 ± 15 nmol/L and 73 ± 16 nmol/L at weeks 8 and 12 of vitamin D_3 supplementation ($p < 0.001$), respectively. As a result, 94% of the vitamin D_3-supplemented participants reached 25(OH)D_3 concentrations of ≥50 nmol/L and thereof 46% attained 25(OH)D_3 levels of ≥75 nmol/L until the end of the study. The extent of the 25(OH)D_3 increase upon vitamin D_3 supplementation depended on 25(OH)D_3 baseline levels, age, body weight and circulating levels of triglycerides. In contrast to 25(OH)D_3, the response of 24,25$(OH)_2D_3$ to the vitamin D_3 treatment was affected only by baseline levels of 24,25$(OH)_2D_3$ and age.
Conclusions: The average improvement of 25(OH)D_3 levels in individuals who received 20 μg vitamin D_3 per day during the winter months was 41 nmol/L compared to individuals without supplementation. As a result almost all participants with the vitamin D_3 supplementation attained 25(OH)D_3 concentrations of 50 nmol/L and higher. The suitability of 24,25$(OH)_2D_3$ as a marker of vitamin D status needs further investigation.
Clinical trial registration number at clinicaltrails.gov: NCT01711905.

1. Introduction

There is an ongoing debate on the necessity of vitamin D supplementation in the healthy population. Assessment of vitamin D status is currently based on measuring circulating 25-hydroxyvitamin D_3 (25(OH)D_3), which is considered as reliable biomarker of the vitamin D status. The classification of 25(OH)D concentrations into deficient, insufficient and adequate is mainly

Abbreviations: BIA, bioelectrical impedance analysis; BMI, body mass index; FGF-23, fibroblast growth factor 23; LC–MS/MS, liquid chromatography coupled with mass spectrometry; LLOQ, lower limit of quantification; 1,25$(OH)_2D$, 1,25-dihydroxyvitamin D; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25(OH)D_3, 25-hydroxyvitamin D_3.

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based on the onset of bone ossification disorders [1]. Although it is generally agreed that serum 25(OH)D concentrations below 30 nmol/L are deficient, the optimum level of 25(OH)D remains controversial. The Institute of Medicine (IOM) classified 25(OH)D concentrations of 50 nmol/L as sufficient to prevent bone disorders [1]. Studies that considered further vitamin D associated diseases such as cancer and cardiovascular diseases, recommend 25(OH)D levels of at least 75 nmol/L [2,3]. Heaney proposed that the vitamin D status to ensure normal cell function had to be higher than that necessary to prevent diseases [4].

Recent data from a random sub-cohort of the German arm of the European Prospective Investigation into Cancer and Nutrition (EPIC) demonstrated pronounced seasonal variations in $25(OH)D_3$ concentrations and showed that, from January to March, 82.2% of the subjects had $25(OH)D_3$ serum concentrations <50 nmol/L and 34.1% were even below 30 nmol/L [5]. With regard to the high prevalence of vitamin D insufficiency, the German Nutrition Society recommends a daily vitamin D intake of 20 μg for healthy adults in periods of limited endogenous vitamin D synthesis, to achieve serum $25(OH)D_3$ concentrations of at least 50 nmol/L [6]. Previous work of Cashman and co-workers clearly showed that the amount of dietary vitamin D necessary to maintain the 25(OH)D concentrations during wintertime depends on the extent of summer sunshine exposure, diet intake of vitamin D and the desired levels of circulating 25(OH)D [7]. Data of that study pointed out that there is a substantial heterogeneity in the individual response of 25(OH)D to vitamin D supplementation [7], although other factors that may explain that phenomenon have not been entirely clarified.

The baseline 25(OH)D level is assumed to be an important determinant (e.g. 8–10), but also genetic variances (e.g. 9, 10), body fat and/or body mass index (BMI) (e.g. 8, 11) are suggested to influence the response. In vitamin D studies, little attention has been paid to age, since most investigations were dedicated to a specific age group, e.g. premenopausal women (19–35 years) [12], postmenopausal women [11] or elderly (≥60 years) [8,10].

The present study aimed: (i) to investigate the efficacy of a daily oral dosage of 20 μg vitamin D_3 to increase the $25(OH)D_3$ serum concentration of healthy volunteers to at least 50 nmol/L during the winter months, (ii) to identify factors that may modify the response of $25(OH)D_3$ to vitamin D_3 supplementation, and (iii) to validate the suitability of 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) as biomarker for vitamin D supplementation. To this end, females and males (≥18 years of age) without restrictions regarding BMI were recruited to participate in the study and serum concentrations of $25(OH)D_3$, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), $24,25(OH)_2D_3$ and biomarkers of calcium and phosphate homeostasis were quantified.

2. Materials and methods

2.1. Study design and study population

This study was conducted as a double-blind, randomized, controlled human intervention trial in parallel groups. The aims of the study were to assess the effect of an 8 and 12 week recommendation-based supplementation of 20 μg/d vitamin D_3 versus a placebo on the serum concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$, and to identify factors that modify the response of these metabolites upon vitamin D_3 supplementation. The study was performed from January 2013 until the beginning of April 2013 in Halle/Saale, Germany (51° of Northern latitude), when endogenous vitamin D synthesis via UVB irradiation was absent. The study was approved by the local ethics committee of the Medical Faculty at the Martin Luther University Halle-Wittenberg. Each participant gave written informed consent prior to the beginning of the study. The study was registered at clinicaltrails.gov (NCT01711905).

Sample size was calculated using G*Power analysis program [13] to find statistically significant differences in the serum vitamin D metabolite response between predictor-based subgroups. Considering one-way ANOVA as statistical test, a mean $25(OH)D_3$ difference of 10 nmol/L between subgroups and a standard deviation of 14 nmol/L, a power of 95% and a significance level of 0.05, a total sample size of 51 subjects ($n = 17$ in each subgroup) was required.

Apparently healthy subjects of both sexes from a minimum age of 18 years were recruited in cooperation with the blood donation center of the Department of Transfusion Medicine (University Hospital Halle, Germany), through newspapers advertisements, personal contacts and public information events. All volunteers underwent a screening visit in autumn 2012. They had to fill in a questionnaire on medical history, body weight and height, lifestyle behaviors (e.g. smoking, usage of sun blocker containing cosmetics) and dietary habits especially focusing on the intake of foods rich in vitamin D. Blood samples were drawn to estimate the serum $25(OH)D_3$ concentrations. The overall health status was assessed by questionnaires on disease history, clinical measurements (e.g. blood pressure) and clinical chemical analysis of markers of renal function, thyroid function and calcium metabolism. Exclusion criteria were use of dietary supplements, vacations in regions with abundant UVB irradiation 8 or less weeks prior to the study begin or during the study period, serum $25(OH)D_3$ concentrations >75 nmol/L, pregnancy or lactation, participation in other clinical studies, intake of prescription medication, deviations in blood count or metabolic disorders.

A total of 106 subjects (age range: 20–71 years) were included in the study. They were randomly assigned into the two groups (placebo group, $n = 52$, vitamin D_3 group, $n = 54$) by block randomization using a computer-generated randomization schedule with serum $25(OH)D_3$ concentration, BMI and sex as stratification criteria. One female participant from the placebo group dropped out because of personal reasons. Therefore, 105 participants completed the study and were included into the statistical analysis.

Study visits were scheduled at baseline, and after 8 and 12 weeks of intervention. At each study visit anthropometric data (height, body weight, waist circumference, body fat mass), blood pressure and heart frequency were recorded, fasting blood samples were drawn and participants were asked for adverse effects in response to the treatment. Body fat mass was determined by bioelectrical impedance analysis (BIA, Data Input, Darmstadt, Germany). After 5 min of rest, blood pressure and heart rate were measured in triplicate at the left arm at heart level with a one minute interval in between (BpTRU Medical Devices, Coquitlam, Canada).

Prior to the start of the study, the participants received their prepacked and numbered (according to the randomization schedule) 12-week ration of either the placebo or the vitamin D_3 supplement. The participants were instructed to take one tablet per day for a total of 12 weeks. The time of consumption was not specified but it was recommended to take the tablet together with a meal. Compliance was ensured by counting the remaining tablets, and achieved 99%. The supplements were manufactured by Vital Products GmbH (Waldsassen, Germany). Vitamin D_3 was received from DSM Nutritional Products Ltd. (Basel, Switzerland). Placebo and vitamin D_3 tablets were outwardly indistinguishable in appearance and taste. Cellulose was used as the placebo. The vitamin D_3 content per tablet was quantified by liquid chromatography coupled with mass spectrometry (LC–MS/MS) in four separate runs [14]. The analyzed vitamin D_3 content of a single vitamin D_3 tablet amounted to 19.6 ± 1.5 μg. An independent

investigator numbered the vitamin D_3 tablet containers, so that all researchers and staff who conducted the study and analyzed the samples and all study participants were unaware of the group assignment. Treatment codes were generated in blocks of two by using a computer-generated randomization schedule. Subjects were enrolled by a physician involved in the trial, but assigned to the intervention by another investigator.

The study was completed as scheduled. After completion of the study, information on the individual vitamin D status and strategies for a vitamin D status improvement were provided to all subjects, and individuals of the placebo group were offered vitamin D supplements to improve the vitamin D status.

2.2. Analytical methods

For biochemical analyses venous blood was collected and centrifuged at 2000 × *g* for 10 min to obtain serum and EDTA-plasma samples. For the quantification of glucose, fluoride-coated tubes were used. Samples were aliquoted, frozen and stored at −80 °C until analyses.

Serum 25(OH)D_3 concentrations at baseline and after 8 and 12 weeks of intervention were quantified by means of a MassChrom® 25-OH Vitamin D_3 reagent kit (Chromsystems GmbH, Munich, Germany) for LC–MS/MS using an API 2000™ system (Applied Biosystems, Darmstadt, Germany) as described elsewhere [15]. The lower limit of quantification (LLOQ) was 10.7 nmol/L and the coefficients of variance were 8.3% (n = 4, inter assay) and 5.0% (n = 3, intra assay) at 39.9 nmol/L. Serum 1,25$(OH)_2$D concentrations were determined using a commercially available ELISA kit (Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany) at baseline and after 12 weeks of intervention.

Serum 24,25$(OH)_2D_3$ concentration at baseline and week 12 was determined by LC–MS/MS (PU-2080 Plus, LG-2080-02, LG-2080-04, Jetstream II, AS-2057 Plus, all Jasco, Gross-Umstadt, Germany; 4000 QTrap system, Applied Biosystems) using a Hypersil ODS column, 150 × 2 mm^2, 5 μm (VDS optilab, Berlin, Germany) at 40 °C and 0.576 mL/min. The mobile phase consisted of (A) 5 mM ammonium formate, 0.1% formic acid in water/acetonitrile (9 + 1, v/v), and (B) acetonitrile (time table: 0–1.9 min 50% B; 5.5 min 56% B; 5.7 min 100% B; 11.8 min 100% B; 12–15 min 50% B). Calculations were based on (*m*/*z*) 574.6/298.4 for 24,25$(OH)_2D_3$ and 582.5/298.4 for 25(OH)D_3-d_6 (Chemaphor Inc., Ottawa, Canada) which was used as internal standard. The coefficients of variance were 11.3% (n = 6, inter assay) and 7.5% (n = 3, intra assay) at 2.11 nmol/L. The LLOQ was 0.26 nmol/L.

Calcium and inorganic phosphate serum concentrations were determined using spectrophotometric assays (Analyticon Biotechnologies AG, Lichtenfels, Germany). Commercial ELISA kits were used to quantify serum concentrations of intact parathyroid hormone (PTH, Biomerica Inc., Irvine, USA) and soluble α-Klotho (IBL Immuno-Biological Laboratories Co., Ltd., Japan) as well as plasma fibroblast growth factor 23 (FGF-23; C-term, Immutopics Inc., San Clemente, USA). Plasma glucose and serum triglycerides concentrations were measured using enzymatic assays (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). All analyses were run in duplicate following the manufacturer's instructions.

2.3. Statistical analyses

Statistical analyses were performed using SPSS Version 22.0 (IBM, Chicago, USA) and SigmaPlot (Systat Software, Inc., San Jose, USA). Data are presented as mean ± SD. The significance level was set at 5%. Normal distribution of the data was verified by the Kolmogorov–Smirnov test, skewed variables (24,25$(OH)_2D_3$, PTH, FGF-23, Klotho) were logarithmically transformed. Differences between groups at baseline were analyzed by a two sample t-test. To assess the effects of the factors time (within-subjects factor) and treatment (between-subjects factor) as well as their interaction a two-way ANOVA with Bonferroni post hoc test was conducted. The number of volunteers was n = 51 in the placebo group, and n = 54 in the vitamin D_3 group.

To identify correlations between plasma vitamin D metabolite concentrations Spearman's correlation coefficients were calculated. Therefore, coefficients between circulating concentrations of 25(OH)D_3, 1,25$(OH)_2$D and 24,25$(OH)_2D_3$ were calculated by considering data from all subjects at baseline and after 12 weeks. To elucidate the impact of an improved vitamin D status on health parameters, correlations between changes (baseline to week 12) of vitamin D metabolites and anthropometric and clinical markers were assessed.

In order to compile a prediction model of the vitamin D_3 supplementation response, a regression analysis was performed. Best subset regression based on the adjusted R^2 and subsequent multivariate linear regression analyses were accomplished to identify predictors for the changes (baseline to week 12) of serum 25(OH)D_3 and 24,25$(OH)_2D_3$ upon vitamin D_3 supplementation representing the dependent variables. For those parameters that significantly affected changes in serum 25(OH)D_3 and 24,25$(OH)_2D_3$, a subgroup analysis was conducted. The subjects were divided into tertiles based on the baseline values of the predictors and a one-way ANOVA with Bonferroni post hoc test was performed to estimate differences between the subgroups.

3. Results

3.1. Baseline characteristics of the subjects and vitamin D status

Characteristics of the subjects at study entry are presented in Table 1. The study collective comprised of 67% females and 33% males. The mean age and BMI at baseline were 39 years and 24.0 kg/m^2, respectively. Anthropometric data, blood pressure, heart rate and concentrations of glucose and triglycerides at baseline did not differ between the two groups. None of the participants were vegan or vegetarian.

Serum concentrations of the vitamin D metabolites at the beginning of the study were also not different between the two groups (Table 2). At baseline, 32% of the individuals had 25(OH)D_3 concentrations lower than 30 nmol/L, 48% had concentrations between 30 and 50 nmol/L, and 20% had concentrations that ranged between 50 and 75 nmol/L. During the study, the serum level of 25(OH)D_3 from individuals of the placebo group decreased significantly from baseline to week 8 ($p < 0.01$), but remained unchanged from week 8 to week 12 (Table 2). In the placebo group the decrease of 25(OH)D_3 within the 12 weeks during winter was on average 6 nmol/L. In the vitamin D_3 group, the serum levels of 25(OH)D_3 increased from baseline to week 8 ($p < 0.001$), without showing any further increase from week 8 to week 12. The 25(OH)D_3 levels could be improved on average by about 35 nmol/L during the 12 week intervention. After 8 and 12 weeks of treatment, individuals from the vitamin D_3 group showed 2.2- and 2.3-fold higher 25(OH)D_3 levels than those of the placebo group. After 12 weeks, 94% of the participants that received vitamin D_3 reached 25(OH)D_3 concentrations higher than 50 nmol/L and 46% even reached concentrations higher than 75 nmol/L. In the placebo group, 53% had 25(OH)D_3 concentrations below 30 nmol/L, 33% ranged between 30 and 50 nmol/L and 14% had concentrations above 50 nmol/L. None of them showed 25(OH)D_3 concentrations higher than 75 nmol/L.

Due to the non-significant changes of 25(OH)D_3 between week 8 and 12 in both groups, the serum levels of 1,25$(OH)_2$D and 24,25$(OH)_2$D were analyzed only at baseline and after 12 weeks of

Table 1
Characteristics of study participants at baseline.

	Placebo group (n = 51)	Vitamin D_3 group (n = 54)	Range (n = 105)
Sex [male/female]	17/34	18/36	
Age [years]	39 ± 14	39 ± 14	20–71
Body weight [kg]	70 ± 14	72 ± 11	49–107
Body mass index [kg/m^2]	24 ± 3	24 ± 3	18–31
Body fat mass (BIA) [%]	19 ± 6	20 ± 7	10–43
Waist circumference [cm]	80 ± 11	83 ± 10	62–106
Blood pressure [mmHg]			
Systolic	114 ± 13	113 ± 13	93–148
Diastolic	75 ± 9	74 ± 9	54–102
Heart rate [beats/min]	69 ± 11	69 ± 9	38–94
Glucose [mmol/L]	4.9 ± 0.6	4.8 ± 0.8	3.4–8.3
Triglycerides [mmol/L]	1.0 ± 0.6	0.9 ± 0.5	0.3–3.4

Values are given as mean ± SD.

intervention. Analysis revealed that the serum levels of 1,25$(OH)_2$D were altered by the treatment but to a lesser extent than 25(OH)D_3 (Table 2). Serum concentrations of 1,25$(OH)_2$D slightly decreased from baseline to week 12 in the placebo group ($p < 0.05$), whereas no changes were observed in the vitamin D_3 group. After 12 weeks, subjects of the vitamin D_3 group had higher serum levels of 1,25$(OH)_2$D than subjects of the placebo group ($p < 0.001$).

As observed for 25(OH)D_3, the 24,25$(OH)_2D_3$ serum concentration was significantly influenced by treatment and time (Table 2). In the placebo group, the serum concentration of 24,25$(OH)_2D_3$ decreased from baseline to week 12 ($p < 0.001$), whereas it increased in the vitamin D_3 group ($p < 0.001$). After 12 weeks of treatment, subjects of the vitamin D_3 group had nearly 3-fold higher 24,25$(OH)_2D_3$ concentrations than those of the placebo group.

3.2. Biomarkers of calcium and phosphate metabolism

Data demonstrated no differences in baseline concentrations of calcium, phosphate, PTH, klotho and FGF-23 between the two groups (Table 2). Serum concentration of calcium was influenced by time, but not by the treatment; there was a decline from baseline to week 12 in both groups (Table 2). The circulating concentration of inorganic phosphate was neither affected by treatment nor by time. The serum concentration of PTH increased from baseline to week 12 in the placebo group ($p < 0.05$), whereas it remained unchanged

Table 2
Concentrations of vitamin D metabolites and parameters of calcium and phosphate homeostasis at baseline as well as after 8 and/or 12 weeks of intervention.

	Placebo group	Vitamin D_3 group	Two-way ANOVA (p-value)		
			Time	Treatment	Time × Treatment
25(OH)D_3 [nmol/L]					
Baseline	38 ± 15^a	38 ± 14^a	<0.001	<0.001	<0.001
8. week	32 ± 14^b	70 ± 15^{b*}			
12. week	32 ± 13^b	73 ± 16^{b*}			
1,25$(OH)_2$D [pmol/L]					
Baseline	110 ± 37^a	119 ± 44	0.66	<0.005	<0.001
12. week	96 ± 34^b	130 ± 35*			
24,25$(OH)_2D_3$ [nmol/L]					
Baseline	1.9 ± 1.1^a	1.8 ± 0.9^a	<0.05	<0.001	<0.001
12. week	1.2 ± 0.8^b	3.4 ± 1.2^{b*}			
Calcium [mmol/L]					
Baseline	2.4 ± 0.1^a	2.4 ± 0.1^a	<0.001	0.20	<0.05
8. week	2.3 ± 0.1^a	2.3 ± 0.1^b			
12. week	2.2 ± 0.1^b	2.3 ± 0.1^b			
Inorganic phosphate [mmol/L]					
Baseline	1.2 ± 0.2	1.2 ± 0.2	0.35	0.17	<0.05
8. week	1.2 ± 0.2	1.2 ± 0.2			
12. week	1.2 ± 0.2	1.2 ± 0.2			
PTH [pmol/L]					
Baseline	6.5 ± 2.1^a	6.4 ± 2.5	<0.05	0.08	<0.001
8. week	7.0 ± 2.3^{a,b}	6.2 ± 1.9			
12. week	7.4 ± 2.6^b	6.1 ± 2.3			
Klotho [pg/mL]					
Baseline	821 ± 738	753 ± 459	0.08	0.82	0.93
8. week	845 ± 795	738 ± 419			
12. week	807 ± 795	716 ± 414			
FGF-23 [RU/mL]					
Baseline	95 ± 53	96 ± 53	0.16	0.91	0.96
8. week	93 ± 58	85 ± 35			
12. week	100 ± 72	96 ± 61			

Data are given as mean ± SD. Bonferroni post hoc test was applied.
^{a,b}Significant differences between time points within a group ($p < 0.05$).
*Significantly different from placebo group at a given time ($p < 0.05$).

in the vitamin D_3 group. The circulating concentrations of klotho and FGF-23 were not affected by treatment and time, respectively.

3.3. Data from correlation analyses

Correlation analysis of vitamin D metabolite serum concentrations from all subjects at baseline and after 12 weeks of treatment are presented in Fig. 1. Data revealed a strong positive association between the serum concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$ (Fig. 1A). The correlation between $25(OH)D_3$ and $1,25(OH)_2D$ was weaker than that between $25(OH)D_3$ and $24,25(OH)_2D_3$ (Fig. 1B). Similar correlation data were observed when taking the changes (from baseline to week 12) of the vitamin D metabolites as a basis (Fig. 1C and D).

Correlation analysis between changes of circulating vitamin D metabolites and changes of the other analyzed parameter is presented in Table 3. Data showed a weak positive correlation between $\Delta 24,25(OH)_2D_3$ and Δwaist circumference and a weak positive correlation between $\Delta 25(OH)D_3$ and Δcalcium. In contrast, all vitamin D metabolites changes were inversely correlated with ΔPTH, in the order of magnitude from the strongest to weakest correlation coefficients: $25(OH)D_3 > 24,25(OH)_2D_3 > 1,25(OH)_2D$.

3.4. Predictors of serum $25(OH)D_3$ and $24,25(OH)_2D_3$ response to vitamin D_3 supplementation

Best subset regression analyses were performed to identify factors that modify the response of $25(OH)D_3$ and $24,25(OH)_2D_3$ serum concentrations to vitamin D_3 supplementation (Table 4). Choice of predictors was based on the adjusted R^2 that considers the number of independent variables and prevents an overfitting in regression-type models.

The variance in $25(OH)D_3$ changes explained by the regression model was 43% (overall p-value of the regression < 0.001, $R^2 = 0.48$, adjusted $R^2 = 0.43$). There was no multi-colinearity between the determinants. The prediction model revealed the baseline $25(OH)D_3$ concentration as the strongest predictor for $\Delta 25(OH)D_3$, followed by age, body weight and baseline serum triglycerides. For $\Delta 24,25(OH)_2D_3$, about 17% of the overall variability were explained by the regression model (overall p-value of the regression < 0.001, $R^2 = 0.20$, adjusted $R^2 = 0.17$). Determinants of the $24,25(OH)_2D_3$ response were baseline $24,25(OH)_2D_3$ concentration and age.

Results from the subgroup analyses are shown in Table 5. The $\Delta 25(OH)D_3$ decreased significantly with increasing levels of baseline $25(OH)D_3$ and with increasing age. Although body weight and baseline triglyceride concentrations were shown to affect $\Delta 25(OH)D_3$, significant differences between subgroups were not demonstrated. Analyses of $\Delta 24,25(OH)_2D_3$ revealed only a trend toward decreasing changes with increasing levels of baseline $24,25(OH)_2D_3$ and increasing age.

4. Discussion

This study aimed to investigate the efficacy of 20 μg vitamin D_3 per day to improve the vitamin D status of individuals during the winter months by analyzing changes of serum $25(OH)D_3$ concentrations. Taking the recommended 25(OH)D level of the IOM as a basis (≥50 nmol/L) [1], 32% of the study participants had a deficient and 48% had an insufficient vitamin D status at baseline. Virtually all participants who were treated with 20 μg vitamin D_3 per day reached serum 25(OH)D levels of at least 50 nmol/L. When taking

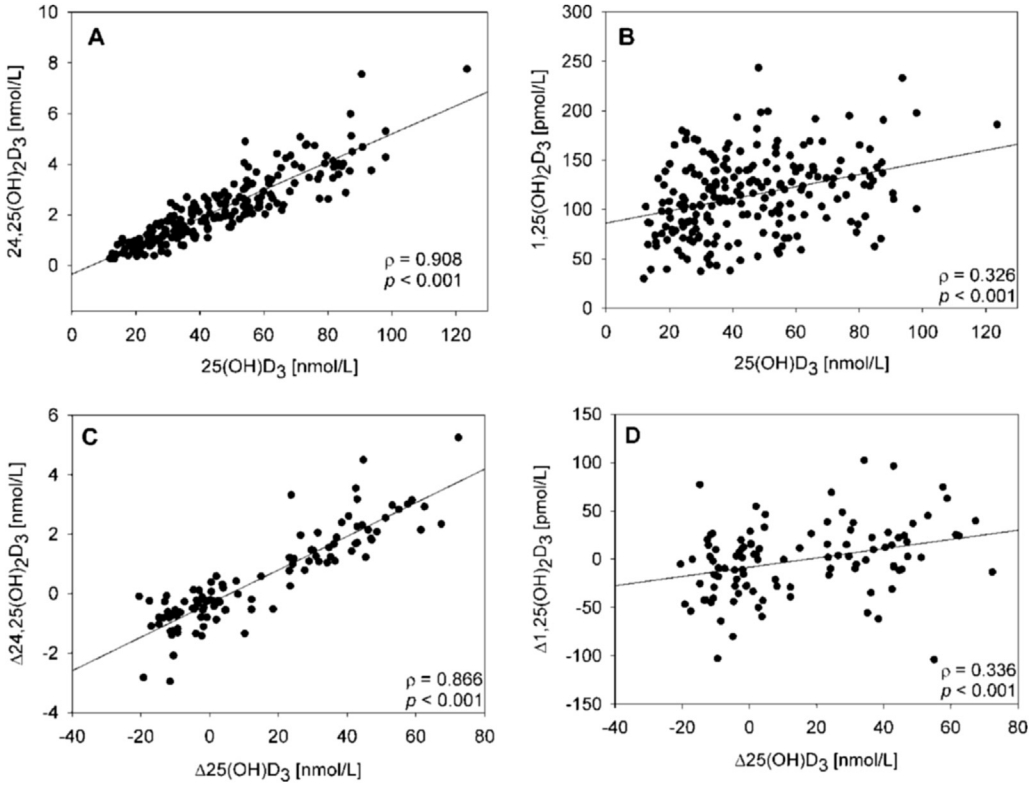

Fig. 1. Spearman's correlation between $25(OH)D_3$ and (A) $24,25(OH)_2D_3$ and (B) $1,25(OH)_2D_3$ serum concentrations comprising data at baseline and after 12 weeks of treatment from both groups (vitamin D_3 and placebo) (n = 210); correlations between $\Delta 25(OH)D_3$ and (C) $\Delta 24,25(OH)D_3$ and (D) $\Delta 1,25(OH)_2D_3$ including changes from baseline to week 12 of treatment from both groups (n = 105).

Table 3
Spearman's correlation analysis of changes in parameters in the vitamin D_3 group.

	Correlation coefficients		
	Δ25(OH)D_3 [nmol/L]	Δ1,25(OH)$_2$D [pmol/L]	Δ24,25(OH)$_2D_3$ [nmol/L]
ΔBody mass index [kg/m^2]	0.09	0.07	0.10
ΔBody fat mass (BIA)[%]	−0.12	−0.13	−0.07
ΔWaist circumference [cm]	0.12	−0.08	0.20*
ΔGlucose [mmol/L]	−0.06	−0.01	0.02
ΔTriglycerides [mmol/L]	−0.10	−0.14	−0.09
ΔCalcium [mmol/L]	0.21*	−0.04	0.18
ΔInorganic phosphate [mmol/L]	0.18	0.08	0.15
ΔParathyroid hormone [pmol/L]	−0.42***	−0.24*	−0.40***
ΔKlotho [pg/mL]	−0.01	0.07	−0.04
ΔFGF-23 [RU/mL]	−0.07	−0.06	−0.03

*$p < 0.05$, ***$p < 0.001$.

Table 4
Identified predictors for supplementation response assessed by best subset regression analysis.

Predictors	Coefficient	*p*-value
Δ25(OH)D_3		
Constant	97 ± 14	<0.001
Baseline 25(OH)D_3 [nmol/L]	−0.67 ± 0.14	<0.001
Age [years]	−0.38 ± 0.14	0.01
Body weight [kg]	−0.43 ± 0.18	<0.05
Baseline triglycerides [mmol/L]	8.8 ± 3.9	<0.05
Δ24,25(OH)$_2D_3$		
Constant	3.5 ± 0.6	<0.001
Baseline 24,25(OH)$_2D_3$ [nmol/L]	−0.50 ± 0.17	<0.01
Age [years]	−0.02 ± 0.01	<0.05

Coefficients are given as mean ± standard error.
Δ25(OH)D_3: *p*-value of the regression < 0.001, R = 0.69, R^2 = 0.48, adjusted R^2 = 0.43; Δ24,25(OH)$_2D_3$: *p*-value of the regression < 0.001, R = 0.45, R^2 = 0.20, adjusted R^2 = 0.17.

75 nmol/L as recommended cut-off level [16], only half of the vitamin D_3 treated participants in our study attained the required 25(OH)D concentrations. Systematic review data demonstrated that the 25(OH)D concentrations to prevent falls, cancer and respiratory infection should be at least 95 nmol/L, 100 nmol/L, and 95 nmol/L, respectively [3]. We conclude that the currently used vitamin D_3 dosage is capable of attaining the recommendations of IOM and the German Nutrition Society but not to reach levels of ≥75 nmol/L. In line with previous findings [17], extended times of administered vitamin D_3 appear not to improve 25(OH)D_3 levels since changes in serum 25(OH)D_3 concentrations between the weeks 8 and 12 were minimal and non-significant.

Since 25(OH)D_3 response to vitamin D_3 showed a great inter-individual variability (Δ25(OH)D ranging from −13 to 72 nmol/L), we tested whether 24,25(OH)$_2D_3$ could provide a more robust biomarker to assess vitamin D status. 24,25(OH)$_2D_3$ is formed from 25(OH)D_3 and 1,25(OH)$_2D_3$ by the action of CYP24A1 and is proposed to be an inactive vitamin D metabolite that is destined for excretion [18]. Most studies that aimed to investigate the vitamin D status did not analyze 24,25(OH)$_2D_3$. Cashman et al. were the first who demonstrated the response of 24,25(OH)$_2D_3$ to 20 μg vitamin D_3, although modifying factors of 24,25(OH)$_2D_3$ and associations to 25(OH)D were not analyzed [19]. Current data show that 24,25(OH)$_2D_3$ levels highly correlated with the 25(OH)D_3 concentration, and also the magnitude of 24,25(OH)$_2D_3$ increase in response to vitamin D_3 supplementation closely resembles to that of 25(OH)D_3. So far, an association between 24,25(OH)$_2$D and 25(OH)D has only been demonstrated in studies that administered extremely high vitamin D doses of 600,000 IU as bolus [20] or 28,000 IU once per week over 8 weeks [21].

To identify determinants that explain the individual differences in the response to the vitamin D_3 supplementation, we applied a best subset regression model and conducted a subgroup analysis. Among the comprised factors, baseline 25(OH)D_3 concentrations, age, body weight and triglyceride concentrations were identified to affect the efficacy of 25(OH)D_3 response to vitamin D_3 intake. The finding, that changes in 25(OH)D_3 following vitamin D_3 treatment were inversely associated with the baseline levels of 25(OH)D_3 is in

Table 5
Subgroup analysis of vitamin D_3 supplementation response (Δ25(OH)D_3 and Δ24,25(OH)$_2D_3$) according to significant predictors.

	1st tertile	2nd tertile	3rd tertile	One-way ANOVA *p*-value
Δ25(OH)D_3				
Baseline 25(OH)D_3 [nmol/L]	≤31	>31–44	>44	<0.01
Δ25(OH)D_3 [nmol/L]	44 ± 13[a]	35 ± 15[a,b]	25 ± 20[b]	
Age [years]	≤29	>29–45	>45	<0.05
Δ25(OH)D_3 [nmol/L]	43 ± 16[a]	34 ± 17[a,b]	27 ± 18[b]	
Body weight [kg]	≤65	>65–76	>76	0.07
Δ25(OH)D_3 [nmol/L]	42 ± 18	30 ± 18	31 ± 14	
Triglycerides [mmol/L]	≤0.8	>0.8–1.0	>1.0	0.13
Δ25(OH)D_3 [nmol/L]	31 ± 13	32 ± 23	40 ± 15	
Δ24,25(OH)$_2D_3$				
Baseline 24,25(OH)$_2D_3$ [nmol/L]	≤1.3	>1.3–2.1	>2.1	0.05
Δ24,25(OH)$_2D_3$ [nmol/L]	1.8 ± 0.8	1.8 ± 1.1	1.0 ± 1.6	
Age [years]	≤29	>29–45	>45	0.09
Δ24,25(OH)$_2D_3$ [nmol/L]	2.0 ± 1.1	1.4 ± 1.3	1.2 ± 1.2	

Data are given as mean ± SD.
[a,b]Significantly different between subgroups (Bonferroni post hoc test).

accordance with other studies [8–10,12] and is suggested to be caused by a negative feedback of 25-hydroxylase activity [22].

Age was identified as the second important predictor that independently influenced the response of 25(OH)D_3 to vitamin D_3 supplementation. We observed an inverse association between age and changes in 25(OH)D_3 concentration to vitamin D_3. Subjects of the lowest age tertile (≤29 years) appeared to be more responsive to supplemented vitamin D_3 than those of the highest age tertile (>45 years). Most of the previous vitamin D studies that found age as non-modifying factor of the 25(OH)D response addressed only a specific age group [10,23]. A recently published meta-regression found age positively associated with the 25(OH)D_3 response [24]. The reason for the contradictory finding in the meta-regression and our study could be the mean age of the volunteers which were on average 66 years (subgroups: <69, 70–79 and >80 years) in the meta-regression study, and 39 years (subgroups: ≤ 29, 29–45 and >45 years) in the current study.

Body weight which was negatively associated with the 25(OH)D_3 response represented a further predictor of the vitamin D_3 supplementation efficacy. Numerous studies found lower 25(OH)D_3 concentrations in overweight/obese subjects than in lean subjects (e.g. [25,26]), and postulated an inverse relation between BMI and changes of 25(OH)D_3 [8–10]. The authors attribute this observation to the differences in body fat mass, as vitamin D is stored in the adipose tissue and hence be less available for hydroxylation [8,27]. Body fat mass and BMI were also included to our regression model, but both were not associated with the Δ25(OH)D_3. We speculate that total body mass rather than fat mass may modulate the 25(OH)D response. Although serum triglycerides contribute to improve the prediction model for Δ25(OH)D_3 in response to vitamin D_3 treatment, there were no pronounced differences between subgroups. Nevertheless, triglyceride levels should be given more attention in future studies on vitamin D.

Based on the best subset regression analysis, we provided first evidence that the response of 24,25$(OH)_2D_3$ to vitamin D_3 administration was also modified by individual factors, although less than that of 25(OH)D_3. The current study found Δ24,25$(OH)_2D_3$ to be affected by baseline 24,25$(OH)_2D_3$ concentrations and age, but not by body weight, triglycerides or baseline 25(OH)D_3 concentrations as demonstrated for 25(OH)D_3. Thus, serum 24,25$(OH)_2D_3$ could possibly provide a more robust marker of vitamin D status than 25(OH)D_3.

We further analyzed PTH as sensitive marker of serum calcium dysbalance, and found an increase of PTH concentration in placebo-treated subjects from baseline to week 12, but no changes in the group supplemented with vitamin D_3. The absence of the PTH response to vitamin D_3 supplementation was an unexpected result since 80% of the participants in this group showed baseline 25(OH)D_3 levels lower than 50 nmol/L and improved their vitamin D_3 status by vitamin D_3 treatment. Since PTH of young adults is known to respond less pronounced than that of older adults [28], we assume that the young age of individuals included in our study could explain the lack of PTH response.

Another unexpected finding was the decrease of serum calcium from baseline to week 12 in both study groups. We fail to explain this phenomenon, but we exclude calcium intake as a causal factor, as data from the Tromsø study showed no association between intake and serum levels of calcium [29]. Moreover, the current data provide no hint for an impact of vitamin D_3 supplementation on FGF-23 and klotho that are both linked to regulation of phosphate homeostasis [30].

However, data interpretation is somewhat restricted due to some limitations of our study. We neither have analyzed activities of enzymes involved in the conversion of vitamin D metabolites nor genetic polymorphisms of hydroxylating enzymes (CYP27B1, CYP2R1, CYP24A1) and vitamin D binding protein, which are known to modulate the 25(OH)D_3 response to vitamin D_3. The intake of minerals and vitamin D which are determinants of vitamin D status and metabolites were not assessed.

In conclusion, the administration of 20 μg vitamin D_3 per day is suitable to improve deficient or insufficient concentrations of 25(OH)D_3 to at least 50 nmol/L during the winter months. The efficacy to increase 25(OH)D_3 serum levels depends on 25(OH)D_3 levels at baseline, age, body weight and circulating triglycerides. The 24,25$(OH)_2D_3$ concentration highly correlates with the 25(OH)D_3 concentration and appears to be less susceptible to modifying factors. However, further investigations are required to validate 24,25$(OH)_2D_3$ as biomarker of vitamin D status.

Statement of authorship

UL conducted the study, processed the samples, contributed to the analyses of 25(OH)D_3, FGF-23, PTH, 1,25$(OH)_2$D, calcium, glucose and triglycerides, reviewed the literature and drafted the manuscript. AR performed the statistical analyses, reviewed the literature and helped to draft the manuscript. FH analyzed 25(OH)D_3, FGF-23 and PTH. CB analyzed klotho, and contributed to measurement and evaluation of plasma parameters and anthropometric data. MG participated in the design of the study. CU conducted blood sampling, controlled the quality of blood analyses and helped to draft the manuscript. ES performed data analysis. CH analyzed 24,25$(OH)_2D_3$. MAG established the method and quality control of 24,25$(OH)_2D_3$. JD participated in the design of the study. GIS conceived the study, prepared the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2015.04.021.

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3.3. Study 3

Efficacy of vitamin D-fortified rainbow trout on vitamin D status

3.3.1. Study Design

The study was conducted as a double blind, randomized, parallel study during November and December, when virtually no UVB irradiation is measurable in Halle and surroundings. Study visits were scheduled at baseline and after 4 weeks. Subjects were block randomized (stratified for body-mass index, sex and 25(OH)D_3-concentrations in serum as obtained during a screening visit 6-8 weeks prior to baseline) to receive either vitamin D_3- fortified or conventional fish. Fillets of trout's with skin were used for the study. The participants received their portion of rainbow trout (100g) six times per week at lunchtime to verify their compliance. The time of consumption was not specified. During each study visit, a venous blood sample was taken for analysis of 25(OH)D_3, PTH, serum calcium, total cholesterol, HDL and LDL cholesterol after a fasting period of at least 4 hours.

The study protocol was evaluated and approved by the ethic committee of the Medical Faculty at the Martin-Luther-University Halle-Wittenberg and each participant gave written, informed consent prior to the start of the study. The study was registered at clinicaltrails.gov, (NCT01696526).

3.3.2. Subjects

Participants were recruited among students and employees (only those who are unrelated to Nutritional science) on the campus through personal contacts and leaflets information in the library, cafeteria or hallways of the University. During a screening in autumn (about 1 months before the study start), the participants filled in a self-administered questionnaire on disease history, weight and height, lifestyle-behaviors (smoking, use of cosmetics containing sunblocker) and dietary habits concerning ingestion of food rich in vitamin D. Exclusion criteria were pregnancy and lactation, use of vitamin D supplements, vacation in regions with UVB light during an interval of 8 weeks before and during the study, subjects with known renal diseases or malignant diseases, participation in other clinical studies. Participants with elevated creatinine (in females ≥1.1 mg/dl, in males ≥ 1.3 mg/dl) were excluded. People who were already

well supplied with vitamin D (25-(OH)D_3-concentrations > 75 nmol/l) were excluded from study participation (n = 7). Five volunteers resigned from participation for personal reasons. In total, 56 subjects were recruited for the intervention study. Due to personal reasons, 3 participants dropped out during the study period. Finally, 53 subjects (age range 20 – 63 years) were included into the study. Characteristics of subjects are provided in Table 5.

3.3.3. Production of bio-fortified fish

The conventional and improved fish were provided and portioned by Forellenhof Thiessen (Coswig, Germany). The enrichment of the fillets with vitamin D_3 via UVB-radiation was held in Institute of Inland Fisheries (Potsdam-Sacrow, Germany). Fillets were irradiated with UVB light (G8 T5E UV-B, Sanyo Denki Germany GmbH, Eschborn, Germany) in vitro for 6 hours on ice. The irradiation intensity was measured in the medium at about 2500 mW/m^2 with distance of 27 cm from the light source. Bio-fortified fish and conventional fish were of identical appearance and indistinguishable from each other.

3.3.4. Methods

Blood samples for analysis were taken at the beginning and at the end of the study and collected in serum- and EDTA-tubes (Becton Dickinson, Heidelberg, Germany). Serum samples were centrifuged at 2000 g for 10 minutes at room temperature. EDTA samples were stored on ice till centrifugation at 2000 g for 10 minutes at 4° C. The samples were separated into aliquots and frozen at -80° C until the time of analysis. Serum concentrations of 25(OH)D_3 were determined by using a liquid chromatography tandem mass spectrometry (LC-MS/MS), MassChrom®25-OH Vitamin D_3 (Chromsystems GmbH, Munich, Germany), on a API 2000 (Applied Biosystems, Carlsbad, CA). The coefficient of variation for 25(OH)D_3 measurements was 6.5%, and the lower level of detection was 2.5 nmol/l. Serum creatinine was determined spectrophotometrically (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). Total cholesterol, HDL cholesterol and LDL cholesterol were quantified spectrophotometrically (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). Serum concentrations of parathyroid hormone (PTH) were measured using a

commercial ELISA Kit (IBL International GmbH, Hamburg, Germany). All measurements were made in duplicate.

3.3.5. Analysis of cholecalciferol concentration in fillet and skin of rainbow trout

Cholecalciferol in rainbow trout was determined by LC-MS/MS according to [Schutkowski et al., 2013] and [Higashi et al., 2008]. Samples were homogenized, mixed with deuterated internal standard (D_3-d_3, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and hydrolyzed under exclusion of oxygen. After extraction with n-hexane, hexane phase was washed with ultrapure water. Samples were fractionated by HPLC (Agilent 1100 HPLC, Agilent Technologies) and derivatized with 4-phenyl-1,2,4-triazolin-3,5-dione (solved in acetonitrile) according to [Mattila et al., 1995]. Ethanol and mobile phase were added to the dried residue and then analyzed by HPLC coupled to a MS system.

3.3.6. Statistical analysis

Statistical analysis was performed with SPSS, version 20.0 (Chicago, IL, USA). All data are expressed as mean ± SD, and a p-level of <0.05 was regarded as significant. The primary outcome variables were 25(OH)D_3-concentrations which were compared between groups at baseline and after 4 weeks by Student`s t-Test. Additionionally,the change in these parameters within groups after 4 weeks to baseline were compared by paired t-test. The statistical power calculation revealed that 22 subjects per group would be required to show a difference of 15 nmol/l in the mean 25(OH)D_3-concentration after 4 weeks of fish consumption between the control und intervention group, (at an assumed standard variation of 15 nmol/l for each group, at a power of 80% and a significance level of 0.05). Only subjects who finished the study according to protocol were included into the analyses.

3.3.7. Results

The baseline characteristics were shown in Table 5. The study population consisted of 24 males and 29 females who were randomly assigned to either intervention (n=26) or control group (n=27). The study groups did not differ in body mass index (BMI) and age. On average, the participants were 29±9.4 years and had a BMI of 23.2±3.0

(kg/m^2). Furthermore no significant differences between both groups were demonstrated in serum calcium, PTH, $25(OH)D_3$, total cholesterol, LDL and HDL cholesterol.

Table 5: Baseline characteristics of the study population

	Intervention group	Control group	p-value
Sex (male/female)	13 / 13	11 / 16	0.508
Age [years]	30.3 ± 11.5	27.7 ± 6.8	0.325
BMI [kg/m^2]	23.1 ± 3.0	23.2 ± 3.0	0.912
25(OH)D_3 [mmol/l]	44.8 ± 14.0	43.0 ± 14.5	0.651
Calcium [nmol/l]	2.3 ± 0.1	2.3 ± 0.1	0.823
Total cholesterol [mmol/l]	4.5 ± 0.6	4.2 ± 0.6	0.090
LDL cholesterol [mmol/l]	2.6 ± 0.6	2.5 ± 0.6	0.477
HDL cholesterol [mmol/l]	1.5 ± 0.4	1.4 ± 0.4	0.394
PTH [pg/ml]	66.5 ± 27.2	66.1 ± 30.0	0.877

Student's t-test was used to compare intervention and control group at baseline

25-$(OH)D_3$-concentrations

At baseline, 25-$(OH)D_3$-concentrations did not differ between control and intervention group (44.8±14.0 vs. 43.0±14.5 nmol/l, p=0.651; respectively). In both groups, the $25(OH)D_3$-concentrations decreased during the study. After four weeks $25(OH)D_3$-concentrations were significantly higher in the intervention compared to control group (42.0±12.2 vs. 33.9±10.6 nmol/l, *p=0.013*; respectively). The $25(OH)D_3$-concentrations after four weeks was significantly different from baseline in both groups (Figure 2). Decrease of $25(OH)D_3$-concentration between baseline and 4 weeks showed a significant difference within the groups (control: -9.1±9.1; intervention: -2.7±6.9; *p=0.004*), with a smaller decline in the intervention group.

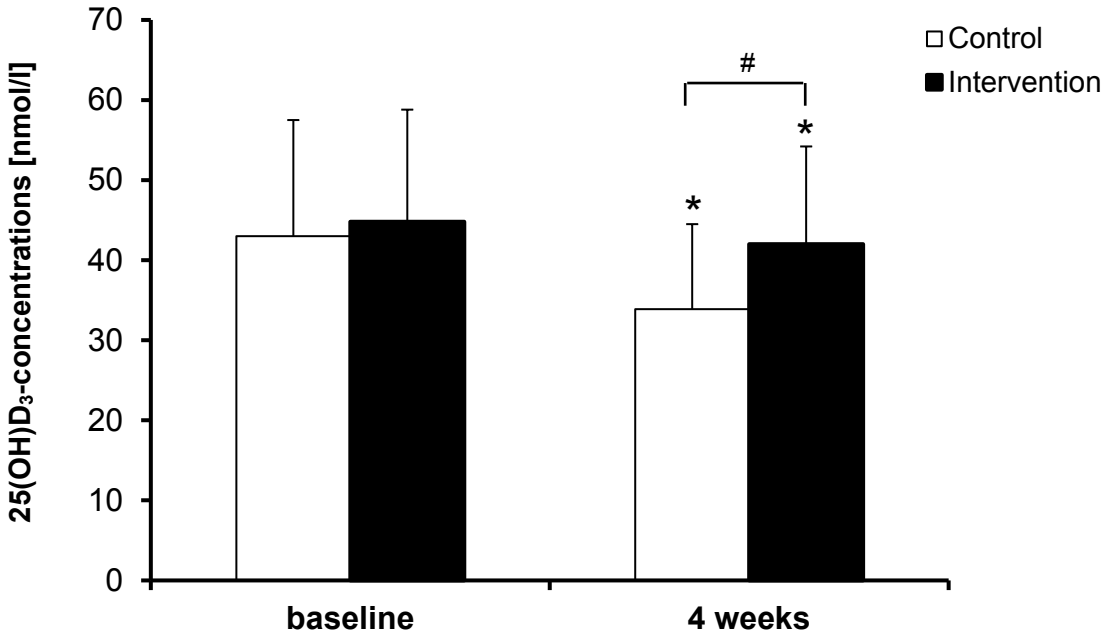

Figure 2: 25(OH)D_3 concentrations [nmol/l] in the control and intervention group at baseline and after four weeks.
A paired *t*-test was used to compare baseline and four weeks values.
Student's t-test was used to compare groups at baseline and four weeks
* significant different from baseline
# significant different between groups

Concentrations of total cholesterol, HDL and LDL cholesterol

At baseline, neither total cholesterol nor HDL or LDL cholesterol was different between control and intervention group. After four weeks, total cholesterol was significantly increased in both groups (*p ≤0.01*), but showed no differences between control and intervention group (4.65±0.57 vs. 4.90±0.82 mmol/l, p=0.212; respectively). Furthermore, HDL cholesterol did not differ between groups after 4 weeks (control: 1.71±0.55, intervention: 1.77±0.54 mmol/l, p=0.713), but was increased significantly in both groups compared to baseline concentrations (*p≤0.01*). After 4 weeks LDL cholesterol did not differ between control and intervention group (2.56±0.60 vs. 2.69±0.76 mmol/l, p=0.657). In addition, LDL concentrations remained unchanged compared to baseline concentrations in both groups (control: p=0.420, intervention: p=0.243).

Concentrations of calcium and parathyroid hormone

Serum calcium concentrations did not show differences between control and intervention group after four weeks at second study visit (2.36±0.12, 2.32±0.15 nmol/l, p=0.823; respectively) and remained unchanged compared to baseline concentrations

in both groups (control: p=0.141, intervention: p=0.546). Intact parathyroid hormone did not differ between groups after 4 weeks (p=0.945). Furthermore it did not change significantly within four weeks neither control nor intervention group (p=0.780, p=0.793; respectively).

3.4. Study 4

Lehmann, U., Gjessing, H.R., Hirche, F., Mueller-Belecke, A., Gudbrandsen, O.A., Ueland, P.M., Mellgran G., Laurizen L., Lindqvist H., Hansen A.L., Erkkilä, A.T., Pot G.K., Stangl G.I., & Dierkes J. (2015). Efficacy of fish intake on vitamin D status: a meta-analysis of randomized controlled trials. *The American journal of clinical nutrition*, *102*(4), 837-847.

Efficacy of fish intake on vitamin D status: a meta-analysis of randomized controlled trials[1,2]

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ABSTRACT

Background: It is well known that fish is the major natural source of vitamin D in the diet; therefore, this meta-analysis investigated the influence of fish consumption in randomized controlled trials (RCTs) on serum 25-hydroxyvitamin D [25(OH)D] concentrations.

Objective: A literature search was carried out in Medline, Embase, Web of Science, and the Cochrane Library (up to February 2014) for RCTs that investigated the effect of fish consumption on 25(OH)D concentrations in comparison to other dietary interventions.

Results: Seven articles and 2 unpublished study data sets with 640 subjects and 14 study groups met the inclusion criteria and were included in this meta-analysis. Compared with controls, the consumption of fish increased 25(OH)D concentrations, on average, by 4.4 nmol/L (95% CI: 1.7, 7.1 nmol/L; $P < 0.0001$, $I^2 = 25\%$; 9 studies).The type of the fish also played a key role: the consumption of fatty fish resulted in a mean difference of 6.8 nmol/L (95% CI: 3.7, 9.9 nmol/L; $P < 0.0001$, $I^2 = 0\%$; 7 study groups), whereas for lean fish the mean difference was 1.9 nmol/L (95% CI: −2.3, 6.0 nmol/L; $P < 0.38$, $I^2 = 37\%$; 7 study groups). Short-term studies (4–8 wk) showed a mean difference of 3.8 nmol/L (95% CI: 0.6, 6.9 nmol/L; $P < 0.02$, $I^2 = 38\%$; 10 study groups), whereas in long-term studies (∼6 mo) the mean difference was 8.3 nmol/L (95% CI: 2.1, 14.5 nmol/L; $P < 0.009$, $I^2 = 0\%$; 4 study groups).

Conclusion: As the major food source of vitamin D, fish consumption increases concentrations of 25(OH)D, although recommended fish intakes cannot optimize vitamin D status. *Am J Clin Nutr* 2015;102:837–47.

Keywords: fish intake, meta-analysis, randomized controlled trial, vitamin D, intervention studies, 25(OH)D, vitamin D status

INTRODUCTION

Vitamin D deficiency is a global problem and is associated with an increased risk of cardiovascular diseases (1–4), autoimmune diseases (5), type 1 diabetes (6, 7), osteoporosis (6), and probably various types of cancer (8–10). Although vitamin D is synthesized in the skin on exposure to UV-B radiation, it is not possible to maintain an adequate vitamin D status during winter at high latitudes when UV-B radiation is absent (11). Fish, egg yolk, cheese, and mushrooms are the only dietary sources that contain natural vitamin D (12). Among these, fish has, in general, the highest content of vitamin D (12, 13) and is the major natural food source in many populations within (14–17) and outside of (18, 19) Europe. Other significant food sources are fortified items such as margarine, skimmed milk, and orange juice (20, 21). Although, in general, fish is a good source of vitamin D, there are considerable differences in vitamin D content between different fish species (13, 22). Other important factors are environmental conditions, such as season, and the fat content of the fish (13), but more research is needed in this area.

In observational studies (23, 24) fish consumption was shown to have a beneficial effect on cardiovascular morbidity and mortality, although it must be considered that these health effects could also be due to other constituents present in fish, such as long-chain n–3 PUFAs, amino acids, iodine, or selenium, in addition to vitamin D. The effects of short- to medium-term fish interventions on PUFAs (25–28, 30), blood lipids (25–27, 29–34), vitamin B-12 and selenium status (28, 35), insulin and leptin concentrations (29), eicosanoids and adhesion molecule concentrations (36), heart rate variability (25, 34), and vitamin D status have been investigated in several randomized controlled trials (RCTs)[13] (25–28, 34, 35, 37), but systematic studies of the

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[2] Supplemental Text and Supplemental Tables 1–3 are available from the "Supplemental data" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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[13] Abbreviations used: LC-MS/MS, liquid chromatography–tandem mass spectrometry; RCT, randomized controlled trial; 25(OH)D, 25-hydroxyvitamin D; $25(OH)D_3$, 25-hydroxycholecalciferol.

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extent to which fish consumption may contribute to dietary status or to biomarkers for nutrient uptake are lacking. To the best of our knowledge, the effect of fish consumption on vitamin D status has not been investigated systematically. Because increased vitamin D intake due to regular fish consumption may be one explanation for the beneficial health effects of fish, the aim of this study was to conduct a meta-analysis of RCTs on the effect of fish consumption on serum 25-hydroxyvitamin D [25(OH)D] concentrations as the outcome.

METHODS

Search strategies and data collection

To identify relevant studies, Medline, Embase, Web of Science, and the Cochrane Library databases were searched between January 1950 and 12 February 2014. The following search terms were used: vitamin D, cholecalciferol, ergocalciferol, hydroxycholecalciferols, dihydroxycholecalciferol, calcitriol, 24,25-dihydroxyvitamin D, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, calcitriol or calcidiol, fishes, seafood, shellfish, clinical trial, and random trial or parallel trial (as shown in **Supplemental Table 1**). Additional studies were identified by manual searches through references or the clinicaltrials.gov database. The search was restricted to studies published in English.

The studies were assessed by 2 independent investigators (UL and JD), taking the inclusion criteria into account. Data on the primary patients were collected by personal communication with the relevant investigators by e-mail. Standard data files were provided for this purpose. Investigators who agreed to collaborate were asked to provide data for each participant, including the measured serum 25(OH)D concentration, the definition of the fish consumption group, age and sex, BMI, and the season in which blood samples were taken.

Study eligibility criteria

Any randomized intervention trial that involved human adults and investigated the effects of fish meals on serum 25(OH)D concentrations was included in the analysis. We excluded studies that used only a food-frequency questionnaire to calculate fish intake and studies with only 1 fish meal/wk as an intervention (38). In addition to studies in healthy participants, those that included patients who had survived a myocardial infarction or overweight subjects were also included in the meta-analysis. Studies that involved children, adolescents, or pregnant or breastfeeding women were excluded. Differences between the extracted studies in daily fish intake, the frequency of meals containing fish, or study duration were not a cause for exclusion.

Data collection

The quality of the included studies was checked manually by careful examination of the original publications. Several studies did not originally intend to evaluate the effect of fish consumption on vitamin D status, and therefore most studies did not adequately report the methods of 25(OH)D measurement or the season of blood collection. Because this meta-analysis was concerned with the effect of real food, the issue of blinding was not applicable to the participants. Indeed, only 1 study (U Lehmann, unpublished data, 2012) was sufficiently blinded to participants, as expected in studies investigating natural food. In most studies, meat was used as the comparator or no food was provided to the participants in the control group. In 2 studies, fish with a low vitamin D content was used as the control intervention (27; U Lehmann, unpublished data, 2012). The accepted quality-control measures, such as the Jadad scale (43) or the CONSORT(Consolidated Standards of Reporting Trials) statement (44), were therefore not appropriate for estimating study quality. The quality of the studies was instead assessed on the basis of compliance, number of dropouts, measurements of the vitamin D content in the fish, season of the intervention, the type of vitamin D analysis, and the type of randomization. One score point was given for each item of information included. Scores of 5–6 denote good quality, 3–4 indicate moderate quality, and 0–2 points denote low quality.

Analysis of the data

Studies were analyzed by using RevMan 5.2, which was provided by the Cochrane Collaboration. After consultation with the relevant authors, we received individual patient data from 6 trials (26–28, 35; U Lehmann, unpublished data, 2012; OA Gudbrandsen, unpublished data, 2014). For each study we recorded the number of subjects and mean (SD) baseline and postintervention 25(OH)D concentrations separately for controls and for the intervention group. The mean change in 25(OH)D was calculated by subtracting the mean baseline 25(OH)D concentration from the mean postintervention 25(OH)D concentration. For calculation of the SD of the change in 25(OH)D we applied a correlation coefficient of 0.82 in the control group and a correlation coefficient of 0.77 in the intervention group. These correlation coefficients were calculated from studies with access to individual data ($n = 6$), according to the Cochrane Handbook (39).

Studies that included >1 intervention group (26–28, 37; OA Gudbrandsen, unpublished data, 2014) were treated by dividing the number of subjects in the control group by the number of comparisons while retaining the mean and SD of the change according to the Cochrane Handbook (40).

The changes in 25(OH)D concentrations were calculated as weighted mean differences with 95% CIs. Statistical heterogeneity between the studies was tested by using the Cochrane Q-test (41). A random-effects model was applied. Publication bias was assessed by a funnel plot (**Figure 1**) (42). In addition to the main analysis, we conducted several sensitivity analyses taking into account study duration, type of fish, mean baseline 25(OH)D concentrations, season of blood collection, access to individual data or calculated data, participants' health status, the amount of fish consumed during the trial, measurements of total 25(OH)D or 25-hydroxycholecalciferol [25(OH)D_3], the determination of the vitamin D content in the fish, and the method of determination of 25(OH)D concentrations [ELISA/radioimmunoassay or liquid chromatography–tandem mass spectrometry (LC-MS/MS)]. Two studies (27; U Lehmann, unpublished data, 2012) compared fish with different vitamin D contents. These 2 fish interventions were compared in an additional separate analysis.

Included studies

In addition to published studies, we included 2 unpublished RCTs involving fish consumption in healthy adults. One of these was conducted at the University of Bergen in Norway and the

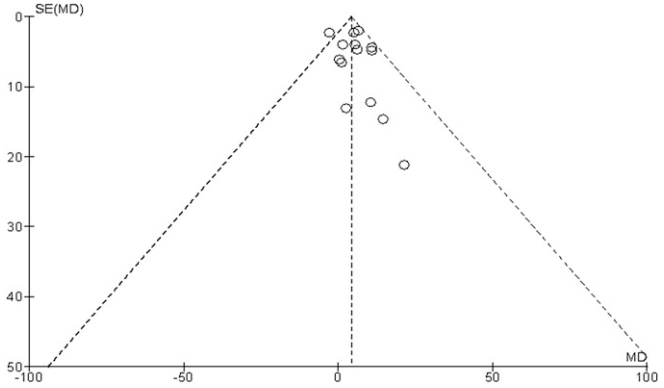

FIGURE 1 Funnel plot with pseudo 95% CIs for the effect of fish intake on serum 25-hydroxyvitamin D concentrations. MD, mean difference.

other at the Martin Luther University of Halle-Wittenberg in Germany. These studies are described briefly below and in greater detail in the **Supplemental Text**.

Lehmann study

The study in Halle (Saale) at the Martin Luther University of Halle-Wittenberg (latitude 51° north) was conducted during the late autumn of 2012. The major aim was to compare the effect of vitamin D–enriched rainbow trout on 25(OH)D_3 concentrations in comparison with conventional rainbow trout in healthy volunteers (n = 53) over a 4-wk period. The participants consumed 6 times/wk 100-g portions of rainbow trout enriched with vitamin D by postmortem irradiation with UV-B or 100-g portions of conventional, untreated rainbow trout fillets. Consumption was usually at lunchtime and was supervised on weekdays. Both participants and investigators were blinded to the type of trout. Blood samples were collected at baseline and after 4 wk for determination of 25(OH)D_3 concentrations by LC-MS/MS (MassChrom 25-OH Vitamin D_3 reagent kit for LC-MS/MS analysis; Chromsystems GmbH) on an API2000 LC-MS/MS system (Applied Biosystems), as described elsewhere (51). Characteristics of participants are provided in **Supplemental Table 2**.

Gudbrandsen study

This was a randomized controlled intervention study with a parallel design and 3 intervention arms: cod, salmon, or chicken in weekly doses of 750 g (5 meals of 150 g)/wk for 4 wk, with study visits at baseline and after 4 wk. The study included 57 participants recruited in Bergen, Norway, and randomly assigned to the intervention groups. Because of the reduced number of blood samples (n = 5 with missing data) and dropouts (n = 3), samples for the 25(OH)D analyses were only available for 19, 18, and 12 participants, respectively. Fasting blood samples were collected at baseline and after 4 wk, and 25(OH)D was determined in serum by LC-MS/MS according to methods of Midttun and Ueland (45). Characteristics of participants are provided in **Supplemental Table 3**.

RESULTS

A systematic search of the literature led to the identification of 3277 possibly relevant articles (**Figure 2**). A first examination identified 61 studies as appropriate to be included in the analysis by reviewing titles and abstracts. After detailed consideration, 54 studies were rejected from the analysis, because they did not measure 25(OH)D as an outcome, were not RCTs, gave no detailed information on amount of fish, or were duplicates of included studies. In total, 7 published and 2 unpublished studies that fulfilled the inclusion criteria were included in the present meta-analysis investigating the effect of fish intake on serum 25(OH)D concentrations.

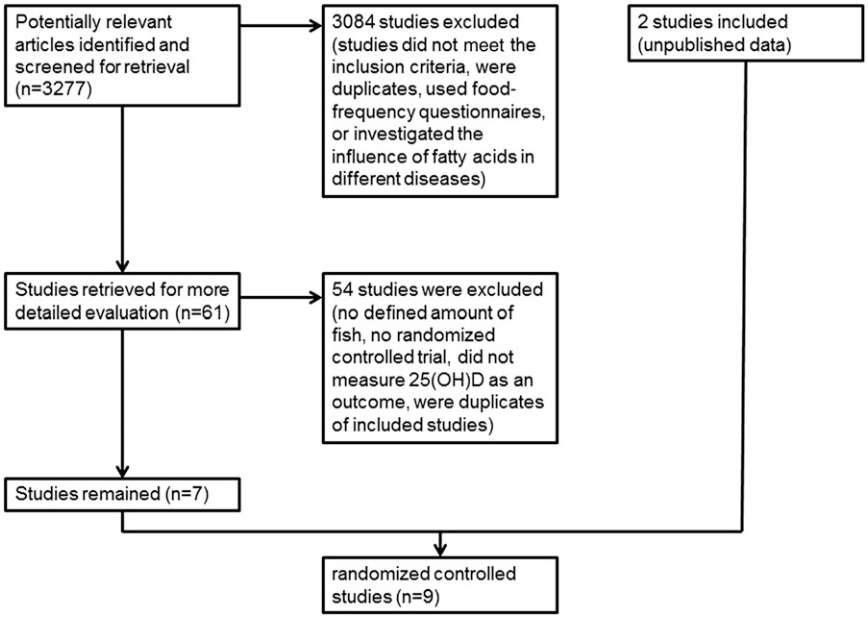

FIGURE 2 Flow diagram for the selection of studies of the effects of fish intake on serum 25(OH)D concentrations in the present meta-analysis, indicating numbers of articles reviewed and later excluded or included for the meta-analysis. 25(OH)D, 25-hydroxyvitamin D.

TABLE 1
Characteristics of the randomized controlled trials included in the present meta-analysis[1]

Authors (ref)	Year	Country	Intervention, dose, and frequency[2]	Included in analysis, *n*	Sex and age	Follow-up	Baseline 25(OH)D (fish group), nmol/L
Erkkilä et al. (26)[3]	2008	Finland	*1*) Control (lean meat or chicken) [10]	33	27 men, 6 women; 61.0 ± 5.8 y	8 wk	—
			2) Lean fish (400–600 g/wk) [11]				96 ± 30[4]
			3) Fatty fish (400–600 g/wk) [12]				124 ± 43
Lucey et al. (37)[3]	2008	Iceland, Ireland, Spain	*1*) Control (sunflower oil, 3 g/d) [66]	210	92 men, 118 women; 20–40 y	8 wk	—
			2) Cod (450 g/wk) [70]				59.0 ± 22.1
			3) Salmon (450 g/wk) [74]				61.9 ± 33.8
Pot et al. (28)[3]	2009	United Kingdom, Netherlands	*1*) Control (dietary advice) [23]	74	37 men, 37 women; 18–79 y	6 mo	—
			2) Cod (300 g/wk) [22]				71.6 ± 30
			3) Salmon (300 g/wk) [29]				71.7 ± 26.5
Hallund et al. (27)[3]	2010	Denmark	*1*) Control (chicken, 1050 g/wk) [22]	68	All men; 40–70 y	8 wk	—
			2) Trout raised on marine feed (1050 g/wk) [23]				45.9 ± 20.9
			3) Trout raised on vegetable-based feed (1050 g/wk) [23]				48.1 ± 22.1
Hansen et al. (34)	2010	Norway	*1*) Control (alternative dinner) [14]	29	All men; 20–60 y	23 wk	
			2) Seafood, mainly salmon (600 g/wk) [15]				48 ± 15
OA Gudbrandsen (unpublished data, 2014)[3]	2013	Norway	*1*) Chicken (750 g/wk) [12]	49	16 men, 33 women; 20–36 y	4 wk	—
			2) Cod (750 g/wk) [18]				66.5 ± 17.8
			3) Salmon (750 g/wk) [19]				77.0 ± 23.1
U Lehmann (unpublished data, 2012)	2013	Germany	*1*) Control (common rainbow trout) (600 g/wk) [27]	53	24 men, 29 women; 20–63 y	4 wk	
			2) Vitamin D–enriched rainbow trout (600 g/wk) [26]				44.8 ± 14
Scheers et al. (35)	2013	Sweden	*1*) Control (650 g pork or 750 g chicken/wk) [21]	21	All men; 35–60 y	2 × 6 wk (crossover)	
			2) Herring (750 g/wk) [21]				66.9 ± 22.1
Hansen et al. (25)	2014	Norway	*1*) Control (3 times meat/wk) [42]	82	All men; 18–61 y	6 mo	
			2) Salmon (900 g/wk over 5 mo, 450 g/wk during the past 4 wk) [40]				85 ± 36

[1]ref, reference; 25(OH)D, 25-hydroxyvitamin D.
[2]*n* in brackets.
[3]These studies included 2 intervention groups (fatty compared with lean fish).
[4]Mean ± SD (all such values).

TABLE 2
Quality evaluation of the 9 randomized controlled studies included in the meta-analysis[1]

Authors, year (ref)	Compliance	Dropouts, *n* (%)	Measured vitamin D content in fish	Intervention period	Analytic method for 25(OH)D	Randomization	Participants	Quality score[2]
Erkkilä et al., 2008 (26)	Checked by self-report and serum fatty acid composition	2 (5.7)	No data	Spring, August–September, October	ELISA	Stratified by sex	Survivors of myocardial infarction	4
Lucey et al., 2008 (37)	86%	34 (13.9)	Data cited but not measured (9.6 μg/100 g)	October–May	ELISA	No information	Overweight; consuming a low-calorie diet	4
Pot et al., 2009 (28)	Serum n–3 very-long-chain PUFAs	22 missing blood samples	No information	November 2004–July 2007	ELISA	Randomization in blocks (n = 6)	Only participants with healthy colon included	5
Hallund et al., 2010 (27)	99% evaluated in study diaries	7 (9.3)	Measured trout raised on marine feed: 0.62 μg/100 g; trout raised on vegetarian feed: <0.1 μg/100 g	Spring or autumn	Chemiluminescence immunoassay	Randomization without taking baseline characteristics into account	Healthy volunteers	6
Hansen et al., 2010 (34)	No information	24 (45.3)	No data	April–November	Radioimmunoassay	No information	Prisoners	3
OA Gudbrandsen (unpublished data, 2014)	Checked by self-report	8 (14)	No data	Winter	LC-MS/MS	Consideration of sex, BMI, and age	Healthy volunteers	4
U Lehmann (unpublished data, 2012)	93%	4 (7)	2.8 μg/100 g in intervention trout; 0.6 μg/100 g in control trout	November–December	LC-MS/MS	Randomization in blocks (n = 2); consideration of sex, BMI, and 25(OH)D_3 concentrations at screening	Healthy volunteers	6
Scheers et al., 2013 (35)	Checked by self-report (24-h recall) and fatty acid concentration in blood	n = 5 dropouts; n = 19 for this analysis who were missing blood samples for 25(OH)D (19.2)	8.5 μg/100 g vitamin D in herring	April–June, September–November	HPLC	No randomization because crossover study	Healthy, overweight	6
Hansen et al., 2014 (25)	No information	11 (11.8)	15 μg/300 g in salmon but no source added	September – February	Chemiluminescence immunoassay	No information	Sex offenders	3

[1]The quality score was based on assessment of compliance, number of dropouts, measurements of vitamin D content in fish, season of intervention, type of vitamin D analysis, and type of randomization. One score point was given for each item of information included. LC-MS/MS, liquid chromatography–tandem mass spectrometry; ref, reference; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D_3, 25-hydroxycholecalciferol.

[2]Scores of study quality: 5–6 denotes good quality, 3–4 indicates moderate quality, and 0–2 points denotes low quality.

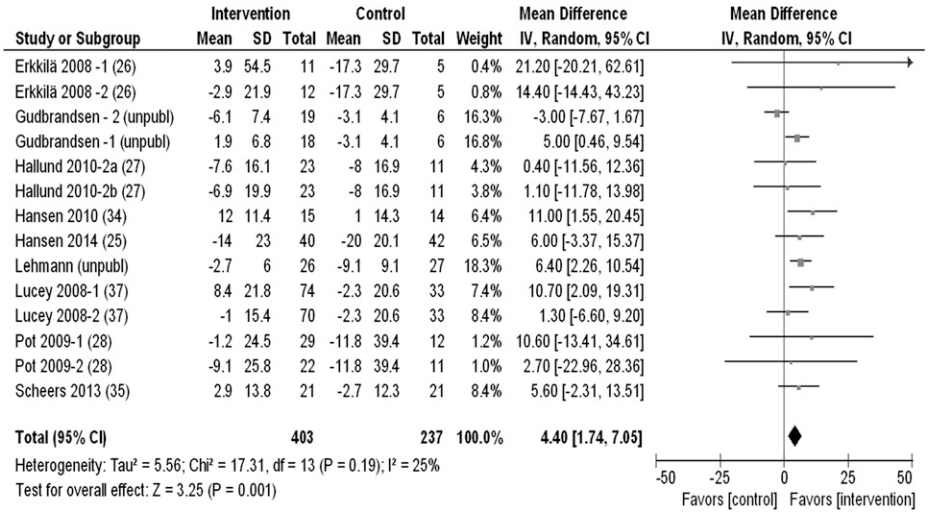

FIGURE 3 Random-effects meta-analysis comparing the effects of fish intervention with the control food on the 25(OH)D concentrations (in nmol/L). The forest plot indicates a significant effect of the absolute change in 25(OH)D concentrations compared with the control food by fish intervention. "Total" denotes the cumulative *n* from all of the included studies. IV, inverse variance; unpubl, unpublished; 25(OH)D, 25-hydroxyvitamin D; −1, study groups who received fatty fish; −2, study groups who received lean fish.

Study characteristics

The 9 studies considered comprised 619 participants [640 participants were included in the meta-analysis on account of 1 crossover study (34)] aged between 18 and 79 y. Descriptive study information is shown in **Table 1**. Sixty-four percent of the study population were men (n = 396) and 36% were women (n = 223). The studies were conducted in Finland, Iceland, Ireland, Spain, the United Kingdom, Netherlands, Denmark, Norway, the United States, Germany, and Sweden. Two studies (28, 37) were multicenter studies. Most of the participants were white, although a number of studies did not specify this explicitly. The change in 25(OH)D concentration served as the primary outcome in only 1 case (U Lehmann, unpublished data, 2012), whereas in the other studies the 25(OH)D concentration was measured as a secondary outcome (25–28, 34, 35) or was measured post hoc (OA Gudbrandsen, unpublished data, 2014).

The interventions differed between the studies in dosage, time, and fish species. We included 3 long-term studies with an intervention period of 6 mo or 23 wk (25, 28, 34). Six studies (26, 27, 35, 37; OA Gudbrandsen, unpublished data, 2014; U Lehmann, unpublished data, 2012) investigated the influence of short-term fish intake (4–8 wk) on 25(OH)D concentrations. The planned amount of fish to be consumed varied from 300 to 1050 g/wk. In 6 studies the weekly fish intake was planned to be between 300 and 600 g (25, 26, 28, 34, 37; U Lehmann, unpublished data, 2012), whereas in 3 studies (27, 35; OA Gudbrandsen, unpublished data, 2014) the intake was between 750 and 1050 g. The selected fish species differed between studies. The intake of fatty fish (salmon, herring) was investigated in 3 studies (25, 34, 35), whereas 4 studies compared both fatty and lean fish (cod, rainbow trout; 26, 28, 37; OA Gudbrandsen, unpublished data, 2014). One study included rainbow trout in the fatty fish group (26), and 2 studies investigated rainbow trout that differed in either the feeding regimen (27) or in postmortem treatment (U Lehmann, unpublished data, 2012). Total serum 25(OH)D concentrations measured by ELISA/immunoassay were reported in 6 studies (25–28, 34, 37), whereas in 3 studies serum 25(OH)D_3 concentrations were measured by chromatographic methods (35; U Lehmann, unpublished data, 2012; OA Gudbrandsen, unpublished data, 2014).

All of the studies were designed as RCTs and included a control group. Because of the visible differences between the meals, 8 studies were not blinded. Only 1 study (U Lehmann, unpublished data, 2012) was double-blinded. Details of the randomization scheme and criteria were reported in 4 cases (27, 28; OA Gudbrandsen, unpublished data, 2014; U Lehmann, unpublished data, 2012), and 1 study had a crossover design (35). All of the studies provided general information on season of the intervention period, but an exact timing (month or season) of the blood collection procedures was usually not possible. Exact compliance rates were reported in only 3 studies (27, 37; U Lehmann, unpublished data, 2012), but the drop-out rates were given in all studies (25–28, 34, 35, 37; OA Gudbrandsen, unpublished data, 2014; U Lehmann, unpublished data, 2012).

Individual data were available for 6 of the 9 studies [298 participants, although 319 individual data are included because of 1 crossover study (35)] to calculate the change in 25(OH)D concentrations (26–28, 35; OA Gudbrandsen, unpublished data, 2014; U Lehmann, unpublished data, 2012), whereas the mean (SD) 25(OH)D concentrations at baseline and during follow-up in the study groups were available in 3 studies (25, 34, 37). The vitamin D_3 concentration in the fish was reported in 3 studies (27, 35; U Lehmann, unpublished results) but was explicitly measured only by Hallund et al. (27) and Lehmann (unpublished data, 2012). Average fish vitamin D_3 concentrations were cited by 2 studies (25, 37).

Results of the study quality assessment are presented in **Table 2**. The quality score was high in 5 studies (27, 28, 35; OA Gudbrandsen, unpublished data, 2014; U Lehmann, unpublished data, 2012) and moderate in 4 studies (25, 26, 34, 37). None of the studies had a low score.

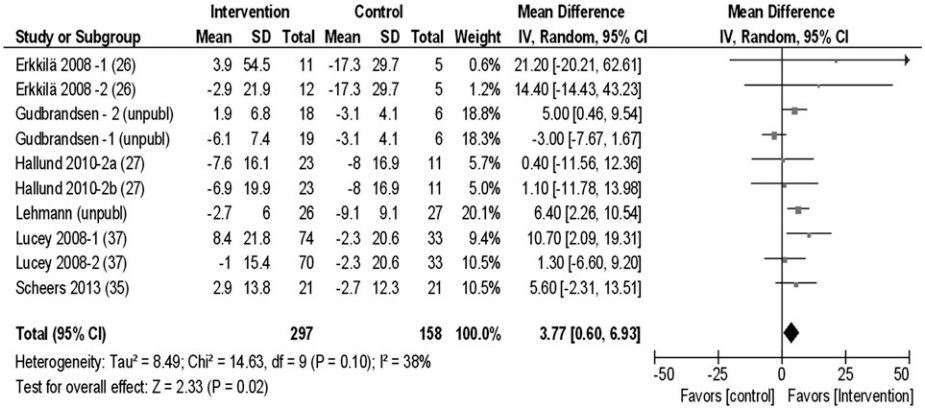

FIGURE 4 Random-effects meta-analysis comparing the effects of short-term (4–8 wk; 10 study groups) fish intervention with the control food on 25(OH)D concentrations (in nmol/L). The forest plot indicates a significant effect of the absolute change in 25(OH)D concentrations compared with the control food by fish intervention. "Total" denotes the cumulative *n* from all of the included studies. IV, inverse variance; unpubl, unpublished; 25(OH)D, 25-hydroxyvitamin D; −1, study groups who received fatty fish; −2, study groups who received lean fish.

Meta-analysis

Comparison of the changes in 25(OH)D concentrations between the fish intervention and the control groups including all studies (n = 9; 14 study groups) resulted in a weighted mean difference of 4.4 nmol/L (95% CI: 1.7, 7.1 nmol/L; $P < 0.001$, $I^2 = 25\%$) (**Figure 3**).

The effect of the fish intervention varied depending on the study duration. Short-term studies (4–8 wk) showed a mean difference of 3.8 nmol/L (95% CI: 0.6, 6.9 nmol/L; $P < 0.02$, $I^2 = 38\%$), whereas long-term studies (~6 mo) showed a mean difference of 8.3 nmol/L (95% CI: 2.1, 14.5 nmol/L; $P < 0.009$, $I^2 = 0\%$) (**Figures 4** and **5**).

The type of fish also had an effect. Fatty fish (salmon, herring; n = 7 study groups) resulted in a mean difference of 6.8 nmol/L (95% CI: 3.7, 9.9 nmol/L; $P < 0.0001$, $I^2 = 0\%$), whereas studies that used lean fish (trout, cod; n = 6 study groups) showed a mean difference of −1.1 nmol/L (95% CI: −4.7, 2.5 nmol/L; $P < 0.55$, $I^2 = 0\%$). When the unpublished study by Lehmann was included (n = 7 study groups), which used lean fish that were biofortified with vitamin D, the weighted mean difference changed to 1.9 nmol/L (95% CI: −2.3, 6.0 nmol/L; $P < 0.38$, $I^2 = 37\%$) (**Figures 6** and **7**).

In 2 studies, different types of rainbow trout were compared: the intervention group received trout that had been biofortified either by the feeding regimen (26) or by postmortem irradiation (U Lehmann, unpublished data, 2012). A separate meta-analysis of these studies showed a weighted mean difference of 5.4 nmol/L (95% CI: 1.6, 9.1 nmol/L; $P < 0.005$, $I^2 = 0\%$) between the intervention groups and controls.

An additional sensitivity analysis was carried out to investigate the influence of the mean baseline 25(OH)D concentration. In 3 studies that included 4 study groups, mean baseline 25(OH)D in the intervention groups was <50 nmol/L (27, 34; U Lehmann, unpublished data, 2012). The weighted mean difference was 6.1 nmol/L (95% CI: 2.7, 9.6 nmol/L; $P < 0.0006$, $I^2 = 0\%$), compared with 3.9 nmol/L (95% CI: 0.4, 7.3 nmol/L; $P < 0.03$, $I^2 = 30\%$) in 6 studies with 10 study groups in which mean baseline 25(OH)D concentrations were >50 nmol/L. A meta-analysis that used individual patients' data that were available from 6 trials (26–28, 35; OA Gudbrandsen, unpublished data, 2014; U Lehmann, unpublished data, 2012) did not show results that were different from those in the analysis of aggregated data (data not shown).

DISCUSSION

In this meta-analysis we investigated whether fish intake increases serum 25(OH)D concentrations in healthy adults under controlled conditions and included 9 RCTs with good or moderate quality. The main result was that the consumption of at least 2 fish meals, corresponding to ~300 g fish/wk over a period of at least 4 wk, was associated with a significant increase in 25(OH)D

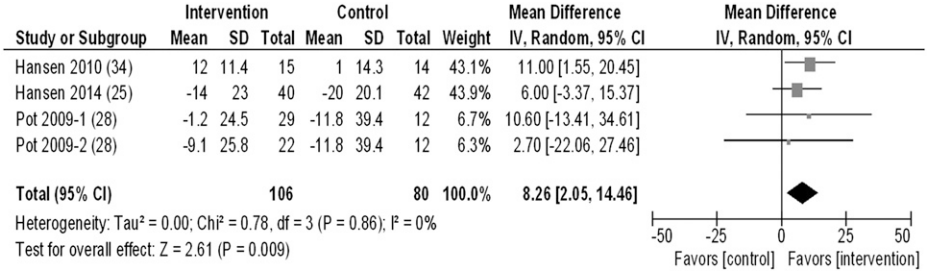

FIGURE 5 Random-effects meta-analysis comparing the effects of long-term (6 mo; 4 study groups) fish intervention with the control food on 25(OH)D concentrations (in nmol/L). The forest plot indicates a significant effect of the absolute change in 25(OH)D concentrations compared with the control food by fish intervention. "Total" denotes the cumulative *n* from all of the included studies. IV, inverse variance; 25(OH)D, 25-hydroxyvitamin D; −1, study groups who received fatty fish; −2, study groups who received lean fish.

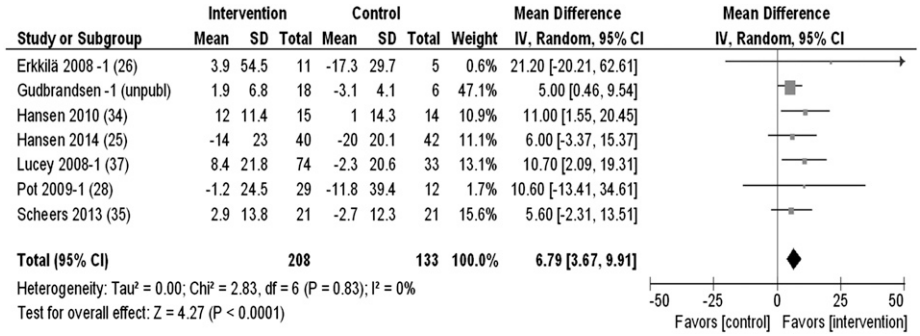

FIGURE 6 Random-effects meta-analysis comparing the effects of fatty fish intervention (7 study groups) with the control food on 25(OH)D concentrations (in nmol/L). The forest plot indicates a significant effect of the absolute change in 25(OH)D concentrations compared with the control food by fish intervention. "Total" denotes the cumulative *n* from all of the included studies. IV, inverse variance; unpubl, unpublished; 25(OH)D, 25-hydroxyvitamin D; −1, study groups who received fatty fish.

concentrations. In addition, fatty fish, longer study durations, and lower baseline 25(OH)D concentrations were associated with larger increases in 25(OH)D concentrations.

Although it has long been known that the consumption of fish is healthy, and this finding is included in most dietary recommendations, so far there have been few quantitative analyses supporting this effect on intermediate endpoints. The present analysis adds to our knowledge of the health-related effects of fish consumption and allows a quantitative estimate of what may be expected from increased fish consumption. This type of analysis is not very common in nutrition research and has not so far been included in recommendations and guidelines.

The present meta-analysis showed that the type of the fish is an important factor. Lean fish, mostly cod, did not increase vitamin D status to a significant extent, although it should be noted that differentiation between lean and fatty may be arbitrary in some species that could also be classified as medium-fatty fish. We observed a significant increase in 25(OH)D concentrations only when biofortified rainbow trout was included in the lean fish group. Thus, the consumption of fatty or biofortified fish should be recommended from the standpoint of improving vitamin D status.

In 2 studies, different types of rainbow trout were investigated: fish that were biofortified with vitamin D either by feeding or by postmortem exposure to UV-B radiation. These studies showed that there is a huge potential for improving the vitamin D content, which is more pronounced by using postmortem irradiation than by feeding. However, both technologies should be developed further, because the absolute amounts of vitamin D in the treated fish were still relatively low. In this respect, it is interesting to note that preliminary data on freshwater fish species also indicate an effect of different living conditions on vitamin D content (46).

One side effect of high fish consumption may be an increased exposure to environmental toxins that accumulate in fish and in seafood. Health authorities such as the Norwegian Scientific Committee for Food Safety therefore recommended in 2007 an upper intake limit of 400 g fatty fish/wk (47). It has been shown that the accumulation of toxins was high in fatty fish species such as herring, salmon, and sprat (48). Within the same fish species contamination may vary depending on age, fat content, and geographic region (49). In our meta-analysis none of the studies investigated the association between fish intake and toxins, but this clearly should be taken into account when recommending high fatty fish intakes to improve vitamin D status and should be explored in future studies.

Our knowledge of the variation in vitamin D content in fish is limited. For example, the vitamin D content of the fish used throughout the study was only measured in 2 studies (27; U Lehmann, unpublished data, 2012). It may be assumed that, even within a given fish species, there is a wide variation in vitamin D content depending on growth, feed, and other factors such as season (13). For example, it has been hypothesized that farmed

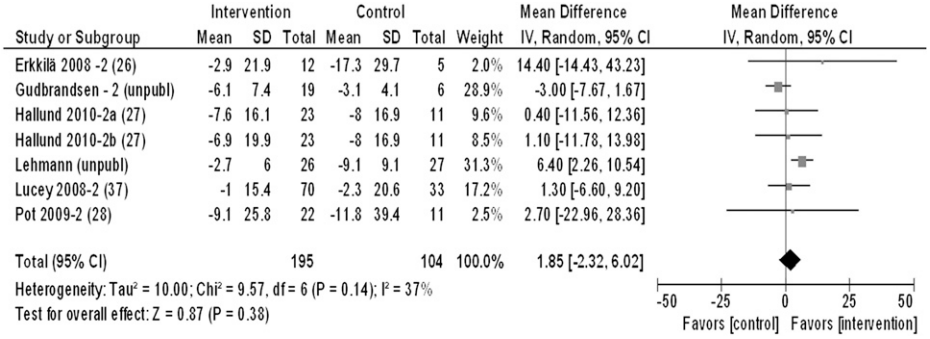

FIGURE 7 Random-effects meta-analysis comparing the effects of lean fish intervention (7 groups) with the control food on 25(OH)D concentrations (in nmol/L). The forest plot indicates a nonsignificant effect of the absolute change in 25(OH)D concentrations compared with the control food by fish intervention. "Total" denotes the cumulative *n* from all of the included studies. IV, inverse variance; unpubl, unpublished; 25(OH)D, 25-hydroxyvitamin D; −2, study groups who received lean fish.

salmon contains less vitamin D than does wild salmon (13, 50). Thus, there is a need for more detailed and accurate data on the determinants of vitamin D content in wild and farmed fish.

Although fish is one of the few foods that contain vitamin D (12), there is still an ongoing discussion whether fish intake contributes to a sufficient supply of vitamin D. Several observational studies (51–53) investigated the relation of fish intake and vitamin D status with the aid of food-frequency questionnaires. With the use of data from EPIC (European Prospective Investigation into Cancer and Nutrition)–Germany, Kühn et al. (51) reported a low, but positive, significant association between fish intake and 25(OH)D concentrations. In the United Kingdom, people who consumed meat and fish had higher 25(OH)D concentrations than did vegetarians and vegans (52). In Swedish women, fatty fish was one important predictor of serum 25(OH)D concentrations (53).

On the basis of all of the available data on fish intake, we observed a mean increase of 4.4 nmol 25(OH)D/L and an increase of 6.8 nmol/L when only fatty fish was considered. The application of the proposed increase of 25(OH)D of 1.97 nmol/L per additional microgram of vitamin D intake (54) suggests an intake of 2.2 μg vitamin D/d for all types of fish and 3.5 μg vitamin D/d for fatty fish such as salmon. According to the available data from food-composition tables, 300 g raw salmon/wk (corresponding to 2–3 portions) would provide 4.3, 6.9, or 2.5 μg vitamin D/d when using Norwegian, German, or UK databases, respectively (55–57). Whether these differences reflect natural variation or differences in analytic methods is unclear at present. Efforts to harmonize food-composition databases have been undertaken, e.g., by European Food Safety Authority or the EuroFIR project (www.eurofir.org).

The above calculations also show clearly that this fish intake is insufficient and does not fulfill the revised recommendations for a daily dietary amount of vitamin D that would improve vitamin D status (58–61). Indeed, it may be misleading to recommend fish consumption alone to improve vitamin D status. A daily intake of 2.2 or 3.5 μg vitamin D—which is achieved by consuming $\sim$300–600 g fish/wk—will not increase serum 25(OH)D concentrations to an optimal level ($>$50 nmol/L) in most people and will only result in increases of 4.4 or 6.8 nmol/L, respectively. Our results are in line with dose-response studies conducted in older adults (54), which showed that subjects supplemented with 5 μg vitamin D/d were able to maintain 25(OH)D concentrations during wintertime, whereas supplementation with 10 μg/d increased 25(OH)D concentrations by $\sim$12 nmol/L, on average. In healthy postmenopausal women, a daily supplement of 800 IU vitamin D (corresponding to 20 μg) was needed to increase 25(OH)D concentrations to $>$50 nmol/L in almost all of the women (62).

Strengths and limitations of the study

A major strength of the study is the inclusion of only RCTs and the collection of individual patients' data for at least some of the studies. All of the studies reached either a high- or moderate-quality score, although the use of established quality scores was prevented by the use of real food and therefore lack of participant blinding in 8 of the 9 studies. Limitations included that, due to the low number of studies, no further sensitivity analyses with respect to the amount or type of fish, length of intervention, or analytic method for determination of 25(OH)D were possible. In particular, the different analytic methods used for 25(OH)D measurements may have affected the results, because only 3 studies used chromatographic (LC-MS/MS or HPLC) methods.

Conclusions

We conclude that fish, as an important food source of vitamin D, increases 25(OH)D concentrations but cannot optimize vitamin D status. The side effects of the accumulation of environmental pollutants must be taken into account and investigated further. It should be clarified which foods are effective in improving vitamin D status; however, it seems to be difficult to increase the vitamin D concentrations sufficiently without using either supplements or fortified food in the absence of UV-B radiation.

The authors' responsibilities were as follows—UL and JD: designed the study and applied for funding; UL, HRG, and JD: performed the literature search and the meta-analysis; FH, AM-B, OAG, PMU, GM, GIS, and JD: were involved in the planning, design, and conduct of the 2 unpublished studies; LL, HL, ALH, ATE, and GKP: provided original data from their studies; and all authors: were substantially involved in the writing process. None of the authors stated a conflict of interest.

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4. Discussion

It has been described earlier that vitamin D deficiency is a major global and national problem. In Germany, daily intake of vitamin D from food is less than 3 µg/d [Max Rubner-Institut, 2008] which deviates enormously to German recommendations [German Nutrition Society, 2012]. This deficit can be compensated through either endogenous vitamin D synthesis, vitamin D supplementation or vitamin D food fortification.
This thesis aimed to investigate dietary measures for the increase of the vitamin D status by either vitamin D supplementation or food intake.

4.1. Efficacy of vitamin D supplementation to optimize vitamin D status in humans

It has been a debate for many years whether vitamin D_2 and D_3 are bioequivalent with respect to the treatment of deficiency, the increase in 25(OH)D-concentrations, and their effect on bone health. Therefore, it was the aim of this thesis to investigate the efficacy of vitamin D_2 and D_3 on vitamin D status (Study 1).

While vitamin D_2 is effective in the treatment of rickets [Jones et al., 1998], the efficacy of increasing the 25(OH)D-concentrations is still unclear. Thus, a number of studies have investigated the ability to correct vitamin D deficiency and to increase the total 25(OH)D-concentration in human. These studies applied different study designs, e.g. high iv. application routes [Romagnoli et al., 2008], daily [Tjellesen et al., 1986; Trang et al., 1998; Holick et al., 2008b, 2008b, 2008a; Glendenning et al., 2009; Biancuzzo et al., 2010; Binkley et al., 2011; Hammami and Yusuf, 2017], weekly [Heaney et al., 2011; Hammami and Yusuf, 2017] or monthly oral application [Binkley et al., 2011; Hammami and Yusuf, 2017] or high single doses of either vitamin D_2 or D_3 [Armas et al., 2004; Romagnoli et al., 2008; Leventis and Kiely, 2009]. However, there was no clear conclusion on the bioequivalence of both forms, which is also due to the fact that many studies were underpowered. Even a recent meta-analysis [Tripkovic et al., 2012] that included one study with intramuscular vs. oral application [Romagnoli et al., 2008]

and seven studies with oral applications only [Trang et al., 1998; Holick et al., 2008b; Glendenning et al., 2009; Biancuzzo et al., 2010; Binkley et al., 2011; Heaney et al., 2011; Hammami and Yusuf, 2017], did not reveal a definitive conclusion whether vitamin D_3 was superior to vitamin D_2 in increasing the 25(OH)D-concentration when applied daily. The results shown in Study 1 clearly demonstrate the superior effect of daily oral vitamin D_3 applications in increasing the total 25(OH)D concentrations, and even more in increasing the 25(OH)D_3-concentrations. Indeed, vitamin D_2 was associated with a significant decrease in 25(OH)D_3-concentrations. This effect was neglected in those studies that measured total 25(OH)D-concentrations by immunoassays [Trang et al., 1998; Romagnoli et al., 2008; Heaney et al., 2011]. Including Study 1 to the meta-analysis of Tripkovic et al. [Tripkovic et al., 2012], the conclusion would change to a clear superiority of vitamin D_3 compared to D_2 by daily supplementation. (Figure 3)

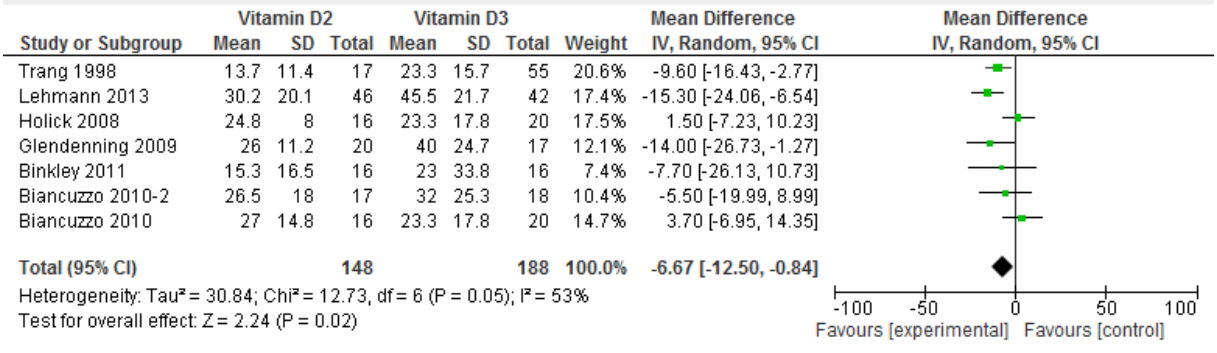

Figure 3: Forest plot on the effect of daily supplementation with either vitamin D_2 or vitamin D_3 on plasma total 25(OH)D concentrations.
experimental = vitamin D_3; control = vitamin D_2. The figure is equivalent to Figure 4 from Tripkovic et al. [2012], only data from the present study are included (data are derived from Table 2B of the article [Lehmann et al., 2013]. Review Manager release 5.2 was used to generate the analysis and the figure.

The different conclusion from the meta-analysis after inclusion of the data of Study 1 might be explained by the increase in the number of subjects included in the meta-analysis. Many prior studies lacked in statistical power and included insufficient sample numbers to draw firm conclusions. This is in contrast to Study 1, that was designed to find a difference in 25(OH)D between vitamin D_3 and D_2 and was thus sufficiently powered.

Currently, it is unclear whether there is also a difference in clinical outcomes between vitamin D_2 and D_3. The group of Bischoff-Ferrari et al. [2009] evaluated in her meta-analysis on vitamin D supplementation and falls supplements containing vitamin D_2

and D_3 separately. While vitamin D_3 supplementation was associated with a 26% decrease of the risk of falls, the effect was only 12% decrease for vitamin D_2 supplementation. There is a lack of studies that compare vitamin D_2 and D_3 for clinical outcomes.

One aspect is of particular interest, and that is the decrease of 25(OH)D_3 upon supplementation with vitamin D_2. It should be noted that only analytical methods using chromatography or mass spectrometry can differentiate between the two compounds, as immunoassays can measure only the total 25(OH)D-concentration. The decrease of 25(OH)D_3 has been also observed by others, [Armas et al., 2004; Glendenning et al., 2009; Binkley et al., 2011; Hammami and Yusuf, 2017]. However, most of these authors did not discuss this specifically. The group of [Holick et al., 2008b] did not observe this effect after 1000 IU vitamin D daily for 11 weeks. Hammami and Yusuf [2017] reported an inverse association of the increase in 25(OH)D_2 with the decrease of 25(OH)D_3 after supplementation with vitamin D_2 with in total 250,000 IU for 140 days (r= -0.48). Even if total amounts and also the observed increase in 25(OH)D_2 are comparable to the results of Study 1 (2000 IU/d for 56 days), the association suggested by the authors is in contrast to the association calculated from the present results (r= -0.07). In agreement with others [Binkley et al., 2011], a large interindividual variation in the response of both 25(OH)D_3 and 25(OH)D_2 upon vitamin D_2 or D_3 supplementation was observed. This may mask the ability to observe a true association between the changes of either metabolite.

Several mechanisms have been suggested to explain the differences between 25(OH)D-concentrations after similar amounts of vitamin D_3 and D_2, including differences in the affinity for transport proteins or enzymatic activity [Trang et al., 1998; Armas et al., 2004]. However, the present results do not reveal differences in 25-hydroxylation as the increase of 25(OH)D_3 or 25(OH)D_2. Consequently, vitamin D_3 or vitamin D_2 do not differ significantly, suggesting, in agreement with others, [Cheng et al., 2003; Shinkyo et al., 2004; Strushkevich et al., 2008] that the 25-hydroxylation for both vitamin D forms is equal and do not cause differences in bioefficiency.

However, several enzymes are capable to hydroxylize vitamin D_3, among them in the liver microsomal fraction *CYP2R1* and *CYP27A1*. However, the CYP27A1 cannot 25-hydroxylize vitamin D_2, but hydroxylates vitamin D_2 at C24 [Tuckey et al., 2019]. Thus, the reduction in circulating 25(OH)D_3 following vitamin D_2 supplementation could be due to substrate competition [Glendenning et al., 2009].

Although vitamin D_2 and D_3 only differ in the side chain, they show different metabolic fates. Obviously, both ergocaliferol or 25(OH)D_2, have lower affinity to DBP than cholecalciferol or 25(OH)D_3 [Nilsson et al., 1972; Hollis, 1984; Hollis et al., 1986; Glendenning et al., 2009]. Differences in the side chain of vitamin D_2 and D_3 might cause the different binding capacity [Hollis, 1984] and lead to higher stability of vitamin D_3 binding with consequently higher half-life [Jones et al., 2014] and increased clearance of vitamin D_2 metabolites [Horst et al., 1986]. Further, Horst et al. [1986] demonstrated a 40% higher degradation rate for 1,24,25-$(OH)_3D_2$ compared to 1,24,25-$(OH)_3D_3$ in rats.

Study 1 demonstrate that vitamin D_3 is more effective than vitamin D_2 in increasing the total serum 25(OH)D- and 25(OH)D_3-concentration, while vitamin D_2 supplementation is associated with a significant decrease in 25(OH)D_3. Therefore, vitamin D_3 should be recommended as preferred supplement to prevent or treat vitamin D deficiency in humans.

After identifying vitamin D_3 as the most promising supplement (Study 1), it was further aimed to investigate whether 20 µg vitamin D_3 representing the recalculated German recommendations are suitable to optimize vitamin D status in healthy volunteers (Study 2).

Dietary recommendations for vitamin D intake have been revised in many countries during the past 10 years [Ross et al., 2011; German Nutrition Society, 2012; Nordic Council of Ministers, 2012; Scientific Advisory Committee on Nutrition, 2016] following the observations of widespread vitamin D deficiency and low vitamin D intake in many countries [Moore et al., 2005; Hyppönen and Power, 2007; Hintzpeter et al., 2008; Totland et al., 2012; Rabenberg et al., 2015; Pilz et al., 2018]. Numerous vitamin D supplementation studies have been published which have been summarized in several meta-analyses [Cranney et al., 2007; Shab-Bidar et al., 2014; Whiting et al., 2015]. Although these meta-analyses differ in inclusion and exclusion criteria, in the publication periods covered, and in the number of studies, the main findings can be summarized as following: First, all meta-analysis reported significant heterogeneity in the increase of 25(OH)D-concentrations among studies, and this is also true for studies using similar doses of vitamin D. Second, the main determinants of the 25(OH)D-response to vitamin D_3 supplementation are dose, age, baseline 25(OH)D-concentrations and trial duration [Shab-Bidar et al., 2014]. Of these factors, dose,

baseline 25(OH)D-concentrations and trial duration were also identified by Whiting et al. [2015]. Third, both Cranney et al [2007] and Whiting et al. [2015] reported that the response to vitamin D can be described by a regression line (calculated as 2.19±0.97 nmol/l increase in 25(OH)D per µg vitamin D by Whiting et al. [2015], and 0.016+19.65 nmol/l increase in 25(OH)D per IU vitamin D by Cranney et al. [2007]). Interestingly, the regression line by Whiting et al. [2015] is quite similar to the slope of the regression line obtained by Cashman et al. [2009] from several dose response studies in elderly (1.97 nmol/l per µg vitamin D intake).

Applying these calculations to the results of Study 2 would display the average increase in 25(OH)D_3-concentrations in the studied population almost perfectly (observed mean increase after 12 weeks: 35 nmol/l, calculated with Whiting et al. [2015]: 44 nmol/l, calculated with Cranney et al. [2007]: 32.6 nmol/l. It has to be taken into account that the response of 25(OH)D to vitamin D_3 supplementation is most likely not linear at higher doses, but can best be described by a curvilinear function [Shab-Bidar et al., 2014; Whiting et al., 2015].

Both meta-analyses identified study duration as a factor for the achieved 25(OH)D level. Studies with study duration of less than 6 months had a lower increase in 25(OH)D-concentrations than studies of longer duration [Shab-Bidar et al., 2014; Whiting et al., 2015]. At present, it is not clear which trial duration is required to achieve a plateau of 25(OH)D concentrations. Study 2 revealed only a small and non-significant increase in 25(OH)D_3-concentrations between 8 and 12 weeks, however, as the trial finished after 12 weeks, it is difficult to estimate whether the mean 25(OH)D_3-concentrations would have further increased with continued vitamin D supplementation.

Even more interesting than the mean concentration would be the development of 25(OH)D_3-concentrations both at the lower end and the upper end. It was one aim of the Study 2 to increase the 25(OH)D_3-concentration in almost all participants to levels exceeding 50 nmol/l. This aim was already achieved after 8 weeks (when 50 out of 54 participants had 25(OH)D_3-concentrations >50 nmol/l). None of the participants in the vitamin D_3 group had 25(OH)D_3 concentrations <40 nmol/l at this time.

Thus, Study 2 confirmed that 20 µg/d of vitamin D_3 are sufficient to increase the 25(OH)D_3-concentrations in almost all healthy subjects during wintertime. However, when comparing the recommendations, it is obvious that in other countries, lower vitamin D intakes are recommended, despite similar or more northern latitude [Nordic Council of Ministers, 2012]. Taking into account the average 25(OH)D-concentration in Germany (mean 45.6 nmol/l [Rabenberg et al., 2015] or median 45 nmol/l [Hintzpeter et al., 2008] and applying the above mentioned regression lines from the meta-analyses by Whiting et al. [2015] or Cranney et al. [2007], the average 25(OH)D concentration would also exceed 50 nmol/l at additional daily intakes of 4 µg vitamin D. When almost all people should exceed 50 nmol/l, these amounts are not sufficient. Hintzpeter et al. [2008] provided the median and interquartile range of the distribution of 25(OH)D. Choosing the 25th percentile from the NVS 1998 which was 30.5 nmol/l in men and 30.7 nmol/l in women [Hintzpeter et al., 2008] and applying the regression line by Whiting et al. [2015] again, these persons would need about 10 µg additional vitamin D per day to achieve 50 nmol/l. Thus, it can be concluded that a substantial part of the population would need more than 10 µg additional vitamin D to achieve 50 nmol/l 25(OH)D-concentrations. However, 20 µg/d, as recommended by DACH in periods with no endogenous synthesis of vitamin D [German Nutrition Society, 2012], might be too high. Additionally, it has to be cosidered that the 25(OH)D-concentrations show huge variations among certain groups of the population, e.g. low 25(OH)D-levels are associated with high BMI, during winter and spring, higher age, and low physical activity and low socio-economic status [Rabenberg et al., 2015]. Thus, certain groups clearly need more additional vitamin D than other, and this would call for personalized nutritional advice (and supplementation strategies) instead of general recommendations.

4.2. Efficacy of fish and food consumption to optimize vitamin D status in humans

In addition to vitamin D supplementation, the consumption of vitamin D-rich foods is important to counteract vitamin D deficiency. Since only a few foods contain vitamin D in significant amounts, the enrichment of vitamin D in natural foods is of growing importance. National dietary surveys indicate fish and fish dishes as the most important source of dietary vitamin D in many countries [Max Rubner-Institut, 2008; Amcoff et al., 2012; Totland et al., 2012] and besides fortified food in some countries

(Netherlands, Finland). However, in population-based investigations on determinants of vitamin D status, fish has often a minor role [Kuhn et al., 2014]. Overall low fish consumption and large proportions of non-consumers in certain populations might cause this. Official recommendations of fish intake, usually to consume fish two or three times a week, are met by less than 40% of the population in many countries [Dutch National Institute for Public Health and the Environment; Max Rubner-Institut, 2008; Totland et al., 2012]. These considerations led to the question whether fish intake contributes to the vitamin D status.

Study 3 demonstrated that enrichment of vitamin D content in fish (rainbow trout) is feasible, but is further insufficient to compensate the seasonal drop of 25(OH)D_3-concentrations. In detail, the regular consumption of vitamin D-fortified rainbow trout reduced the decrease of 25(OH)D_3-concentrations that was observed in participants in the conventional fish group during the study period, leading to significantly different 25(OH)D_3-concentrations after 4 weeks. Interestingly, although fish is an important food providing vitamin D, there are no other intervention studies with fish and 25(OH)D_3-concentrations as primary outcome. There are several fish intervention studies with 25(OH)D-concentrations as secondary outcome which are included in the meta-analysis (Study 4) and discussed below.

Thus, the effect of vitamin D-enriched rainbow trout (Study 3) will be compared to low-dose supplement studies in adults, although there only limited number of studies available with supplements that provided 5 µg [Viljakainen et al., 2006; Cashman et al., 2008; Cashman et al., 2009] and none with lower doses. These studies have shown that daily 5 µg vitamin D supplements could either increase 25(OH)D-concentrations in elderly [Viljakainen et al., 2006], maintaine 25(OH)D-concentrations in elderly [Cashman et al., 2009], or was associated with a decrease of 25(OH)D-concentrations in adults [Cashman et al., 2008]. Reasons for the different findings may be more related to the higher baseline 25(OH)D-concentrations in the adults than in the elderly [Cashman et al., 2008; Cashman et al., 2009]. Baseline levels in Study 3 resembled closer the baseline levels in the Finnish study, but the amount of vitamin D provided by the fish was less than 5 µg [Viljakainen et al., 2006].

As RCTs are the gold standard to investigate such a hypothesis, we aimed to summarize available studies from the literature. The systematic literature search revealed nine relevant studies (Study 4). It is noteworthy to mention that only Study 3 had the effect of fish consumption on vitamin D status as primary outcome. The other included studies measured vitamin D status as secondary outcome, and reported details of the 25(OH)D-concentration on request (personal communication). The meta-analysis (Study 4) clearly showed that regular fish consumption is associated with an increase in 25(OH)D-concentrations, and fatty fish and study duration were of importance. Indeed, fatty fish like salmon, herring and mackerel have much higher vitamin D contents than lean fish like cod, pollock and freshwater fish like pike and pikeperch. However, comparing the different food databases, large differences in the vitamin D content of fish species among databases become evident (Table 4, Introduction). It is unclear at present whether these differences reflect true differences due to season, habitat or feeding, or whether these differences are due to analytical methods used or other reasons. However, these differences are impeding transnational comparisons on vitamin D intake from fish, and also add insecurity to estimate the amount of fish needed to achieve serum 25(OH)D-concentrations exceeding 50 nmol/l.

Further, it was found that study duration had a strong effect on the results (Study 4). The longest observation period, was 6 months, and the increase of 25(OH)D-concentration was higher in these studies, compared to studies lasting 4-8 weeks. This is particularly interesting as the food based dietary guidelines are intended for life-long use, and are aimed at regular fish consumption as part of regular meals. Thus, it cannot be excluded that the present meta-analysis underestimated the effect of regular fish consumption on 25(OH)D-concentrations in the long term. On the other hand, it is also not known whether vitamin D intake from fish would be high enough to result in a plateau of 25(OH)D-concentrations, as seen in supplement studies, even at low vitamin D dose [Viljakainen et al., 2006]. The uncertainty of vitamin D content of fish, the different vitamin D content of fish species and the dependence of the total 25(OH)D increase on baseline levels makes it difficult to estimate the required amount of fish to increase 25(OH)D-concentrations to specific levels for the general population.

Nevertheless, Study 3 showed that post-mortem radiation of rainbow trout fillet can increase the vitamin D content of this type of fish creating a kind of “functional food”. The efficacy of this principle (UVB-radiation of food to increase vitamin D content) has also been shown in mushrooms [Urbain et al., 2011] thus increasing vitamin D_2 content, and also in living animals like hens which led to increased vitamin D_3 and 25(OH)D_3 content in their eggs [Kühn et al., 2014]. Additional UVB exposure of free-range hens increased vitamin D_3 content by 2.58 µg per egg (3.8 µg in total). During Study 3 content of vitamin D-enriched trout was 2.8 µg/100g fish, less than in eggs containing 3.8 µg per egg following UVB-radiation [Kühn et al., 2014]. It is not known whether upscaling of vitamin D-enriched eggs would lead to same challenges than in fish or could be a more promising strategy with comparable amounts of vitamin D even in higher quantities. Further, the efficacy of vitamin D-enriched eggs on vitamin D-status in humans remains unclear so far, but would lead to doubling of current vitamin D intake in the German population [Max Rubner-Institut, 2008].

For other foods such as UVB-exposed mushrooms a meta-analysis [Cashman and Kiely et al., 2016] showed small increase of 25(OH)D-concentrations in terms of low baseline 25(OH)D-concentrations in humans. The number of RCTs included in this meta-analysis were small (n=6), showed large heterogeneity and inconsistent results. It can be assumed, that vitamin D food fortification via UVB-radiation is a promising strategy. So far, it can be stated that fish is suitable to reduce seasonal 25(OH)D decrease. Further intervention studies on bio fortified foods (e.g. UVB-exposed eggs and mushrooms) targeting vitamin D status are needed to derive recommendations for food fortification.

4.3. Effects of vitamin D supplementation on vitamin D metabolites and cardiovascular risk factors

Further the present results indicated a significant increase of vitamin D-metabolites (24,25$(OH)_2D_3$) following or a significant difference between placebo and vitamin D_3-group after 12 weeks of vitamin D_3 supplementation (1,25$(OH)_2D_3$-concentrations). Even other vitamin D supplementation studies showed significant increases of vitamin D-metabolites [Binkley et al., 2017; Saleh et al., 2017; Ketha et al., 2018; Vaes et al., 2018; Martucci et al., 2019]. This was presented for daily [Vaes et al., 2018] and high

single dose [Saleh et al., 2017]. One study comparing single and daily doses showed significant higher 24,25$(OH)_2D_3$-concentrations for a high single dose, but did not showed differences after 28 days [Ketha et al., 2018].
As a secondary outcome the effect of vitamin D_3 supplementation on cardiovascular risk factors like PTH and FGF-23 (Study 2) was evaluated. There was no significant decrease of PTH concentrations following 12 weeks of vitamin D_3 supplementation.
This is in line with a meta-analysis [Moslehi et al., 2015] which demonstrated high heterogeneity among PTH response according to vitamin D supplementation. This study suggested that PTH response depend on calcium dose, trial duration, baseline PTH-levels, BMI, age and sex. Further they conclude that >75 µg vitamin D per day for at least 12 months a necessary to reach a PTH plateau [Moslehi et al., 2015]. Even another meta-analysis indicated higher dosages and 25(OH)D-concentrations (>86 nmol/l) to decrease PTH concentrations [Mirhosseini et al., 2018].

Studies on the effect on FGF-23 showed different results: While the EVITA-Trial demonstrated an increase of FGF-23 concentrations after 3 years of daily 100µg Vitamin D supplementation [Zittermann et al., 2018], another study did not find a significant change of FGF-23 with a high single oral dose (300.000 IU) [Chitalia et al., 2014]. Even another study using different doses (2.000 IU or 40.000 IU) or time points of vitamin D supplementation (daily or monthly) did not find a significant reduction of FGF-23 concentrations [Mager et al., 2017]. It has to be taken into account that FGF-23 concentrations were mainly investigated in association with patients with cardiovascular diseases [Zittermann et al., 2018] or in patients with chronic kidney disease [Seibert et al., 2013; Mager et al., 2017] and not in healthy adults as we elucidated in Study 2.

4.4. Health and adverse effects of vitamin D supplementation and fish consumption

The meaning of high 25(OH)D-concentrations for health is discussed controversially. While levels around 100 nmol/l have been suggested by meta-analysis to prevent falls, cancer and respiratory infections [Spedding et al., 2013] such levels have recently been associated with increased risk for mortality in patients with coronary heart disease [Degerud et al., 2018] and increased need for mechanical circulatory support

implantation in patients with heart failure [Zittermann, 2017]. However, in Study 2 only one participant had 25(OH)D_3-concentrations exceeding 100 nmol/l and two participants had concentrations exceeding 95 µmol/l both after 8 weeks and after 12 weeks of supplementation. It would have been interesting to study the effect of ongoing supplementation, and more attention to the effect of vitamin D supplementation on high 25(OH)D_3-concentrations should be paid.

Results of Study 4 clearly showed that fish consumption increases concentrations of 25(OH)D, although recommended fish intakes cannot optimize vitamin D status. High intake of fish exceeding recommendation of two to three portions per week might be associated with higher 25(OH)D-concentrations but will also be associated with other health effects. In particular, increased consumption of fatty fish also leads to a high intake of dioxins. Dioxins show negative health effects and promotes cellular growth and differentiation [Döhr et al., 1994] and inhibits estrogen receptor [Tian et al., 1998]. In addition, high intake of dioxins increases insulin resistance and consequently increases diabetic risk [Ruzzin et al., 2010]. The consumption of lean fish might be associated with lower intake of dioxin but is further accompanied with lower intake of vitamin D.
For the environment, increased fish consumption poses the risk of overfishing. Therefore, both, health effects and adverse effects of fish, have to be taken into account while discussing increased fish intake exceeding the recommendations.

4.5. Limitations of vitamin D supplementation and food enrichment

Results showed (Study 2) that the response of 25(OH)D_3-concentrations on supplementation depends on various factors such as baseline vitamin D levels and revealed large heterogeneity among the study participants. This has been described in other studies which additionally identified age [Chen et al., 2008], doses [Heaney et al., 2003] and BMI [Gallagher et al., 2012] as predictors of vitamin D response. People with lower baseline 25(OH)D-concentrations benefit more from vitamin D supplementation than people with higher baseline concentrations [Trang et al., 1998; Aloia et al., 2008; Mazahery et al., 2015].

Results of Study 1 further showed that the decrease of 25(OH)D_3 due to vitamin D_2 supplementation may be of particular importance for the growing part of the population that follows a vegan diet, as this diet does not contain vitamin D_3. Thus, vegans and most vegetarians are dependent on either sun exposure or vitamin D supplements. As vegetarians and especially vegans want to avoid food and nutrients of animal origin, their vitamin D would be mainly vitamin D_2 from either supplements or the few foods (mushrooms, and yeast) that contain vitamin D_2. Although vitamin D_2 improves vitamin D status, it is obviously less effective than vitamin D_3 in comparable amounts (Study 1). Therefore, it is recommended to tailor recommendations according to individual needs and living conditions for optimization of vitamin D status.

The enrichment of foods with vitamin D using UVB-radiation has been identified as a promising strategy for the supply of the population in the future. Indeed, the vitamin D content of fortified fish in Study 3 was unexpectedly low (2.8 µg/100g fillet). Pre-trial measurements had indicated higher vitamin D values in the fillets (18.1 µg/100g). This demonstrates the challenges associated with upscaling food technological processes from the experimental scale to intermediate scale. Irradiation of food using UVB is also a very cost- and time-consuming process that has to be considered in relation to benefits of food enrichment.
Furthermore, vitamin D content in fish showed large variations depending on species [Lu et al., 2007], feeding [Mattila et al., 1997] and saison [Mattila et al., 1995] which has to be taken into account while enriching fish with vitamin D.
For the German population fish consumption per capita is low [BLE, 2018], while eggs, which also have been identified as a promising target for UVB-radiation [Kühn et al., 2014], are widely used in German diets. However, if fish will be successfully fortified with vitamin D (*based on preliminary results of Study 3*) and in higher quantities, low amounts of fish would be needed to fulfill vitamin D recommendations.

4.6. Suggestions for future research

During the studies of this thesis, it became apparent that the response to supplementation is mainly dependent on both, the dose of vitamin D and the baseline vitamin D status. Differences in baseline 25(OH)D-concentrations may explain to a large proportion the heterogeneity of the response to similar doses of vitamin D. However, baseline vitamin D status was not included into the regression formula by neither Whiting et al. [2015] or Cashman et al. [2008]. Thus, there is a need for a recalculation of the response to supplemental or dietary vitamin D with consideration of the baseline 25(OH)D-concentrations.

Even though it is well known that there is a large seasonal variation in total 25(OH)D-concentration, it is not clear whether the decrease in 25(OH)D-concentrations during winter season can be predicted by summer or autumn 25(OH)D-concentrations. More longitudinal data are needed, which also take into account potential health effects of high summer and low winter values.

Although, it is now established that vitamin D_2 is associated with a decrease in 25(OH)D_3, this form is further used as a supplement. This applies especially for the growing part of the population who follows a vegan or plant based diet. Long-term effects of vitamin D_2 supplementation in this special group should be investigated.

Work for this thesis also revealed that there are substantial gaps of knowledge in the vitamin D content of foods. Here, international comparisons and application of validated methods both for analysis and sampling should be applied. This would be also a prerequisite for the implementation and monitoring of food fortification strategies, which are more promising than supplementation for the improvement of the vitamin D status of the general population and especially of vulnerable groups.

4.7. Conclusion

The main conclusion derived from the bioavailability study on vitamin D_2 and D_3 is the higher effectiveness of vitamin D_3 in comparison to vitamin D_2 in increasing the 25(OH)D-concentrations in healthy individuals. Thus, vitamin D_3 should be preferred as supplement and for food fortification instead of vitamin D_2. Biochemical, physiological and health effects of long-term vitamin D_2 use, for example in vegans, warrant further investigations.

The second study showed that 12 weeks of supplementation with 20 µg/d vitamin D_3 were efficient to increase the 25(OH)D_3-concentration in wintertime in nearly all participants without increasing the concentrations beyond levels of 100 nmol/l. It became apparent that this dose is safe, but may not be required by all, as seen by the large variation in the achieved concentrations. Thus, a more personalized approach considering individual baseline vitamin D status, dietary habits, health status and lifestyle habits would be warranted to achieve an optimized result in the individual person.

It was shown that the biofortification of fish with vitamin D was successful and efficient, although the achieved vitamin D content was lower than expected. Fish is a major source of dietary vitamin D, and regular fish intake, especially fatty fish can increases the 25(OH)D_3-concentration.

Biofortification seems to be a promising strategy to increase the dietary vitamin D intake and should be promoted more intensely.

This thesis demonstrates the possibility to optimize the vitamin D status by vitamin D supplementation, and also partly by fish intake. Even though recent randomized clinical studies (EVITA, VITAL) did not show improvements in clinical outcomes [Zittermann et al., 2017; Manson et al., 2020], this does not preclude the prevention and treatment of vitamin D deficiency through either dietary fortification or supplementation.

5. Zusammenfassung

Eine unzureichende Vitamin D-Versorgung ist ein weltweites Problem und ist assoziiert mit einem erhöhten Risiko für Bluthochdruck, Diabetes, kardiovaskuläre Erkrankungen und begünstigt weiterhin die Entstehung verschiedener Krebserkrankungen. Es war das Ziel der vorliegenden Arbeit in 3 Studien zu untersuchen inwieweit Vitamin D-Supplemente und der Verzehr von Fisch als wertvolle Vitamin D-Quelle zur Optimierung des Vitamin D-Status bei gesunden Probanden beitragen. Weiterhin sollte der Einfluss von Fischverzehr auf den Vitamin D-Status systematisch untersucht werden.

In Studie 1 wurde die Effekte einer täglichen Dosis von 50 µg Vitamin D_2 und D_3 auf den Vitamin D-Status über einen Zeitraum von 8 Wochen untersucht. Dabei konnte gezeigt werden, dass Vitamin D_3 die 25(OH)D-Konzentration signifikant erhöht und eine Vitamin D_2-Supplementierung zu einer signifikanten Minderung der 25(OH)D_3-Konzentration führt.

In Studie 2 wurde die Effektivität der neuen Vitamin D-Empfehlungen der DGE von täglich 20 µg Vitamin D_3 auf eine Optimierung des Vitamin D-Status nach 12 Wochen untersucht. Die Ergebnisse konnten zeigten, dass in den Vitamin D_3-supplementierten Probanden die 25(OH)D_3-Konzentration signifikant anstieg und 94% der Probanden Konzentrationen > 50 nmol/l erreichten.

Im Rahmen der Studie 3 wurde der Einfluss eines 4-wöchigen Verzehrs von Vitamin D-angereichertem Fisch auf die 25(OH)D-Konzentration untersucht. Die Ergebnisse zeigten, dass nach Verzehr des Vitamin D-angereicherten Fisches die 25(OH)D-Konzentration signifikant weniger sank als durch den Verzehr von konventionellem Fisch. Eine systematische Literatursuche und Meta-Analyse von Fischinterventionsstudien (Studie 4) konnte zeigen, dass der Verzehr von Fisch, insbesondere Fettfisch, zu einer Erhöhung der Vitamin D-Konzentration beiträgt, aber nicht für eine Optimierung des Vitamin D-Status ausreichend ist.

Die Ergebnisse der vorliegenden Arbeit konnten zeigen, dass eine Optimierung des Vitamin D-Status durch Vitamin D-Supplementierung und teilweise durch Fischverzehr möglich ist. Die Anreicherung von Lebensmitteln mit Vitamin D ist dabei eine vielversprechende Strategie zur Verbesserung der Vitamin D-Aufnahme über die Nahrung. Eine personalisierte Betrachtung von Vitamin D-Ausgangswerten, der

Nahrungszufuhr und Lebensstilfaktoren könnte zusätzlich den Effekt von Vitamin D-Supplementierungen beeinflussen.

6. Summary

Inadequate vitamin D supply is a worldwide problem, is associated with increased risk of high blood pressure, diabetes, cardiovascular diseases, and further promotes development of various types of cancer. It was the aim of the present work to investigate the extent to which vitamin D supplements and the consumption of fish as a valuable source of vitamin D contributing to the optimization of vitamin D status in healthy volunteers in three different studies. Furthermore, the influence of fish consumption on vitamin D status was systematically examined.

Study 1 investigated the effects of a daily dose of 50 µg vitamin D_2 and D_3 on vitamin D status over a period of 8 weeks. It was shown that vitamin D_3 significantly increases the 25(OH)D-concentrations and that vitamin D_2 supplementation significantly decreases 25(OH)D_3-concentrations.

Study 2 examined the effectiveness of the new German recommendations for vitamin D intake (20 µg vitamin D_3 daily) to optimize vitamin D status after 12 weeks. Subjects receiving vitamin D_3 showed significantly increases 25(OH)D_3-concentration of which 94% reached concentrations >50 nmol/l.

Study 3 investigated the influence of a 4-week intake of vitamin D-enriched fish on the 25(OH)D-concentration. The results showed that 25(OH)D-concentrations decreased significantly less in the group receiving vitamin D-enriched fish, than in the group consuming conventional fish. A systematic literature search and meta-analysis of fish intervention studies (Study 4) showed that the consumption of fish, particularly fatty fish, contributes to an increase in vitamin D concentration, but is not sufficient to optimize the vitamin D status.

The results of the present work were able to show that an optimization of the vitamin D status is possible through vitamin D supplementation and partly through fish consumption. Enriching foods with vitamin D is a promising strategy for improving vitamin D intake through food. Personalized consideration of baseline vitamin D concentrations, food intake and lifestyle factors could additionally influence vitamin D supplementation

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Unterschrift

Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und diese nicht bereits für eine Promotion oder ähnliche Zwecke an einer anderen Universität eingereicht habe. Weiterhin versichere ich, dass ich die verwendeten wissenschaftlichen Arbeiten und Hilfsmittel genau und vollständig angegeben habe.

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