UVB-exposed plant oils as potential sources of vitamin D – studies on bioavailability and metabolism of vitamin D metabolites

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„Es ist nicht genug zu wissen, man muss auch anwenden;

es ist nicht genug zu wollen, man muss auch tun.”

Johann Wolfgang von Goethe (1749 – 1832)

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Abbreviations

$1,25(OH)_2D$	1,25-Dihydroxyvitamin D
$24,25(OH)_2D$	24,25-Dihydroxyvitamin D
25(OH)D	25-Hydroxyvitamin D
7-DHC	7-Dehydrocholesterol
ABC	Adenosine triphosphate-binding cassette
CD36	Cluster of differentiation 36
CYP	Cytochrome P450
D-A-CH	Nutrition societies of the countries Germany (D), Austria (A) and Switzerland (CH)
DBP	Vitamin D binding protein
DMM	Dietary mixed micelles
EFSA	European Food Safety Authority
EPIC	European Prospective Investigation into Cancer and Nutrition
FGF23	Fibroblast growth factor 23
IOM	Institute of Medicine
NPC1L1	Nieman-pick C1-like protein 1
PTH	Parathyroid hormone
SCARB1	Scavenger receptor, class B, member 1
UV	Ultraviolet light
VDR	Vitamin D receptor
WGO	Wheat germ oil

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1 Introduction

1.1 Vitamin D metabolism and status in the population

The main function of vitamin D is the maintenance of calcium and bone homeostasis by regulation of the intestinal absorption, renal excretion and osseous mineralization and bone turn-over of calcium and phosphate (Bikle, 2012; DeLuca, 2004; Jones et al., 1998). Thus, vitamin D deficiency is classically linked to severe diseases of the musculoskeletal system, namely rickets in children and osteomalacia in adults. Other milder symptoms of insufficient vitamin D levels are secondary hyperparathyroidism, osteoporosis, mineralization defects, fractures and myopathy (Lips, 2001). The actions of vitamin D are mediated by the interaction of the active vitamin D hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$, calcitriol) with the vitamin D receptor (VDR). VDR is a steroid receptor of the nuclear receptor family and functions as a transcription factor. In 2010, 2667 VDR DNA-binding sites have been identified, stimulating 229 genes in response to $1,25(OH)_2D$ (Ramagopalan et al., 2010). The finding that the VDR is expressed ubiquitous in nearly every tissue (Pike et al., 2017), indicates that vitamin D has an impact on multiple extra-skeletal biologic processes. That is why there have been several studies associating insufficient vitamin D levels with a series of diseases. Among them, increased risks for cancer (Garland et al., 2006), cardiovascular diseases (Artaza et al., 2009; Gaksch et al., 2017; Schmidt et al., 2012; Schmidt et al., 2014), respiratory tract infections, cognitive (Maretzke et al., 2020), neurological (Moretti et al., 2018) and autoimmune disorders (Altieri et al., 2017; Yang et al., 2013). Moreover, vitamin D seems to play a pivotal role in the innate and adaptive immune response (Adams et al., 2009; Liu et al., 2006; Prietl et al., 2013). However, whether a higher vitamin D status is beneficial in extra-skeletal health is still a matter of debate, since results of placebo controlled megatrials showed no effect of supplemental vitamin D on cancer and cardiovascular events (Bischoff-Ferrari et al., 2020; Manson et al., 2020; Scragg, 2019).

In nature, the secosteroid vitamin D can be found predominantly in two forms, vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Both are synthesized from their precursors (provitamin D) by exposure to ultraviolet (UV) B-light (290 – 320 nm) (Holick, 1994). Through UVB-exposure, provitamin D is converted in previtamin D, which in turn becomes vitamin D in a thermic-regulated process (Tian et al., 1993) (**Figure 1**). The precursor of vitamin D_2 is ergosterol, a steroid of fungal origin. In vertebrates, vitamin D_3 is formed from 7-dehydrocholesterol (7-DHC), an intermediate from the cholesterol synthesis. Vitamin D_2 differs from vitamin D_3 only in the sidechain, with a double bond between C-positions 22 and 23 and an additional CH_2-group at C24.

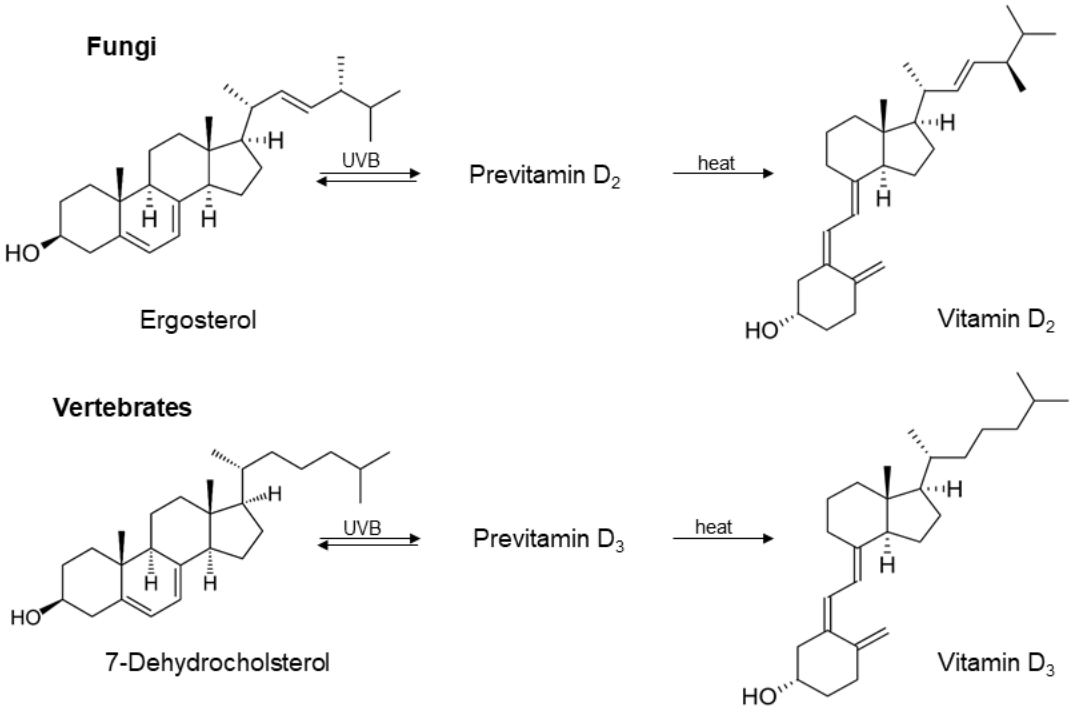

Figure 1 The synthesis of vitamin D_2 and vitamin D_3 from their precursors in fungi and vertebrates.

Exposure of ergosterol and 7-dehydrocholesterol with ultraviolet-light type B (UVB) led to the formation of previtamin D_2 and previtamin D_3, which are then converted to vitamin D_2 and vitamin D_3 by a thermic-regulated process (Bikle, 2014).

Both vitamin D forms are biologically inert and have to be activated by hydroxylation in the body. First, vitamin D is hydroxylated in the liver at C-position 25 to 25-hydroxyvitamin D (25(OH)D). This reaction is mainly catalyzed by the cytochrome P450 (CYP) enzymes CYP27A1 (Axén et al., 1994; Guo et al., 1993), CYP2R1 (Cheng et al., 2004) and CYP3A4 (Gupta et al., 2004). 25(OH)D is then transported via the blood to the kidneys for further activation. During the circulation, most of the 25(OH)D is bound to the vitamin D binding protein (DBP) (Daiger et al., 1975). The DBP-25(OH)D complex is then taken up by the proximal tubular cell via a megalin-mediated endocytosis (Nykjaer et al., 1999). In the cell, 25(OH)D undergoes a second hydroxylation to $1,25(OH)_2D$ by the 1α-hydroxylase CYP27B1. CYP27B1 is tightly regulated by the parathyroid hormone (PTH), the fibroblast growth factor 23 (FGF23) and $1,25(OH)_2D$ itself (Blau and Collins, 2015; Takeyama et al., 1997). Another regulation mechanism is the activity of the 24-hydroxylase (CYP24A1), which also utilizes 25(OH)D as a substrate and is therefore in direct competition with CYP27B1. The main function of CYP24A1 is to prevent the accumulation of toxic vitamin D levels by formation of biologically inactive vitamin D metabolites, e.g. 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) (Sakaki et al., 2000).

Since the 25-hydroxylation is not strongly regulated, the main circulating vitamin D form is 25(OH)D. Additionally, the half-life of 25(OH)D is much longer than of 1,25$(OH)_2$D (2–3 weeks vs. 4 hours) (Holick et al., 2011). Therefore, vitamin D status is generally assessed by the blood concentration of 25(OH)D (Seamans and Cashman, 2009). However, 25(OH)D levels indicating a vitamin D deficiency are widely discussed in literature. The assessment of the cut-off value is derived mostly from the inverse relationship between the parathyroid hormone (PTH) and 25(OH)D, since PTH increases, when 25(OH)D levels drop below the cut-off value (Lips et al., 1983; Lips, 2004). It is generally agreed, that 25(OH)D concentrations below 50 nmol/L are indicative of vitamin D deficiency (Bacon et al., 2009; Lips, 2004; Lips et al., 2019; Ross et al., 2011). Moreover, some authors state 25(OH)D levels below 75 nmol/L as insufficient (Bischoff-Ferrari et al., 2006; Holick et al., 2011). Therefore, the term *vitamin D insufficiency* has been introduced to describe lower levels of 25(OH)D, which are associated with extraskeletal disease outcomes (Thacher and Clarke, 2011). Vitamin D insufficiency and deficiency are common all over the world. In 2014, Hilger et al. meta-analyzed that 88.1 % and 37.3 % of the global population had mean 25(OH)D values below 75 nmol/L and 50 nmol/L, respectively. The 25(OH)D values were statistically lower in Europe than in North America (Hilger et al., 2014). In the USA, the prevalence of 25(OH)D values below 50 nmol/L was 24 % (Schleicher et al., 2016), while it was 40.4 % in Europe (Cashman et al., 2016a). In Germany, a subcohort of the *European Prospective Investigation into Cancer and Nutrition* (EPIC) study had mean 25(OH)D values of 46.9 nmol/L. Overall 59.9 % of the cohort showed sub-optimal vitamin D levels (<50 nmol/L), with a significantly higher prevalence in winter than in summer months (82.2 % vs. 33.2 %) (Kühn et al., 2014).

In vertebrates, most of the vitamin D is synthesized in the skin through exposure to sunlight. However, in the northern hemisphere, endogenous vitamin D synthesis is non-existent in the winter months. In Germany (latitude 47 - 55° N), this "*vitamin D winter*" lasts 4 months (O'Neill et al., 2016). Moreover, the vitamin D synthesis is limited all year round due to insufficient exposure to the sun, the use of sun protection (Matsuoka et al., 1987), skin melanin content (Chen et al., 2007), age (MacLaughlin and Holick, 1985), time and length of the day (Wacker and Holick, 2013), smog and cloudy weather (Spina et al., 2005). In the case of an absent vitamin D synthesis in the skin, vitamin D has to be ingested through the diet. The *U.S. Institute of Medicine* (IOM) recommends a daily oral intake of 15 µg vitamin D (Ross et al., 2011). In 2012, the D-A-CH reference values (nutrient recommendations for the countries Germany (D), Austria (A) and Switzerland (CH)) were adjusted from 5 µg to 20 µg per day (German Nutrition Society, 2012). Though, to raise 25(OH)D levels consistently above 75 nmol/L, vitamin D intakes of up

to 50 µg per day may be required (Holick et al., 2011). However, natural food sources are scarce, as fatty sea fish is the main source of nutritional vitamin D_3 (Jakobsen et al., 2019) and the only known natural source of vitamin D_2 are wild mushrooms (Mattila et al., 1994). Therefore, the current daily intake of vitamin D falls far short of the recommendations. The mean vitamin D intake of the German adult population was 3.8 µg (males) and 2.9 µg (females) per day in 2008 (Max-Rubner-Institut, 2008). Similar values have been reported for Europe (males, 4.9 µg/day; females, 3.4 µg/day), with highest concentrations in Sweden (Jenab et al., 2009). Alimentary vitamin D intake could be enhanced by the use of supplements, which are produced from sheep wool and lichens (vitamin D_3) or by UVB-exposure of ergosterol from yeasts (vitamin D_2) (Hirsch, 2011; Wang et al., 2001). However, the acceptance of supplements is limited (Hintzpeter et al., 2008) and the improper use of supplements includes the risk of overdosing (Pilz et al., 2018). Thus, there is the need for food-based strategies to bridge the gap between the current daily vitamin D intake and the recommended intake.

1.2 Vitamin D fortification strategies

Systematic food fortification has been proven to be an effective approach to enhance vitamin D intake and subsequently, to combat vitamin D deficiency (Black et al., 2012; Kiely and Black, 2012; O'Donnell et al., 2008). In some countries, fluid-milk products are either mandatorily (Canada and Sweden) (Health Canada, 2021; Itkonen et al., 2018a) or voluntarily (e.g. USA) fortified with either vitamin D_3 or vitamin D_2 (Calvo et al., 2004; Institute of Medicine, 2011), which is very likely the reason for higher 25(OH)D levels in these countries. However, due to the high-prevalence of lactose intolerance and milk allergies, fortification of milk-products alone is not sufficient to combat vitamin D deficiency (Cashman and Kiely, 2016; Tangpricha et al., 2003). Hence, attempts have been made to fortify non-dairy food sources. Vitamin D fortified bread (Natri et al., 2006) and orange juice (Biancuzzo et al., 2010; Tangpricha et al., 2003) are promising approaches, since they were able to enhance 25(OH)D blood levels as efficient, as vitamin D supplements. An example for a successful food-fortification strategy is the public health policy of Finland. In 2003, the *Finnish National Nutrition Council* enacted a policy to voluntarily fortify not only fluid-milk products, but also fat-spreads with vitamin D. In 2010, the fortification recommendations were doubled to 1 µg/100 g for the dairy-products and 20 µg/100 g for the fat-spreads (Raulio et al., 2017). As a result, the vitamin D intake from dietary sources (without supplements) nearly doubled from 2002 (men and women, 7 µg/day) to 2011 (men, 14 µg/day; women, 12 µg/day). Besides, the prevalence of 25(OH)D levels below 50 nmol/L was significantly reduced (58.5 % vs. 13.7 %) (Jääskeläinen et al., 2017).

In Finland and other European countries, the addition of vitamins and minerals to foods is settled in the regulation of the European Union (Regulation (EC) Nr. 1925/2006, 2006). In Germany, food fortification is additionally restricted by national law, which only permits vitamin D fortification for plant-based spreads or foods intended for use as or in place of a meal (LMvitV, 1942). Although there are some vitamin D enriched foods available in Germany (e.g. juices and milk), since they can be imported or authorized by a special permit (LFGB, 2005), vitamin D fortified staple foods are still scarce. Thus, alternative strategies for vitamin D enrichment of dietary sources are needed. Vitamin D fortification can also be achieved by means of "*bio-fortification*" (or "*bio-addition*"), which is the enrichment of foods by other means rather than simply adding exogenous vitamin D (Pilz et al., 2018), e.g. by manipulation of food sources post-harvesting or pre-processing (Calvo and Whiting, 2013). An example for bio-fortification is the UVB-treatment of chickens, to enhance the vitamin D_3 content of the eggs (Kühn et al., 2015; Kühn et al., 2019; Schutkowski et al., 2013). Other examples to enhance the vitamin D_3 content in animal products are the UVB-exposure of pigs (Barnkob et al., 2019; Larson-Meyer et al., 2017) and cows (Jakobsen et al., 2015) to enrich meat and milk. Milk has also been UVB-exposed post-milking (EFSA, 2016). Recent studies focus on the UVB-treatment of yeasts and mushrooms to enhance foods with vitamin D_2, as this may be cost-effective and more easily accessible to a wider part of the population (Itkonen et al., 2018b). UVB-exposed baker's yeast has been used commercially for many years (Degre et al., 2007) and has been considered to be a safe novel food ingredient by the *European Food Safety Authority* (EFSA, 2014). The vitamin D content of UVB-exposed mushrooms has been studied intensively (Huang et al., 2015; Jasinghe and Perera, 2005; Kalaras et al., 2012a; Keegan et al., 2013; Ko et al., 2008; Koyyalamudi et al., 2011; Nölle et al., 2017; Simon et al., 2011; Urbain and Jakobsen, 2015; Won et al., 2018) and the UV-treatment of mushrooms is already implemented in some cultivation farms, successfully producing enriched mushrooms with approximately 10 µg vitamin D_2 per 100 g fresh weight (Food Safety Authority of Ireland, 2016, 2017). For example, a 100 g-serving of these mushrooms would meet up to 50 % of the D-A-CH recommendations of 20 µg vitamin D per day. The UVB-treated mushrooms are considered to be safe (EFSA, 2020) and bioavailable in rodents (Jasinghe et al., 2005; Koyyalamudi et al., 2009) and humans (Keegan et al., 2013; Mehrotra et al., 2014; Urbain et al., 2011). In general, it would be preferable to have a range of vitamin D enriched foods to accommodate nutritional diversity and to prevent overdosing by single food sources (Kiely and Black, 2012). Hence, other potential food sources are sought.

1.3 Plant oils as potential sources of vitamin D

Plants do also contain several sterols. The origin of those is the cyclization of squalene via 2,3-oxidosqualene which forms either cycloartenol or lanosterol (**Figure 2**). While animals and fungi convert lanosterol to either cholesterol or ergosterol, respectively (Ohyama et al., 2009), plants convert cycloartenol and also small amounts of lanosterol to various phytosterols, e.g. stigmasterol or sitosterol. It is suggested, that plants are also capable of synthesizing cholesterol from cycloartenol via 7-DHC (Jäpelt and Jakobsen, 2013), as they do generally contain cholesterol in concentrations accounting of 1-2 % of the total sterol content (Moreau et al., 2002). Plants from the *Solanaceae* family are especially rich in cholesterol, with concentrations accounting to 5 % of the total sterol content (Jäpelt et al., 2011b; Moreau et al., 2002; Whitaker, 1988, 1991). Since cholesterol and vitamin D_3 share the same intermediate, plants can also synthesize vitamin D_3 by photoconversion of 7-DHC.

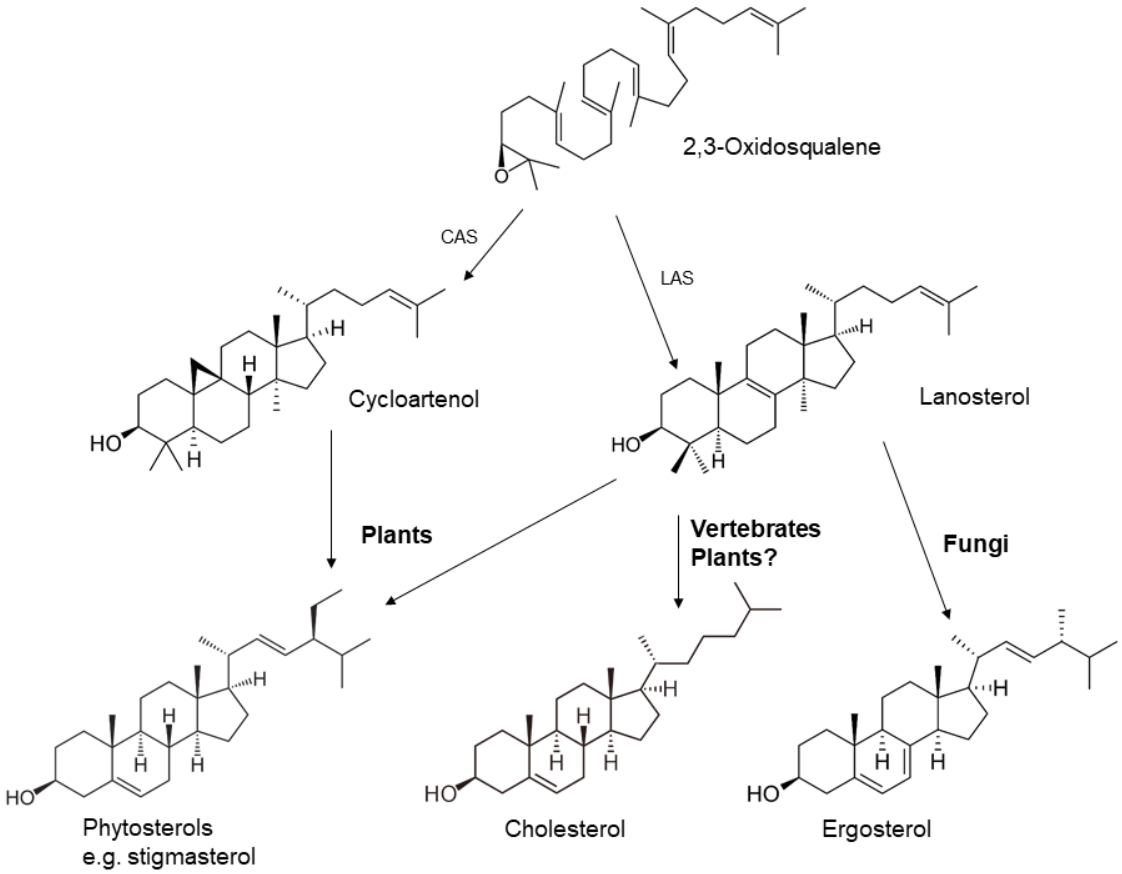

Figure 2 Cyclization steps of 2,3-oxidosqualene in plants, vertebrates and fungi.

2,3-Oxidosqualene is either cyclized to cycloartenol or lanosterol. Plants then convert cycloartenol and small amounts of lanosterol in various phytosterols e.g. stigmasterol, while lanosterol is converted to cholesterol in vertebrates and to ergosterol in fungi (Ohyama et al., 2009). It is hypothesized that plants are also capable of converting lanosterol to cholesterol (Jäpelt and Jakobsen, 2013). CAS, cycloartenol synthase; LAS, lanosterol synthase.

First evidence of vitamin D active compounds in plants came in 1924 by two independent research groups (Hess and Weinstock, 1924; Steenbock, 1924). Steenbock irradiated rats and also their air and food in their cages with UV-light, and found that not only the exposure of the rats but also the consumption of the irradiated food was able to prevent and cure rickets (Steenbock and Black, 1924). He attributed this to the presence of a fat-soluble compound which is activated by UV-light, and also patented the UV-treatment process (Steenbock, 1924). Later this antirachitic compound was identified to be vitamin D by Adolf Windaus, who was awarded the "*Nobel Prize in Chemistry*" in 1928 for his work on the structure of sterols and the connection to vitamins (Nicholls, 2019). *Trisetum flavescens* (yellow oat grass) from the *Poaceae* family is known to cause calcinosis in grazing cattle due to high concentrations of vitamin D (Dirksen et al., 1970; Peterlik et al., 1977; Rambeck et al., 1979). Peterlik et al. attributed this to the presence of a water-soluble 1,25$(OH)_2D_3$-glycoside (Peterlik et al., 1977). Other calcinogenic plants which have been shown to contain 1,25$(OH)_2D_3$-glycosides are *Solanum malacoxylon* (Peterlik et al., 1976), *Cestrum diurnum* (Hughes et al., 1977) and *Solanum glaucophyllum* (Zimmerman et al., 2015) from the *Solanaceae* family. Other vitamin D metabolites, like 7-DHC and vitamin D_3, also have been found in plants of the *Fabaceae* (Horst et al., 1984), the *Solanaceae* (Aburjai et al., 1998; Jäpelt et al., 2011b; Skliar et al., 1992) and the *Cucurbitaceae* families (Aburjai et al., 1996; Aburjai et al., 1998). Ergosterol and vitamin D_2 were analyzed in several plants (Hughes et al., 2018; Hurst et al., 2016; Jäpelt et al., 2011a), among them, plants from the *Poaceae* (Jäpelt et al., 2011a) and *Fabaceae* (Horst et al., 1984) families. Although, vitamin D_2 is often referred to as a plant sterol, any vitamin D_2 that is found in plants is suggested to be caused by a fungal infection and the subsequent photoconversion of the fungal ergosterol (Jäpelt and Jakobsen, 2013).

Since vitamin D and vitamin D precursors are fat-soluble, it can be assumed that the vitamin D metabolites can also be found in oils of these plants. The finding by Hess and Weinstock in 1924, that plant oils treated with a mercury vapor lamp had antirachitic-potentials in rats corroborates this theory (Hess and Weinstock, 1924). Very recently, 50 cold-pressed oils of sun-dried seeds were analyzed for their vitamin D_3 concentrations, which ranged from 0.63 µg/g (green amaranth oil) to 1 288 µg/g (angled loofah oil) (Yuenyong et al., 2021). However, there is still a lack of quantitative data about the concentrations of vitamin D_3, vitamin D_2 and their precursors in edible plant oils intended for the use in human nutrition. As plant oils are usually consumed daily by many people, with mean intakes of 9 g (females) to 15 g (males) per day (Max-Rubner-Institut, 2013), they are also considered to be good food vectors for vitamin D fortification.

1.4 Bioavailability of vitamin D

Orally ingested vitamin D_2 or vitamin D_3 have to be absorbed from the intestine. For a long time, it was assumed that vitamin D is absorbed by passive diffusion (Hollander et al., 1978). Though, there is evidence that the intestinal cholesterol transporters Nieman-pick C1-like protein 1 (NPC1L1), cluster of differentiation 36 (CD36) and scavenger receptor class B member 1 (SCARB1) are also involved (Kiourtzidis et al., 2020c; Kiourtzidis et al., 2020b; Reboul et al., 2011). After passage through the enterocyte, the absorbed vitamin D is transported from chylomicrons via the lymphatic system into the blood (Dueland et al., 1985). The bioavailability of oral vitamin D is assumed to be very efficient (Cipriani et al., 2013), but varies individually. The response in 25(OH)D concentrations after oral intake of vitamin D depends on physiological factors such as age (Lehmann et al., 2016), genetics (Didriksen et al., 2013), body mass index (Didriksen et al., 2013; Gallagher et al., 2012) and baseline 25(OH)D levels (Didriksen et al., 2013; Lehmann et al., 2016). Food ingredients (Diarrassouba et al., 2015; Goncalves et al., 2011; Hollander et al., 1978; Kühn et al., 2016) and matrices (Itkonen et al., 2018b; Lipkie et al., 2016) have also been discussed to influence the bioavailability of vitamin D. Monounsaturated fatty acids, and in particular oleic acid, enhanced the intestinal vitamin D uptake (Goncalves et al., 2013). In 2016, the vitamin D_3 precursor 7-DHC was found to be a modulator of the vitamin D metabolism. Here, dietary relevant dosages of oral 7-DHC increased the body vitamin D storages significantly, but without changing the circulating 25(OH)D concentrations (Kühn et al., 2016). It is tempting to speculate, that ergosterol as the vitamin D_2 precursor is also able to affect the vitamin D metabolism. However, it is not yet known, whether the dietary consumption of ergosterol modulates vitamin D absorption, storages and metabolism in the body. Although this is especially interesting, since ergosterol can not only be found in apparent food sources, like mushrooms and bakery products made with yeasts, but also in significant amounts in plant-based fat spreads, cheese and to a lesser amount in some meats and poultry (Seeburg, 2014). Moreover, plant oils can also contribute to a high dietary intake of ergosterol.

Historically, the biological activity of vitamin D_2 and vitamin D_3 was considered to be equal, since both forms are able to cure rickets (Park, 1940). Although, this was confirmed by some authors (Biancuzzo et al., 2010; Binkley et al., 2011; Holick et al., 2008), the equality of the two vitamin D forms in raising 25(OH)D levels is still somewhat controversial (Houghton and Vieth, 2006; Lanham-New et al., 2010). Today, vitamin D_2 is believed to be less efficient than vitamin D_3, as this has been shown by a series of studies (Armas et al., 2004; Glendenning et al., 2009; Heaney et al., 2011; Jakobsen et

al., 2017; Lehmann et al., 2013; Leventis and Kiely, 2009; Romagnoli et al., 2008; Shieh et al., 2016; Trang et al., 1998; Tripkovic et al., 2017), especially when given over a long period (Logan et al., 2013) or as a bolus (Binkley et al., 2011). Armas et al. showed that the potency of vitamin D_2 was less than one third of that of vitamin D_3 after a single dosage of 50 000 IU (or 1 250 µg) vitamin D in healthy males (Armas et al., 2004). Lehmann et al. who administered 50 µg vitamin D_2 or vitamin D_3 daily found higher total 25(OH)D (25(OH)D_2 + 25(OH)D_3) concentrations under vitamin D_3 than under vitamin D_2 supplementation (89.2 ± 22.1 nmol/L vs. 67.8 ± 20.1 nmol/L). This was due to a significant decline of the 25(OH)D_3 concentrations in response to vitamin D_2, which was more pronounced than in the placebo-treated control group for a, so far unknown, reason (Lehmann et al., 2013). It is possible that the differential responses in 25(OH)D after vitamin D_2 and vitamin D_3 supplementation are due to a differential intestinal absorption, hepatic hydroxylation, or renal activation or degradation. Differences in the metabolic routes of vitamin D_2 and vitamin D_3 would be best elucidated by analysis of the tissue distribution of vitamin D metabolites. However, since most available data regarding the bioavailability of the two forms are from human intervention studies, tissue data are scarce. It is therefore mandatory to investigate whether orally provided vitamin D_3 and vitamin D_2 are differently stored and metabolized.

2 Aims

Overall, this PhD-thesis aims to elucidate plant oils as potential sources of vitamin D by addressing the following questions:

(1) Are edible plant oils potential sources of vitamin D or vitamin D precursors?
(2) Is the UVB-treatment of plant oils a successful strategy to enhance the vitamin D concentration?
(3) Is vitamin D from UVB-exposed plant oils bioavailable?
(4) Is ergosterol a modulator of the vitamin D metabolism?
(5) Is there a difference in the metabolic routes of vitamin D_2 and vitamin D_3?

The questions (1) and (2) are addressed in **Study 1**, in which eight edible plant oils were selected and analyzed for their concentrations of vitamin D_2, vitamin D_3 and their precursors ergosterol and 7-DHC. Additionally, three selected oils were exposed to UVB-light to investigate whether the vitamin D precursors can be converted to vitamin D. To elucidate, whether the UVB-exposure was accompanied by detrimental effects on oil quality, parameters of autoxidation and organoleptic tests of an UVB-treated oil were also included in this study. Moreover, the susceptibility to thermal treatment and prolonged storage durations was considered, since cooking and storage have been shown to effect vitamin D concentrations in UVB-treated mushrooms. Question (3) is considered in both, **Study 1** and **Study 2**. First, the bioavailability of vitamin D from UVB-exposed wheat germ oil (WGO) in comparison to a vitamin D supplement was investigated in mice (**Study 1**). Later, the UVB-exposed WGO was examined for its ability to enhance 25(OH)D serum levels in vitamin D deficient, healthy volunteers in a randomized controlled intervention study (**Study 2**). As plant oils contain considerable quantities of ergosterol, the impact of this sterol on vitamin D metabolism was investigated (question (4)). Therefore, different concentrations of ergosterol were used in a mouse model and in human hepatoma cells in **Study 3**. Finally, possible differences in the metabolic routes of vitamin D_2 and vitamin D_3 were addressed (question (5)), since vitamin D_2 and vitamin D_3 have been shown to increase 25(OH)D differently by unknown reasons. Here, not only the activation, but also the cellular uptake and tissue distributions were examined in **Study 4** by use of a mice model and human and murine hepatoma cells. **Study 1, Study 3 and Study 4** were submitted to international journals and published in a peer-review process. **Study 2** is drafted as a manuscript and submitted for publication.

3 Original Research Articles

3.1 Study 1

Baur, A.C., Brandsch C., König, B., Hirche F., and Stangl, G.I. (2016). Plant Oils as Potential Sources of Vitamin D. *Frontiers in Nutrition* 3, 29.

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Plant Oils as Potential Sources of Vitamin D

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To combat vitamin D insufficiency in a population, reliable diet sources of vitamin D are required. The recommendations to consume more oily fish and the use of UVB-treated yeast are already applied strategies to address vitamin D insufficiency. This study aimed to elucidate the suitability of plant oils as an alternative vitamin D source. Therefore, plant oils that are commonly used in human nutrition were first analyzed for their content of vitamin D precursors and metabolites. Second, selected oils were exposed to a short-term UVB irradiation to stimulate the synthesis of vitamin D. Finally, to elucidate the efficacy of plant-derived vitamin D to improve the vitamin D status, we fed UVB-exposed wheat germ oil (WGO) for 4 weeks to mice and compared them with mice that received non-exposed or vitamin D_3 supplemented WGO. Sterol analysis revealed that the selected plant oils contained high amounts of not only ergosterol but also 7-dehydrocholesterol (7-DHC), with the highest concentrations found in WGO. Exposure to UVB irradiation resulted in a partial conversion of ergosterol and 7-DHC to vitamin D_2 and D_3 in these oils. Mice fed the UVB-exposed WGO were able to improve their vitamin D status as shown by the rise in the plasma concentration of 25-hydroxyvitamin D [25(OH)D] and the liver content of vitamin D compared with mice fed the non-exposed oil. However, the plasma concentration of 25(OH)D of mice fed the UVB-treated oil did not reach the values observed in the group fed the D_3 supplemented oil. It was striking that the intake of the UVB-exposed oil resulted in distinct accumulation of vitamin D_2 in the livers of these mice. In conclusion, plant oils, in particular WGO, contain considerable amounts of vitamin D precursors which can be converted to vitamin D *via* UVB exposure. However, the UVB-exposed WGO was less effective to improve the 25(OH)D plasma concentration than a supplementation with vitamin D_3.

Keywords: ergosterol, 7-dehydrocholesterol, vitamin D, plant oils, wheat germ oil, ultraviolet light irradiation, bioavailability, mice

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INTRODUCTION

Food sources of vitamin D are scarce. Although oily fish is considered to be a good source of vitamin D_3 (1, 2), its consumption and its vitamin D content is not high enough to significantly improve the vitamin D status of humans (3). Besides fish, mushrooms are often considered as another valuable source of vitamin D, in particular of vitamin D_2. However, the major natural vitamin D metabolite in fungi and yeast is the vitamin D precursor ergosterol, which can be converted to vitamin D_2

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; 7-DHC, 7-dehydrocholesterol; LLOQ, lower limit of quantification; WGO, wheat germ oil.

by UVB irradiation (4). The UVB-exposed baker's yeast, which has been approved by the European Food Safety Authority as a reliable ingredient to enrich bakery products with vitamin D, is a prominent example for a successful application of UVB irradiation to enhance vitamin D in natural foods (5). However, less data are available on vitamin D precursors and metabolites in plants. Yellow oat grass (*Trisetum flavescens*) is well described for its capability to synthesize bioactive vitamin D. It contains vitamin D glycosides which can be hydrolyzed in the gut or by the gastrointestinal microflora to the biologically active 1,25-dihydroxyvitamin D (6–8). Other so-called calcinogenic plants that contain active vitamin D forms are *Solanum malacoxylon*, *Cestrum diurnum*, and *Nierembergia veitchii* of the *Solanaceae* family (6–8). These plants are presumed to cause calcinosis in grazing animals due to the hypercalcemic effect of toxic 1,25-dihydroxyvitamin D levels (9). Vitamin D metabolites were also found in *Cucurbitaceae*, *Fabaceae*, and *Poaceae* (10–12). Besides that, certain plants are associated with fungal endophytes (13, 14) or are capable to produce the vitamin D_3 precursor 7-dehydrocholesterol (7-DHC) on its own *via* the lanosterol pathway (15). Based on these data, we hypothesized that plant oils could also contain vitamin D precursors or metabolites. The main aims of this investigation were [1] to identify and quantify precursors and metabolites of vitamin D in plant oils that are used in human nutrition and [2] to investigate whether a short-term exposure of selected oils to UVB light could increase their vitamin D content. To elucidate possible adverse effects of UVB exposure on the quality of the oils, we analyzed oxidative biomarkers and tested the sensory quality of the UVB-exposed oils. Additional tests were conducted to assess the stability of these vitamin D metabolites subsequent to thermal treatment and storage of the UVB-exposed oil. Finally, we aimed to elucidate the efficacy of plant-derived vitamin D to improve the vitamin D status by feeding an UVB-exposed plant oil to mice.

MATERIALS AND METHODS

Characterization of Vitamin D Metabolites in the Plant Oils

Avocado oil, linseed oil, olive oil, pumpkinseed oil, rapeseed oil, soya oil, sunflower oil, and wheat germ oil (WGO) were used to characterize and quantify their vitamin D precursors and metabolites. From each type of oil, three commercially available representatives were obtained from local supermarkets and used for the analyses. The oil samples selected for analyses were flushed with N_2 after the first opening, to avoid oxidation processes and stored at 4°C until further analyses.

UVB Exposure of Selected Oils

Rapeseed oil, avocado oil, and WGO were used for the UVB treatments and exposed to UVB light. In the first approach, aliquots of the three oils were placed into plastic vessels (thickness of the oil layer 1.0 mm) and exposed to UVB light for 0 (control), 4, and 8 min at room temperature. The UVB-emitting lamp (650 µW/cm_2, in a distance of 15 cm, UV-8M, Heroloab GmbH, Wiesloch, Germany) was placed 10 cm above the oil surface. During that treatment, the oils were flushed with N_2. In a second approach, WGO was used to investigate the impact of the oil layer thickness on the efficacy of vitamin D formation through UVB irradiation. Therefore, different volumes of the oil were filled in glass vessels to reach a layer thickness of either 1.6 or 3.2 mm, to be UVB-exposed for 10 min at room temperature. During that time, the oil samples were constantly stirred by a magnetic stirrer under N_2. The oil samples were stored at −20°C until analyses of vitamin D_2, vitamin D_3, and tocopherols. In addition to that, the peroxide and the acid values were analyzed in the 10 min UVB-exposed WGO and compared with those of the non-exposed oil of the same batch. The analyses were complemented by organoleptic tests. The UVB-treated oil (exposure time: 10 min, oil layer thickness: 3.2 mm) which was intended for use in the mouse study was analyzed for vitamin D metabolites and stored at −20°C until preparation of the diet. All diets were stored at −20°C until their administration.

Thermal Treatment and Storage of UVB-Exposed Wheat Germ Oil

To estimate the stability of the UVB-exposed oils, aliquots of the 10-min UVB-exposed WGO (1.6 mm layer) were (1) heated at 100 or 180°C for 10 min and (2) stored for 1 day, 2 weeks, or 4 weeks at room temperature in the dark. After the thermal treatment and storage terms, the WGOs were flushed with N_2 and stored at −20°C until further analysis. Aliquots of untreated oil samples of the same batch were used as a reference. Besides the concentration of the vitamin D metabolites, the concentration of tocopherols were analyzed to gain information about oxidation processes. The thermally treated and the 4 weeks-stored WGOs were analyzed to record the peroxide and the acid values and subjected to organoleptic tests.

Analysis of Vitamin D Metabolites in Oils

The concentrations of ergosterol, 7-DHC, vitamin D_2, and vitamin D_3 were analyzed by high performance liquid chromatography (HPLC) coupled with a tandem mass spectrometry system (LC-MS/MS), as described elsewhere (16). In brief, aliquots of the oils (300 mg) were mixed with internal deuterated standards (7-DHC-d_6, Chemaphor Incorporation, Ottawa, ON, Canada; vitamin D_2–d_3, vitamin D_3–d_3, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany). After an overnight hydrolysis, the D vitamers were extracted with *n*-hexane, washed with ultrapure water, solved in *n*-hexane/isopropanol (99/1, v/v), and fractionated using an Agilent 1100 HPLC-System with a LiChrospher Si 60 column (250 mm × 4.0 mm, 5 µm particle size; Agilent Technologies, Waldbronn, Germany) (17). Subsequent to drying the fractions, 100 µl of 4-phenyl-1,2,4-triazoline-3,5-dione (0.15 mg/ml acetonitrile, Sigma Aldrich) were added for derivatization (18). After evaporation of the solvent, the samples were resolved in methanol, mixed with 10 mM ammonium formate solution (4/1, v/v), and analyzed using HPLC (Agilent 1100) with a Hypersil ODS-column (150 mm × 2.0 mm, 5 µm particle size; VDS Optilab Chromatographie Technik GmbH, Berlin, Germany) coupled with a MS system (API 2000, Sciex, Darmstadt, Germany). Two mixtures with a gradient flow were used as mobile phase (A: 1 mM methylamine in acetonitrile; B: 1 mM methylamine and 5 mM ammonium formate in acetonitrile/ultrapure water, 1/1, v/v). External standards (ergosterol, 7-DHC, vitamin D_2, vitamin D_3, Sigma Aldrich) were used for calibration. The multiple reaction monitoring (MRM) was used

for quantification at the following mass transitions: ergosterol, 603 > 377; 7-DHC, 591 > 365; 7-DHC-d_6, 597 > 371; vitamin D_2, 603 > 298; vitamin D_2–d_3, 606 > 301; vitamin D_3, 591 > 298 and vitamin D_3–d_3, 594 > 301.

Except for the analysis of the vitamin D metabolites in rapeseed, avocado, and WGO after UVB exposure (1.0 mm layer), all analyzes were run in duplicate. The lower limit of quantification (LLOQ, signal-to-noise ratio = 10) in oils was 4.3 ng/g for all vitamin D metabolites. The coefficients of variation were 10.1% for ergosterol, 12.8% for 7-DHC, 5.9% for vitamin D_2, and 9.2% for vitamin D_3 ($n = 10$).

Analysis of Autoxidation Markers in Oils

The concentrations of the tocopherols were measured by a modified HPLC method of Coors (19). Prior to the quantification of the tocopherols, aliquots of the oils were solved in *n*-hexane (1/100, w/v), mixed thoroughly, and separated isocratically by HPLC (Agilent 1100) using a LiChrospher Si 60 column (250 mm × 4.0 mm, 5 µm particle size; Agilent Technologies). A mixture of *n*-hexane and isopropanol (99/1, v/v) was used as mobile phase (flow rate: 1 ml/min). The α-, β-, and γ-tocopherols were detected by a fluorescent detector (emission: 330 nm, excitation: 295 nm). External standards (α-, β-, γ-tocopherols, Supelco, Bellefonte, PA, USA) were used for calibration. The peroxide and the acid values of the oils were determined according to the German official methods (20, 21).

Organoleptic Characterization of the Oils

The UVB-exposed, the thermally treated, the 4 weeks-stored, and the -untreated WGOs were evaluated by a trained panel (ÖHMI Analytik GmbH, Magdeburg, Germany) in a blinded fashion. Taste, aroma, color, and transparency of the oils were judged at 40°C (22), and the oils were ranked according to its organoleptic quality (23).

Mouse Study

The experimental procedures described below followed the established guidelines for the care and handling of laboratory animals according to the National Research Council (24) and were approved by the local government (Landesverwaltungsamt Sachsen-Anhalt, Germany; approval number 42502-5-34). All mice were housed in pairs on a 12-h light, 12-h dark cycle in a room controlled for temperature (22 ± 2°C) and relative humidity (50–60%). Food and water were provided *ad libitum*.

Forty-two 4-week-old male mice (C57BL/6NCrl, Charles River Laboratories, Sulzfeld, Germany) were used. Five weeks prior to the actual treatment, the mice received a vitamin D-free semi-synthetic basal diet (20% casein, 20% sucrose, 38.8% starch, 10% WGO, 6% vitamin-mineral-mixture, 5% cellulose, and 0.2% DL-methionine) to reduce their vitamin D status. Except for the vitamin D, all other vitamins and minerals were supplemented according to the recommendations of the AIN (25). After the 5-week, six mice were sacrificed to determine the vitamin D status of these animals at baseline. The remaining 36 mice (mean body weight: 13.9 ± 0.8 g) were allotted to 3 groups of 12 mice each and fed the basal vitamin D-free diet with 10% of either the 10 min UVB-exposed WGO (3.2 mm-layer, WGO-UV), the untreated WGO, or WGO that was supplemented with synthetic vitamin D_3 (WGO-D_3) in comparable amounts to the total vitamin D content analyzed in the UVB-exposed oil. In the experimental diet fed to the WGO-UV group, a mean vitamin D_2 concentration of 87.3 µg/kg diet was measured, whereas no vitamin D_3 was found. The diet fed to the WGO-D_3 group had a mean analyzed vitamin D_3 concentration of 80.0 µg/kg and no vitamin D_2, while in the diet fed to the WGO group neither vitamin D_2 nor vitamin D_3 could be detected. The experimental diets were fed to the mice for 4 weeks. Individual body weights and mean food intake per cage were recorded weekly. Finally, the mice were sacrificed after a 4-h food deprivation under light anesthesia with diethyl ether. Blood was collected into heparin tubes (Sarstedt, Nümbrecht, Germany). Plasma was separated by centrifugation at 3000 × *g* at 4°C for 20 min and stored at −20°C until analysis of the vitamin D metabolites. The livers were harvested, immediately snap-frozen in liquid N_2, and stored at −80°C until analysis of the vitamin D metabolites.

Analysis of Vitamin D Metabolites in Plasma, Diet, and Liver

The plasma concentrations of ergosterol, 7-DHC, vitamin D_2, vitamin D_3, 25-hydroxyvitamin D [25(OH)D], in particular 25(OH)D_2 and 25(OH)D_3, were measured using LC-MS/MS (16). Plasma aliquots (100 µl) were mixed with potassium hydroxide, ascorbic acid, sodium sulfide, and internal standards (7-DHC-d_6, vitamin D_2–d_3, vitamin D_3–d_3, and 25(OH)D_3–d_6, Chemaphor Incorporation) and flushed with N_2. After incubation for 3 h at 37°C, followed by 17 h incubation at room temperature, the D vitamers were extracted two times with *n*-hexane and mixed with 4-phenyl-1,2,4-triazoline-3,5-dione for derivatization. Subsequently, the extracts were dried, resolved in methanol and 10 mM ammonium formate (4/1, v/v), and analyzed *via* LC-MS/MS as described before for the oils samples. Mass transitions for the hydroxylated metabolites were 25(OH)D_2, 619 > 298; 25(OH)D_3, 607 > 298; and 25(OH)D_3-d_6, 613 > 298. In plasma samples, the LLOQ of ergosterol, vitamin D_2, and vitamin D_3 was 1.25 nmol/l, that of 25(OH)D_2 and 25(OH)D_3 was 4.2 nmol/l.

The vitamin D_2 and D_3 concentrations in the diets and liver samples were analyzed as already described for the oil samples. The vitamin D_2 and vitamin D_3 concentrations of the diets were analyzed in aliquots of 1 g in triplicate. In the diets, the LLOQ for both vitamin D metabolites was 4.3 ng/g. Liver aliquots of 200 mg were analyzed for their concentrations of ergosterol, 7-DHC, vitamin D_2, vitamin D_3, 25(OH)D_2, and 25(OH)D_3. In the liver samples, the LLOQ was 5.0 ng/g for vitamin D_2, 10.5 ng/g for vitamin D_3, 0.3 ng/g for 25(OH)D_2, and 2.1 ng/g for 25(OH)D_3.

Analysis of Tocopherols in Plasma

To analyze the α-tocopherol concentrations in plasma, aliquots (30 µl) were mixed with pyrogallol solution (1% in ethanol, absolute) and saturated sodium hydroxide solution for hydrolysis. Subsequently, the samples were incubated at 70°C for 30 min, and tocopherols were extracted with *n*-hexane and ultrapure water. The supernatant was directly applied to the HPLC (26). HPLC conditions were the same as described for the tocopherol analysis of the oils.

Statistical Analysis

Data concerning the characterization of the plant oils were not subjected to statistical analysis. Values of the *in vivo* experiment are presented as means ± SD. If values were below the LLOQ, randomly generated values (between 0 and the appropriate LLOQ) were used for statistical treatment analyses. Statistical analyses were conducted using SPSS statistical software (SPSS 22, IBM; Armonk, NY, USA). All data were subjected to a normality test using the Shapiro–Wilk test. If the data followed a normal distribution, differences between the groups were analyzed by one-way analysis of variances (ANOVA), and subsequently subjected to the Levene's test for homoscedasticity. In case of homogeneity of variance, the three treatment groups were compared by the Tukey's test, in case of unequal variances by the Games–Howell test. If the data were not normal distributed, the Kruskal–Wallis test was used to analyze differences between the groups and the Mann–Whitney *U* test was conducted for *post hoc* comparisons of the three treatment groups (corrected by Bonferroni). Differences were considered to be significant at $P < 0.05$.

RESULTS

Vitamin D and Vitamin D Precursors in Selected Plant Oils

Eight commercially available plant oils for human nutrition were characterized for their vitamin D precursors and vitamin D contents. Analysis revealed that the concentrations of the vitamin D precursors ergosterol and 7-DHC varied strongly between the different oils, but all oils had a markedly higher concentration of ergosterol than of 7-DHC (**Figure 1**). The highest ergosterol concentration was found in the WGOs (22.1–34.2 µg/g) followed by the avocado oils (4.2–23.4 µg/g) and the sunflower oils (7.9–17.4 µg/g). Oils derived from rapeseed, soya, and linseed had lower ergosterol concentrations that ranged from 4.1 to 9.5 µg/g; the lowest concentrations were found in olive and pumpkinseed oils (<4.5 µg/g). Analyses revealed that the WGOs had the highest concentrations of 7-DHC (638–669 ng/g), while other oils had very low quantities of 7-DHC (**Figure 1**). The 7-DHC concentration in the linseed oils ranged between 71.7 and 97.5 ng/g; the other oils had 7-DHC concentrations between 10.7 and 47.9 ng/g. Vitamin D_2 and D_3 were not quantifiable in the eight analyzed plant oils.

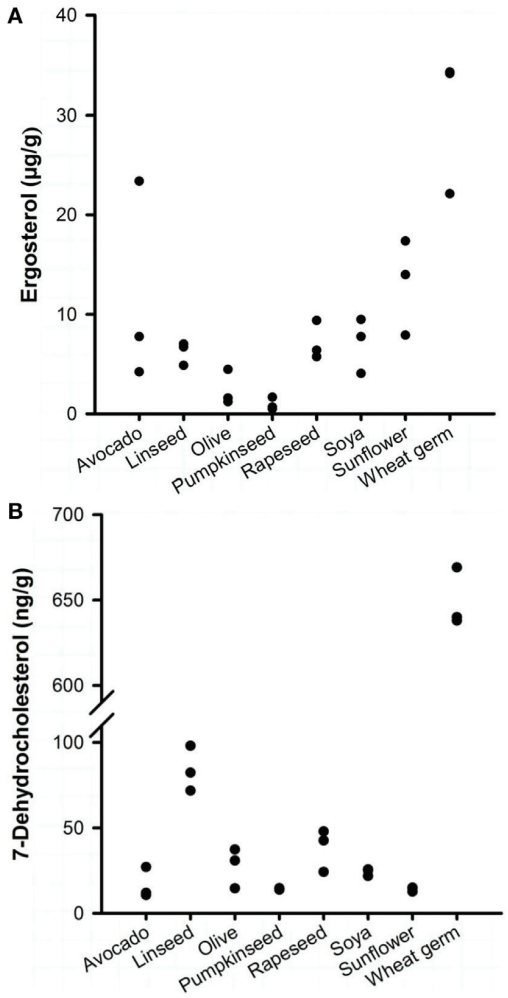

FIGURE 1 | Concentrations of (A) ergosterol and (B) 7-dehydrocholesterol of eight plant oils. Each dot represents one oil from one manufacturer. Analyses were run in duplicate.

Formation of Vitamin D in the UVB-Exposed Oils

To elucidate the impact of a short-term UVB irradiation on the formation of vitamin D in the plant oils, we exposed rapeseed oil, avocado oil, and WGO that differed widely in their amounts of vitamin D precursors to UVB light. The UVB exposure of rapeseed, avocado, and WGO increased the vitamin D concentrations in these oils in a time-dependent manner (**Figure 2**). The amount of vitamin D_2 produced by UVB irradiation was higher in the wheat germ and the avocado oil than in the rapeseed oil. The amount of the vitamin D_3 increased only in the WGO upon UVB exposure, but not in the rapeseed and the avocado oil, which was probably due to the higher 7-DHC concentration in the WGO (**Figure 2**).

The data further showed a significant impact of the layer thickness on the efficacy of the UVB exposure to increase the vitamin D content. The concentrations of vitamin D_2 and vitamin D_3 in the 1.6 mm-layer of WGO, which was UVB-exposed for 10 min, were 1035 and 37.0 ng/g, respectively. UVB-exposed WGO with a 3.2 mm-oil layer thickness had still high concentrations of vitamin D_2 and vitamin D_3, reaching 82 and 94% of the concentrations observed in the 1.6 mm-oil layer.

Changes in Quality Parameters of the Oils upon UVB Exposure

To elucidate the impact of the UVB treatment on the oil quality, the tocopherol concentrations and markers of autoxidation were measured in the UVB-exposed oils. The tocopherol concentrations of the 8-min UVB-exposed wheat germ and avocado oil (1.0 mm-layer) were not different from those of the untreated

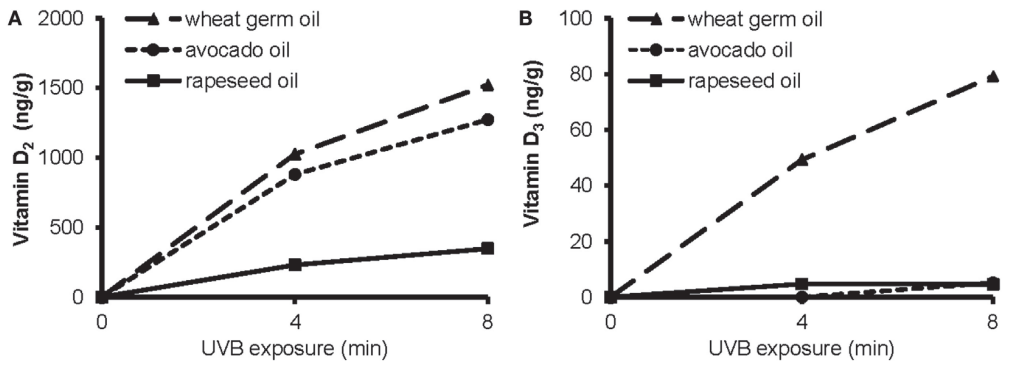

FIGURE 2 | Concentrations of (A) vitamin D_2 and (B) vitamin D_3 in wheat germ oil, avocado oil, and rapeseed oil before and after a 4 min- and an 8 min-UVB exposure. UVB exposure conditions: oil layer thickness, 1.0 mm; UVB lamp distance, 10 cm.

TABLE 1 | Influence of UVB exposure[a] on tocopherol concentrations of selected plant oils.

Oil	UVB exposure[a] time (min)	Tocopherols (mg/100 g)		
		α	β	γ
Rapeseed	0	19.1	0	36.8
	4	18.7	0	36.4
	8	16.8	0	36.0
Avocado	0	20.8	0.8	1.4
	4	20.0	0.6	1.3
	8	18.8	0.7	1.4
Wheat germ	0	140	51.1	14.0
	4	136	50.8	14.0
	8	136	51.5	11.3

[a]*UVB exposure conditions: oil layer thickness, 1.0 mm; UVB lamp distance, 10 cm.*

TABLE 2 | Influence of thermal treatment and storage on tocopherol concentrations, peroxide, and acid values of untreated and UVB-exposed[a] wheat germ oil.

Exposure	Treatment	Tocopherols (mg/100 g)			Peroxide value (mEq O_2/kg)	Acid value (g KOH/kg)
		α	β	γ		
–	–	165	62.9	8.1	7.0	10.3
UVB	–	166	64.3	8.2	5.0	10.4
–	100°C, 10 min	164	63.2	8.3	8.8	9.8
UVB	100°C, 10 min	163	63.8	8.1	7.1	10.8
–	180°C, 10 min	159	59.3	7.7	1.0	10.2
UVB	180°C, 10 min	160	59.5	7.7	1.0	9.5
–	1 day storage (RT)	166	65.0	8.2	n. a.	n. a.
UVB	1 day storage (RT)	166	63.8	8.1	n. a.	n. a.
–	2 weeks storage (RT)	178	68.0	8.5	n. a.	n. a.
UVB	2 weeks storage (RT)	163	63.0	7.9	n. a.	n. a.
–	4 weeks storage (RT)	163	63.4	8.1	20.6	10.0
UVB	4 weeks storage (RT)	160	61.8	7.8	21.4	10.1

[a]*UVB exposure conditions: exposure time, 10 min; oil layer thickness, 1.6 mm; UVB lamp distance, 13 cm.*
n. a., not analyzed; RT, room temperature.

oils (**Table 1**). In the rapeseed oil, a slight decrease in the α- and γ-tocopherol contents upon UVB exposure was observed. A 10-min-UVB exposure of WGO (1.6 mm-layer) had again no effect on the tocopherol concentrations, and also the peroxide and the acid value were not affected (**Table 2**). Organoleptic analyses revealed that the UVB-exposed WGO had a slightly more off-flavor than the non-exposed oil sample of the same batch (**Table 3**).

Susceptibility of UVB-Exposed Oils to Thermal Treatment and Storage

To elucidate the stability of a 10-min UVB-exposed WGO, we analyzed the concentrations of vitamin D metabolites and tocopherols, the peroxide and acid values, and the organoleptic quality after thermal treatment and after storage of the oil samples.

The heating of UVB-exposed WGO at 100°C for 10 min resulted in a 50% increase of vitamin D_2 ($\Delta = 521$ ng/g) and a 66% increase in the vitamin D_3 ($\Delta = 24.4$ ng/g) concentration compared with the non-heated UVB-exposed WGO. In contrast, heating the oil at 180°C for 10 min resulted in a slight reduction of the vitamin D_2 ($\Delta = -47.0$ ng/g) and vitamin D_3 ($\Delta = -2.8$ ng/g) concentrations (**Figure 3**). Thermal treatment of the UVB-exposed and untreated WGO at 100°C had no effect on the analyzed markers for oxidation, while a thermal treatment at 180°C resulted in a slight reduction of the tocopherol concentrations and in a decrease in the peroxide value; the acid value remained unchanged (**Table 2**). Thermal treatment also affected the taste of the oil: the higher the treatment temperature, the lower was the organoleptic quality. The UVB exposure *per se* had only a small effect on the taste when the oil was heated at 180°C (**Table 3**).

The storage of UVB-exposed oil at room temperature also resulted in a rise of the vitamin D_2 and the vitamin D_3 concentrations (**Figure 3**). The highest vitamin D_2 concentration was measured after the 2-week storage ($\Delta = 1157$ ng/g; **Figure 3A**). The vitamin D_3 concentration rose continuously during the 4-week storage and reached the highest values after

TABLE 3 | Influence of thermal treatment and storage on taste and aroma of UVB-exposed[a] and non-exposed wheat germ oil.

Exposure	Treatment	Taste[b]	Points[c]	Aroma[b]	Points[c]	Rank
–	–	Not quite neutral	4	Specific, neutral	5	1
UVB	–	Slightly rancid	3	Specific, neutral	5	2
–	100°C, 10 min	Slightly old	3	Specific, neutral	5	3
UVB	100°C, 10 min	Slightly old	3	Specific, neutral	5	4
–	180°C, 10 min	Rancid	2	Slightly dull, hay/ seed	3	7
UVB	180°C, 10 min	Rancid, scratchy	1	Slightly dull, hay/ seed	3	8
–	4 weeks storage (RT)	Slightly green	3	Specific, neutral	4	5
UVB	4 weeks storage (RT)	Slightly green	3	Specific, neutral	5	6

[a]UVB exposure conditions: exposure time, 10 min; oil layer thickness, 1.6 mm; UVB lamp distance, 13 cm.
[b]The oil samples were tempered to 40°C.
[c]Correspondent: 1, poor; 2, insufficient; 3, satisfactory; 4, good; 5, excellent.
RT, room temperature.

4 weeks (**Figure 3B**). The tocopherol concentrations in the UVB-exposed oil decreased slightly with the storage time; those of the untreated oil remained unchanged (**Table 2**). Both, the UVB-exposed and the -untreated oil showed increased peroxide values after the 4-week storage, no changes were observed for the acid values (**Table 2**). Organoleptic tests showed that the 4-week storage lead to deteriorated taste of the UVB-exposed and the -untreated oils, without showing any substantial difference between the UVB-exposed and the -untreated oil (**Table 3**). The aroma of the oils was not affected by the UVB exposure or the 4-week storage (**Table 3**).

Efficacy of the UVB-Exposed Wheat Germ Oil to Improve the Vitamin D Status of Mice

To evaluate the efficacy of UVB-exposed WGO to improve the vitamin D status, a feeding study with mice was conducted. The analyzed concentrations of vitamin D precursors and vitamin D in the WGO demonstrate that the applied UVB treatment was capable of increasing the vitamin D_2 and vitamin D_3 in this oil (**Table 4**). The tocopherol concentrations in the untreated and the UVB-exposed oils were comparable (**Table 4**). Mice of the three groups did not differ in their daily food intake (WGO: 3.03 ± 0.23 g, WGO-UV: 3.01 ± 0.14 g, and WGO-D_3: 3.11 ± 0.07 g) and final body mass (WGO: 31.5 ± 3.0 g, WGO-UV: 31.8 ± 2.9 g, and WGO-D_3: 32.1 ± 1.6 g).

Because any changes in 25(OH)D upon feeding vitamin D are usually becoming the higher the lower the vitamin D status is at baseline (27), all mice received a vitamin D-free diet 5 weeks prior to the treatment with the UVB-exposed or vitamin D_3-supplemented WGO. The plasma concentrations of total 25(OH)D [25(OH)D_2 + 25(OH)D_3] after feeding the vitamin D-free basal diet for 5 weeks was below the LLOQ ($n = 6$). Feeding mice the diet with UVB-exposed oil (WGO-UV) or with vitamin D_3 supplemented oil (WGO-D_3) for 4 weeks resulted in markedly higher plasma concentrations of total 25(OH)D compared with feeding the untreated WGO-based diet without any vitamin D supplementation (WGO) ($P < 0.001$). However, the increase in the total plasma concentration of 25(OH)D was stronger in the WGO-D_3 group than in the WGO-UV group ($P < 0.001$). The predominant form of the plasma 25(OH)D in the WGO-UV group was 25(OH)D_2; the predominant form in the WGO-D_3 group was 25(OH)D_3 (**Figure 4**). The plasma concentration of ergosterol was below the LLOQ in all groups of mice (**Table 5**). All mice had comparable plasma concentrations of 7-DHC. Mice from the WGO and WGO-D_3 groups had plasma concentrations of vitamin D_2 that were below the LLOQ, whereas mice from the WGO-UV group had more than 10-fold higher LLOQ values (**Table 5**). By contrast, the WGO-D_3 group showed a markedly higher plasma concentration of vitamin D_3 than the WGO-UV group ($P < 0.001$); the plasma concentration of vitamin D_3 in the WGO group was below the LLOQ. No differences between the three groups were observed in the plasma concentrations of α-tocopherol (**Table 5**).

Analysis of the D vitamer concentrations in the livers of the mice revealed no significant differences in the concentration of ergosterol (WGO: 12.0 ± 19.6 ng/g, WGO-UV: 3.92 ± 1.27 ng/g, and WGO-D_3: 5.40 ± 2.73 ng/g) and 7-DHC (WGO: 94.3 ± 31.6 ng/g, WGO-UV: 84.7 ± 16.5 ng/g, and WGO-D_3: 102 ± 46 ng/g). However, data showed distinct differences in the liver concentrations of vitamin D (**Figure 5**). Livers of mice from the WGO-UV group were characterized by extremely high vitamin D_2 concentrations and high levels of 25(OH)D_2, whereas the livers of mice from the WGO-D_3 group had significantly higher vitamin D_3 and 25(OH)D_3 concentrations than those of mice from the two other groups (**Figure 5**).

DISCUSSION

The presented studies demonstrated that plant oils contain high amounts of ergosterol, but comparatively low amounts of 7-DHC. It was striking that the ergosterol concentrations in the plant oils were on average 100 times higher than the 7-DHC concentrations. It is assumed that plants are *per se* not capable of producing ergosterol or vitamin D_2 (28), and that any of these metabolites are synthesized by endophytic fungi or by superficial fungal infections (13, 14, 29). Regarding 7-DHC, the analyses revealed 10 times higher concentration of this cholesterol precursor in the WGO than in the other oils. 7-DHC is an intermediate of the cholesterol synthesis pathway. It is well described that plants from the *Solanaceae*, *Fabaceae*, and *Poacaea* families are capable of producing cholesterol (30, 31), which is assumed to be used for the synthesis of glycoalkaloids and ecdysteroids (32, 33). The 7-DHC has also been proposed to function as an UV light protector (34), because the 7-DHC absorbs UVB irradiation that would otherwise damage the ribonucleic acids. The detectable amounts of 7-DHC in the linseed, rapeseed, and pumpkinseed oil suggest that cholesterol is also synthesized in plants from the *Linaceae*,

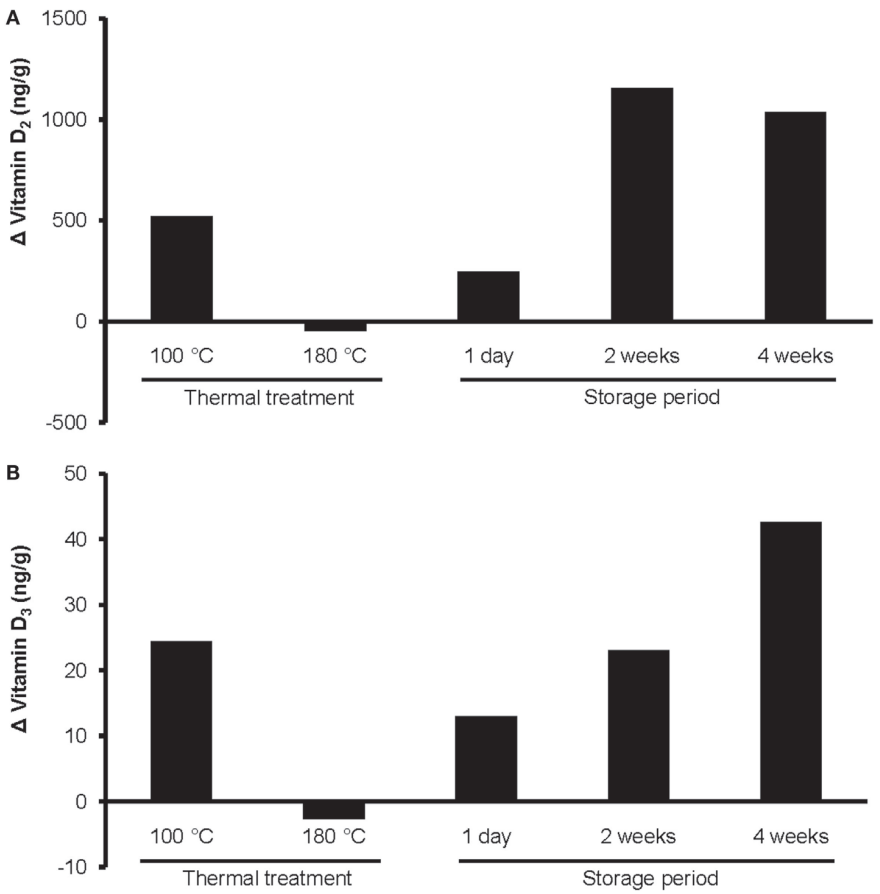

FIGURE 3 | Changes in the concentrations of (A) vitamin D_2 and (B) vitamin D_3 in UVB-exposed wheat germ oil after thermal treatment at 100 or 180°C for 10 min, and after a storage of 1 day, 2 weeks, or 4 weeks at room temperature in darkness. UVB exposure conditions: exposure time, 10 min; oil layer thickness, 1.6 mm; UVB lamp distance, 13 cm. Analyses were run in duplicate.

TABLE 4 | Characterization of the untreated and UVB-exposed wheat germ oils that were used in the mouse study.

	Wheat germ oil	
	Untreated	**UVB-exposed[a]**
Ergosterol (μg/g)	42.2	42.3
7-DHC (ng/g)	960	921
Vitamin D_2 (ng/g)	<LLOQ	850
Vitamin D_3 (ng/g)	<LLOQ	34.8
Tocopherols (mg/g)		
α	1.32	1.34
β	0.51	0.50
γ	0.12	0.15

[a]*UVB exposure conditions: exposure time,10 min; oil layer thickness, 3.2 mm; distance of the UVB-emitting lamp, 13 cm.*

7-DHC, 7-dehydrocholesterol; LLOQ, lower limit of quantification (4.3 ng/g).

Brassicaceae, and *Cucurbitaceae* families. However, in contrast to other researchers, who measured vitamin D in certain parts of the plant (12, 31, 35–37), we were not able to detect vitamin D in untreated plant oils.

The detection of vitamin D precursors in the plant oils prompted us to speculate that exposure of oils to UVB irradiation could convert ergosterol and 7-DHC into vitamin D_2 and vitamin D_3, respectively. Among the analyzed plant oils, the highest levels of vitamin D_2 and vitamin D_3 in response to an UVB irradiation were found in the WGO. After an 8-min exposure of thin-layered WGO, 1 g of this oil contained 1.5 μg vitamin D_2 and 0.08 μg vitamin D_3. We further found that the conversion rate of vitamin D precursors to vitamin D in the WGO was reduced by 40% if the oil layer thickness was increased from 1.0 to 3.2 mm. One gram of this thick-layered WGO provided in total a vitamin D content of 885 ng. With an average consumption of 12 g oil/day (38), a total of 10.6 μg vitamin D could be supplied by intake of UVB-exposed WGO, which matches 50% of the recommended daily vitamin D intake (1).

An interesting finding of this study was that the vitamin D content in the oils increased with the time of storage and a moderate thermal treatment. It is well described that the UVB photon converts the precursors, 7-DHC and ergosterol, to previtamin D which in turn isomerizes to vitamin D by a thermal

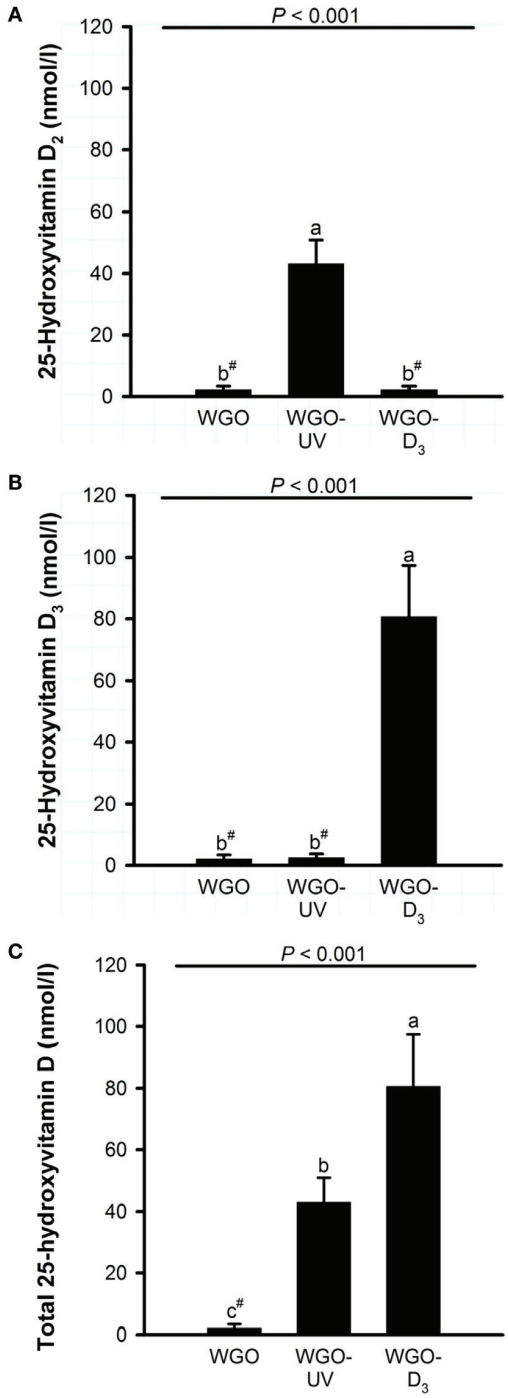

FIGURE 4 | Plasma concentrations of (A) 25-hydroxyvitamin D_2, (B) 25-hydroxyvitamin D_3, and (C) total 25-hydroxyvitamin D (25-hydroxyvitamin D_2 + 25-hydroxyvitamin D_3) of mice fed diets with 10% of either wheat germ oil (WGO), UVB-exposed wheat germ oil (WGO-UV), or wheat germ oil that was supplemented with vitamin D_3 (WGO-D_3) for 4 weeks. Data represent means ± SD, $n = 12$. [a–c]Means not sharing a letter are significantly different ($P < 0.05$, Mann–Whitney U test). [#]Values were below the lower limit of quantification (4.2 nmol/l).

TABLE 5 | Plasma concentrations of D vitamers and α-tocopherol of mice fed diets with UVB-treated (WGO-UV) and vitamin D_3-supplemented wheat germ oil (WGO-D_3) compared with those fed the diet with non-treated wheat germ oil (WGO) for 4 weeks.

Diet	WGO	WGO-UV	WGO-D_3	*P* values
Ergosterol (nmol/l)	<LLOQ	<LLOQ	<LLOQ	–
7-DHC (nmol/l)	85.9 ± 18.8	83.3 ± 18.2	90.2 ± 28.8	n. s.
Vitamin D_2 (nmol/l)	$0.55^{b,d}$ ± 0.38	16.8^{a} ± 2.5	$0.50^{b,d}$ ± 0.40	<0.001
Vitamin D_3 (nmol/l)	$0.36^{c,d}$ ± 0.28	1.44^{b} ± 1.34	20.5^{a} ± 3.9	<0.001
Total vitamin D (nmol/l)	$0.55^{c,d}$ ± 0.38	16.8^{b} ± 2.5	20.5^{a} ± 3.9	<0.001
α-Tocopherol (μg/ml)	27.8 ± 3.8	29.9 ± 9.9	28.1 ± 5.9	n. s.

Data represent means ± SD, n = 12.
WGO, 10% wheat germ oil; WGO-UV, 10% UVB-exposed wheat germ oil; WGO-D_3, 10% wheat germ oil supplemented with vitamin D_3; 7-DHC, 7-dehydrocholesterol; LLOQ, lower limit of quantification); n. s., not significant.
[a–c]Means not sharing a superscript letter are significantly different (P < 0.05, Mann–Whitney U test).
[d]Values were below the LLOQ (1.25 nmol/l).

reaction (34, 39). Therefore, we assume that the preformed pre-vitamin D can convert to vitamin D in conditions with absent UVB irradiation. Our data further indicate that taste and aroma, and also biomarkers that are indicative of autoxidation such as the tocopherol concentration, peroxides, and free acids were not significantly influenced by a short-term exposure of the plant oils to UVB irradiation. This makes the short-term UVB treatment of plant oils to a safe and reliable technique to produce vitamin D supplements.

To evaluate the efficiency of UVB-exposed plant oils to improve the vitamin D status *in vivo*, we conducted a study with mice that were fed diets with either UVB-exposed WGO, untreated WGO, or WGO with supplemented vitamin D_3. Here, we found that the UVB-exposed WGO is suitable to improve the vitamin D status of the mice as the group fed the UVB-exposed oil developed higher 25(OH)D plasma levels than the group fed the untreated oil. Compared with the group fed the vitamin D_3-supplemented WGO, the UVB-exposed oil was less effective in increasing the 25(OH)D plasma concentrations. However, it should be noted that the livers of mice that received the UVB-exposed WGO stored huge amounts of vitamin D_2 in comparison to that of mice fed the vitamin D_3 supplemented oil. The increased storage of hepatic vitamin D_2 in combination with the reduced plasma concentration of 25(OH)D_2 in the group fed the UVB-exposed oil suggests that vitamin D_2 is less appropriate as a substrate for hepatic hydroxylation than vitamin D_3. It has been a debate for many years whether both forms of vitamin D are bioequivalent. A series of studies has shown that vitamin D_2 does not increase 25(OH)D serum concentrations to the same amount as vitamin D_3 does (40–42). The current data confirm the different efficacy of both vitamin D isoforms. However, we cannot exclude at this stage, that photo-isomers that are produced by the UVB treatment may also impact the bioavailability of the vitamin D form in UVB-exposed oil.

To conclude, plant oils that are commonly used in human nutrition contain considerable quantities of ergosterol, but small amounts of 7-DHC. Among the different analyzed oils, WGO has the highest amounts of vitamin D precursors. A short-term UVB irradiation was successful in increasing the vitamin D content of

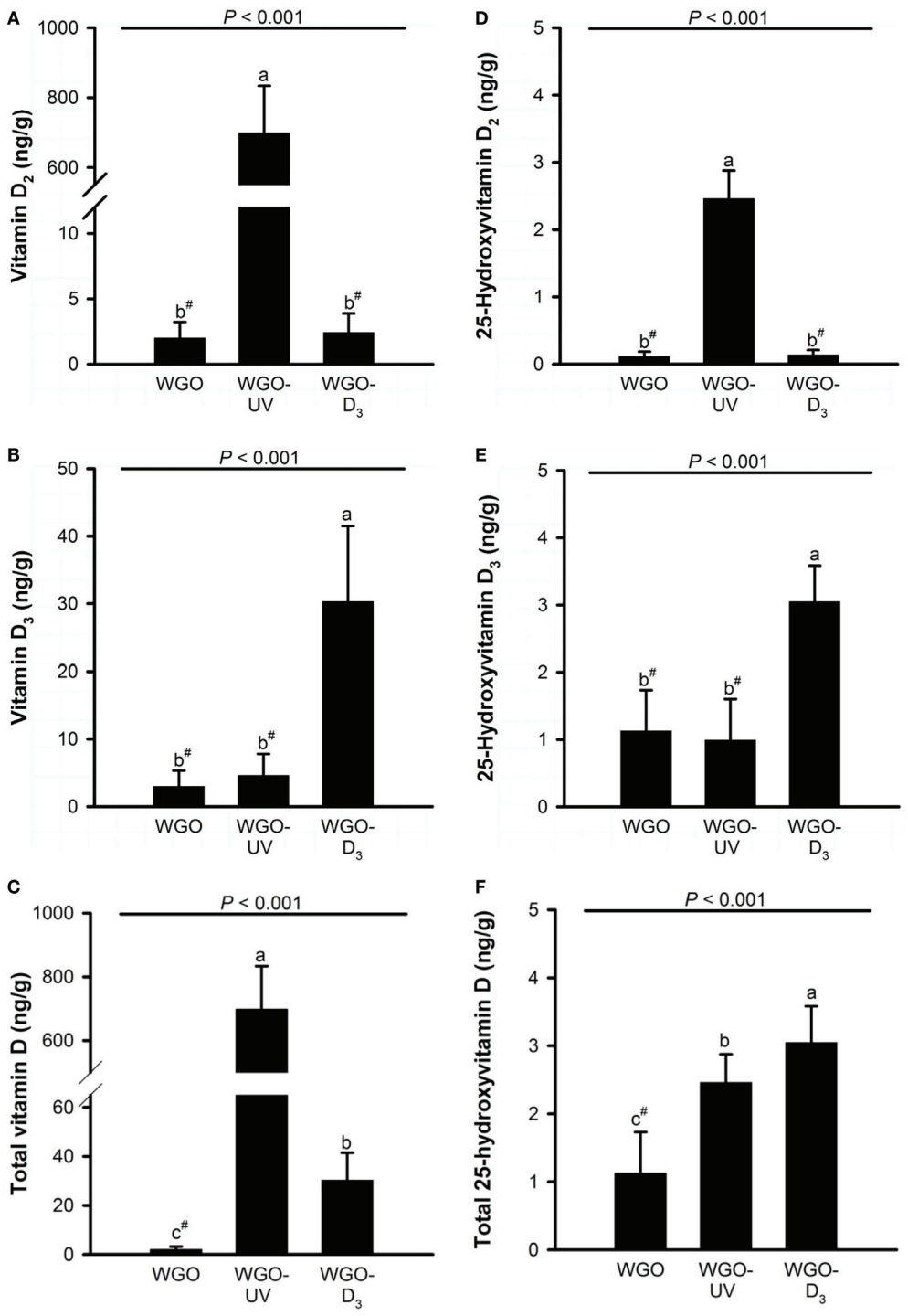

FIGURE 5 | Liver concentrations of (A) vitamin D_2, (B) vitamin D_3, (C) total vitamin D (vitamin D_2 + vitamin D_3), (D) 25-hydroxyvitamin D_2, (E) 25-hydroxyvitamin D_3, and (F) total 25-hydroxyvitamin D (25-hydroxyvitamin D_2 + 25-hydroxyvitamin D_3) in mice fed diets with 10% of either wheat germ oil (WGO), UVB-exposed wheat germ oil (WGO-UV) or wheat germ oil that was supplemented with vitamin D_3 (WGO-D_3) for 4 weeks. Data represent means ± SD, $n = 12$. $^{a-c}$Means not sharing a letter are significantly different ($P < 0.05$, Mann–Whitney U test for vitamin D_2, total vitamin D, 25-hydroxyvitamin D_2, and 25-hydroxyvitamin D_3; Games–Howell test for vitamin D_3; Tukey test for total 25-hydroxyvitamin D). $^{\#}$Values were below the lower limit of quantification (vitamin D_2, 5.0 ng/g; vitamin D_3, 10.5 ng/g; 25-hydroxyvitamin D_2, 0.3 ng/g; 25-hydroxyvitamin D_3, 2.1 ng/g).

the selected oils. The *in vivo* study has shown that UVB-exposed WGO can improve the vitamin D status, although less effective than vitamin D_3.

AUTHOR CONTRIBUTIONS

CB, BK, and GS conceived and designed the experiment. AB performed the experiment. AB and FH analyzed the data. AB, CB, and GS wrote the manuscript. BK and FH critically reviewed the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2 Study 2

Baur, A.C., Philipp, S., Staudt, S., Weidauer, T., Lorkowski, S., Stangl, G.I., and Dawczynski, C. (in Revision). UVB-exposed wheat germ oil increases serum concentrations of 25-hydroxyvitamin D_2 but decreases 25-hydroxyvitamin D_3 in humans. *European Journal of Nutrition*.

UVB-exposed wheat germ oil increases serum 25-hydroxyvitamin D_2 without improving overall vitamin D status – a randomized controlled trial

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Abstract

Purpose: This study investigated whether UVB-exposed wheat germ oil (WGO) is capable to improve the vitamin D status in healthy volunteers.

Methods: A randomized controlled human-intervention trial in parallel design was conducted in Jena (Germany) during the winter. Ultimately, 46 healthy males and females with insufficient vitamin D-status (mean 25-hydroxyvitamin D (25(OH)D), 34.9 ± 10.6 nmol/L) were randomized into three groups receiving either no oil (control, n = 14), 10 g non-exposed WGO per day (-UVB WGO, n = 16) or 10 g WGO, which was exposed for 10-minutes to ultraviolet B-light (UVB) and provided 23.7 µg vitamin D (22.9 µg vitamin D_2 and 0.89 µg vitamin D_3) (+UVB WGO, n = 16). The study period was 6 weeks and the participants visited at baseline, after 3 and 6 weeks. Blood was obtained for analysis of vitamin D metabolites via LC-MS/MS.

Results: Participants who received the UVB-exposed WGO were characterized by an increase of circulating 25(OH)D_2 after 3 and 6 weeks of intervention, which was accompanied by substantial decreases of 25(OH)D_3. Finally, the total 25(OH)D concentration (sum of 25(OH)D_2 and 25(OH)D_3) in the +UVB WGO group was moderately lower than that of the control group. In contrast, circulating vitamin D (vitamin D_2 + vitamin D_3) was higher in the +UVB WGO group than in the control group.

Conclusion: UVB-exposed WGO is not able to substantially improve the vitamin D status of vitamin D-insufficient subjects and is not recommended as a potential vitamin D source.

ClinicalTrials.gov: NCT03499327 (registered, April 13, 2018).

Keywords Vitamin D_2, vitamin D status, healthy subjects, wheat germ oil, UVB-exposure, randomized controlled trial

Abbreviations 25(OH)D, 25-hydroxyvitamin D; 7-DHC, 7-dehydrocholesterol; ANOVA, analysis of variance; FAME, fatty acid methyl esters; FFP, food frequency protocol; HDL, high-density lipoprotein; LA, linoleic acid; LDL, low-density lipoprotein; LOQ, limit of quantification; MUFA, monounsaturated fatty acid; PTH, parathyroid hormone; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TAG, triacylglycerols; TC, total cholesterol; UVB, ultraviolet B light; WGO, wheat germ oil.

1. Introduction

Vitamin D deficiency is widespread among the population worldwide [1, 2]. In times of inadequate endogenous synthesis, e.g. by absent ultraviolet B light (UVB) exposure, recommendations for intake are 15 to 20 µg vitamin D per day [3, 4]. Vitamin D occurs in two forms in nature, vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol), but food sources of vitamin D are scarce. Relevant amounts of vitamin D are only found in fatty fish [4]. Inefficient endocrine synthesis and inadequate intake of vitamin D result in suboptimal vitamin D status, which is assessed by the analysis of 25-hydroxyvitamin D (25(OH)D), a metabolite of vitamin D which is commonly used as status marker in serum or plasma [5]. Cut-off values indicating insufficient vitamin D status are discussed to be 50 nmol/L [4] or 75 nmol/L [6]. In the U.S. National Health and Nutrition Examination Survey (NHANES) study, the prevalence of individuals who have vitamin D concentrations below 50 nmol/L was 24 % [7]. In Europe, the prevalence of insufficient vitamin D concentrations is even higher and estimated to be 40.4 % [8]. The intake of vitamin D supplements is one option to combat vitamin D deficiency [9–11]. However, vitamin D supplements are not widely used and therefore are not suitable to improve vitamin D status in large populations [12]. New food sources of vitamin D could be a more efficient strategy to prevent vitamin D insufficiency. The exposure of foods such as yeast, edible mushrooms or milk to UVB light is a promising approach to increase the vitamin D concentrations in foods and diets [13–17]. Nowadays, UVB-exposed foods are commercially available and considered to be safe [17–19].

Plant oils have recently been discovered to be a potential source of vitamin D_2 and vitamin D_3 precursors, namely ergosterol and 7-dehydrocholesterol (7-DHC) [20]. In particular, wheat germ oil (WGO) showed relevant concentrations of ergosterol and 7-DHC, ranging from 22.1 – 34.2 µg/g and 0.638 – 0.669 µg/g, respectively. Following a 10 min-UVB-exposure, the vitamin D concentrations increased from non-detectable in the non-treated oil to 1035 ng/g vitamin D_2 and 37.0 ng/g vitamin D_3, respectively. It has been shown that the UVB-exposed WGO was able to significantly raise serum 25(OH)D concentrations in vitamin D-depleted mice [20]. However, data on the bioavailability of vitamin D from UVB-exposed WGO in humans are not yet available. The here presented intervention study aimed to elucidate the potential of UVB-exposed WGO in humans to improve their vitamin D status. The study was conducted in Jena (Germany, 51 °N) during winter time and the bioavailability of the vitamin D was assessed by measurements of 25(OH)D blood levels. Oxidation markers in the oils and blood levels of lipids and tocopherols served as safety markers or reference parameters to explain differences in plasma levels of vitamin D metabolites between the groups.

2. Materials and Methods

2.1. Study design and wheat germ oil

The study protocol has been approved by the Ethics Committee of the Friedrich Schiller University Jena (No. 5417-01/18). The study was registered at clinicaltrials.gov (NCT03499327).

The trial was conducted as a randomized controlled study in a three-armed parallel design during February, March and April 2018, when UVB irradiation in Jena and the surrounding region was low. The intervention period lasted six weeks, and the participants were scheduled to visit the study center at baseline and after three and six weeks. The participants received either no WGO (control, n = 14), non UVB-treated wheat germ oil (-UVB WGO, n = 17) or UVB-treated WGO (+UVB WGO, n = 17) and were instructed to consume 10 g of the respective oil per day (**Fig. 1**). The intervention was blinded (except for the control group which received no oil), and participants were not informed about the oil they received. All investigators and physicians were unaware of the group assignment.

The WGO was acquired commercially (vomFass, Waldburg, Germany) and UVB-treated at the Martin Luther University Halle-Wittenberg under food-safe conditions. Therefore, UVB-emitting lamps (UV-15M, Herolab, Wiesloch, Germany, analyzed intensity 500-630 µW/cm^2) were placed approximately 19 cm above the oil surface (diameter of the oil surface, 3.5 mm) for 10 min. During UVB-exposure, the oil was constantly stirred by a magnetic stirrer and flushed with nitrogen to avoid oxidation. The oil was UVB-exposed 2 weeks prior to the beginning of the study and had final vitamin D_2 and vitamin D_3 concentrations of 2.19 ± 0.36 µg/g and 0.08 ± 0.01 µg/g, respectively. With a consumption of 10 g oil per day, the participants met their recommended daily intake of vitamin D [4]. The non-exposed WGO was treated, except UVB exposure, in the same way as the UVB-exposed WGO and had vitamin D concentrations below the limit of quantification (LOQ). The participants were instructed to refrigerate the oil during the study period. They were also instructed to consume the oil pure or e.g., stirred in yogurt.

During each visit, anthropometric data and blood samples were collected for the determination of vitamin D metabolites (vitamin D_2, vitamin D_3, 25(OH)D_2, 25(OH)D_3), parathyroid hormone (PTH) (primary outcome measures), fatty acid distribution, lipids (triacylglycerols (TAG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol) and tocopherols (secondary outcome measures). The participants documented their normal nutritional habits over 7 days in a food frequency protocol (FFP, originated from PRODI® 5.4 software, Nutri Science, Freiburg, Germany) prior to the baseline and the 6-week visit.

115

116 *2.2. Subjects*

117 Male and female participants were recruited through newspaper advertisements, information in public institutions
118 and personal contacts in February 2018 in Jena (Germany). Inclusion criteria were: age between 20 and 70 years
119 and serum levels of 25(OH)D < 75 nmol/L. Exclusion criteria were: chronic diseases, medications, consumption
120 of supplements (e.g. vitamin supplements or fish oil capsules), relevant food allergies and visits of sun beds or
121 travel to areas with abundant UVB irradiation during or three months prior to the study.

122 Prior to the study, 69 participants were assessed for eligibility. A total of 48 participants (age range, 22 - 66 years)
123 met the inclusion criteria and were randomized in three groups (control, n = 14; -UVB WGO, n = 17; +UVB WGO,
124 n = 17; **Fig. 1**). The participants were individually allocated to one of the three study groups, generated by a
125 randomization list with a block size of 8. The allocation ratio of the study oil groups was 1:1.

126

2.3. Blood collection

After a 12-hour fasting period, blood samples were drawn by venipuncture into tubes (Sarstedt, Nümbrecht, Germany). For the analysis of vitamin D metabolites and tocopherols, serum was separated by centrifugation for 10 min at 2,000 × g. For the analysis of PTH, fatty acids and blood lipids, plasma was separated by centrifugation for 10 min at 1,300 × g and 4 °C and aliquoted. All samples were stored at -80 °C until further analysis.

2.4. Analytical methods

The concentration of vitamin D_2 and vitamin D_3 in WGO was analyzed via liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Sample preparation was in accordance with Baur et al. [20]. Analysis was conducted with a QTRAP 5500 MS-system with ESI^+ ionization (Sciex, Darmstadt, Germany) coupled to a reverse phase HPLC (Agilent 1200, Agilent Technologies, Waldbronn, Germany) equipped with a *Kinetex®* *Phenyl-Hexyl* column (100 x 2.1 mm, particle size: 2.6 µm, Phenomenex Incorporation, Torrance, CA, USA). The mobile phase consisted of (A) acetonitrile and (B) aqueous acetonitrile (1/1, v/v) with 5 mmol/L ammonium formate and 0.1 % formic acid and a gradient was used for separation (0.0 - 2.1 min, 85 % B; 2.1 - 7.0 min, 45 % B; 18.0 min, 35 % B; 22 min, 10 % B; 24 - 26 min, 0 % B; 28 min, 100 % B; 28.5 – 30 min, 85 %B) with a flow rate of 225 µL/min. MS settings and mass transitions have been reported before [21, 22]. The LOQ was 0.3 µg/g for vitamin D_2 and 0.03 µg/g for vitamin D_3. The fatty acid composition of the oils was performed using gas chromatography (GC-17 V3; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and an autosampler (AOC-5000), as described by Dawczynski et al. 2007 [23].

The concentrations of α-, β-, γ- and δ-tocopherol in the WGO were analyzed via LC with fluorescence detector as described before [20]. The acid value was determined according to the German official method [24]. An organoleptic characterization of the oils was conducted by a blinded panel (n = 3; ÖHMI Analytik, Magdeburg, Germany). Color, taste, flavor and viscosity were evaluated [25]. The taste of the oil was ranked by a blinded panel (n = 3; ÖHMI Analytik), whereby the best-tasting oil was given the lowest rank (1, 2 or 3) [26]. To rule out the sole influence of the storage period of the WGO, a fresh oil was included as a control.

To analyze the serum concentrations of vitamin D_2 and vitamin D_3, the samples were prepared in accordance to Baur et al. [21] and were analyzed by LC-MS/MS as described above for the WGO. The serum concentrations of $25(OH)D_2$ and $25(OH)D_3$ were analyzed via the commercially available *Mass Chrom® 25-OH Vitamin D_3/D_2 reagent kit for LC-MS/MS* (Chromsystems, Gräfelfing, Germany) by use of an Agilent 1200 HPLC system and a

156 QTRAP 5500 MS-System with APCI ionization. The LOQ was 0.25 nmol/L for vitamin D_2, 1.3 nmol/L for vitamin
157 D_3, 5.3 nmol/L for 25(OH)D_2 and 7.5 nmol/L for 25(OH)D_3.

158 The concentration of PTH in plasma was analyzed via ELISA according to the manufacturer's instruction
159 (*Immutopics Human Bioactive PTH 1-84,* TECOmedical, Sissach, Switzerland).

160 The fatty acid distribution in plasma lipids was analyzed according to Dawczynski et al. [27] by use of gas
161 chromatography with flame ionization detection (GC-17 V3, Shimadzu, Tokyo, Japan), equipped with a fused
162 silica capillary column (DB-225MS, 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm, Agilent
163 Technologies). Fatty acid concentrations were expressed as percentage of the total area of all fatty acid methyl
164 esters (% of total fatty acid methyl esters, FAME) using GC solution software version 2.3 (Shimadzu).

165 Plasma lipids (TAG, TC, HDL-cholesterol, LDL-cholesterol) were measured by using an Abbott Architect CI
166 16200 analyzer (Abbott, Wiesbaden, Germany) according to the manufacturer's recommendations.

167 High performance LC with fluorescent detection was used to analyze the concentration of α-tocopherol in serum
168 as described before [20].

169 All analyses were run in duplicate.

170

171 *2.5. Statistical analysis*

172 Sample size calculation was based on previously published data of Seibert et al. [28], who showed an increase in
173 25(OH)D concentrations from 38 ± 14 nmol/L to 70 ± 15 nmol/L (Δ25(OH)D, 32 nmol/L; 84 % rise) after 8 weeks
174 of supplementation with 20 µg vitamin D_3 daily during winter time. In contrast, 25(OH)D concentrations in the
175 placebo-treated control group decreased from 38 ± 15 nmol/L to 32 ±14 nmol/L. Initially assuming that a daily
176 consumption of 10 g UVB-treated WGO would provide 10 µg vitamin D per day, we hypothesized an increase of
177 42 % to approximately 54 nmol/L in the UVB WGO group. Thus, we calculated a sample size of 14 participants
178 per group with an effect size of 1.47, a 95 % power and a significance level of 0.05 (G*Power version 3.1.9.2). To
179 take potential dropouts in the WGO consuming groups into account, 17 participants were included in each WGO
180 group.

181 Statistical analyses were conducted using SPSS statistics version 24 (IBM, Armonk, NY, USA). For all statistical
182 tests $\alpha = 0.05$ was used to decide if the test result is significant or not. If values were below the LOQ, the appropriate
183 LOQ was used for statistical analysis. To compare the values of the three groups and the absolute changes between

baseline and week 6, the data were tested for normal distribution using the Shapiro-Wilk test. Given a normal distribution for all three groups, comparison was done with Welch’s one-way analysis of variance (ANOVA) test. Individual differences were investigated with Games-Howell test. Otherwise, the Kruskal-Wallis test was used and differences between individual groups were investigated using pairwise Mann-Whitney U tests with Bonferroni correction.

In case of normal distribution (Shapiro-Wilk test) and equal variances (Mauchly's sphericity test), time-depending differences within a group were compared by repeated measurement ANOVA with post-hoc comparison by Bonferroni. Otherwise, the Friedman test was used and the P values were corrected by Bonferroni.

3. Results

3.1. Characterization of the wheat germ oils

Since the vitamin D concentrations in UVB-treated WGO are known to increase during storage conditions [20], the concentration of vitamin D in WGO was assessed during the study period. In the non-treated WGO, the concentration of vitamin D (vitamin D_2 and vitamin D_3) remained below the LOQ during the whole study period. In the UVB-treated WGO, the vitamin D_2 concentration ranged from 2.06 to 2.70 µg/g (mean, 2.29 ± 0.18 µg/g, n = 9) and the vitamin D_3 concentration ranged from 0.079 to 0.099 µg/g (mean, 0.089 ± 0.008 µg/g, n = 9) (**Table 1**).

The composition of characteristic fatty acids was similar between the non-treated and UVB-exposed WGOs and did not change during the study period (**Table 1**). To elucidate, whether the UVB-exposure was accompanied by an increased oxidation of the fatty acids in WGO, the acid value and the concentrations of α-, β-, γ- and δ-tocopherol were analyzed at baseline and at the end of the study period. However, no obvious time- and treatment-dependent differences were observed (**Table 1**). Organoleptic tests revealed UVB-exposure induced changes in the flavor of the WGO, because the UVB-exposed WGO achieved the highest number of points, followed by the non-exposed WGO and the fresh WGO (**Table 1**).

3.2. Baseline characteristics

Forty-eight subjects were enrolled in this study. The baseline characteristics are given in **Table 2**. Two subjects did not complete the study, due to personal reasons (dropout rate 4.2 %). In total, 46 participants (age range, 22-65 years; 19 males/27 females) completed the 6-week-intervention (**Fig. 1**). The average baseline concentration of 25(OH)D was 35.5 ± 10.4 nmol/L, and therefore much lower than the aimed values of <75 nmol/L. After the study, the subjects were asked for their compliance in daily oil consumption. From the 32 participants which had to consume the oil daily, 11 subjects (+UVB WGO, n = 8; -UVB WGO, n = 4) admitted that they did not consume the oils during three or four days.

3.3. Nutrient intake assessed by food frequency protocols

The mean daily intake of energy, fat and PUFAs was higher in the two groups which received the WGO (data in **Supplementary Table S1**). The average daily vitamin D intake was 3.20 ± 2.73 µg vitamin D at baseline and 2.72 ± 2.24 µg at week 6. The vitamin D intake with the background diet (without the WGO) was not different between the groups at any time (**Table S1**).

3.4. Concentrations of vitamin D status markers

To elucidate the potential of UVB-exposed WGO to improve vitamin D status, the circulating serum concentrations of 25(OH)D were analyzed. At baseline, the $25(OH)D_2$ concentration was below the LOQ in all subjects except one (-UVB WGO group). In subjects treated with the +UVB WGO the concentration of $25(OH)D_2$ increased from baseline to week 3 and 6, respectively ($P < 0.001$ for both time points). The mean level of $25(OH)D_2$ in the control and -UVB WGO groups remained below the LOQ (**Table 3**). The concentration of $25(OH)D_3$ in the three groups was comparable at baseline and changed during the intervention. The $25(OH)D_3$ levels in the +UVB WGO group decreased steadily during the intervention period, resulting in significant lower levels after 3 and 6 weeks in the +UVB WGO group compared to both other groups ($P < 0.001$ for both time points, **Table 3**). In contrast, the control and the -UVB WGO group showed a moderate rise in their $25(OH)D_3$ concentrations over the study period ($P < 0.001$ for both groups). To elucidate the net effect of the UVB-exposed WGO on vitamin D status, the total 25(OH)D concentrations were calculated by summing up $25(OH)D_2$ and $25(OH)D_3$. Data show that the total 25(OH)D concentrations in the +UVB WGO group remained unchanged, while the concentration of total 25(OH)D moderately increased in the control and in the -UVB WGO groups over the study period ($P < 0.001$ for both groups; **Table 3**). Finally, the concentration of total 25(OH)D was lower in the +UVB WGO group than in the control group.

In comparison to the 25(OH)D levels, the vitamin D concentrations in serum were noticeably lower. Vitamin D analysis revealed that the circulating concentration of vitamin D_2 in the +UVB WGO group increased from baseline to week 6 of the study ($P < 0.001$), in contrast to the two other groups. Data on plasma vitamin D_3 showed a heterogenous picture. The concentration of vitamin D_3 rose from baseline to week 6 in the two WGO groups ($P < 0.001$ for both groups), but not in the control group (**Table 3**). No difference in the final plasma vitamin D_3 concentration was seen between the +UVB WGO group and the -UVB WGO group. Calculation of the total vitamin D (vitamin D_2 + vitamin D_3), revealed a time-dependent rise in in the -UVB WGO and in the +UVB WGO group ($P < 0.001$ for both groups), while the total vitamin D concentration in control group remained unchanged (**Table 3**). The absolute changes of the vitamin D metabolites after 6 weeks of intervention compared to baseline are given in **Table 4**.

To elucidate whether the vitamin D source affects the hydroxylation of vitamin D to 25(OH)D, we calculated the ratio of $25(OH)D_2$ to vitamin D_2, of $25(OH)D_3$ to vitamin D_3 and of total 25(OH)D to total vitamin D (**Table 5**). Hydroxylation of vitamin D_3, and as a result of total vitamin D, was reduced by the daily consumption of both WGOs. However, this effect was much more pronounced in the +UVB WGO than in the -UVB WGO group.

In contrast to vitamin D metabolites, the PTH concentrations did not differ between the groups at any time. After 3 weeks of intervention, the PTH concentrations were higher in the control and in the -UVB WGO groups compared to baseline ($P < 0.05$ and $P < 0.01$, respectively), but not in the + UVB WGO group. However, the PTH concentrations were finally not different after 6 weeks of intervention compared to baseline in all three groups (**Table 3**).

3.5. Concentrations of plasma lipids

To ensure that changes in blood vitamin D concentrations were not caused by changes in plasma lipids, we analyzed the fatty acid profile, and the plasma concentrations of TAGs and cholesterol. Data show no differences in the profiles of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) between the groups after the intervention (**Table 6**). Since WGO contains high concentrations of linoleic acid (LA), the concentration of LA was analyzed as compliance marker in plasma of the subjects at baseline and after 6 weeks. The plasma LA concentration in the -UVB WGO and the +UVB WGO groups increased from baseline to week 6 of the intervention (Δ baseline vs. week 6: +1.44 ± 2.61 and +1.71 ± 2.45 %, respectively; $P < 0.05$), while the LA concentration in the control group slightly but not statistically significant declined (Δ: -0.43 ± 2.22 %). Finally, the LA concentration did not differ between the three groups (**Table 6**). The concentrations of TAGs, total, HDL- and LDL-cholesterol and their ratio were similar between the groups ($P < 0.1$, **Table 6**).

To investigate whether the intake of UVB-treated WGO was accompanied by changes in plasma antioxidants, we analyzed α-tocopherols, but found no time- and group-specific differences (**Table 6**).

269 **Table 1** Characteristics of the wheat germ oils at baseline and at the end of the intervention period

	Control (Fresh WGO)		**-UVB WGO**		**+UVB WGO**	
	Baseline	End	Baseline	End	Baseline	End
Vitamin D, µg/g						
Vitamin D_2	n.a.		<0.3	<0.3	2.34	2.36
Vitamin D_3	n.a.		<0.03	<0.03	0.086	0.099
Total Vitamin D	-		-	-	2.43	2.46
Fatty acids, % FAME						
C16:0 palmitic acid	n.a.		15.2	15.1	15.2	15.1
C18:1 n-9 oleic acid	n.a.		16.5	16.3	16.6	16.2
C18:2 n-6 linoleic acid	n.a.		58.0	58.0	57.8	58.0
C18:3 n-3 α-linolenic acid	n.a.		6.24	6.27	6.26	6.23
Oxidative markers						
Acid value (g KOH/kg)	11.2	11.2	11.2	10.6	11.2	11.0
Tocopherols (mg/100 g)						
α	152	151	151	150	151	149
β	62.2	60.0	61.8	59.3	61.2	58.6
γ	19.8	20.2	20.2	20.6	20.6	20.4
δ	1.47	1.40	1.38	1.37	1.42	1.33
Organoleptic test[1]						
Color	Yellow/Orange, Slightly dull		Yellow/Orange, Slightly dull		Yellow/Orange, Slightly dull	
Aroma	Characteristic		Characteristic		Characteristic	
Flavor	Weak		Moderate		Strong	
Viscosity	Liquid, Slightly viscous		Liquid, Slightly viscous		Liquid, Slightly viscous	
Rank sum[2]	3		6		9	

270 Data are presented as means of duplicate measures (except for the organoleptic test). Fresh WGO, control wheat
271 germ oil; -UVB WGO, non-UVB exposed wheat germ oil; +UVB WGO, UVB-exposed wheat germ oil; n.a., not
272 analyzed; FAME, fatty acid methyl esters. [1] The organoleptic test was done five weeks after the UVB-exposure
273 of the oils by a blinded panel (n = 3). [2] Sum of given ranks (1,2 or 3) by a panel (n = 3), with the best-tasting oil
274 was given the lowest rank.

275

276 **Table 2** Characteristics of the study participants at baseline

Parameters	**Control (no WGO)**	**-UVB WGO**	**+UVB WGO**	***P*-Value[1]**
n	14	17	17	-
Age (years)	34 ± 12	34 ± 13	30 ± 5	0.868[1]
Sex (m/f)	3/11	8/9	8/9	-
Weight (kg)	66.9 ± 8.5	70.5 ± 9.0	71.4 ± 12.9	0.417[2]
Body mass index (kg/m^2)	23.4 ± 2.2	23.4 ± 2.6	22.7 ± 2.5	0.645[2]
Systolic blood pressure (mm Hg)	119 ± 11	124 ± 15	124 ± 16	0.464[2]
Diastolic blood pressure (mm Hg)	79.5 ± 7.0	80.5 ± 8.7	79.1 ± 12.7	0.918[2]
Pulse rate (beats per minute)	72.4 ± 11.8	64.1 ± 8.7	63.9 ± 13.2	0.088[2]

277 Data are presented as means ± SD. Participants consumed no wheat germ oil (Control), 10 g non-UVB-exposed
278 wheat germ oil (-UVB WGO) or UVB-exposed wheat germ oil (+UVB WGO) per day. Statistical analysis was
279 conducted by [1] Kruskal Wallis or [2] Welch's ANOVA.

280 **Table 3** Blood levels of vitamin D status markers at baseline and after 3 and 6 weeks of intervention

	Control (no WGO)	-UVB WGO	+UVB WGO	P-value
n	14	16	16	
25(OH)D_2, nmol/L				
Baseline	<LOQ	<LOQ	<LOQ^{z}	n.a.
3 weeks	<LOQ^{b}	<LOQ^{b}	11.6 ± 3.0$^{a, y}$	<0.001[1]
6 weeks	<LOQ^{b}	<LOQ^{b}	14.6 ± 3.9$^{a, x}$	<0.001[2]
Repeated measure analysis	n.a.	n.a.	<0.001[3]	
25(OH)D_3, nmol/L				
Baseline	32.7 ± 10.2^{y}	28.3 ± 11.5$^{z, y}$	29.9 ± 9.5^{x}	0.530[2]
3 weeks	33.2 ± 8.7$^{a, y}$	30.6 ± 13.0$^{a, y}$	21.7 ± 5.6$^{b, y}$	<0.001[2]
6 weeks	42.3 ± 10.5$^{a, x}$	37.7 ± 15.6$^{a, x}$	20.5 ± 6.1$^{b, y}$	<0.001[2]
Repeated measure analysis	<0.001[4]	<0.001[3]	<0.001[3]	
Total 25(OH)D, nmol/L				
Baseline	38.0 ± 10.2^{y}	33.6 ± 11.6^{y}	35.2 ± 9.5	0.535[2]
3 weeks	38.5 ± 8.7^{y}	35.9 ± 13.0^{y}	33.3 ± 5.7	0.177[2]
6 weeks	47.6 ± 10.5$^{a, x}$	43.0 ± 15.7$^{ab, x}$	35.1 ± 7.2^{b}	0.003[2]
Repeated measure analysis	<0.001[4]	<0.001[3]	0.829[3]	
Vitamin D_2, nmol/L				
Baseline	<LOQ	<LOQ	<LOQ^{y}	n.a.
3 weeks	<LOQ^{b}	<LOQ^{b}	1.46 ± 0.76$^{a, x}$	<0.001[1]
6 weeks	<LOQ^{b}	<LOQ^{b}	1.58 ± 1.05$^{a, x}$	<0.001[1]
Repeated measure analysis	n.a.	n.a.	<0.001[4]	
Vitamin D_3, nmol/L				
Baseline	1.75 ± 1.22	1.35 ± 0.19^{y}	1.85 ± 1.36^{y}	0.235[1]
3 weeks	3.05 ± 2.60	2.29 ± 2.69^{y}	2.12 ± 0.87^{y}	0.220[1]
6 weeks	2.24 ± 1.05^{b}	5.29 ± 5.51$^{ab, x}$	5.00 ± 2.44$^{a, x}$	0.002[1]
Repeated measure analysis	0.164[3]	<0.001[3]	<0.001[4]	
Total vitamin D, nmol/L				
Baseline	2.00 ± 1.22	1.60 ± 0.19^{y}	2.10 ± 1.36^{z}	0.235[1]
3 weeks	3.30 ± 2.60$^{ab, xy}$	2.54 ± 2.69$^{a, y}$	3.57 ± 1.23$^{b, y}$	<0.001[1]
6 weeks	2.49 ± 1.05$^{b, y}$	5.54 ± 5.51$^{ab, x}$	6.58 ± 2.65$^{a, x}$	<0.001[1]
Repeated measure analysis	0.164[3]	<0.001[3]	<0.001[3]	
Parathyroid hormone, pmol/L				
Baseline	4.06 ± 4.22^{y}	5.87 ± 6.34^{y}	4.26 ± 2.71	0.402[1]
3 weeks	5.29 ± 6.72^{x}	7.67 ± 10.6^{x}	4.33 ± 2.06	0.159[1]
6 weeks	4.71 ± 5.17^{xy}	5.64 ± 5.99^{y}	5.61 ± 6.88	0.811[1]
Repeated measure analysis	0.046[3]	0.009[3]	0.144[3]	

281 Data are presented as means ± SD. Participants consumed no wheat germ oil (Control), 10 g non-UVB-exposed
282 wheat germ oil (-UVB WGO) or UVB-exposed wheat germ oil (+UVB WGO) per day. Differences between the
283 groups were compared by [1] Kruskal Wallis test or [2] Welch's ANOVA. ^{abc} Different superscript letters indicate
284 significant differences between the groups ($P < 0.05$). Differences between the appointment times were compared
285 by [3] Friedman test or [4] ANOVA. ^{xyz} Different superscript letters indicate significant differences between the
286 appointment times ($P < 0.05$). LOQ, limit of quantification (25(OH)D_2, 5.3 nmol/L; vitamin D_2, 0.25 nmol/L);
287 25(OH)D, 25-hydroxyvitamin D; total 25(OH)D, sum of 25(OH)D_2 and 25(OH)D_3; total vitamin D, sum of
288 vitamin D_2 and vitamin D_3; n.a., not analyzed.

289 **Table 4** Changes in the serum concentrations of hydroxylated and non-hydroxylated vitamin D metabolites after 6 weeks of intervention compared to baseline

	Control	-UVB WGO	+UVB WGO	P-value
n	14	16	16	
Δ 25(OH)D_2 to baseline, nmol/L	n.a.	n.a.	+9.27 ± 3.9	<0.001
Δ 25(OH)D_3 to baseline, nmol/L	+9.55 ± 6.84[a]	+9.43 ± 6.09[a]	-9.40 ± 8.48[b]	<0.001
Δ Total 25(OH)D to baseline, nmol/L	+9.55 ± 6.84[a]	+9.44 ± 6.10[a]	-0.13 ± 11.2[b]	0.015
Δ Vitamin D_2 to baseline, nmol/L	n.a.	n.a.	+1.33 ± 1.05	<0.001
Δ Vitamin D_3 to baseline, nmol/L	+0.49 ± 1.15[a]	+3.93 ± 5.43[b]	+3.15 ± 2.77[b]	0.006
Δ Total vitamin D to baseline, nmol/L	+0.49 ± 1.15[a]	+3.93 ±5.43[b]	+4.48 ± 3.06[b]	0.001

290 Participants consumed no wheat germ oil (control), 10 g non-UVB-exposed wheat germ oil (-UVB WGO) or UVB-exposed wheat germ oil (+UVB WGO) per day. Presented are
291 means ± SD. 25(OH)D, 25-hydroxyvitamin D; n.a., not analyzed. Different superscript letters indicate significant differences between the treatment groups ($P < 0.05$).

292 **Table 5** Ratio of 25(OH)D to vitamin D at baseline and after 3 and 6 weeks of intervention

	Control (no WGO)	-UVB WGO	+UVB WGO	P-value
n	14	16	16	
Ratio 25(OH)D_2 to vitamin D_2				
Baseline	n.a.	n.a.	n.a.^{y}	-
3 weeks	n.a.^{b}	n.a.^{b}	9.7 ± 4.6$^{a, x}$	<0.001[1]
6 weeks	n.a.^{b}	n.a.^{b}	12.5 ± 6.9$^{a, x}$	<0.001[1]
Repeated measure analysis	-	-	<0.001[3]	
Ratio 25(OH)D_3 to vitamin D_3				
Baseline	22.4 ± 8.9	20.7 ± 7.8^{x}	19.6 ± 8.6^{x}	0.708[2]
3 weeks	18.1 ± 10.6^{ab}	19.0 ± 9.1$^{a, x}$	11.3 ± 3.7$^{b, x}$	0.006[2]
6 weeks	21.0 ± 6.4^{a}	12.5 ± 8.2$^{b, y}$	4.6 ± 1.5$^{c, y}$	<0.001[2]
Repeated measure analysis	0.420[4]	0.005[4]	<0.001[3]	
Ratio total 25(OH)D to total vitamin D				
Baseline	22.0 ± 7.9	20.8 ± 6.5^{x}	19.7 ± 7.6^{x}	0.718[2]
3 weeks	17.9 ± 9.7^{a}	19.1 ± 8.1$^{a, xy}$	10.2 ± 3.4$^{b, x}$	<0.001[2]
6 weeks	20.8 ± 5.6^{a}	13.0 ± 7.9$^{b, y}$	6.0 ± 2.0$^{c, y}$	<0.001[2]
Repeated measure analysis	0.374[4]	0.004[4]	<0.001[3]	

293 Data are presented as means ± SD. Participants consumed no wheat germ oil (control), 10 g non-UVB-exposed
294 wheat germ oil (-UVB WGO) or UVB-exposed wheat germ oil (+UVB WGO) per day. Differences between the
295 groups were compared by [1] Kruskal Wallis test or [2] Welch's ANOVA. [abc] Different superscript letters indicate
296 significant differences between the groups ($P < 0.05$). Differences between the appointment times were compared
297 by [3] Friedman test or [4] ANOVA. [xyz] Different superscript letters indicate significant differences between the
298 appointment times ($P < 0.05$). 25(OH)D, 25-hydroxyvitamin D; total 25(OH)D, sum of 25(OH)D_2 and 25(OH)D_3;
299 total vitamin D, sum of vitamin D_2 and vitamin D_3; na, not analyzed.

300 **Table 6** Concentrations of fatty acids, lipids, and tocopherol in plasma

	Control (no oil)	-UVB-WGO	+UVB-WGO	P-value
n	14	16	16	
Σ SFA, % FAME				
Baseline	29.9 ± 2.1[ab]	28.6 ± 2.0[b]	30.7 ± 1.9[a]	0.020[1]
6 weeks	28.4 ± 2.2	28.9 ± 2.3	28.8 ± 3.0	0.675[2]
Σ MUFA, % FAME				
Baseline	25.4 ± 2.9	26.2 ± 2.7	24.9 ± 2.3	0.385[1]
6 weeks	25.6 ± 3.7	25.0 ± 2.2	24.5 ± 2.3	0.630[1]
Σ PUFA, % FAME				
Baseline	41.6 ± 4.2	41.9 ± 3.2	41.1 ± 3.3	0.811[1]
6 weeks	42.8 ± 5.3	43.3 ± 2.6	43.9 ± 3.7	0.801[1]
Linoleic acid, % FAME				
Baseline	29.4 ± 3.7	30.5 ± 3.1	30.2 ± 3.7	0.664[1]
6 weeks	29.0 ± 4.2	32.0 ± 2.7	31.9 ± 3.6	0.076[1]
Triacylglycerols, mmol/L				
Baseline	0.98 ± 0.42	1.02 ± 0.44	0.93 ± 0.30	0.974[2]
6 weeks	1.09 ± 0.71	1.00 ± 0.46	0.95 ± 0.31	0.962[2]
Total cholesterol, mmol/L				
Baseline	4.95 ± 1.08	4.97 ± 1.02	4.59 ± 0.72	0.630[2]
6 weeks	4.99 ±1.12	4.65 ± 1.10	4.50 ± 0.65	0.732[2]
HDL-cholesterol, mmol/L				
Baseline	1.61 ± 0.38	1.51 ± 0.24	1.62 ± 0.34	0.543[1]
6 weeks	1.72 ± 0.42	1.47 ± 0.27	1.61 ± 0.31	0.134[1]
LDL-cholesterol, mmol/L				
Baseline	2.78 ± 0.82	3.02 ± 0.94	2.61 ± 0.69	0.399[1]
6 weeks	2.74 ± 0.86	2.79 ± 0.94	2.51 ± 0.57	0.531[1]
LDL-cholesterol-/HDL-cholesterol-ratio				
Baseline	1.83 ± 0.69	2.04 ± 0.70	1.71 ± 0.62	0.414[2]
6 weeks	1.69 ± 0.74	1.97 ± 0.83	1.65 ± 0.57	0.388[2]
α–Tocopherol, µg/mL				
Baseline	11.0 ± 3.7	10.7 ± 2.3	9.54 ± 2.1	0.235[1]
6 weeks	10.7 ± 2.5	11.1 ± 3.2	10.3 ± 1.8	0.669[1]

301 Data are presented as means ± SD. Participants consumed no wheat germ oil (Control), 10 g non-UVB-exposed
302 wheat germ oil (-UVB WGO) or UVB-exposed wheat germ oil (+UVB WGO) per day. SFA, saturated fatty acids;
303 MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; FAME, fatty acid methyl esters; HDL,
304 high-density lipoprotein; LDL, low density lipoprotein. Differences between the groups were compared by [1]
305 Welch's ANOVA or [2] Kruskal Wallis test. [ab] Different superscript letters indicate significant differences between
306 the groups ($P < 0.05$).

Supplementary Table S1 Daily intake of nutrients in the background diet as evaluated by the food frequency protocols 7-days prior to baseline and week 6

	Control (no oil)	-UVB-WGO	+UVB-WGO	P-value
n	14	16	16	
Energy (kcal)				
Baseline	2019 ± 883	3045 ± 2844	2209 ± 633	0.138[1]
6 weeks	1850 ± 461[b]	2320 ± 714[a]	2253 ± 438[a]	0.034[1]
Carbohydrates (g)				
Baseline	242 ± 126	338 ± 315	223 ± 53	0.110[1]
6 weeks	204 ± 70	240 ± 69	236 ± 44	0.310[2]
Protein (g)				
Baseline	75.6 ± 26.3	120 ± 131	80.3 ± 27.7	0.198[1]
6 weeks	70.5 ± 16.1	85.4 ± 35.2	79.0 ± 16.2	0.449[1]
Fat (g)				
Baseline	69.8 ± 31.7[a]	114 ± 101[ab]	96.9 ± 41.3[b]	0.025[1]
6 weeks	70.2 ± 23.2[b]	100 ± 40[a]	94.2 ± 20.0[a]	0.013[1]
SFA (g)				
Baseline	27.5 ± 11.6[b]	48.7 ± 44.0[ab]	42.0 ± 18.1[a]	0.016[1]
6 weeks	30.2 ± 9.6	39.5 ± 14.2	37.6 ± 9.0	0.115[1]
MUFA (g)				
Baseline	21.7 ± 11.0[b]	37.2± 29.0[a]	33.5 ± 16.3[a]	0.008[1]
6 weeks	24.2 ± 9.3	32.2 ± 14.9	31.2 ± 9.2	0.146[1]
PUFA (g)				
Baseline	11.9 ± 8.0	15.6 ± 12.4	12.6 ± 7.4	0.403[1]
6 weeks	10.7 ± 5.1[b]	21.6 ± 9.9[a]	18.7 ± 4.7[a]	<0.001[1]
Vitamin D (µg)				
Baseline	3.36 ± 2.39	3.20 ± 3.16	3.07 ± 2.72	0.752[1]
6 weeks	2.12 ± 1.26	3.20 ± 3.03	2.77 ± 1.98	0.518[1]

Data are presented as means ± SD. Participants consumed no wheat germ oil (Control), 10 g non-UVB-exposed wheat germ oil (-UVB WGO) or UVB-exposed wheat germ oil (+UVB WGO) per day. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Differences between the groups were compared by [1] Kruskal Wallis test or [2] Welch's ANOVA. [ab] Different superscript letters indicate significant differences between the groups.

4. Discussion

This study investigated the efficacy of UVB-treated WGO to improve the vitamin D status of healthy subjects during the wintertime, when the endogenous vitamin D synthesis is reduced. Firstly, we were able to markedly increase the vitamin D content of WGO by the exposure of this oil to UVB light. The resulting vitamin D content of WGO, which mainly comprised vitamin D_2, amounted to 2.37 ± 0.16 µg/g (n = 9), so that a daily intake of 10 g UVB-treated WGO provided 23.7 µg vitamin D. This intake can be considered as safe, although it is moderately higher (1.6-times) than the recommended daily intake for vitamin D [4]. In addition, the unchanged concentrations of circulating α-tocopherols are not indicative of any oxidative stress associated with the consumption of UVB-treated WGO. However, the UVB-exposure did negatively affect the taste of the oil, as shown by the organoleptic tests.

The major finding of the current study was that UVB-exposed WGO leads to an increase of the plasma levels of 25(OH)D_2, without improving the total 25(OH)D concentrations. The latter resulted from the finding that the treatment with UVB-exposed WGO had lowered 25(OH)D_3 disproportionately stronger than the treatment with the unexposed WGO. There may be multiple reasons for the strong decline in 25(OH)D_3 after the consumption of UVB-treated WGO. Firstly, a few human studies which compared the efficacy of vitamin D_2 and vitamin D_3 to increase 25(OH)D, also found a strong reduction of 25(OH)D_3 in vitamin D_2 treated groups that was stronger than in groups that received no vitamin D [9, 29–31]. Although both isoforms of vitamin D are considered equally in the treatment of rickets [32], Lehman et al. found substantially lower levels of 25(OH)D in the group supplemented with vitamin D_2 than in the vitamin D_3 supplemented group [9]. The lower 25(OH)D levels in the vitamin D_2 group were caused by a marked decline in 25(OH)D_3 in comparison to the vitamin D_3 group. A phenomenon, which has also been demonstrated vice versa [33]. Although the reason for the strong decline of 25(OH)D_3 concentrations in response to vitamin D_2 from the UVB-exposed WGO in the present study remains unclear, the data are indicative for a reduced hepatic hydroxylation of vitamin D. This could be due to a competition of vitamin D_2 and vitamin D_3 for 25-hydroxylase. Alternatively, the degradation of 25(OH)D_3 as a result of an upregulated expression of catabolic enzymes by vitamin D_2 could be enhanced. So far, three enzymes of the cytochrome P450 family (CYP) are known to 25-hydroxylate vitamin D in the liver. While CYP2R1 can hydroxylate both vitamin D isoforms at C-position 25 [34], CYP27A1 is capable of hydroxylating only vitamin D_3 [35] and CYP3A4 only vitamin D_2 [36]. The latter is also known to degrade vitamin D_3, by mono-hydroxylation of 25(OH)D_3 at several other positions, including C-positions 23, 24, 26 and in particular C4 [37]. Thus, we speculate that the vitamin D_2 and D_3 can activate the various hydroxylases in different ways, thereby influencing the 25(OH)D_2 and 25(OH)D_3

profile. Secondly, the UVB treatment of WGO could have resulted in the formation of vitamin D photoisomers such as lumisterol or tachysterol, which in turn may affect the metabolism of the vitamin D_3 isoforms. In this regard, UVB-treated mushrooms (providing 17.1 µg vitamin D_2) were also not able to increase total 25(OH)D levels, since the 25(OH)D_3 concentrations decreased by 10.3 ± 1.75 nmol/L after 3 weeks and by 20.6 ± 14.6 nmol/L after 6 weeks of intervention [38]. By that, the decrease of 25(OH)D_3 was higher as the decrease in the current study, which was 8.27 ± 6.19 nmol/L and 9.40 ± 8.48 nmol/L after 3 and 6 weeks, respectively. Recently, oral intake of lumisterol has been shown to markedly reduce the circulating 25(OH)D_3 concentrations in mice [39]. Lumisterol, which is formed during UVB-exposure, is likely to be present in the UVB-exposed WGO. Although we did not quantify photoisomers in the oil, randomly selected plasma samples of the +UVB WGO-treated subjects did not show measurable lumisterol concentrations of lumisterol or tachysterol (data not shown). Thus, we assume that photoisomers formed during the UVB treatment of the WGO were not responsible for the strong decline in 25(OH)D_3. Thirdly, it is possible that small amounts of vitamin D_3 and 25(OH)D_3 were synthesized endogenously in the late intervention period, and we cannot exclude that the production was accidentally higher in the -UVB WGO group than in the +UVB WGO group.

Because the reduced formation of 25(OH)D_3 from vitamin D_3 would have resulted in an accumulation of vitamin D_3, we analyzed the plasma level of this D vitamer, but found no differences between the +UVB WGO and the -UVB WGO group (**Table 3**). Compared to the control group, the total vitamin D concentrations were higher in the two groups, which received the WGO, although this was only statistically significant in the +UVB-WGO group. This could be due to high concentrations of ergosterol in the WGO, which has been shown before to increase the vitamin D concentrations in circulation and tissue storages [21].

The unequal gender distribution in the control group (males, 3; females, 11) compared to both other groups (males, 8; females 8) is a limitation of this study. The higher proportion of males in the two WGO receiving groups may be the reason for the higher intake of energy, fat and polyunsaturated fatty acids assessed by the FFP [40, 41]. To elucidate, whether the differences in 25(OH)D_3 were attributed to differences in plasma lipids, we measured plasma fatty acids and other lipids, but found no significant differences between the groups after the intervention. Thus, we suggest that the plasma lipids were not attributable to the observed differences of vitamin D_3 metabolites. Analysis of plasma PTH revealed no differences between the groups, which indicates the inability of the UVB-treated WGO to improve the vitamin D status.

5. Conclusion

UVB-light exposure of WGO can significantly increase the vitamin D_2 concentrations. Although the UVB-exposed WGO was able to increase the 25(OH)D_2 levels in vitamin D insufficient healthy individuals, it resulted in a concurrent decrease of 25(OH)D_3 levels which was higher than those observed in the -UVB WGO group. Ultimately, UVB-exposed WGO was not able to raise total 25(OH)D levels in humans, and is therefore not suitable for improving vitamin D status. The reason for the strong reduction of 25(OH)D_3 following ingestion of +UVB WGO remains elusive.

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Declarations

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Conflict of interest All authors declare that they have no relevant conflict of interest.

Availability of data and material Not applicable.

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Authors' contributions Anja C. Baur: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. Sophie Philipp: Validation, Investigation; Shabnam Staudt: Validation, Investigation; Thomas Weidauer: Formal analysis; Michael Kiehntopf: Investigation, Resources, Writing - review & editing; Stefan Lorkowski: Resources, Supervision, Writing - review & editing; Gabriele I. Stangl: Conceptualization, Resources, Writing - review & editing, Supervision; Christine Dawczynski: Conceptualization, Investigation, Resources, Writing - review & editing, Supervision, Project administration.

Ethics approval The study design was approved by the Ethics Committee of the Friedrich Schiller University Jena (No. 5417-01/18) and registered at clinicaltrials.gov (NCT03499327).

Consent to participate Written consent was obtained from all participants before enrollment in this study

Consent for publication Not applicable.

Figure captions

Fig. 1 Flow diagram of the participants. Sixty-nine subjects were enrolled in this study. Twenty-one subjects were excluded, since they did not meet the inclusion criteria or declined to participate. Forty-eight subjects were randomized into three groups. As two subjects discontinued the intervention, 46 participants completed the 6 weeks intervention study

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3.3 Study 3

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Intake of ergosterol increases the vitamin D concentrations in serum and liver of mice

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ABSTRACT

Factors that can modify the bioavailability of orally administered vitamin D are not yet widely known. Ergosterol is a common fungal sterol found in food which has a chemical structure comparable to that of vitamin D. This study aimed to investigate the effect of ergosterol on vitamin D metabolism. Therefore, 36 male wild type-mice were randomly subdivided into three groups (n = 12) and received a diet containing 25 µg vitamin D_3 and either 0 mg (control), 2 mg or 7 mg ergosterol per kg diet for 6 weeks. To elucidate the impact of ergosterol on hepatic hydroxylation of vitamin D, human hepatoma cells (HepG2) were treated with different concentrations of ergosterol. Concentrations of vitamin D_3 and 25-hydroxyvitamin D_3 ($25(OH)D_3$) in cells, livers and kidneys of mice and additionally 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) in serum were quantified by LC-MS/MS. The concentration of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in serum was analyzed by commercially-available enzyme immuno assay. The concentrations of cholesterol and triglycerides were analyzed in livers of mice by photometric assays.

Analyses revealed that mice receiving 7 mg/kg ergosterol with their diet had 1.3-, 1.7- and 1.5-times higher concentrations of vitamin D_3 in serum, liver and kidney, respectively, than control mice ($P < 0.05$), whereas no significant effects were observed in mice fed 2 mg/kg ergosterol. The hydroxylation of vitamin D remained unaffected by dietary ergosterol, since the concentration of 25-hydroxyvitamin D_3 in serum and tissues and the concentrations of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ in serum were not different between the three groups of mice. The lipid concentrations in liver were also not affected by dietary ergosterol. Data from the cell culture studies showed that ergosterol did not influence the conversion of vitamin D_3 to $25(OH)D_3$. To conclude, ergosterol appears to be a modulator of vitamin D_3 concentrations in the body of mice, without modulating the hydroxylation of vitamin D_3 in liver.

1. Introduction

Vitamin D supplements or food fortified with vitamin D are efficient strategies to prevent or combat vitamin D insufficiency in humans. However, numerous studies have shown that there is a strong inter-individual variation in the improvement of the vitamin D status in response to a given dose of vitamin D [1,2]. Currently, a large number of endogenous and environmental factors, such as genetics [3,4], body weight and body fat [1,2,4,5], age [2], the basal serum concentration of 25-hydroxyvitamin D (25(OH)D) [2–4] and circulating concentrations of triglycerides [2] have been found to be predictors in the response to vitamin D supplementation. It is suggested that approximately 50% of the variation in vitamin D status after supplementation with vitamin D is attributed to unknown factors [6]. Recent data showed that dietary fatty acids can promote or interfere with the intestinal absorption of vitamin D by influencing the cellular uptake and efflux of vitamin D [7,8] and by modifying the mRNA abundance of transporters involved in vitamin D absorption such as the Niemann Pick C1-like-1 receptor (*Npc1l1*), the scavenger receptor BI (*Scarb1*), the microsomal triglyceride transfer protein and the adenosine triphosphate-binding cassette (Abc) A1 transporter [8]. In 2016, our group identified 7-dehydrocholesterol (7-DHC) as a sterol that can impact the vitamin D

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; $25(OH)D_3$, 25-hydroxyvitamin D_3; 7-DHC, 7-dehydrocholesterol

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concentration in the body [9]. This study showed a substantial dose-dependent increase of vitamin D_3 in the liver and kidney of mice fed dietary 7-DHC, without altering serum and tissue concentrations of 25(OH)D_3. Ergosterol has a structure similar to 7-DHC. In contrast to 7-DHC the side chain carries a CH_2 group at C24 and a double bound between C22 and C23. Ergosterol is a component of yeast and other fungal cell membranes [10,11]. Analytical studies that compared 10 types of mushrooms found ergosterol contents ranging from 26.3 to 84.9 mg/100 g [10]. Recently published data showed that plant oils intended for human nutrition also contain considerable amounts of ergosterol [12]. Based on the findings that lipid-soluble molecules, such as dietary fatty acids and sterols, may impact vitamin D status, we hypothesized that ergosterol could also influence the concentration of vitamin D in the body. Here, we investigated the impact of two doses of orally administered ergosterol on the concentrations of vitamin D_3 and hydroxylated vitamin D_3 metabolites in serum and tissues of mice. The ergosterol concentrations which were used in this study corresponded to amounts found in typical diets. To examine, the impact of ergosterol on cellular uptake of vitamin D and conversion of vitamin D to 25(OH) D, human hepatoma cells were treated with vitamin D and different concentrations of ergosterol.

2. Materials and methods

2.1. Mouse study

All mice included in our investigation were kept in pairs in a windowless room (no form of natural ultraviolet light (UV) source) controlled for temperature (22 ± 2 °C), light (12-h light, 12-h dark cycle; non UV-emitting lamps) and relative humidity (50–60%). The experimental procedures described below followed the established guidelines for the care and handling of laboratory animals according to the US National Research Council [13] and were approved by the animal welfare committee of the Martin Luther University Halle-Wittenberg (approval number: H1-4/T1-15).

Thirty-six 4-week-old male C57BL/6NCrl mice (Charles River Laboratories, Sulzfeld, Germany) with an initial body weight of 19.4 ± 1.5 g were randomly allotted to three groups (n = 12), and were fed either 0 mg, 2 mg or 7 mg ergosterol (Labor Dr. Ehrenstorfer-Schäfers, Augsburg, Germany) per kg diet for 6 weeks. The chosen concentrations of ergosterol in the mouse diets correspond to the daily amount of ergosterol ingested with 1–10 g plant oils in the human diet [12]. The concentrations of ergosterol in the diets were confirmed by analyses via LC–MS/MS (0 mg/kg: 0.16 ± 0.05 mg/kg; 2 mg/kg: 2.11 ± 0.42 mg/kg; 7 mg/kg: 7.16 ± 0.09 mg/kg, n = 5). All diets were supplemented with adequate amounts of vitamin D_3 (Sigma Aldrich, Munich, Germany), to meet the requirements of vitamin D for mice (mean analyzed concentration: 14.6 ± 1.4 µg/kg) [14]. The basal diet contained (per kg): 288 g starch, 200 g sucrose, 200 g casein, 194 g lard, 6 g linseed oil (4.88 µg ergosterol per g oil), 50 g cellulose, 60 g vitamin-mineral mixture (which comprised minerals and vitamins according to the recommendations of the US National Research Council [14]) and 2 g DL-methionine. All mice had free access to their diets and water.

The individual body weights and the mean food intake per two mice and cage were recorded weekly. After 6 weeks, the mice were sacrificed by decapitation after a 4 h food deprivation period and an anesthesia with diethyl ether. Blood was collected in tubes (Sarstedt, Nümbrecht, Germany) and serum was separated by centrifugation (10,000 × g, 10 min, 20 °C). Liver, kidney and intestinal mucosa samples were harvested and immediately snap-frozen in liquid nitrogen. All samples were stored at −80 °C until further analyses.

2.2. Cell culture experiments

To elucidate the effect of ergosterol on vitamin D_3 uptake and the conversion of vitamin D_3 to 25(OH)D_3, two cell culture experiments with human hepatoma cells were conducted. Liver carcinoma HepG2 cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were cultivated in RPMI 1640 + GlutaMAX® medium (Gibco, Life Technologies, Darmstadt, Germany), supplemented with 10% fetal calve serum (FCS, Invitrogen, Karlsruhe, Germany) and 0.5% Gentamicin (Gibco, 10 mg/ml) at 37 °C in a humidified atmosphere (95% air and 5% CO_2). Prior to treatments, HepG2 cells were seeded in T25-cell culture flasks with a density of 7.5×10^5 per flask and were used after reaching confluency. In the first experiment, cells were incubated with 1 µmol/l vitamin D_3 and different concentrations of ergosterol (0, 1, 2 or 10 µmol/l) for 48 h. In the second experiment, cells were incubated with 1 µmol/l vitamin D_3 and 0 or 10 µmol/l ergosterol for 20 min, 60 min and 360 min, to investigate time depended effects. The incubation medium consisted of FCS-free RPMI medium supplemented with 0.5% gentamicin (10 mg/ml) and pluronic®F127 (100 µg/ml, Sigma Aldrich), a surfactant, to improve the solubility of vitamin D_3 and ergosterol in aqueous culture media [15]. Vitamin D_3 and ergosterol used for the treatments of cells were dissolved in ethanol. The final ethanol concentration in the incubation medium did not exceed 0.1% (v/v). Cells incubated with medium and the appropriate ethanol concentration as vehicle were used as control. Cell viability was assessed by the MTT assay [16] and was not altered by the used concentrations of vitamin D_3 and ergosterol.

Subsequent to the treatment, cells were washed 2-times with phosphate buffered saline (PBS) supplemented with pluronic®F127 (100 µg/ml) and then 2-times with pure PBS. Afterwards cells were harvested with trypsin, suspended in PBS and centrifuged at 900 x g for 2 min at 20 °C. Cell pellets and the cell culture media before and after incubation were stored at -20 °C until further analysis. Aliquotes of the cell pellets were used for the determination of the protein concentration by the Bradford protein assay as described elsewhere [17]. Each cell culture experiment was repeated 3-times and analyzes from each experiment were run in duplicate. Data are presented as mean values.

2.3. Analysis of vitamin D metabolites

2.3.1. Serum

To analyze the serum concentrations of ergosterol, vitamin D_3 and vitamin D_2, aliquots were mixed with deuterated 7-DHC (26,26,26,27,27,27-d_6; 7-DHC-d_6, Chemaphor Chemical Services, Ottawa, Canada) and deuterated vitamin D_3 (6,19,19-d_3; vitamin D_3-d_3, Sigma-Aldrich) as internal standards. The mixtures were hydrolyzed with potassium hydroxide for 3 h at 37 °C, extracted with *n*-hexane, washed with ultrapure water and derivatized with 4-phenyl-1,2,4-triazolin-3,5-dion (PTAD, Sigma Aldrich) as described elsewhere [9]. The derivatized samples were dissolved in methanol and mixed with a 10 mmol/l ammonium formate solution (4/1, v/v, Sigma Aldrich). To analyze the serum concentrations of 25(OH)D_3 and 24,25-dihydroxyvitamin D_3 (24,25(OH)$_2$$D_3$), proteins were precipitated with acetonitril mixed with deuterated 25(OH)D_3 (26,26,26,27,27,27-d_6, 25(OH) D_3-d_6, Chemaphor) as internal standard. After extraction with *n*-hexane and washing with ultrapure water, the solvent was evaporized and the residues were derivatized with PTAD.

The concentrations of ergosterol, vitamin D_2, vitamin D_3, 25(OH)D_3 and 24,25(OH)$_2$$D_3$ were analyzed by high performance liquid chromatography (HPLC; Agilent 1260, Agilent Technologies, Waldbronn, Germany) coupled to a tandem mass spectrometry system (MS/MS; QTRAP 5500, Sciex, Darmstadt, Germany). The HPLC was equipped with a Hypersil ODS column (2.0 × 150 mm^2, particle size: 5 µm; VDS Optilab, Berlin, Germany). The column temperature was set to 40 °C and the flow rate to 576 µl/min. The mobile phase consisted of (A) acetonitrile and (B) water/acetonitrile (1/1, v/v) with 5 mmol/l ammonium formate and 0.1% formic acid. A gradient was used to separate the vitamin D metabolites (0.00–3.10 min, 85.0% B; 4.00 min, 83.5% B;

5.00 min, 65.0% B; 8.00 min, 40.0% B; 18.0 min, 23.5% B; 20.0–23.0 min, 0.00% B; 24.0 min, 95% B; 25.0–30.0 min, 85.0% B). The MS settings were as follows: curtain gas, 241 kPa; ion spray voltage, 5500 V; temperature, 550 °C; gas 1, 414 kPa; gas 2, 483 kPa. Positive electrospray was used for ionization and the data were collected by multiple reaction monitoring with the following mass transitions (quantifier ions) presented as PTAD adducts [M + PTAD + H^+]: ergosterol, 572 > 377; vitamin D_2, 572 > 298; vitamin D_3, 560 > 298; 25(OH)D_3, 576 > 298; 24,25$(OH)_2D_3$, 592 > 298; 7-DHC-d_6, 566 > 371; vitamin D_3-d_3, 563 > 301 and 25(OH)D_3-d_6, 582 > 298. All mass transitions of the analytes were verified by qualifier ions (ergosterol, 572 > 395; vitamin D_2, 572 > 280; vitamin D_3, 560 > 280; 25(OH)D_3, 576 > 558; and 24,25$(OH)_2D_3$, 592 > 280). Retention times of the vitamin D metabolites were: ergosterol, 12.8 min; vitamin D_2, 12.1 min; vitamin D_3, 12.2 min; 25(OH)D_3, 2.9 and 3.2 min and 24,25$(OH)_2D_3$, 1.2 and 1.4 min (both latter ones were quantified as sum of 6R- and 6S-isomers of PTAD). The peaks were identified by means of internal standard peaks with the following retention times: 7-DHC-d_6, 12.6 min; vitamin D_3-d_3, 12.2 min and 25(OH)D_3-d_6, 2.9 and 3.2 min (6R and 6S, respectively). Standard solutions of ergosterol, vitamin D_2, vitamin D_3, 25(OH)D_3 (all from Sigma Aldrich) and 24,25$(OH)_2D_3$ (Enzo Life Science GmbH, Lörrach, Germany) were spiked with internal standard that contained the deuterated vitamin D metabolites (as described above) and were used for calibration. The calibration curve was constructed in a linear regression model (r > 0.99) by plotting the ratio of the analyte peak area to the internal standard peak area versus the concentration of the analytes. The coefficients of variation were 3.9% for ergosterol, 2.1% for vitamin D_3 and 5.9% for 25(OH)D_3 (n = 8). The precision of the 25(OH)D_3 concentration was additionally assessed by using MassCheck® controls (Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany).

The concentration of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) in serum was analyzed by using a commercial ELISA kit (IDS, Immundiagnostic Systems GmbH, Frankfurt am Main, Germany) according to the manufacturer's protocol. Prior to the analysis, samples were diluted with isotonic saline (1/2, v/v) and 50 µl of the degreased samples were used for immunoextraction.

2.3.2. Tissues

To analyze the concentrations of vitamin D_3 and 25(OH)D_3 in liver and kidney, aliquots were minced on ice and mixed with internal standards (vitamin D_3-d_3 and 25(OH)D_3-d_6), hydrolyzed over night with potassium hydroxide and extracted with *n*-hexane. The extract was washed, evaporated and resolved in *n*-hexane/isopropanol (99/1, v/v). To purify and separate the vitamin D metabolites, a normal phase HPLC (Agilent 1100, Agilent Technologies) with UV-detection (265 nm) equipped with a LiChrospher Si 60 column (250 × 4.6 mm^2; particle size: 5 µm; Agilent Technologies, column temperature 25 °C) was used. *n*-Hexane/isopropanol with a flow rate of 1.5 ml/min served as mobile phase and the gradient was in accordance to the first step of semi-preparative HPLC analysis which was described by Mattila et al. [18]. Vitamin D_3 and 25(OH)D_3 were collected in separate fractions with a Water Fraction Collector II (Waters GmbH, Eschborn, Germany). The fractions were dried and subjected to PTAD-derivatization. The concentrations of vitamin D_3 and 25(OH)D_3 were analyzed by LC–MS/MS as described above. The coefficients of variation for tissue samples were 2.5% for vitamin D_3 and 14.8% for 25(OH)D_3 (n = 4).

2.3.3. Cell culture

To analyze the concentrations of vitamin D_3 and 25(OH)D_3 in HepG2-cells and incubation media, the complete cell pellet or 100 µl of incubation media were mixed with internal standards (vitamin D_3-d_3 and 25(OH)D_3-d_6) and hydrolyzed for 3 h as described for serum samples. The samples were extracted with *n*-hexane, washed with ultrapure water and fractionated via normal phase HPLC as described above for tissue samples. After PTAD-derivatization the concentration of vitamin D_3 was analyzed via LC–MS/MS as described above. To significantly improve separation of overlapping peaks in the cell culture samples, the 25(OH)D_3 concentration was analyzed via HPLC (Agilent 1260) equipped with a Poroshell 120 column (4.6 × 50 mm^2, particle size: 2.7 µm, Agilent Technologies) coupled to a MS/MS (QTRAP 5500) with settings and mobile phases as described above with the following gradient: 0.00–2.10 min, 100% B; 4.00 min, 76.5% B; 6.00 min, 76.0% B; 10.0 min, 40.0% B; 12.0–15.0 min, 0.00% B; 17.0–20.0 min, 100%. The analyzed concentration of vitamin D metabolites in cells are referred to their protein concentration.

2.4. Analysis of cholesterol and triglycerides in liver of mice

Commercial photometric assays were used to quantify liver concentrations of cholesterol (*Cholesterol FS*, Diagnostic Systems GmbH, Holzheim, Germany) and triglycerides (*Triglyceride FS*, Diagnostic Systems). Prior to the analysis, the liver samples were extracted with *n*-hexane/isopropanol (3/2, v/v) for 18 h [19]. After evaporation of the solvents, the lipids were dissolved in chloroform/Triton X-100 (1/1, w/w). The chloroform was evaporated, before analysis of cholesterol and triglycerides.

2.5. Analysis of relative mRNA expression in liver and intestine of mice

The relative mRNA abundance of genes involved in the hydroxylation and cellular transport of vitamin D and sterols in liver and intestinal mucosa was analyzed by real-time RT-PCR. Therefore, total RNA was isolated with peqGOLD TriFast™ kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. The RNA concentration was measured photometrically at 260 nm and purity was verified by agarose gel electrophoreses. cDNA was synthesized using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Real-time-PCR was conducted as described elsewhere [20] using GoTaq® Flexi DNA-Polymerase (Promega) and the Rotorgene 6000 system (Corbett Research, Mortlake, Australia). The amplification of a single product of the expected size was confirmed by agarose gel electrophoresis. The method of Pfaffl [21] was used to calculate the relative mRNA abundance of target genes, which were expressed as fold change to the 0 mg/kg group. Beta-2-microglobulin and ribosomal protein, large, P0 served as appropriate reference genes. Primer sequences of target and reference genes are shown in Table 1.

2.6. Statistical analysis

Sample size calculation for the mouse study was based on data obtained from a study that investigated the effects of 7-dehydrocholesterol (provitamin D_3) on the vitamin D_3 content in tissue samples of mice [9]. Assuming an effect size (f) of 1.3, an α error probability of 0.05 and a power (1-β) of 0.8, we calculated a sample size of 12 animals per group (G*Power, Heinrich Heine University Düsseldorf, Germany).

Values are presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS statistical software (version 22, IBM, Armonk, USA). All data were subjected to Shapiro-Wilk normality test. The differences between the three groups of mice were analyzed using one-way analysis of variance (ANOVA, 1-WA). In the second cell culture experiment, time depended differences between two groups were analyzed using two-way ANOVA (2-WA) with the factors time, ergosterol treatment and their interaction. Homoscedasticity was tested by Levene's test. Since all data showed homoscedasticity, post-hoc comparison was conducted with Tukey's test. The mRNA data were compared by the Student's t-test. Differences were considered to be significant at $P < 0.05$.

Table 1
Primer sequences of target and reference genes, used in the analysis of mRNA expression in liver or intestinal mucosa of mice.

	Forward primer (5' to 3')	Reverse primer (5' to 3')	Accession number	Product size (bp)
Abca1[a]	ACTGGAGACACCCCTGTGAC	GGAGAGCTTTCGTTTGTTGC	NM_013454.3	285
Abcg5[a]	TGGATCCAACACCTCTATGCTAAA	GGCAGGTTTTCTCGATGAACTG	NM_031884.1	77
Abcg8[a]	TGCCCACCTTCCACATGTC	ATGAAGCCGGCAGTAAGGTAGA	NM_026180.2	73
Cd36[a]	CTTGAAGAAGGAACCACTGC	GTTCTTTGCCACGTCATCTG	NM_001159556.1	108
Cyp27a1[b]	CTTTCTCTTCCCCAAGAATAC	TTATCAGCCTCTTTCTTCCTC	NM_024264.4	126
Cyp2r1[b]	CAAAAATGGGAGGCTTACTC	GTTATCAGTTGCTTGAGGTC	NM_177382.4	196
Cyp3a11[b]	CTGACACCAGTATATGAGATG	GGCTTTATGAGAGACTTTGTC	NM_007818.3	186
Npc1l1[a]	ATCCTCATCCTGGGCTTTGC	GCAAGGTGATCAGGAGGTTGA	NM_207242.2	76
Scarb1[a]	GTCCGCATAGACCCGAGCAG	CCAGCGCCAAGGTCATCATC	NM_016741.1	361
B2m[a,c]	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC	NM_009735.3	104
Rplp0[a,c]	GAAACTGCTGCCTCACATCCG	CTGGCACAGTGACCTCACACG	NM_007475.5	146

[a] Obtained from Eurofins MWG Synthesis GmbH, Ebersberg, Germany.
[b] Obtained from Sigma Aldrich Chemie GmbH, Taufkirchen, Germany.
[c] Reference Gene. Annealing temperature: 60 °C. *Abca1*: adenosine triphosphate (ATP)-binding cassette A1; *Abcg5*: ATP-binding cassette G5; *Abcg8*: ATP-binding cassette G8; *Cd36*: cluster of differentiation 36; *Cyp27a1*: sterol-27-hydroxylase; *Cyp2r1*: vitamin D-25 hydroxylase; *Cyp3a11*: cytochrome P450, family 3, subfamily A, polypeptide 11; *Npc1l1*: niemann-pick C1-like 1; *Scarb1*: scavenger receptor class B member 1; *B2m*: beta-2-mikroglobulin; *Rplp0*: ribosomal protein, large, P0; bp: base pair.

3. Results

3.1. Body weight and food intake of mice

Final body weight of the mice (0 mg/kg: 31.6 ± 2.8 g; 2 mg/kg: 31.8 ± 2.8 g; 7 mg/kg: 32.0 ± 2.6 g; $P = 0.943$) and mean daily food intake per two mice and cage (0 mg/kg: 5.3 ± 0.4 g; 2 mg/kg: 5.2 ± 0.2 g; 7 mg/kg: 5.3 ± 0.2 g; $P = 0.804$) did not differ between the three groups.

3.2. Vitamin D metabolites in serum and tissues of mice

To assess the bioavailability of orally administered ergosterol, serum concentrations of ergosterol were analyzed. No detectable amount of ergosterol was found in serum of control mice. In mice fed 2 mg/kg, the serum concentration was below the limit of quantification (LOQ, 3.0 nmol/l), and in mice fed 7 mg/kg it was 5.7 ± 2.2 nmol/l.

To elucidate whether the intake of ergosterol can alter serum concentrations of vitamin D metabolites, we analyzed the concentrations of vitamin D_3, 25(OH)D_3, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$. Mice fed 7 mg/kg ergosterol with the diet had 1.3-times higher serum concentrations of vitamin D_3 than mice of the control group ($P < 0.05$; Fig. 1). In mice fed 2 mg/kg, the serum concentration of vitamin D_3 did not differ from that of the control mice. The serum concentrations of the hydroxylated forms of vitamin D_3 (25(OH)D_3, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$) were not influenced by dietary ergosterol (Fig. 1).

To elucidate whether an oral intake of ergosterol is able to influence the vitamin D metabolite concentrations in tissues, liver and kidney samples were analyzed for their vitamin D_3 and 25(OH)D_3 concentrations. In comparison to the control group, mice fed 7 mg/kg ergosterol had 1.7- and 1.5-times higher vitamin D_3 concentrations in liver and kidney, respectively ($P < 0.05$; Fig. 2). Mice fed 2 mg/kg ergosterol had vitamin D_3 concentrations in between. The 25(OH)D_3 concentrations in liver and kidney did not differ between the three groups of mice (Fig. 2). The vitamin D_2 concentrations in serum, liver and kidney were below the LOQ of 1.0 nmol/l or 25 pmol/g, respectively, in all samples analyzed.

3.3. Cholesterol and triglycerides in liver of mice

To preclude the possibility that different effects of ergosterol on the liver content of vitamin D were due to differences in liver fat content, cholesterol and triglyceride concentrations in liver were analyzed. However, neither cholesterol (0 mg/kg: 2.83 ± 0.37 mg/g; 2 mg/kg: 2.75 ± 0.41 mg/g; 7 mg/kg: 2.83 ± 0.50 mg/g; $P = 0.863$) nor triglycerides (0 mg/kg: 21.0 ± 6.5 mg/g; 2 mg/kg: 22.1 ± 7.5 mg/g; 7 mg/kg: 21.7 ± 6.5 mg/g; $P = 0.919$) showed differences between the three groups.

3.4. Relative mRNA abundance of genes involved in the regulation of vitamin D and sterol metabolism in liver and intestinal mucosa of mice

Because differences in vitamin D metabolites were only observed between the groups fed 0 and 7 mg ergosterol per kg diet, gene expression analysis were conducted in tissue samples of these two groups. To elucidate a possible mechanism for the observed accumulation of vitamin D_3 in serum and tissues of mice fed 7 mg/kg ergosterol, we analyzed the mRNA abundance of genes involved in the hydroxylation and cellular transport of sterols and vitamin D. Here, we found no differences in the relative mRNA abundance of the analyzed hydroxylases in the liver of mice fed with 7 mg/kg ergosterol compared to mice of the control group (Table 2). We further found no impact of ergosterol on the mRNA abundance of the analyzed transporters in liver or intestinal mucosa of mice (Table 2).

3.5. Concentrations of vitamin D metabolites in cells treated with ergosterol

To assess whether ergosterol has a dose-dependent effect on the cellular uptake of vitamin D, HepG2 cells were treated with 1 µmol/l vitamin D_3 and 0, 1, 2 or 10 µmol/l ergosterol for 48 h. Irrespective of the treatment, all cells had comparable vitamin D_3 concentrations (Fig. 3), suggesting that there was no impact of ergosterol on the vitamin D uptake. Vehicle treated control cells (without vitamin D_3 or ergosterol) had vitamin D_3 concentrations below the LOQ (30 nmol/l). The cellular concentration of 25(OH)D_3 was also not different between the treatments, indicating that the conversion of vitamin D_3 to 25(OH)D_3 was not impaired by ergosterol (Fig. 3).

The vitamin D_3 concentrations in the culture media after incubation did not differ between the treatments (0 µmol/l ergosterol: 182 ± 9 nmol/l; 1 µmol/l ergosterol: 178 ± 43 nmol/l; 2 µmol/l ergosterol: 182 ± 40 nmol/l; 10 µmol/l ergosterol: 182 ± 26 nmol/l). The concentration of 25(OH)D_3 was below the LOQ (9.4 nmol/l) in all culture media.

To elucidate whether effects of ergosterol on cellular uptake and hydroxylation of vitamin D_3 are time dependent, we analyzed vitamin D_3 and 25(OH)D_3 concentrations in cells and culture media after treatment of cells with 1 µmol/l vitamin D_3 and 0 or 10 µmol/l ergosterol for 20 min, 60 min and 360 min in a second experiment. Here we found that cellular uptake of vitamin D_3 and the conversion of vitamin D_3 to 25(OH)D_3 largely depended on the incubation time, with

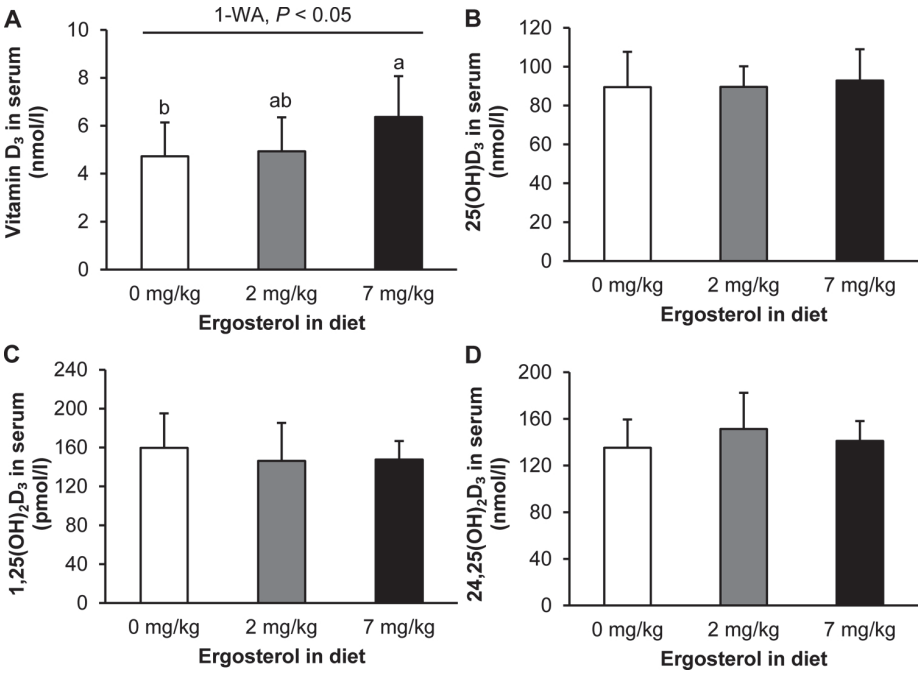

Fig. 1. Ergosterol increases the serum concentrations of vitamin D_3 in mice, but not the concentrations of the hydroxylated vitamin D metabolites.
Data are presented as mean ± SD, groups of 12 mice each were fed diets with either 0, 2 or 7 mg/kg ergosterol for 6 weeks. 1-WA: One-Way ANOVA. [ab] Different superscripts indicate significant differences between the groups (Tukey's test). $25(OH)D_3$: 25-hydroxyvitamin D_3; $1,25(OH)_2D_3$: 1,25-dihydroxyvitamin D_3; $24,25(OH)_2D_3$: 24,25-dihydroxyvitamin D_3.

considerable increased concentrations of both vitamin D metabolites after 360 min (time effect: $P < 0.001$, 2-WA; Fig. 4). As observed in experiment 1, ergosterol did not alter the cellular concentrations of vitamin D_3 and $25(OH)D_3$. In contrast, the vitamin D_3 concentration in the culture medium dropped with the duration of treatment, without having an effect of ergosterol on vitamin D_3 (Fig. 4). The $25(OH)D_3$ concentration in the cell culture media was below the LOQ (9.4 nmol/l).

4. Discussion

This study aimed to elucidate the impact of dietary ergosterol on vitamin D metabolism in mice. Here, we found that dietary ergosterol in physiological amounts caused an increase of vitamin D_3 in serum, liver and kidney of mice. This finding corresponds to the recently observed ability of dietary 7-DHC to increase tissue concentrations of vitamin D [9], although the magnitude of rise in vitamin D following ergosterol intake was lower than that observed with 7-DHC [9]. We hypothesized that ergosterol could have caused this effect by (i) reducing the conversion of vitamin D_3 to $25(OH)D_3$, because ergosterol has been

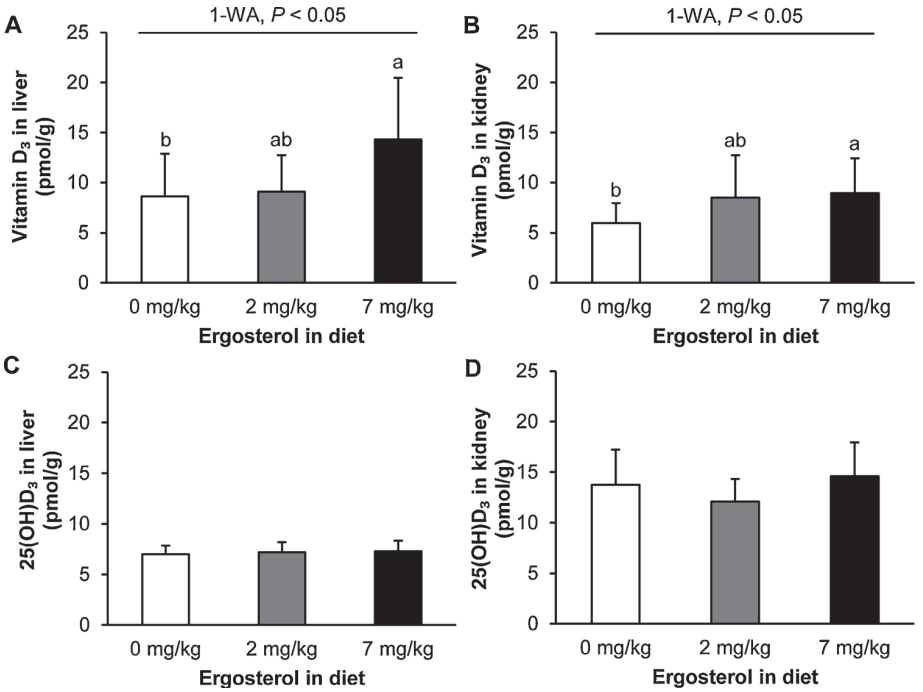

Fig. 2. Ergosterol increases the concentrations of vitamin D_3 in liver and kidney of mice, but not the $25(OH)D_3$ concentrations in these tissues.
Data are presented as mean ± SD, groups of 12 mice each were fed diets with either 0, 2 or 7 mg/kg ergosterol for 6 weeks. 1-WA: One-Way ANOVA. [ab] Different superscripts indicate significant differences between the groups (Tukey's test). $25(OH)D_3$: 25-hydroxyvitamin D_3.

Table 2
Relative mRNA abundance of genes involved in vitamin D hydroxylation and cellular uptake of sterols in response to dietary ergosterol in mice.

	Liver		Intestinal mucosa	
	Ergosterol per kg diet		Ergosterol per kg diet	
	0 mg	7 mg	0 mg	7 mg
Hydroxylation of vitamin D				
Cyp27a1	1.00 ± 0.13	1.05 ± 0.11	n.a.	n.a.
Cyp2r1	1.00 ± 0.10	1.02 ± 0.18	n.a.	n.a.
Cyp3a11	1.00 ± 0.35	1.24 ± 0.40	n.a.	n.a.
Sterol and vitamin D transport				
Abca1	n.a.	n.a.	1.00 ± 0.34	1.09 ± 0.41
Abcg5	1.00 ± 0.19	1.27 ± 0.42	1.00 ± 0.13	0.98 ± 0.16
Abcg8	1.00 ± 0.28	1.36 ± 0.51	1.00 ± 0.15	0.94 ± 0.09
Cd36	n.a.	n.a.	1.00 ± 0.18	1.12 ± 0.21
Npc1l1	n.a.	n.a.	1.00 ± 0.18	0.95 ± 0.12
Scarb1	1.00 ± 0.30	0.90 ± 0.24	1.00 ± 0.58	0.96 ± 0.42

Data are presented as mean ± SD, groups of 12 mice each were fed diets with either 0, 2 or 7 mg/kg ergosterol for 6 weeks. n.a. not analyzed.
Cyp27a1: sterol-27-hydroxylase; *Cyp2r1*: vitamin D-25-hydroxylase; *Cyp3a11*: cytochrome P450, family 3, subfamily A, polypeptide 11; *Abca1*: adenosine triphosphate (ATP)-binding cassette A1; *Abcg5*: ATP-binding cassette G5; *Abcg8*: ATP-binding cassette G8; *Cd36*: cluster of differentiation 36; *Npc1l1*: Niemann-Pick C1-like 1; *Scarb1*: Scavenger receptor class B member 1.

identified as a novel substrate of *Cyp27a1* [22], which is crucial for the hydroxylation of vitamin D_3 to 25(OH)D_3 [23–25], or (ii) by increasing the uptake of vitamin D_3 into liver and kidney.

To test the first hypothesis, we analyzed the 25(OH)D_3 concentrations in serum, liver and kidney of mice, but found no impact of ergosterol on the levels of this vitamin D metabolite. Additionally, the mRNA abundance of hepatic cytochrome P450 (*Cyp*) *27a1*, *Cyp2r1* and *Cyp3a11* (murine homolog to human *CYP3A4*), important enzymes that catalyze the formation of 25(OH)D remained unaffected by dietary ergosterol. The finding was corroborated by the *in vitro* data, which showed that the conversion of vitamin D_3 to 25(OH)D_3 was not affected by ergosterol treatment. The ratio of synthesized 25(OH)D_3 from vitamin D_3 in HepG2 cells was by means 2.60 ± 0.06% and was not influenced by the different treatments (Fig. 3). Thus, it can be assumed that the observed increase of vitamin D_3 in serum, liver and kidney of ergosterol-fed mice was not caused by an impaired conversion of vitamin D_3 to 25(OH)D_3.

To test the second hypothesis, we investigated the uptake of vitamin D_3 into liver cells. We used the human carcinoma cell line HepG2, which has been shown to have a normal vitamin D metabolism [26]. However, data from two cell culture experiments, one using different doses of ergosterol and the other investigating different periods of ergosterol treatment, were not indicative of an altered cellular vitamin D uptake in response to ergosterol. Recently published data showed that dietary ergosterol had influenced the lipid metabolism by decreasing the hepatic triglyceride concentration in mice [27]. As changes in hepatic lipid concentrations could have modified the vitamin D concentrations, we analyzed cholesterol and triglyceride concentrations in livers of mice fed diets with different doses of ergosterol. Because concentrations of cholesterol and triglycerides were not significantly different between the three groups of mice, it can be excluded that ergosterol treatment changed hepatic vitamin D concentrations by modulating liver lipid concentrations. However, it needs to be emphasized that Gil-Ramirez and coworkers [27] used an ergosterol dose (800 mg/kg diet) that was significantly higher than that used in the current study (7 mg/kg diet).

Differences in the degradation of vitamin D metabolites can also be excluded as a plausible explanation for the modulation of vitamin D by ergosterol, because the serum concentration of 24,25$(OH)_2D_3$, which is a biomarker for vitamin D catabolism [28], did not differ between the groups. Furthermore, we observed no reduction of the 1,25$(OH)_2D_3$, suggesting that the bioactivation of vitamin D remained unaffected by ergosterol.

Mice, which received higher doses of dietary ergosterol had detectable serum ergosterol concentrations, which indicates a certain bioavailability of ergosterol. Normally, intestinal absorption of sterols, like ergosterol or vitamin D, requires the formation of micelles and the transport of vitamin D through enterocytes. It is suggested that the intestinal absorption of vitamin D requires sterol transporters such as *Npc1l1*, cluster of differentiation 36 (*Cd36*), *Scarb1* and *Abca1* [29]. At the same time it must be noted that luminal efflux proteins such as the Abc-transporters *Abcg5/8* limit the sterol absorption [30–32]. To elucidate whether dietary ergosterol had influenced the mRNA expression of intestinal sterol transporters, we analyzed the mRNA abundance of *Abca1, Abcg5, Abcg8, Cd36, Npc1l1* and *Scarb1*, but found no effect of ergosterol on these genes at the transcription level. However, to elucidate the role of ergosterol for intestinal vitamin D uptake, models are necessary that are suitable to investigate the effect of ergosterol on micelle formation and composition, including vitamin D affinity of micelles and the intestinal uptake and reverse transport of vitamin D.

To conclude, ergosterol might act as a modulator of vitamin D levels in mice. However, data from human hepatoma cells do not indicate a role of ergosterol in modulating the cellular uptake of vitamin D_3 and its hydroxylation.

Author contributions

ACB, JK and GIS conceived and designed the mice experiment. ACB performed the mice experiment. CB designed the cell experiments. ACB and FH analyzed the data. ACB and GIS wrote the manuscript. JK, CB and FH critically reviewed the manuscript.

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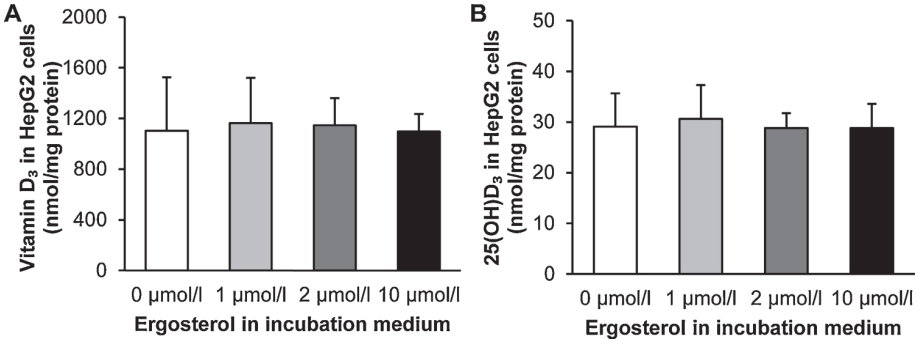

Fig. 3. HepG2 cells treated with different concentrations of ergosterol did not differ in their concentrations of vitamin D_3 and 25(OH)D_3. Cells were incubated with FCS-free medium supplemented with 0.5% gentamicin (10 mg/ml), pluronic®F127 (100 µmol/l), 1 µmol/l vitamin D_3 and different concentrations of ergosterol for 48 h. Data are presented as mean ± SD, n = 3. 25(OH)D_3: 25-hydroxyvitamin D_3.

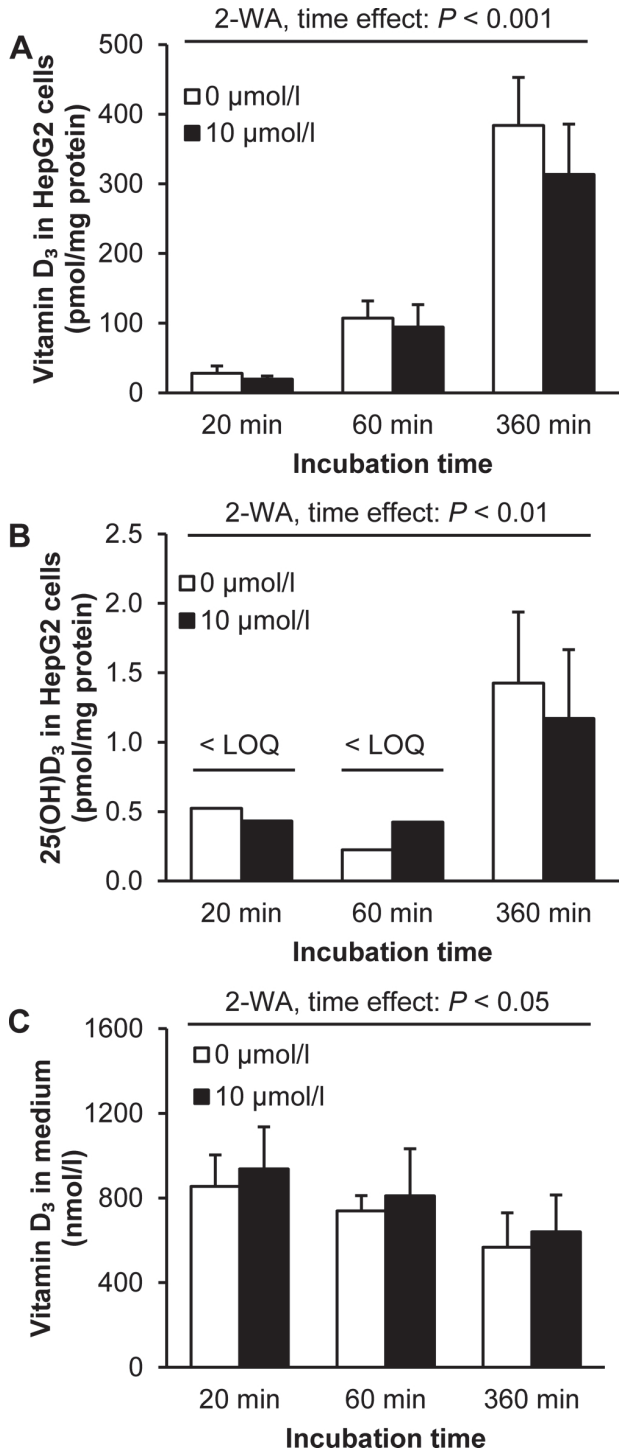

Fig. 4. Irrespective of the ergosterol concentration, the incubation period influenced the concentrations of vitamin D_3 and 25(OH)D_3 in HepG2 cells. Cells were incubated with FCS-free medium supplemented with 0.5% gentamicin (10 mg/ml), pluronic®F127 (100 µmol/l), 1 µmol/l vitamin D_3 and 10 µmol/l ergosterol for 20, 60 and 360 min. Data are presented as mean ± SD, n = 3. 25(OH)D_3: 25-hydroxyvitamin D_3. < LOQ: values were below the limits of quantification (0.75 pmol/mg protein). 2-WA: two-way ANOVA with time and ergosterol treatment as classification factors and their interaction.

agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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3.4 Study 4

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Differential effects of vitamin D_3 vs vitamin D_2 on cellular uptake, tissue distribution and activation of vitamin D in mice and cells

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ABSTRACT

To combat vitamin D deficiency, vitamin D_3 and vitamin D_2 are commonly used as a supplement or to fortify food sources. Human data show that the response of 25-hydroxyvitamin D (25(OH)D) to supplementation with vitamin D_3 is higher than to vitamin D_2. To elucidate the metabolic route of both vitamers, we conducted a study with vitamin D-depleted mice, which were allotted into three groups (n = 12) and received equal doses of either deuterated vitamin D_3, deuterated vitamin D_2 or both for 4 weeks. To further investigate the hepatic uptake and hydroxylation of both D-vitamers to 25(OH)D, we conducted cell culture experiments with murine and human hepatoma cells (Hepa1-6 and HepG2). The vitamin D metabolite concentrations in serum, tissues and cells were analyzed by LC-MS/MS or ELISA.

In mice, vitamin D_2 resulted in lower serum and tissue concentrations of vitamin D ($P < 0.001$) than vitamin D_3, while the group which received both D-vitamers showed values in between. Interestingly, vitamin D_2 fed mice had 1.9-times and 2.9-times higher serum concentrations of total and free 25(OH)D ($P < 0.001$) than mice fed vitamin D_3, while the concentration of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) was 1.8-times lower ($P < 0.001$). The gene and protein expression of enzymes, involved in the hydroxylation and renal uptake of vitamin D remained largely unaffected by the D-vitamer. In contrast to the mice data, hepatoma cells preferred vitamin D_3 for 25-hydroxylation over vitamin D_2 ($P < 0.001$). In general, the formation of 25(OH)D was much more pronounced in human than in murine hepatoma cells ($P < 0.001$).

To conclude, in contrast to humans, vitamin D_2 was more efficient in increasing 25(OH)D than vitamin D_3 in mice, although this difference was not caused by a preferential hydroxylation of vitamin D_2 in the liver. The metabolic routes of D_3 and D_2 in mice differ, showing lower circulating $1,25(OH)_2D$ and tissue vitamin D concentrations in D_2- than in D_3-fed mice.

1. Introduction

In nature, vitamin D can primarily be found in two forms. The animal derived vitamin D_3 (D_3, cholecalciferol) is synthesized in the skin through ultraviolet (UV) light-exposure from 7-dehydrocholesterol. Vitamin D_2 (D_2, ergocalciferol) is the fungal form of vitamin D, which is commonly found in wild mushrooms [1,2] or cultivated mushrooms and yeasts irradiated with UV-B light [2–4]. Plants can also contain remarkable levels of D_2, probably through fungal infection [5]. Both vitamers are sterol compounds which differ only in their sidechain, with an additional CH_2-group at C-position 24 and a double bond between C-positions 22 and 23 for D_2. Once ingested or synthesized through UV-light exposure, vitamin D has to be activated by enzymatic hydroxylation to 25-hydroxyvitamin D (25(OH)D) in the liver and

Abbreviations: $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; Cyp, cytochrome P450; D_3, vitamin D_3; D_2, vitamin D_2; DBP, vitamin D binding protein.

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subsequently to 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) in the kidney. The major carrier protein of these vitamin D metabolites in the circulation is the vitamin D binding protein (DBP) [6].

To combat vitamin D deficiency in the human population, multiple strategies are used. In Europe the most common preparation to treat vitamin D deficiency is D_3 supplementation. In the USA, D_2 is largely used as a supplement or to fortify food. Although both vitamers are effective in the prevention and treatment of rickets [7], a series of studies have found a lower response of plasma 25(OH)D levels to treatment with D_2 than with D_3 [8–13]. Lehmann et al. found that D_2 supplementation of healthy individuals was accompanied by a disproportionate decline in the 25$(OH)D_3$ levels [11]. To elucidate possible mechanisms explaining the differences in the 25(OH)D response to D_3 and D_2 supplementation, tissue data are mandatory. First evidence that the metabolic route of D_3 and D_2 may differ, came from Lipkie et al. (2013) who found lower vitamin D levels in adipose and liver tissues of rats fed D_2 than those fed D_3 [14]. Based on these data and the different human 25(OH)D responses to D_3 and D_2, we hypothesized that D_2 is less efficient in raising serum 25(OH)D levels due to its distinct tissue metabolism than D_3. This study aimed to elucidate presumed differences in the metabolic routes of orally supplemented D_3 and D_2, by analysis of the D-vitamer metabolites in serum and tissues. Hence, we conducted a mouse study elucidating the tissue uptake of deuterium labeled D_3 and D_2 and their hydroxylation to 25(OH)D and 1,25$(OH)_2$D. Additionally, we assessed the serum concentration of free 25(OH)D (not bound to DBP), which has been discussed as a novel marker for the vitamin D status [15]. As liver plays a pivotal role in 25(OH)D synthesis, we additionally investigated the uptake and 25(OH)D formation in hepatoma cells treated with D_3 or D_2. By comparing murine and human hepatoma cells we aimed to figure out the comparability of both species-specific cell lines in response to treatment with both D-vitamers.

2. Materials and methods

First, we conducted a mouse study, in which vitamin D-depleted mice were fed adequate amounts of deuterium-labeled D_3, D_2 or both vitamers for 4 weeks. Focus of this study was to analyze the tissue uptake and distribution of D_3 and D_2 and the activation of these D-vitamers to 25(OH)D and 1,25$(OH)_2$D. By conducting a supplementary cell culture study, we aimed to take a closer look at the hydroxylation processes in liver cells treated with different doses of D_3 and D_2. Special emphasize was placed on the comparison of murine and human hepatocytes reacting to D_2 and D_3.

2.1. Mouse study

All experimental procedures described below followed the guidelines for the care and handling of laboratory animals according to the US National Research Council (NRC) [16] and were approved by the local government (Landesverwaltungsamt Sachsen-Anhalt; approval number: 42502-2-1391 MLU). During the feeding study, mice were kept in pairs in a room controlled for light (12 h-dark, 12 h-light cycle; lamps were not emitting UV light), temperature (22 ± 2 °C) and relative humidity (55 ± 10 %). The access to food and water was not limited during the whole experiment.

Forty-two 4-week-old, male C57BL/6NCrl mice were obtained from Charles River Laboratories (Sulzfeld, Germany). All mice received an initial vitamin D-free basal diet containing: 28.8 % starch, 20 % sucrose, 20 % casein, 17.5 % coconut fat, 2.5 % soya oil, 5 % cellulose, 0.2 % DL-methionine and 6 % vitamin-mineral-mixture, which was designed in accordance to the recommendations of the NRC [17], except for vitamin D. After 4 weeks, six mice were sacrificed as described below, to analyze the initial 25(OH)D levels in serum. The remaining mice (mean body weight: 25.5 ± 1.5 g) were randomly allotted into three groups (n = 12). The first group received the vitamin D-free basal diet supplemented with 25 µg deuterium-labeled vitamin D_3-d_3 (6,19,19-d_3, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) per kg diet (D_3-d_3); the second group received the same basal diet supplemented with 25 µg vitamin D_2-d_3 (D_2-d_3) (6,19,19-d_3, Sigma-Aldrich). To elucidate potential interactions between the two D-vitamers, we included a third group which received the basal diet, supplemented with 12.5 µg vitamin D_3-d_3 and 12.5 µg vitamin D_2-d_3 (D_3-d_3+D_2-d_3). The dosage of deuterium-labeled vitamin D in the diets met the recommendations of the NRC [17]. The diets were fed for 4 weeks. Mean food intake per cage and individual body weights of mice were recorded weekly. After 3 weeks, 6 mice of each group were transferred into individual metabolic cages (Tecniplast GmbH, Hohenpeissenberg, Germany) for 3 days, to collect feces daily for analyzes of non-absorbed vitamin D.

After the experimental period all mice were food-deprived for 4 h, anesthetized with diethyl ether and sacrificed by decapitation. Blood was collected in serum tubes (Sarstedt, Nümbrecht, Germany), allowed to set for 20 min at room temperature and separated by centrifugation (10,000 *g*, 10 min). The gastrocnemius muscle of the right hind leg, intestinal mucosa, liver, retroperitoneal fat tissue and kidneys were harvested for further analyses. To obtain the body homogenates for vitamin D analysis, the gastrointestinal tract, the gonads, the lungs, the spleen, the heart and the brain were removed from the body and the remaining body was homogenized. Samples were immediately snap frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.2. Cell culture study

To elucidate possible differences in utilization of vitamin D between mice and humans, we conducted cell culture experiments. The murine liver carcinoma cell Hepa1-6 (LGC Standards GmbH, Wesel, Germany) and the human liver carcinoma cell line HepG2 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultivated in *Dulbecco's Modified Eagle's Medium* (DMEM) and RPMI 1640 Gluta®MAX medium (Gibco, Life Technologies, Darmstadt, Germany), respectively, supplemented with 10 % fetal calf serum (FCS, Invitrogen, Karlsruhe, Germany) and 0.5 % gentamicin (w/v, 10 mg/mL, Gibco) at 37 °C in a humidified atmosphere (95 % air and 5 % CO_2). For gene expression analysis cells were seeded in 24-well-plates with a density of 1.0×10^5 and for analysis of vitamin D metabolites in T25-cell culture flasks with a density of 7.5×10^5. Cells were used when reaching confluence. Both cell lines have been shown to exhibit a normal vitamin D metabolism [18,19].

To compare the response of murine and human hepatoma cells to D_3 and D_2, Hepa1-6 and HepG2 cells were incubated with 1 µM D_3 or D_2 for 6 h (gene expression analysis) and 48 h (vitamin D metabolite analysis), respectively. To investigate dose-dependent effects, Hepa1-6 and HepG2 cells were incubated with D_3 or D_2 in final concentrations of 1 µM, 5 µM or 10 µM for 48 h, before analysis of vitamin D metabolites.

In all experiments, the vehicle medium consisted of FCS-free medium with 0.5 % gentamicin (w/v, 10 mg/mL) and 10 % pluronic™ F127 (w/v, Sigma Aldrich Chemie GmbH). Pluronic™ F127 was used to improve the solubility of vitamin D in the aqueous cell culture media [20]. Vitamin D_3 and D_2 stock solutions were prepared in absolute ethanol. The final ethanol concentration in the incubation media did not exceed 0.1 % (v/v). Cells treated with the vehicle medium and 0.1 % ethanol were used as control. The viability of the incubated cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT)-test [21] and was not impaired by any incubation condition.

For analysis of vitamin D metabolites, cells were washed 2-times with phosphate buffered saline (PBS) supplemented with 10 % pluronic™ F127 and 2-times with pure PBS. Cells were harvested with trypsin, suspended in PBS, and centrifuged. The separated cell pellets were stored at −20 °C until further analysis.

An aliquot of the harvested cells was used for the determination of the protein concentration, which was conducted via Bradford protein assay as described elsewhere [22]. Each cell culture experiment was repeated 4-times independently. Analyzes from each experiment were

run in duplicate (gene expression analysis) or triplicate (vitamin D metabolite analysis).

2.3. Analysis of vitamin D metabolites and vitamin D binding protein

The concentrations of D_3-d_3, D_2-d_3, and their hydroxylated forms, 25-hydroxyvitamin D_3-d_3 (25(OH)D_3-d_3) and 25-hydroxyvitamin D_2-d_3 (25(OH)D_2-d_3), were analyzed in serum, intestinal mucosa, liver, fat tissue, kidney and body homogenate of all mice. Feces, collected over a period of three days, were freeze dried and homogenized before analysis of deuterated vitamin D. Cell pellets were analyzed for their concentrations of D_3, D_2, 25(OH)D_3 and 25(OH)D_2. Sample preparation and analysis of the vitamin D metabolites were done in accordance to Baur et al. [23]. In brief, serum, tissues, feces and cell pellets were mixed with internal standards (D_3-d_7, Toronto Research Chemicals Inc., BIOZOL, Eching, Germany and 25(OH)D_3-d_6, Chemaphor Chemical Services, Ottawa, Canada). The samples were hydrolyzed with potassium hydroxide, extracted with n-hexane and washed with ultrapure water. After evaporating the solvent, tissue, feces and cell pellets were resolved in n-hexane/isopropanol (99/1, v/v) and fractionated by a high performance liquid chromatography (HPLC) system (Agilent 1100, Agilent Technologies, Waldbronn, Germany) equipped with a Zorbax Rx-SIL column (250 × 4.6 mm; particle size: 5 µm; Agilent Technologies). All samples were derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione (Sigma Aldrich) [24]. Derivatized samples were dried and resolved in methanol/ammonium formate (10 mM) (4/1, v/v), before analysis by tandem mass spectrometry (MS/MS, QTRAP 5500, Sciex, Darmstadt, Germany) with ESI^+ ionization coupled to a reverse phase HPLC system (Agilent 1200, Agilent Technologies). The reverse phase HPLC was equipped with a Hypersil ODS column (150 × 2.0 mm, particle size: 5 µm; VDS Optilab, Berlin, Germany) or a Poroshell 120 column (4.6 × 50 mm, particle size: 2.7 µm, Agilent Technologies) and pure MS-grade solvents (A: acetonitrile, B: aqueous acetonitrile (1/1, v/v) with 5 mM ammonium formate and 0.1 % formic acid) were used, with a gradient as described before [23]. The following mass transitions were recorded in multiple reaction monitoring (quantifier ions): D_3-d_3, 563 > 301; D_3, 560 > 298; D_2-d_3, 575 > 301; D_2, 572 > 298; D_3-d_7, 567 > 298; 25(OH)D_3-d_3, 579 > 301; 25(OH)D_3, 576 > 298; 25(OH)D_2-d_3, 591 > 301; 25(OH)D_2, 588 > 298; 25(OH)D_3-d_6, 582 > 298. The mass transitions of all analytes were verified by qualifier ions: D_3-d_3, 563 > 283; D_3, 560 > 280; D_2-d_3, 575 > 283; D_2, 572 > 280; 25(OH)D_3-d_3, 579 > 561; 25(OH)D_3, 576 > 558; 25(OH)D_2-d_3, 591 > 164; 25(OH)D_2, 588 > 341.

The limit of quantification (LOQ) was defined as a signal-to-noise ratio above 10. In serum, the LOQ was 1.3 ng/mL for D_3-d_3, 0.03 ng/mL for D_2-d_3 and 0.6 ng/mL for 25(OH)D_3-d_3 and 25(OH)D_2-d_3. The LOQ in tissue samples for D_3-d_3 and D_2-d_3, respectively, was for both 5.4 ng/g in intestinal mucosa and muscle, 2.1 and 0.7 ng/g in liver, and for both 0.2 ng/g in kidney, fat tissue and body homogenate. The LOQ of 25(OH)D-d_3 was 10.0 ng/g for all tissues and both D-vitamers. The LOQ in the cell pellets was 1.0 ng/mg protein for D_3, 3.0 ng/mg for D_2, 0.2 ng/mg for 25(OH)D_3 and 0.1 ng/mg for 25(OH)D_2.

The serum concentration of total 1,25(OH)$_2$D (the sum of 1,25(OH)$_2D_3$ and 1,25(OH)$_2D_2$), was analyzed via enzyme immunoassay (IBL International GmbH, Hamburg, Germany) after extraction with diisopropylether, cyclohexane and ethylacetate in accordance with the manufacturers protocol. Prior to the assay, the serum was diluted in isotonic saline (1/8, v/v). The cross-reactivity for 1,25(OH)$_2D_3$ and 1,25(OH)$_2D_2$ was 114 % and 108 %, respectively, as described in the manual.

Analysis of free 25(OH)D was conducted by use of a commercially available ELISA kit (DiaSource ImmunoAssays S.A, Louvain-la-Neuve, Belgium). This assay has a cross-reactivity of 77 % for 25(OH)D_2, as stated by the manufacturer.

The concentration of DBP in mice serum was determined via a sandwich ELISA, designed with the Mouse Vitamin D BP Duo Set (R&D Systems, Bio-Techne GmbH, Wiesbaden, Germany). Prior to analysis, serum was diluted in isotonic saline (1/200 000, v/v). Absorption was measured at 450 nm with reference wavelength set to 550 nm. A linear regression was used to analyze the data. Mean recovery of spiked serum samples was 84.6 ± 6.1 % at a concentration of 790 µg/mL.

Table 1
Primer of target and reference genes, used in the analysis of mRNA expression in liver or kidney of mice and hepatoma cell lines.

	Obtained from	Accession number	Product size (bp)
Mice			
Cyp24a1	Sigma Aldrich	NM_009996.3	109
Cyp27a1	Sigma Aldrich	NM_024264.4	126
Cyp27b1	Sigma Aldrich	NM_010009.2	91
Cyp2r1	Sigma Aldrich	NM_177382.4	196
Cyp3a11	Sigma Aldrich	NM_007818.3	186
Gapdh[1]	Eurofins MWG Synthesis	XM_001473623.1	177
Hprt[1]	Sigma Aldrich	NM_013556.2	116
Lrp2	Sigma Aldrich	NM_001081088.1	99
Rplp0[1]	Eurofins MWG Synthesis	NM_007475.5	146
Human			
CYP27A1	Sigma Aldrich	NM_000784.4	147
CYP2R1	Sigma Aldrich	NM_024514	195
CYP3A4	Sigma Aldrich	NM_017460	129
GAPDH[1]	Eurofins MWG Synthesis	NM_002046.3	453
RPLP0[1]	Eurofins MWG Synthesis	NM_001002.3	223

[1] Reference Gene. Sigma Aldrich Chemie GmbH, Taufkirchen, Germany (KiCqStart® SYBR® Green Primers), Eurofins MWG Synthesis GmbH, Ebersberg, Germany. *Cyp24a1,* vitamin D 24-hydroxylase; *Cyp27a1/CYP27A1,* sterol 27-hydroxylase; *Cyp27b1,* vitamin D 1α-hydroxylase; *Cyp2r1/CYP2R1,* vitamin D 25-hydroxylase; *CYP3A4*, cytochrome P450, family 3, subfamily A, polypeptide 4; *Cyp3a11,* cytochrome P450, family 3, subfamily A, polypeptide 11; *Gapdh/GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *Hprt,* hypoxanthine guanine phosphoribosyl transferase; *Lrp2,* low density lipoprotein receptor-related protein 2 (megalin); *Rplp0/RPLP0*, ribosomal protein lateral stalk unit.

2.4. Relative mRNA abundance of genes involved in vitamin D hydroxylation and uptake in liver and kidney of mice and hepatoma cell lines

The relative hepatic and renal mRNA abundance of genes involved in the hydroxylation and renal uptake of vitamin D was analyzed via real-time RT-PCR as described elsewhere [25]. In brief, total RNA was extracted from tissue samples, Hepa1-6- and HepG2-cells using Tri-Fast™ (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. The RNA concentration was determined photometrically at 260 nm and integrity was verified via agarose gel electrophoresis. cDNA was synthesized by reverse transcriptase (M-MLV Reverse Transcriptase, Promega, Madison, WI, USA) and real-time PCR was conducted using GoTaq® Flexi DNA-Polymerase (Promega) and a Rotorgene 6000 cycler (Corbett Research, Mortlake, Australia). The cDNA was amplified over 25–30 cycles, with denaturation at 95 °C, annealing at 58–60 °C and elongation at 72 °C. Amplification and product size was verified by melting curve analysis and agarose gel electrophoresis. The relative mRNA abundance of target genes was calculated by a method of Pfaffl [26] and was expressed as fold-change to the D_3 supplemented group. Hypoxanthine-guanine-phosphoribosyl-transferase (*Hprt*), glyceraldehyd-3-phosphate-dehydrogenase *Gapdh/GAPDH* and ribosomal protein lateral stalk subunit (*Rplp0/RPLP0*) were used as reference genes. Primer manufacturer, accession numbers and expected product sizes for all genes are shown in Table 1.

2.5. Analysis of the relative expressions of proteins involved in vitamin D hydroxylation and uptake in intestinal mucosa, liver and kidney of mice

The relative protein expressions of enzymes involved in intestinal and hepatic hydroxylation (cytochrome P450 (Cyp) 2r1, Cyp27a1 and Cyp3a11, the murine homologue to Cyp3a4) and in renal hydroxylation (Cyp27b1) and uptake (Lrp2, also known as megalin) of vitamin D were

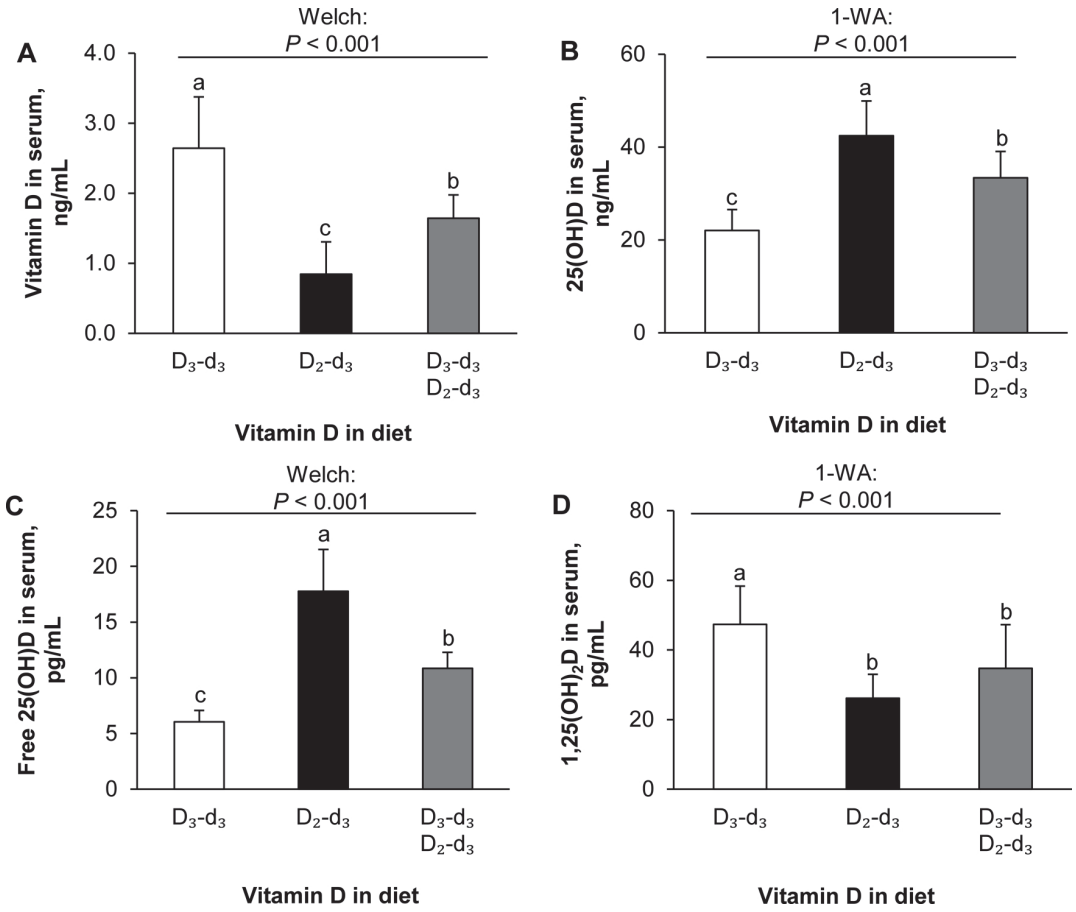

Fig. 1. Concentrations of vitamin D (A), total and free 25(OH)D (B and C) and 1,25(OH)$_2$D (D) in serum of mice fed deuterated vitamin D (either vitamin D$_3$, vitamin D$_2$, or both).
Data are presented as means ± SD, n =12. Mice were fed diets with either 25 µg vitamin D$_3$-d$_3$, 25 µg vitamin D$_2$-d$_3$ or 12.5 µg vitamin D$_3$-d$_3$ + 12.5 µg vitamin D$_2$-d$_3$ per kg diet for 4 weeks. Statistical analysis was conducted via one-way analysis of variance (1-WA) or Welch-ANOVA. [abc] Different superscripts indicate significant differences between the groups (P < 0.05). 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.

analyzed by western blotting as described before [27]. Prior to the analysis, intestinal mucosa, liver and kidney samples were homogenized in RIPA-buffer with a Mixer Mill (MM400, Retsch GmbH, Haan, Germany). After centrifugation, the supernatant was used for protein concentration determination via bicinchoninic acid protein assay. Twenty µg of the protein lysate was used for SDS-PAGE, before transferring to nitrocellulose by semi-dry blotting. Actin or Gapdh, which were not influenced by the treatment, were used for normalization of protein expression data. The following antibodies were used: anti-actin (ab6276, Abcam, Cambridge, UK), anti-Gapdh (#5174, New England Biolabs, Frankfurt am Main, Germany), anti-Cyp27a1 (ab126785, Abcam), anti-Cyp2r1 (ab80101, Abcam), anti-Cyp3a11 (ab197053, Abcam), anti-Cyp27b1 (ab206655, Abcam) and anti-Lrp2 (ab76969; Abcam). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, (#7074, New England Biolabs) or anti-mouse IgG (#7076, New England Biolabs). Bands were detected using ECL Prime western blotting detection reagent (GE Healthcare, Munich, Germany) and the density of each specific band was measured using a computer-assisted imaging analysis system (G: BOX, Gene Tools, Syngene, Cambridge, UK). The protein expression was expressed as fold-change to the D$_3$ supplemented group.

2.6. Statistical analysis

Values are presented as means ± standard deviation (SD). The data of the mouse study were statistically analyzed with SPSS Statistics 25 (IBM, Armonk, USA). All data were tested for normal distribution (Shapiro-Wilk test) and homoscedasticity of variance (Levene's test). If the data were not normally distributed, differences between the three groups of mice were analyzed by the non-parametric Kruskal-Wallis test. If the data followed a normal distribution, differences between the groups were treated by analysis of variance (ANOVA) followed by post-hoc comparison of the means. In case of homoscedasticity, a one-way analysis of variance (1-WA) with the Tukey's test as post-hoc comparison was conducted. In case of unequal variances, Welch's ANOVA (Welch), followed by Games-Howell test was used.

The data of the cell culture studies were analyzed with the SAS 9.4 software package (SAS Institute Inc., Cary, NC, USA). The data were tested for normal-distribution (Shapiro-Wilk) and homoscedasticity of variance (Levene's test). The concentrations of vitamin D metabolites were analyzed by use of two-way ANOVA (2-WA) with the factors cell line, D-vitamer (vitD) and their interaction (cell line x vitD). Dose-dependent effects of the cell culture data were analyzed via 2-WA with the factors concentration (conc), D-vitamer (vitD) and their interaction (conc x vitD). In case of significant interactions between the factors, post-hoc test via Tukey was conducted. Furthermore, if variance heterogeneity was present, a repeated statement was added in the mixed procedure for the interaction effect to model and adjust for unequal variances including Satterthwaite degree of freedom approximation. The gene expression data from the two different cell lines were analyzed

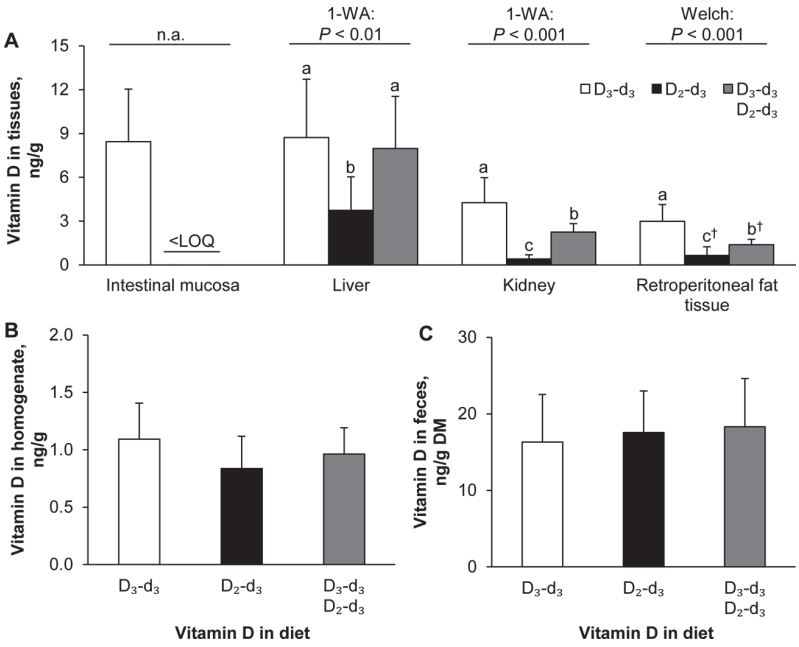

Fig. 2. Vitamin D concentrations in tissues (intestinal mucosa, liver, kidney and retroperitoneal fat tissue) (A), rest of the body homogenates (B) and feces (C) of mice fed deuterated vitamin D (either vitamin D_3, vitamin D_2, or both).
Data are presented as means ± SD, n = 12. Mice were fed diets with either 25 μg vitamin D_3-d_3, 25 μg vitamin D_2-d_3 or 12.5 μg vitamin D_3-d_3 + 12.5 μg vitamin D_2-d_3 per kg diet for 4 weeks. Statistical analysis was conducted via one-way analysis of variance (1-WA) or Welch-ANOVA. [abc] Different superscripts indicate significant differences between the groups ($P < 0.05$). [<LOQ] Data were below the limit of quantification; [†] Data were partly below the limit of quantification (D_2-d_3, n = 2; D_3-d_3 + D_2-d_3, n = 9); DM, dry matter; n.a. not analyzed.

by *t*-test in cases of normal distribution, otherwise with the Mann-Whitney-U test. Differences with $P < 0.05$ were considered to be significant.

Because mice and cell culture samples which received either D_3 or D_2 had only the corresponding D-vitamer, statistical analyses were conducted with the total vitamin D concentration, calculated as the sum of D_3 and D_2. If the D_3 or D_2 concentration of one group, due to lack of oral intake or addition to the incubation media, were below the LOQ, the data were not included in the calculation of the total vitamin D content. If the concentration of a group which received the corresponding D-vitamer was below the LOQ, randomly generated values (between 0 and the appropriate LOQ) were used for statistical treatment analysis.

3. Results

3.1. Mouse study

The final body mass of the mice was not affected by the form of vitamin D provided with the diet (D_3-d_3: 29.2 ± 2.0 g; D_2-d_3: 29.0 ± 2.7 g, D_3-d_3+D_2-d_3: 30.2 ± 1.2 g; $P = 0.235$). The mice, which received only D_3-d_3 or D_2-d_3 with their diets, had either D_3-d_3 and 25(OH)D_3-d_3 or D_2-d_3 and 25(OH)D_2-d_3. The group, which received both D-vitamers with their diets, had metabolites of both. To minimize an interaction with remaining vitamin D in the body of the mice, we fed a vitamin D-free diet prior to the treatment, and analyzed the levels of non-deuterated vitamin D in the serum of the mice. After feeding the vitamin D-free diet for 4 weeks, all mice showed 25(OH)D levels below the LOQ (5.3 nmol/l).

3.1.1. Vitamin D metabolites and vitamin D binding protein in serum and tissue

Mice treated with D_2-d_3 had lower concentrations of total vitamin D-d_3 (D_2-d_3 + D_3-d_3) in serum than mice fed D_3-d_3 or those that received D_2-d_3 + D_3-d_3 ($P < 0.001$, Fig. 1A). In contrast, the serum levels of total and free 25(OH)D-d_3 (25(OH)D_3-d_3 + 25(OH)D_2-d_3) were markedly higher in the D_2-d_3 supplemented group than in the D_3-d_3 supplemented group; the group receiving both D-vitamers had values in-between ($P < 0.001$, Fig. 1B,C). In contrast, the serum concentration of total 1,25$(OH)_2$D (1,25$(OH)_2D_3$ + 1,25$(OH)_2D_2$) was significantly lower in the group fed the D_2-d_3 diet compared to both other groups ($P < 0.001$, Fig. 1D).

Table 2
Relative mRNA expression of genes involved in the hydroxylation of sterols in liver and kidney and the renal sterol uptake of mice fed deuterated vitamin D (either vitamin D_3, vitamin D_2, or both).

	Vitamin D_3-d_3	Vitamin D_2-d_3	Vitamin D_3-d_3 + Vitamin D_2-d_3	*P* Value (1-WA)
Hydroxylation of vitamin D in the liver[1]				
Cyp27a1	1.00 ± 0.16	1.06 ± 0.15	0.89 ± 0.29	0.499
Cyp2r1	1.00 ± 0.36	0.86 ± 0.24	1.05 ± 0.12	0.572
Cyp3a11	1.00 ± 0.40	1.00 ± 0.26	1.05 ± 0.29	0.915
Hydroxylation and uptake of vitamin D in the kidney[2]				
Cyp24a1	1.00 ± 0.42	1.24 ± 0.40	1.04 ± 0.46	0.273
Cyp27b1	1.00 ± 0.47	0.71 ± 0.23	0.74 ± 0.16	*0.064*
Lrp2	1.00 ± 0.46	0.71 ± 0.23	0.75 ± 0.16	*0.063*

Data are presented as means ± SD, n = 12. Mice were fed diets with either 25 μg vitamin D_3-d_3 or 25 μg vitamin D_2-d_3 per kg diet for 4 weeks. Statistical analysis was conducted via one-way analysis of variance (1-WA). *Cyp24a1*, vitamin D 24-hydroxylase; *Cyp27a1*, sterol 27-hydroxylase; *Cyp27b1*, vitamin D 1α-hydroxylase; *Cyp2r1*, vitamin D 25-hydroxylase; *Cyp3a11*, cytochrome P450, family 3, subfamily A, polypeptide 11; *Lrp2*, low density lipoprotein receptor-related protein 2 (megalin). [1] Reference genes were hypoxanthine-guanine-phosphoribosyl-transferase (*Hprt*) and glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*). [2] Reference genes were *Gapdh* and ribosomal protein lateral stalk subunit (*Rplp0*).

Intake of D_2-d_3 resulted in lower concentrations of total vitamin D-d_3 in liver ($P < 0.01$) and kidney ($P < 0.001$, Fig. 2A) of mice than intake of D_3-d_3 or the combination of D_2-d_3 and D_3-d_3. Remarkably, in the intestinal mucosa and most of the retroperitoneal fat tissue vitamin D-d_3 was only quantifiable in the D_3-d_3 supplemented mice ($P < 0.001$, Fig. 2A). In the muscle samples, no vitamin D-d_3 was quantifiable at all (below the LOQ). The analysis of vitamin D in the body homogenate ($P = 0.092$, Fig. 2B) and feces ($P = 0.845$, Fig. 2C) revealed no significant differences between the three groups. The concentrations of 25(OH)D were below the LOQ in all analyzed tissue samples (intestinal mucosa, liver, kidney, fat and muscle) as well as in the body homogenates.

The serum concentration of DBP did not differ between the three

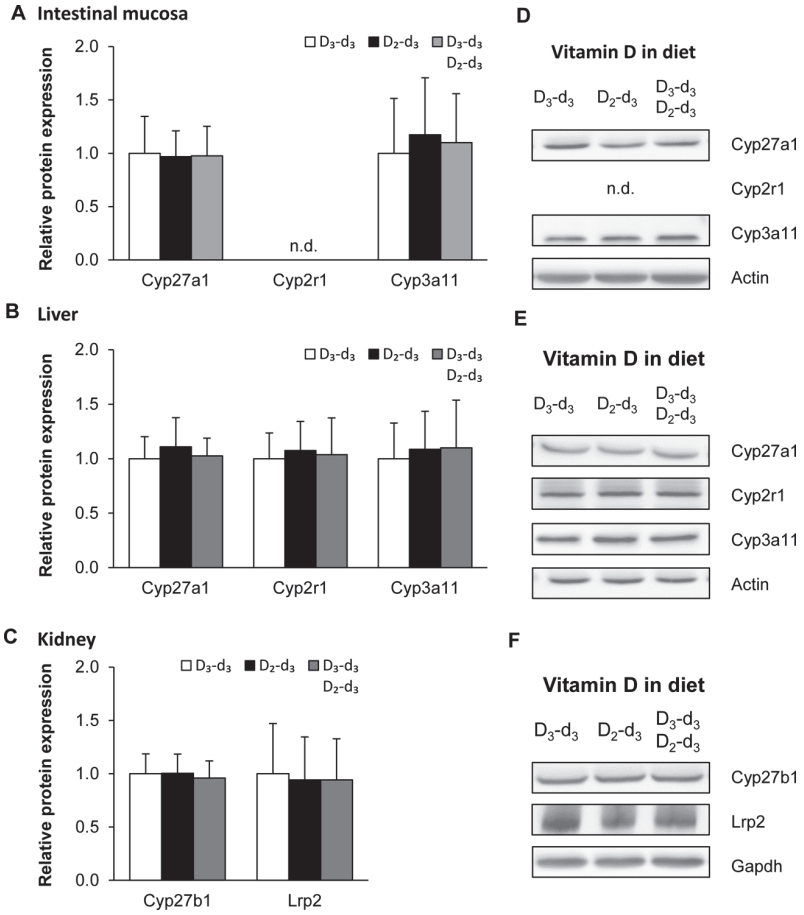

Fig. 3. Relative protein expressions of Cyp27a1, Cyp2r1 and Cyp3a11 in intestinal mucosa (A) and liver (B) and of Cyp27b1 and Lrp2 in kidney (C) of mice fed deuterated vitamin D (vitamin D_3, vitamin D_2, or both). (D, E, F) Representative western blot images.
Data are presented as means ± SD, n = 12. Mice were fed diets with either 25 μg vitamin D_3-d_3, 25 μg vitamin D_2-d_3 or 12.5 μg vitamin D_3-d_3 + 12.5 μg vitamin D_2-d_3 per kg diet for 4 weeks. Cyp27a1, sterol 27-hydroxylase; Cyp27b1, vitamin D 1α-hydroxylase; Cyp2r1, vitamin D 25-hydroxylase; Cyp3a11, cytochrome P450, family 3, subfamily A, polypeptide 11; Lrp2, low density lipoprotein receptor-related protein 2 (megalin); Gapdh, glyceraldehyde-3-phosphate-dehydrogenase. n.d.: not detectable.

groups of mice (D_3-d_3: 164 ± 31 μg/mL, D_2-d_3: 170 ± 31 μg/mL, D_3-d_3+D_2-d_3: 171 ± 31 μg/mL; $P = 0.883$).

3.1.2. Gene and protein expressions of hepatic and renal genes involved in the vitamin D hydroxylation and renal uptake

The relative mRNA abundance of the hepatic genes *Cyp27a1*, *Cyp2r1* and *Cyp3a11* (murine homolog to *CYP3A4*), which are involved in the 25-hydroxylation of vitamin D, did not differ between the three groups (Table 2). The relative mRNA abundance of the renal *Cyp27b1* and *Lrp2* (megalin) tended to be lower in mice which received D_2-d_3 than in mice which were exclusively treated with D_3-d_3 ($P < 0.1$, Table 2). The renal mRNA abundance of *Cyp24a1* was not different between the three groups of mice (Table 2).

Western blot analysis revealed no differences in the protein expression of the intestinal and hepatic hydroxylases Cyp27a1, Cyp2r1 and Cyp3a11 and the renal Cyp27b1 and Lrp2 between the three groups of mice (Fig. 3A-C).

3.2. Cell culture experiments

3.2.1. Vitamin D metabolite concentrations in murine and human liver cells

To compare the response of murine and human hepatic vitamin D metabolism to D_3 and D_2, we analyzed the D_3, D_2, 25(OH)D_3 and 25(OH)D_2 concentrations in murine Hepa1-6 and human HepG2 cells after incubation with 1 μM D_3 or 1 μM D_2. Analyses revealed that the cellular uptake of D_2 was higher than that of D_3 in both types of cells. Interestingly, the uptake of D_2 in HepG2 cells was markedly higher than in the Hepa1-6 cells, whereas the uptake of D_3 was comparable between the two cell lines (Fig. 4A). Irrespective of the D-vitamer, the HepG2 cells showed higher cellular concentrations of 25(OH)D than Hepa1-6 cells (Fig. 4B). The concentrations of synthesized 25(OH)D was considerably lower after treatment with D_2 than D_3 in both types of cells.

To access whether the D-vitamer differently affects the dose-dependent vitamin D uptake and hydroxylation in Hepa1-6 or HepG2 cells, cells were treated with 1 μM, 5 μM or 10 μM of D_3 or D_2 for 48 h. Irrespective of the D-vitamer, the concentration of cellular vitamin D in both cell lines increased with increasing treatment doses of vitamin D ($P < 0.001$, 2-WA; Fig. 5A, B). The cellular concentrations of 25(OH)D also increased dose-dependently in both cell lines ($P < 0.01$, 2-WA, Fig. 5C, D). However, the increase in 25(OH)D was more pronounced after treatment with D_3 than D_2. As observed in the first cell culture study, HepG2 cells showed significantly higher cellular concentrations of 25(OH)D than Hepa1-6 cells.

3.2.2. mRNA abundance in hepatoma cells of genes responsible for vitamin D hydroxylation

To elucidate potential effects of the D-vitamers on vitamin D hydroxylation enzymes in hepatoma cells, we analyzed the mRNA abundance of the enzymes involved in the hydroxylation of vitamin D, but found no difference between the treatment with D_3 and D_2 (Table 3).

4. Discussion

To elucidate possible mechanisms that may explain the different responses of 25(OH)D to supplementation of D_3 and D_2 in humans, we conducted a mouse study and compared the effect of orally administered D_3 versus D_2 on cellular uptake, tissue distribution and hydroxylation of vitamin D. Interestingly, mice fed D_2 had lower serum and tissue levels

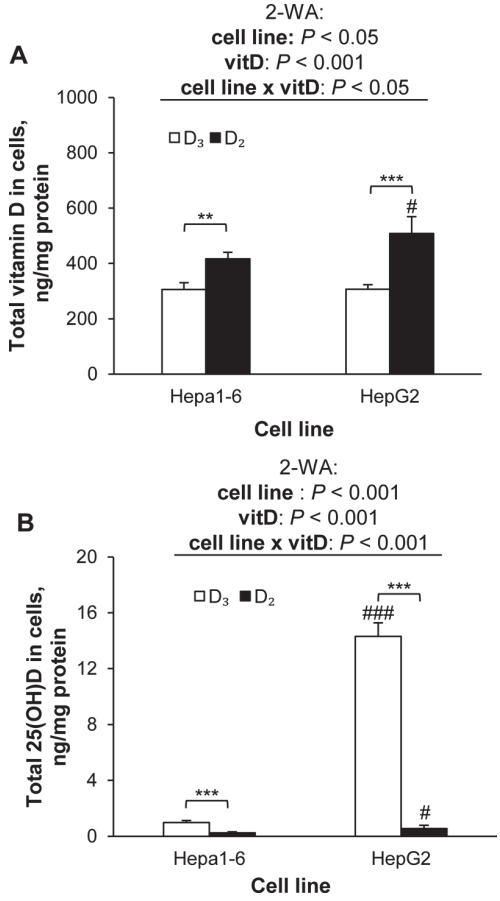

Fig. 4. Concentrations of vitamin D (A) and 25(OH)D (B) in murine Hepa1-6 and human HepG2 cells after incubation with vitamin D (either vitamin D_3 or vitamin D_2).

Data are presented as means ± SD, n = 4. Cells were incubated with 1 μM vitamin D_3 or 1 μM vitamin D_2 in FCS-free medium supplemented with 0.5 % gentamicin (10 mg/mL) and 10 % pluronic® F127 for 48 h. Statistical analysis was conducted via two-way analysis of variance (2-WA) with cell line and D-vitamer (vitD) as classification factors and their interaction (cell line x vitD). ** significantly different ($P < 0.01$); *** significantly different ($P < 0.001$); # significantly different from corresponding Hepa1-6 cells ($P < 0.05$); ### significantly different from corresponding Hepa1-6 cells ($P < 0.001$); 25(OH)D, 25-hydroxyvitamin D.

of vitamin D, higher circulating levels of total and free 25(OH)D, and markedly lower levels of $1,25(OH)_2D$ than mice fed D_3. Mice fed both D-vitamers had values in between, suggesting no mutual influence of the two D-vitamers.

In order to elucidate whether D_2 is absorbed less than D_3, we analyzed fecal concentrations of deuterated vitamin D, but found no differences between the treatment groups. Thus, it can be assumed that both D vitamers are absorbed in comparable quantities. The results indicate that the observed differences in serum and tissue levels of vitamin D, 25(OH)D and $1,25(OH)_2D$ after treatment with D_3 and D_2 were caused by differences in the formation and/or degradation of vitamin D metabolites. In contrast to data observed from human studies, the current findings are indicative of a higher 25(OH)D synthesis after supplementation with D_2 than with D_3, assuming that the response of mice to D_3 and D_2 differs from that of humans. Vitamin D is mainly activated to 25(OH)D in the liver by three different cytochrome-P450 (Cyp) enzymes, whereby Cyp2r1 can convert both, D_3 and D_2 [28], while Cyp27a1 specifically converts D_3 [29] and Cyp3a4 D_2 [30]. Here we found no differential effects of both D-vitamers on the mRNA and protein expressions of these enzymes, assuming that the increased level of 25(OH)D in mice fed D_2 was caused by other factors than alterations in enzyme expression. Surprisingly, data from our cell culture studies clearly indicate that D_2 has a lower affinity to the hydroxylases than D_3, because treatment of murine and human hepatoma cells with D_2 resulted in a considerably lower production of 25(OH)D than treatment with D_3. Thus, data from the cell studies are diametrically opposed to the findings from the mouse study. We therefore assume that the observed differences in circulating 25(OH)D in mice of the D_3 and D_2 groups cannot be explained by vitamer-specific effects on hepatic hydroxylation.

Vitamin D hydroxylases such as Cyp27a1 [31], Cyp2r1 and Cyp3a11 [32] are also expressed in the intestine. To elucidate, whether the differential effects of D_3 and D_2 on serum levels of 25(OH)D are caused by differences in intestinal hydroxylation, we analyzed the protein expression of Cyp2r1, Cyp27a1 and Cyp3a11 in the intestinal mucosa. Here, we found no detectable intestinal Cyp2r1 and no differences in the expressions of intestinal Cyp27a1 and Cyp3a11 between the groups fed D_3, D_2 or both vitamers, assuming that the expression of intestinal hydroxylating enzymes also provides no explanation for the observed differences in circulating 25(OH)D.

Interestingly, mice of the D_2 group had higher concentrations of both, the total (free and protein-bound) and the free 25(OH)D alone than mice of the D_3 group. The higher levels of free 25(OH)D after feeding D_2 is probably a result of a reduced binding of $25(OH)D_2$ to DBP, and has been previously found by several other authors [33–35]. Binding of 25(OH)D to the DBP is crucial for vitamin D uptake in kidneys. This could be responsible for a lower renal synthesis of $1,25(OH)_2D$ from 25(OH)D and explain the lower levels of $1,25(OH)_2D$ in serum of mice fed the D_2. In addition, the renal mRNA abundance of megalin (*Lrp2*), which is crucial for the uptake of vitamin D in the kidney [36], and of *Cyp27b1* (1α-hydroxylase) in the kidney tended to be lower in the D_2 group than in the D_3 group ($P = 0.064$ and $P = 0.063$, respectively).

Interestingly, we found an inverse correlation between circulating levels of 25(OH)D and $1,25(OH)_2D$ in mice ($r = -0.490$; $P = 0.002$; Pearson-correlation). Thus, it is possible that the lower level of the bioactive $1,25(OH)_2D$ in mice fed D_2 instead of D_3 had caused a compensatory increase in 25(OH)D synthesis by not the expression, but the activities of vitamin D hydroxylases.

Both, 25(OH)D and $1,25(OH)_2D$ are catabolized through Cyp24a1 in the kidney [37] or Cyp3a4 (Cyp3a11 in mice) in the liver and intestine [38]. Current findings are not indicative of any differences in the renal Cyp24a1 mRNA abundance and the Cyp3a4 expression in liver and intestine between the D_3 and D_2 groups. Because Cyp3a4 is known to degrade $1,25(OH)_2D_2$ faster than $1,25(OH)_2D_3$ [39], we speculate that D_2 was catabolized more strongly than D_3, which could have caused the lower 1,25(OH)D and tissue vitamin D levels, while production of 25(OH)D was increased to compensate vitamin D deficiency. As deuterated standards of vitamin D degradation products are not commercially available, we were not able to quantify the serum concentrations of 24,25-dihydroxyvitamin D or 1,24,25-trihydroxyvitamin D, the major metabolites in the C24-catabolic pathway of 25(OH)D.

As several human data show that D_2 has not the same potential to increase serum levels of 25(OH)D as D_3 [8–13], we compared the hepatic formation of 25(OH)D from D_3 and D_2, respectively, by using murine and human hepatoma cells. Despite a general higher 25(OH)D formation in the human hepatoma cells than in the murine cells, we found that both types of cells showed comparable responses to treatments with D_3 and D_2. This finding suggests that livers of humans and mice respond similarly to the increasing doses of both D-vitamers, although the rate of 25(OH)D formation was markedly higher in human liver cells than in murine liver cells. In both cell lines, the formation of 25(OH)D was higher in response to treatment with D_3 than with D_2. However, in line with the mouse study, gene expression analysis in both cell lines revealed no differences in the mRNA abundance of the hydroxylation enzymes (Cyp27a1, Cyp2r1 and Cyp3a11 or Cyp3a4,

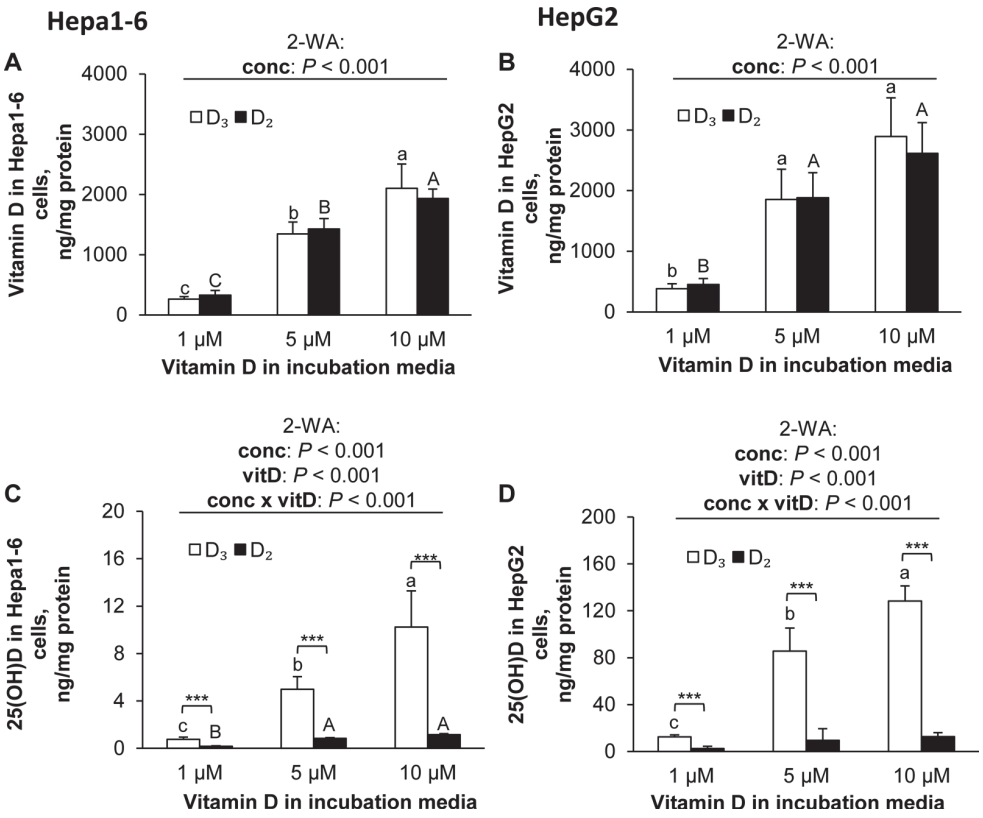

Fig. 5. Concentrations of vitamin D and 25(OH)D in murine Hepa1-6 cells (A and C) and human HepG2 cells (B and D) after incubation with different concentrations of vitamin D (either vitamin D_3 or vitamin D_2).
Data are presented as means ± SD, n = 4. Cells were incubated with 1 µM, 5 µM and 10 µM vitamin D_3 or vitamin D_2 in FCS−free medium supplemented with 0.5 % gentamicin (10 mg/mL) and 10 % pluronic® F127 for 48 h. Statistical analysis was conducted via Two-way analysis of variance (2-WA) with concentration (conc) and D-vitamer (vitD) as classification factors and their interaction (conc x vitD). *** statistically different between the D-vitamer ($P < 0.001$); [abc] or [ABC] Different superscripts indicate significant differences between the concentrations ($P < 0.05$). 25(OH)D, 25-hydroxyvitamin D.

Table 3
Relative mRNA expression of genes responsible for vitamin D hydroxylation in murine and human hepatoma cell lines after incubation with vitamin D (either vitamin D_3 or vitamin D_2).

	Vitamin D_3	Vitamin D_2	*P* Value
Murine Hepa1-6 cells[1]			
Cyp2r1	1.00 ± 0.13	1.08 ± 0.06	0.149[a]
Cyp27a1	1.00 ± 0.14	0.98 ± 0.09	0.773[b]
Cyp3a11	1.00 ± 0.12	0.85 ± 0.07	*0.072*[b]
Human HepG2 cells[2]			
CYP2R1	1.00 ± 0.20	1.16 ± 0.12	0.225[b]
CYP27A1	1.00 ± 0.53	0.98 ± 0.46	0.965[b]
CYP3A4	1.00 ± 0.24	0.94 ± 0.20	0.691[b]

Data are presented as means ± SD, n = 4. [a] Statistical analysis was conducted via *t*-test. [b] Statistical analysis was conducted via Mann-Whitney-U test. Cells were incubated with 1 µM vitamin D_3 or 1 µM vitamin D_2 in FCS-free medium supplemented with 0.5 % gentamicin (10 mg/mL) and 10 % pluronic® F127 for 6 h. *Cyp27a1/CYP27A1,* sterol 27-hydroxylase; *Cyp2r1/CYP2R1,* vitamin D 25-hydroxylase; *CYP3A4*, cytochrome P450, family 3, subfamily A, polypeptide 4; *Cyp3a11,* cytochrome P450, family 3, subfamily A, polypeptide 11. [1]Reference genes were glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) and ribosomal protein lateral stalk subunit (*Rplp0*). [2] Reference genes were *GAPDH* and *RPLP0*.

respectively) in response to D_3 and D_2. As mice showed higher serum levels of 25(OH)D after feeding D_2 than after feeding D_3, it must be emphasized that data from cell culture studies do not necessarily reflect *in vivo* levels of serum 25(OH)D.

To conclude, the current data demonstrate that mice show distinct responses to orally administered D_3 and D_2. In contrast to the effects observed in human intervention studies, mice showed higher serum levels of total and free 25(OH)D after the intake of D_2 than D_3. This effect can not be explained by differences in the rate of hepatic hydroxylation, because murine and human hepatoma cells in culture appear to prefer D_3 as substrate for 25(OH)D synthesis. Interestingly, the formation of 25(OH)D was more pronounced in human hepatoma cells than in the murine hepatoma cells. Besides 25(OH)D, mice fed D_3 or D_2 with their diets differ in the levels of bioactive vitamin D hormone in serum and tissue concentrations of vitamin D, in such a way that the D_2 fed group had noticeably lower levels of circulating $1,25(OH)_2D$ and tissue vitamin D than D_3 fed mice.

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CRediT authorship contribution statement

Anja C. Baur: Conceptualization, Investigation, Validation, Formal analysis, Writing - original draft. **Corinna Brandsch:** Methodology, Writing - review & editing. **Benita Steinmetz:** Investigation, Validation. **Alexandra Schutkowski:** Methodology, Investigation. **Monika Wensch-Dorendorf:** Formal analysis. **Gabriele I. Stangl:** Supervision, Conceptualization, Writing - original draft.

Declaration of Competing Interest

The authors report no declarations of interest.

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4 Discussion

4.1 Plant oils as potential sources of vitamin D

To combat vitamin D deficiency, alternative vitamin D sources are needed. As part of this thesis, eight selected plant oils (avocado, linseed, olive, pumpkinseed, rapeseed, soya, sunflower oil and WGO) were identified to have considerable amounts of the vitamin D precursors ergosterol and 7-DHC (**Study 1**).The mean ergosterol concentration was 9.9 µg/g (range: 0.5 – 34.4 µg/g) and was lowest in the pumpkinseed oils and highest in WGOs. In all oils, the concentrations of 7-DHC were much lower than of ergosterol, with a mean 7-DHC concentration of 0.11 µg/g (range: 0.01-0.67 µg/g). WGO was identified to have high concentrations of ergosterol (30.2 ± 7.0 µg/g) and 7-DHC (0.65 ± 0.02 µg/g). Plants are per se not capable of the production of ergosterol, therefore ergosterol is often used as a marker for fungal growth in plant materials and grains, especially when in storage or in processed products (Perkowski et al., 2008; Schnürer and Jonsson, 1992; Seitz et al., 1979). Boarelli et al. measured ergosterol in degraded olives and found highest values in the visible molded ones. They also found ergosterol concentrations in oils of these olives ranging from <0.1 µg/g to 39.9 µg/g, with concentrations of <3 µg/g for organoleptic high-quality oils (Boarelli et al., 2020). 7-DHC is known to occur in plants that apparently synthesize cholesterol from lanosterol (Jäpelt and Jakobsen, 2013). Because plants (Horst et al., 1984; Jäpelt et al., 2011b; Jäpelt et al., 2011a) and plant oils (Yuenyong et al., 2021) have also been shown to contain vitamin D, we analyzed the concentration of vitamin D_2 and vitamin D_3 in the eight plant oils, but were not able to detect any. Since the conversion of vitamin D precursors to vitamin D requires UVB-light, it is suggested that the oil plants were not exposed to any source of UVB-light post-harvesting. To further investigate, whether the vitamin D concentration can be enhanced through short-term exposure to UVB-light, we selected three oils with low, medium and high concentrations of vitamin D precursors, respectively, and treated them for 4 or 8 minutes with UVB-light (**Study 1**). Here, the concentration of vitamin D increased steadily with increasing UVB-exposure times, depending on the initial concentration of vitamin D precursors, the thickness of the oil layer, the distance of the UVB-emitting lamp from the oil surface (**Study 1**) and the intensity of the UVB lamp (**Study 1**, **Study 2**). In **Study 1** and **Study 2** the total vitamin D concentrations (vitamin D_2 + vitamin D_3) of the UVB-exposed WGOs after a ten-minute UVB-exposure were 0.89 µg/g and 2.37 µg/g, respectively. Hence, twelve grams of these WGO would provide approximately 10 µg or 30 µg vitamin D, respectively, which would meet 50 or 150 % of the D-A-CH recommendations of 20 µg vitamin D per day (German Nutrition Society, 2012).

4.2 Quality, stability and safety of the UVB-exposed oils

WGO is generally rich in health-promising substances, such as flavonoids (Pietrzac et al., 1996) and phytosterols (Anderson et al., 1926; Dulf et al., 2010). In particular, WGO is associated with high concentrations of tocopherols (Trela and Szymańska, 2019). An average daily consumption of 12 g WGO (Max-Rubner-Institut, 2013) would provide approximately 19.2 – 22.8 mg α-tocopherol equivalents (**Study 1**, **Study 2**), which meets the recommended dietary allowances of 15 mg α-tocopherol per day (Traber and Manor, 2012). Additionally, WGO is rich in unsaturated fatty acids, like oleic acid (C18:1, n-9) and polyunsaturated fatty acids, like linoleic acid (C18:2, n-6) and linolenic acid (C18:3, n-3) (Barnes, 1982; Wang and Johnson, 2001). Especially polyunsaturated fatty acids are susceptible for autoxidation (Frankel et al., 1959; Yin and Porter, 2005). Hence, UVB-exposed WGOs were analyzed for their concentrations in markers for autooxidation, antioxidants and sensory quality. Oils treated with UVB-light were not exceptionally susceptible for autoxidation, as there were no differences between UVB-exposed WGO and untreated WGO in the peroxide and acid values (**Study 1**, **Study 2**). Additionally, concentrations of the antioxidant tocopherols were not altered by an UVB-treatment. However, organoleptic tests revealed a slightly lesser taste of the UVB-treated WGO than of the untreated counterparts in **Study 1** and **Study 2**. An effect of an UV-treatment on oxidative and sensory qualities, has been shown in other food stuffs. In mushrooms, an UVB-treatment showed no effect in color and texture (Ko et al., 2008; Koyyalamudi et al., 2009), but decreased the concentrations of antioxidants slightly (Gallotti and Lavelli, 2020). In general, a variety of food sources are exposed to UV-light, typically at 254 nm (UVC-light), to reduce the number of harmful microorganisms (Csapó et al., 2019). Especially, fluids like juices and milk are often preserved by UV-light exposure (Choudhary and Bandla, 2012). Some authors found UVC-light exposure to be accompanied by oxidative degradation processes (Csapó et al., 2019) and for example, milk has long been proven to show an off-flavor after exposure to sunlight (Bradley, 1980). On the contrary, Delorme et al. found that a treatment of milk and dairy products with UVC-light is an effective method to inactivate pathogenic microorganism without negatively affecting the nutritional or sensory quality (Delorme et al., 2020) and therefore might be an efficient alternative to thermal treatment, like e.g. pasteurization, which is usually accompanied by nutritional and sensory quality losses (Buchin et al., 1998). An effect of a thermal treatment and pro-longed storage was also observed in **Study 1**. While the concentrations of tocopherols were not altered through the treatment, the peroxide values of the oils were tremendously negatively affected by the thermal

treatment and pro-longed storage, independently from the UVB-exposure. In addition, organoleptic tests showed an off-flavor of all thermal processed WGOs (**Study 1**).

In general, vitamin D shows a high stability after cooking in eggs (vitamin D_3, retention 94-99 %) (Mattila et al., 1999), fish (vitamin D_3, 75-114 %) (Bhuiyan et al., 1993; Elmadfa et al., 2006; Ložnjak and Jakobsen, 2018; Mattila et al., 1999), mushrooms (vitamin D_2, 67-100 %) (Ložnjak and Jakobsen, 2018; Mattila et al., 1999) and fortified sunflower oil (vitamin D_3, 70-96 %; vitamin D_2, 72-97 %) (Ložnjak and Jakobsen, 2018) and is stable under storage conditions (Jafari et al., 2016; Temova and Roškar, 2017). In **Study 1**, the vitamin D concentration increased under thermal treatment and prolonged storage. The retentions for vitamin D_2 and vitamin D_3 in the UVB-exposed WGO after thermal treatment were 95–150 % and 93–166 %, respectively. Further, the retentions upon storage in darkness at room temperature were exceedingly high for vitamin D_2 (124 – 212 %) and vitamin D_3 (135 – 215 %), with highest total vitamin D concentrations after 2 weeks of storage. Other UVB-exposed foods, like mushrooms, have also been shown to increase in their vitamin D contents during storage (Guan et al., 2016; Koyyalamudi et al., 2009). Since the tolerable upper intake level of vitamin D is 100 µg per day (Ross et al., 2011), it is crucial to implement upper limits for the vitamin D concentrations in the UVB-exposed oils to prevent overdosing, especially in terms of thermal treatment and storage. Upper limits for vitamin D concentrations have already been introduced for UVB-exposed mushrooms and yeasts (EFSA, 2014, 2020).

The finding that the vitamin D concentration increases under thermal treatment and storage conditions is presumably due to significant amounts of previtamin D in freshly UVB-exposed foods, which then converts to vitamin D (Kotwan et al., 2021b) (**Figure 3**). In addition, the formation of vitamin D from provitamin D under UVB-exposure also leads to the formation of photoisomers such as lumisterol and tachysterol (**Figure 3**). In the skin, only small amounts of the instable intermediate previtamin D_3 are converted to vitamin D_3 (Holick et al., 1981). To prevent vitamin D intoxication under pro-longed UVB-exposure, previtamin D_3 is also transformed in tachysterol$_3$ and lumisterol$_3$ in a quasi-stationary state, with lumisterol$_3$ being quantitative more relevant than tachysterol$_3$ (Holick et al., 1981; Wacker and Holick, 2013). Similarly, the UVB-induced transformation of ergosterol to vitamin D_2 is associated with the forming of the photoisomers lumisterol$_2$ and tachysterol$_2$ (Keegan et al., 2013). Therefore, these photoisomers have been found in UVB-exposed foods, like yeasts (EFSA, 2014; Kotwan et al., 2021a) and mushrooms (EFSA, 2020; Kalaras et al., 2012b; Keegan et al., 2013; Krings and Berger, 2014; Wittig et al., 2013). In their safety opinions of novel foods, the EFSA stated that the concentration of photoisomers in UVB-exposed foods is too low to raise any concerns (EFSA, 2014, 2020). However, the concentration of photoisomers may be of health

interest (Schümmer et al., 2021), especially since their concentrations depend strongly on the intensity of the UVB-light source and the duration of the treatment (Holick et al., 1981; Keegan et al., 2013). For example, Kalaras et al. found 10-times higher concentrations of tachysterol$_2$ (2.79 vs. 24.7 µg/g dry matter (DM)) and lumisterol$_2$ (3.05 vs. 30.5 µg/g DM) in mushrooms, when the UVB-exposure was prolonged from only 1 s to 20 s (Kalaras et al., 2012b). Wittig et al. showed tachysterol$_2$ and lumisterol$_2$ concentrations, which account for 30 % and 50 % of the vitamin D$_2$ concentrations in sliced oyster mushrooms after a 60-min UVB-exposure (24.1 ± 0.5 and 41.1 ± 0.7 µg/g DM, respectively, vs. 78.8 ± 3.0 µg/g DM) (Wittig et al., 2013). The assumption that photoisomers are biologically inactive (Holick et al., 1981) was questioned by Slominski et al., who showed that hydroxylated lumisterol can interfere with the non-genomic side of the VDR (Slominski et al., 2017). Indicative for an biological effect of the photoisomers is that vitamin D$_2$ from UVB-exposed yeasts do not raise the 25(OH)D$_2$ levels as efficiently as equal amounts of semi-synthetic vitamin D$_2$ in rats (Itkonen et al., 2018b), humans (Itkonen et al., 2016) and *in vitro*-digestibility studies (Lipkie et al., 2016). Recently, Kotwan et al. demonstrated that lumisterol$_2$ affects the vitamin D$_3$ metabolism, since tissue and circulating 25(OH)D$_3$ and circulating 1,25(OH)$_2$D$_3$ concentrations were substantially lowered in mice, which received high oral doses of lumisterol$_2$. In contrast, the tissue vitamin D$_3$ concentrations were higher (Kotwan et al., 2021a). Finally, Kotwan and coworkers fed UVB-exposed yeasts to mice and observed higher tissue levels of vitamin D than in mice fed iso-amounts of vitamin D$_2$, although the concentrations of hydroxylated vitamin D were similar between the groups (Kotwan et al., 2021a). These findings fit well with the observations made in **Study 1**. Here, the circulating total 25(OH)D levels of mice receiving vitamin D$_2$ from UVB-exposed WGO were lower compared to mice receiving the vitamin D$_3$ supplemented oil, while the hepatic concentration of total non-hydroxylated vitamin D was tremendously higher (699 ± 135 ng/g vs. 30.3 ± 11.2 ng/g) (**Study 1**). The findings from **Study 2** corroborate this theory, since analysis revealed higher non-hydroxylated vitamin D concentrations in serum of subjects treated with UVB-exposed WGO, than in serum of non-treated control subjects. Unfortunately, the concentration of photoisomers was not assessed in **Study 1** or **Study 2**. Random analyzes of samples from the UVB-exposed WGO receiving participants from **Study 2** revealed no detectable concentrations of lumisterol and tachysterol in the serum. However, the building of such and the subsequent oral intake has to be considered when implementing UVB-exposed foods for human nutrition.

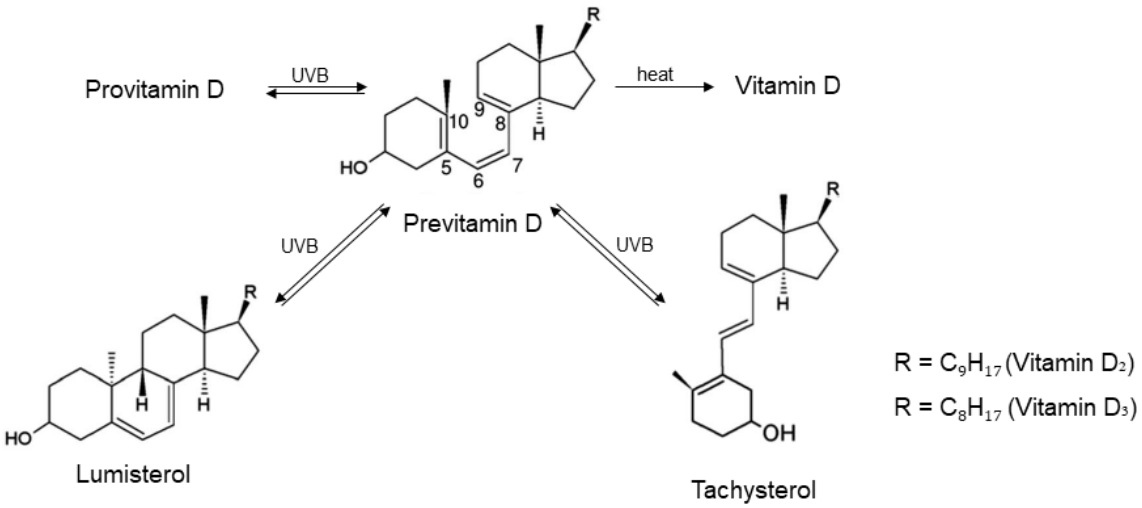

Figure 3 Photosynthesis of lumisterol and tachysterol from previtamin D.

Previtamin D, an intermediate from the UVB-light induced formation of vitamin D out of provitamin D (ergosterol and 7-dehydrocholesterol), is converted to lumisterol and tachysterol under prolonged UVB-light exposure. Modified from Cisneros et al. (2017).

4.3 Efficiency of UVB-exposed wheat germ oils to improve the vitamin D status

The bioavailability of vitamin D from UVB-exposed WGO was first assessed in mice (**Study 1**) and later in a randomized controlled intervention study with healthy humans (**Study 2**). The current data showed that UVB-exposed WGO was able to increase the 25(OH)D_2 serum concentrations significantly in mice and humans. However, the vitamin D status, assessed by the total 25(OH)D concentration was only improved in mice but not in humans. In **Study 1**, the 25(OH)D concentrations in the vitamin D-depleted mice (baseline 25(OH)D, < 4.2 nmol/L) of the control group remained unchanged and undetectable, while the mice, which received UVB-exposed WGO, had 25(OH)D_2 concentrations of 46.3 ± 8.5 nmol/L. However, the increase in 25(OH)D_2 was much lower than the increase in 25(OH)D_3 of the mice which received the same daily dosage of vitamin D_3 (81.6 ± 16.8 nmol/L nmol/L). In **Study 2**, the UVB-exposed WGO increased the 25(OH)D_2 but, due to a decrease in 25(OH)D_3, not the total 25(OH)D serum concentrations of vitamin D insufficient but otherwise healthy humans. At the end of the study, participants, who consumed the UVB-exposed WGO, had even lower concentrations of 25(OH)D than the participants of the control group, who received no oil (**Study 2**).

It has been demonstrated before that in rats, the circulating 25(OH)D_2 levels can be significantly increased by a consumption of UVB-treated mushrooms (Calvo et al., 2013; Jasinghe et al., 2005; Koyyalamudi et al., 2009) and yeasts (Hohman et al., 2011; Itkonen et al., 2018b). However, results from human intervention studies are inconsistent. While some studies indicate a good bioavailability of vitamin D_2 from UVB-exposed mushrooms (Outila et al., 1999; Urbain et al., 2011), others report no significant changes in total 25(OH)D compared to baseline levels after consuming UVB-exposed mushrooms (Nieman et al., 2013; Stephensen et al., 2012; Stepien et al., 2013) or yeasts (Itkonen et al., 2016) (Supplementary **Table S1**). A meta-analysis of various human intervention studies regarding the effectiveness of UVB-exposed mushrooms concluded that the increase of total 25(OH)D was only evident in cohorts with insufficient vitamin D levels at the beginning of the interventions (Cashman et al., 2016b). However, in **Study 2** UVB-exposed WGO was not able to increase the total 25(OH)D levels, although the healthy volunteers had insufficient vitamin D levels at study baseline. In further consideration of the data from the human intervention study, it can be seen, that the unchanged levels of total 25(OH)D were due to decreased 25(OH)D_3 concentrations, although the 25(OH)D_2 concentrations increased. An effect which was not observable in the mice of **Study 1**, since they were fed a vitamin D-null diet prior to the study, and therefore no systemic vitamin D_3 was left and no decrease of 25(OH)D_3 would be observable after a treatment with vitamin D_2 from UVB-exposed WGO. Decreases in 25(OH)D_3 have been reported before in several studies in response to vitamin D_2 originated from UVB-exposed foodstuffs (Cashman et al., 2016b; Nieman et al., 2013; Shanely et al., 2014; Stephensen et al., 2012) (Supplementary **Table S1**) or semi-synthetic vitamin D_2 (Armas et al., 2004; Binkley et al., 2011; Glendenning et al., 2009; Lehmann et al., 2013; Tjellesen et al., 1986). For example, Binkley and coworkers found a substantial decrease in 25(OH)D_3 concentrations of approximately 30 nmol/L when given a vitamin D_2 instead of vitamin D_3 supplement (Binkley et al., 2011). This effect was unrelated to the dosage form (daily vs. bolus), and was most pronounced after 9 months of treatment. However, higher vitamin D_2 intakes might be more beneficial in improving the vitamin D status, as the increases in 25(OH)D_2 might compensate the decreases in 25(OH)D_3 (Cashman et al., 2016b; Urbain et al., 2011). Furthermore, decreases in the formation of 25(OH)D_2 from vitamin D_2 in response to a vitamin D_3 supplementation have also been shown (Hammami et al., 2019; Hymøller and Jensen, 2011).

The reason for the decline of 25(OH)D_3 after consumption of vitamin D_2 has not been sufficiently clarified yet. Results from **Study 2** assume that (1) the hepatic hydroxylation of vitamin D_3 to 25(OH)D_3 in response to a vitamin D_2 administration is reduced, as they

could compete for hydroxylating enzymes or (2) catabolic enzymes are upregulated through vitamin D_2, which accelerates the metabolic degradation of 25(OH)D_3. Conclusions regarding the first hypothesis can be drawn from **Study 4**. Here, vitamin D-depleted mice were fed diets containing iso-amounts of deuterated vitamin D_2, deuterated vitamin D_3 or both (50 % vitamin D_2, 50 % vitamin D_3). Assuming an impaired hydroxylation of vitamin D_3 to 25(OH)D_3 in response to a simultaneous intake of vitamin D_2, it would be expected that the group, which received both vitamin D forms, would show disproportionately low 25(OH)D levels compared to the vitamin D_3 group. The serum concentrations of deuterated 25(OH)D in mice, which received solely vitamin D_2, was 1.9-times higher than in mice receiving solely vitamin D_3 (102 ± 18 nmol/L vs. 54.5 ± 11.3 nmol/L, respectively). In the serum of mice, which received both vitamin D forms simultaneously, the concentration of deuterated 25(OH)D_2 was 2.2-times higher than of deuterated 25(OH)D_3 (55.9 ± 10.0 nmol/L vs. 25.2 ± 4.6 nmol/L, respectively). These findings suggest that the 25-hydroxylation of vitamin D_3 is not substantially affected by the simultaneously administration of vitamin D_2. In addition, gene and protein expression analysis of hepatic enzymes involved in vitamin D hydroxylation revealed no differences between the groups (**Study 4**). Thus, the second hypothesis, which suggested an enhanced degradation of 25(OH)D_3 in response to vitamin D_2, might be more relevant. The vitamin D catabolism is mostly mediated by the CYP24A1 enzyme, which catalyzes the conversion of 25(OH)D and 1,25$(OH)_2$D into water-soluble products (Jones et al., 2012). The catabolism of 25(OH)D_3 and 1,25$(OH)_2D_3$ by the CYP24A1 is either started with 24-hydroxylation, which finally leads to the production of calcitroic acid or with 23-hydroxylation, resulting in a cyclic-lactone (**Figure 4**) (Lohnes and Jones, 1992; Reddy and Tserng, 1989). The affinity of the CYP24A1 has been shown to be higher for bioactive vitamin D_3 than for vitamin D_2 metabolites (Urushino et al., 2009). Because of the differences in the side chain, catabolism of vitamin D_2-metabolites by CYP24A1 is solely mediated by hydroxylation at C-position 24 to a limited series of poly-hydroxylated degradation products (Reddy and Tserng, 1986; Urushino et al., 2009). In addition, some authors described the side-chain cleavage of the 1,25$(OH)_2D_2$-degradation products, resulting in calcitroic acid (Zimmerman et al., 2001), by a so far unknown pathway, seemingly facilitated by CYP24A1 in humans (Urushino et al., 2009) (**Figure 4**). Catabolism of vitamin D_3 metabolites is additionally feasible through CYP3A4, a multifunctional enzyme, which is involved in the xenobiotic metabolism. CYP3A4 is also known to 25-hydroxylate vitamin D_2, but not vitamin D_3 (Gupta et al., 2004). Further, CYP3A4 catabolically hydroxylates 1,25$(OH)_2D_3$ at C23 and C24 (Xu et al., 2006), and 25(OH)D_3 at e.g. C4 and others (Wang et al., 2012) (**Figure 4**). Additionally, an enhanced expression of CYP24A1 and CYP3A4 is also discussed to be the reason for the

occurrence of drug-induced osteomalacia (Pascussi et al., 2005; Xu et al., 2006; Zhou et al., 2006). Hence, it is suggested that vitamin D_2 induces the expression or activity of CYP24A1 and CYP3A4, which then catabolizes vitamin D_3 metabolites more efficiently than vitamin D_2 metabolites via CYP24A1 and CYP3A4.

Although both vitamin D_2 and vitamin D_3 are biologically effective in the cure and treatment of rickets (Park, 1940), the 25(OH)D_3-lowering effect of vitamin D_2 is most likely the reason for the observed lesser efficiency in raising 25(OH)D concentrations of a vitamin D_2 than a vitamin D_3 supplementation (Tripkovic et al., 2012). This is consistent with the findings from **Study 1**, where mice fed vitamin D_2 from UVB-exposed WGO had significantly lower levels of total 25(OH)D, than mice fed a vitamin D_3 supplemented WGO (46.3 ± 7.7 vs. 81.6 ± 16.8 nmol/L). Moreover, the current data of **Study 2** showed impressively, that vitamin D_2 from UVB-exposed WGO is not able to improve the vitamin D status of vitamin D insufficient, but otherwise healthy humans. Thus, UVB-exposed plant oils are not recommended as potential new sources of vitamin D in humans, even though UVB-exposed WGO is rich in vitamin D_2. This finding confirms other studies, which used UVB-exposed foods (Supplementary **Table S1**). This is especially of interest since some UVB-exposed mushrooms and yeasts are already available in food retail stores. Conclusively, vitamin D_2 from UVB-exposed WGO was unable to improve the vitamin D status of healthy participants (**Study 2**) and was less efficient to increase 25(OH)D levels of mice (**Study 1**), which hypothetically could be due to (1) an impact on vitamin D metabolism of other sterols present in the WGO, e.g. ergosterol, as they are found in high concentrations in WGO or (2) differences in the metabolic routes of the two D-vitamers, vitamin D_2 and vitamin D_3. Furthermore, vitamin D may be metabolized differentially in mice than in humans, explaining the differences of UVB-exposed WGO in raising 25(OH)D levels in **Study 1** and **Study 2**. These hypotheses are elucidated in the following chapters.

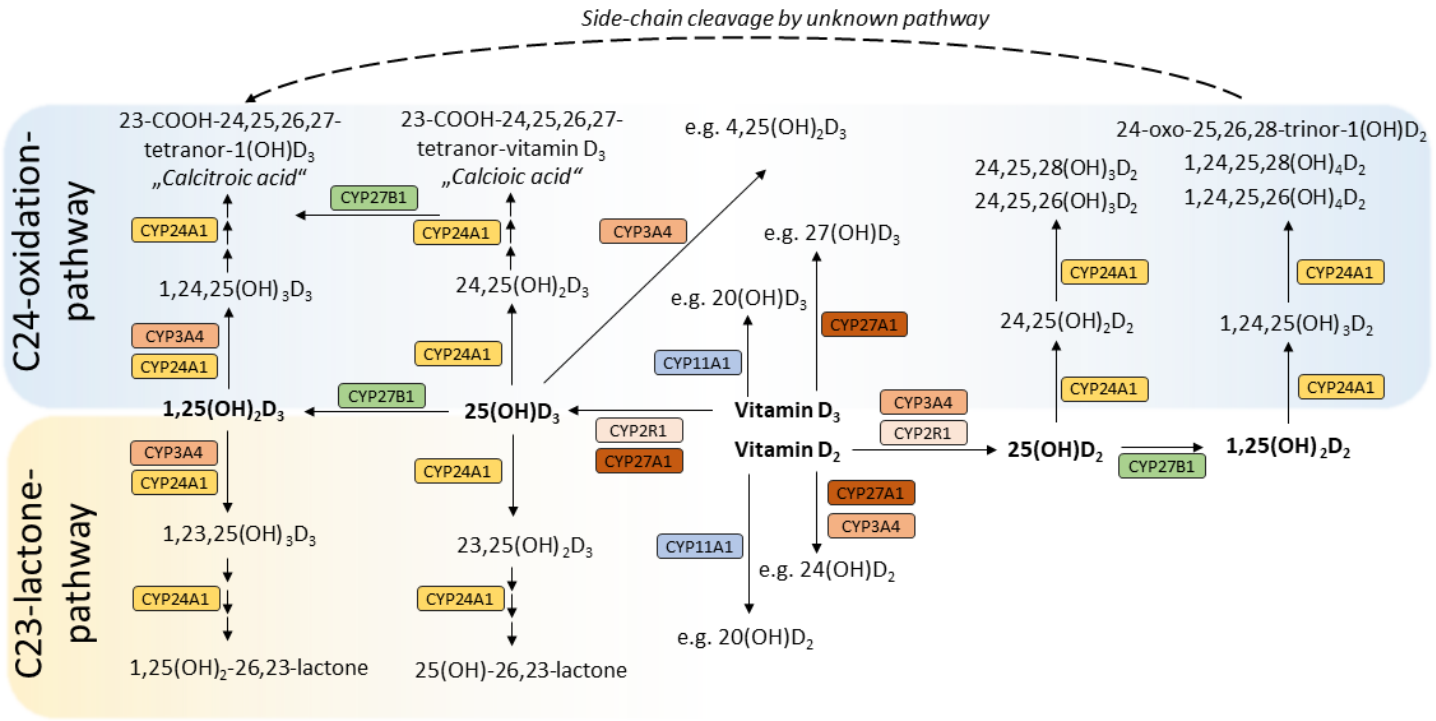

Figure 4 Activation and degradation of vitamin D_3 and vitamin D_2 by cytochrome P450 (CYP) enzymes.

Vitamin D_3 is hydroxylated by CYP2R1 and CYP27A1 to 25-hydroxyvitamin D_3 ($25(OH)D_3$). Then, $25(OH)D_3$ is activated by hydroxylation through CYP27B1 to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). $1,25(OH)_2D_3$ and $25(OH)D_3$ are catabolized by multiple hydroxylation steps facilitated by CYP24A1, starting with either C24-hydroxylation (C24-oxidation-pathway) or with C23-hydroxylation (C23-lactone-pathway). The C24-oxidation pathway either ends in the formation of calcitroic acid ($1,25(OH)_2D_3$ as substrate) or calcioic acid ($25(OH)D_3$ as substrate). Intermediates in the formation of calcioic acid can be hydroxylated at C-position 1 by CYP27B1, entering the pathway in the formation of calcitroic acid. The C23-lactone pathway ends in the formation of either $1,25(OH)_2$-26-23-lactone ($1,25(OH)_2D_3$ as substrate) or 25(OH)-26-23-lactone ($25(OH)D_3$ as substrate). Further, $25(OH)D_3$ and vitamin D_3 can be hydroxylated at other C-positions forming e.g. $4,25(OH)_2D_3$ and $20(OH)D_3$ or $27(OH)D_3$ by CYP3A4 and CYP11A1 or CYP27A1, respectively. Vitamin D_2 is hydroxylated by CYP2R1 and CYP3A4 to $25(OH)D_2$ and further to $1,25(OH)_2D_2$ by CYP27B1. $25(OH)D_2$ and $1,25(OH)_2D_2$ are catabolized by CYP24A1 in the C24-oxidation pathway, forming a more limited series of catabolic end products. A cleavage of the side-chain resulting in calcitroic acid has also been reported for degradations products of vitamin D_2. Vitamin D_2 can also be hydroxylated at other positions e.g. $20(OH)D_2$ and $24(OH)D_2$ by CYP11A1 and CYP27A1 or CYP3A4, respectively (Tuckey et al., 2019; Xu et al., 2006; Yu and Arnold, 2016). Metabolites involved in the metabolic activation of vitamin D are written in bold.

4.4 Ergosterol as modulator of the vitamin D metabolism

In **Study 1** plant oils, especially WGO have been identified to have high concentrations of the vitamin D_2 precursor ergosterol. For example, an average daily consumption of 12 g olive oil (Max-Rubner-Institut, 2013), an oil which showed only small amounts of ergosterol in **Study 1** (1.22 – 4.46 µg/g), would still provide 14.6 – 53.5 µg ergosterol per day. WGO was identified to have high concentrations of ergosterol, ranging from 22.1 – 34.4 µg/g (**Study 1**) and would therefore provide 265 – 413 µg ergosterol per day. The mean ergosterol concentration of the WGO in **Study 2** was 87.4 µg/g (range, 65 – 105 µg/g, n = 6 measurements in duplicate over the study period), providing 874 µg ergosterol per 10 g WGO per day. In **Study 2**, the serum vitamin D concentration was not only higher in participants, who received the UVB-exposed WGO, but also in those who received the untreated WGO compared participants of the control group, who received no oil (5.54 ± 5.51 vs. 2.49 ± 1.05 nmol/L). Although the higher serum vitamin D concentrations in the participants receiving untreated WGO, compared to the control group, did not reach statistical significance, it could have been caused by high concentrations of ergosterol in the WGO. To test, whether a daily consumption of ergosterol in moderate concentrations could affect the vitamin D metabolism, mice were fed diets containing either 2 mg or 7 mg ergosterol in addition to 25 µg vitamin D_3 per kg diet, as recommended by the U.S. *National Research Council* (National Research Council (U.S.), 1995) (**Study 3**). Here, the high dose of ergosterol led to higher concentrations of vitamin D_3 in serum (1.3-times) and in tissue storages (liver, 1.7-times; kidney, 1.5-times) compared to control mice, while the 25(OH)D concentrations remained unchanged. With a mean daily feed intake of 2.64 ± 0.12 g per mouse and day, approximately 5.3 µg (low dose) or 18.5 µg (high dose) ergosterol were consumed daily by these mice. In consideration of the differences in the body surface area between humans and mice, as suggested by Reagen-Shaw et al. (Reagan-Shaw et al., 2008), these doses would be equivalent to approximately 16.5 µg or 57.7 µg ergosterol per day for humans (mean body weight of the mice in **Study 3**, 26.0 ± 4.0 g; estimated body weight of an average human, 60 kg). This dosage would be easily achievable through the consumption of plant oils.

The vitamin D_2 precursor ergosterol shows a structural similarity to other sterols, such as 7-DHC, cholesterol and plant sterols (referred to as "*phytosterols*"). Because of this structural similarity, ergosterol is often counted among the phytosterols, although it is derived from fungi. Comparable to the data of the current study, Kühn and coworkers revealed the vitamin D_3 precursor 7-DHC to be a modulator of the vitamin D metabolism, since an oral application thereof increased the concentration of non-hydroxylated

vitamin D in tissue storages of mice in a dose-dependent manner (Kühn et al., 2016). Later they showed that mice fed supplemental cholesterol in the diet had also higher serum and tissue concentrations of vitamin D (Kühn et al., unpublished manuscript, 2021). However, these data are somewhat controversial, as cholesterol was also shown to interfere with the vitamin D metabolism by reducing the incorporation of vitamin D in dietary mixed micelles (DMM) and the uptake of vitamin D in intestinal carcinoma-cells (Caco2) in culture (Goncalves et al., 2011). Other compounds that have been shown to interfere with the vitamin D metabolism are dietary fatty acids (Goncalves et al., 2015a; Hollander et al., 1978), fat soluble vitamins like tocopherol and retinol (Goncalves et al., 2015b; Hymøller et al., 2016) and various phytosterols (Goncalves et al., 2011).

The metabolism of the vitamin D increasing effect of ergosterol remains elusive, but is not based on differences in the activation or degradation of vitamin D, since there were no differences in the $25(OH)D_3$, $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ concentrations (**Study 3**). Suggesting that ergosterol alters the vitamin D metabolism by rather increasing not decreasing the vitamin D absorption into the enterocyte or hepatocyte, we analyzed the concentration of the intestinal and/or hepatic sterol transporters NPC1L1, CD36 and SCARB1 (**Figure 5A**), but found no differences in the mRNA expression of the genes encoding for these proteins. In addition to the contribution in intestinal sterol absorption, Scarb1 has also been shown to efflux vitamin D back to the lumen through the brush border membrane (Reboul et al., 2011). Other intestinal sterol-effluxing transporters are the adenosine triphosphate-binding cassette (ABC) G5 and G8 (ABCG5/G8). The ABCG5/8 transporters function as heterodimers and are involved in the efflux of sterols from the cell (Wang et al., 2006). Therefore, they play a pivotal role in the transintestinal cholesterol excretion (TICE) and in the hepatic elimination of sterols by biliary excretion (van der Veen et al., 2009; Yu et al., 2002). Additionally, the ABCG5/G8-transporters are crucial for the elimination of xenosterols e.g. phytosterols from the enterocyte (Patel et al., 2018). The relevance of the ABCG5/G8 transporters in preventing the accumulation of phytosterols in the body can be seen in patients suffering from "*sitosterolemia"*, a disease caused by a mutation in either the ABCG5 or the ABCG8 gene loci (Berge et al., 2000; Lee et al., 2001). Sitosterolemia was first described in 1974 and is characterized by hypercholesterolemia, premature atherosclerosis and extremely high tissue and serum levels of phytosterols e.g. sitosterol, which is eponymous for this disease (Bhattacharyya and Connor, 1974). Recently, Kiourtzidis and coworkers found that mice lacking in the ABCG5/G8-gene had higher serum and liver concentrations of vitamin D, suggesting that ABCG5/G8 does also function as an efflux-transporter for vitamin D (Kiourtzidis et al., 2020b). An enhanced expression of ABCG5/G8 is also associated with the well-known lipid-lowering effect of phytosterols (Brufau et al., 2011; Calpe-Berdiel et

al., 2010). Ergosterol has also been shown to function anti-lipidemic (Gil-Ramírez et al., 2016; Hu et al., 2006; Schneider et al., 2011). Gil-Ramírez and coworkers showed that ergosterol and ergosterol-rich extracts from button mushrooms increase the intestinal ABCG5-mRNA expression, reduce the concentrations of hepatic triacyl-glycerols in mice and decrease the inccoporation of cholesterol into DMM (Gil-Ramírez et al., 2014; Gil-Ramírez et al., 2016). Suggesting that ergosterol might have altered the ABCG5/G8-expression level, which then impacted the vitamin D and/or lipid metabolism, we analyzed the murine intestinal and hepatic mRNA abundances of the ABCG5/G8 heterodimeric transporter in **Study 3**. Here, mice fed 7 mg ergosterol per kg diet only showed a tendency to have higher hepatic ABCG5 gene expressions ($P = 0.072$), but showed no differences in the hepatic cholesterol or triacyl-glycerol concentrations between the groups of mice. Furthermore, analysis of blood lipids of the participants in **Study 2**, also revealed no differences between the control group and the groups which received ergosterol as part of the WGO. Finally, it is assumed that the differences in the vitamin D concentrations are not based on ergosterol-induced differences of the lipid composition.

Very recently, Kühn and coworkers found that cholesterol increases tissue vitamin D levels by increasing the synthesis of hydrophobic bile acids in response to a cholesterol-containing diet (Kühn et al., unpublished manuscript). Bile acids are steroid compounds formed from cholesterol through the CYP7A1 and, to a lesser degree, CYP27A1 (Chiang, 2013) (**Figure 5B**). Subsequently, bile acids and their conjugated bile salts are secreted through the ABCB11 transporter into the bile, from which they can (re-)enter the small intestine (often referred to as "*enterohepatic circulation"*) (Chiang, 2004). In the aqueous environment of the intestine, the bile salts form soluble DMM with lipids (Roepke and Mason, 1940), whereby the composition of the micelles affects the availability of the lipids for absorption through the enterocyte (Amiot et al., 2011; Goncalves et al., 2015a). In general, phytosterols are known to increase the bile acid synthesis and modulate the DMM compositions (Carr et al., 2002; Gleize et al., 2016). In particular, ergosterol has been shown to affect the DMM composition by displacing incooperated cholesterol (Gil-Ramírez et al., 2014). Therefore, it is tempting to speculate that ergosterol also increases the vitamin D concentrations in tissues, as seen in **Study 3**, by stimulating the bile acid synthesis, modulating the composition of the DMM and finally increasing the intestinal absorption of vitamin D. However this theory is somewhat controversial as findings of He et al. showed a decreased fecal excretion of bile acids and a downregulation of the CYP7A1 in ergosterol-fed mice (He et al., 2020) (**Figure 5B**). In addition, the phytosterol sitosterol has been proven to rather reduce the absorptions of cholesterol (Mel'nikov et al., 2004), carotenoids (Plat et al., 2005) and vitamin D (Goncalves et al., 2011).

Ergosterol, when entering the body, can be reduced by the 7-dehydrocholesterol (DHCR7) reductase to brassicasterol, a plant sterol mainly found in algae (Ohtsubo et al., 2016; Shefer et al., 1998; Tsugawa et al., 1992) (**Figure 5B**). As mentioned above, some of the orally ingested ergosterol may be immediately effluxed by the ABCG5/G8 transporter in the enterocyte. However, high doses of ergosterol still enter the body, as mice receiving 7 mg/kg ergosterol showed quantifiable serum concentrations of ergosterol (5.7 ± 2.2 nmol/L, **Study 3**). However, the absence of ergosterol in randomly analyzed livers of mice in **Study 3** (data not shown), suggests that hepatic ergosterol was reduced to brassicasterol. Hence, the higher vitamin D concentrations observed in mice fed ergosterol could be caused by brassicasterol instead of ergosterol.

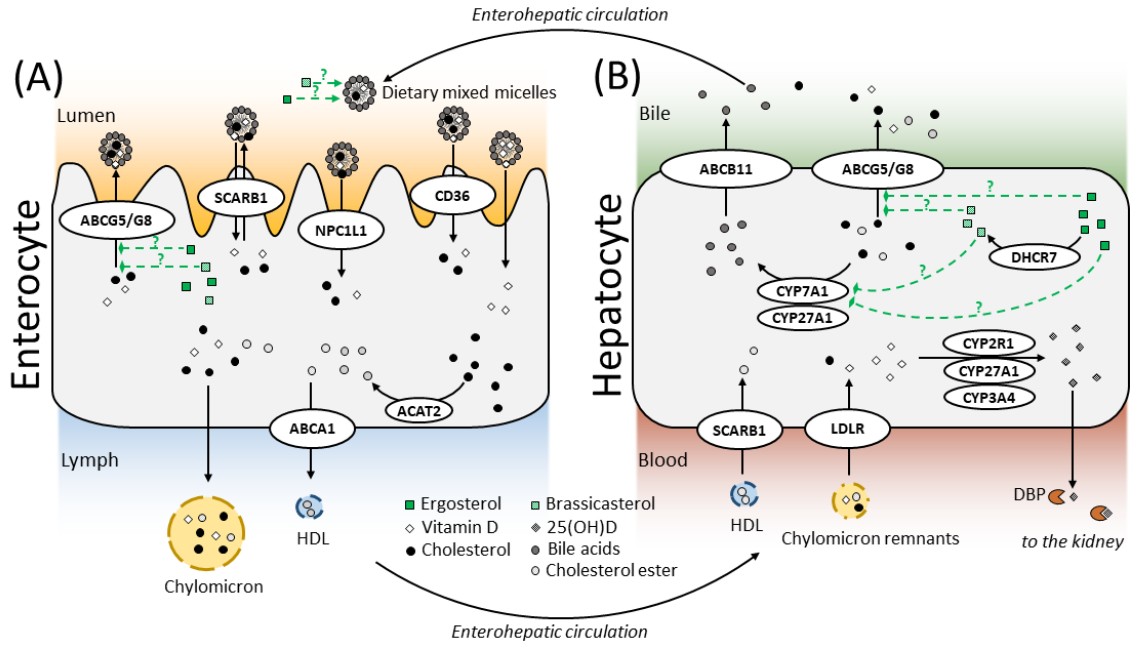

Figure 5 Sterol absorption in the intestine (A) and metabolism in the liver (B).

Cholesterol and vitamin D, incorporated in dietary mixed micelles, arrive at the brush border membrane and are taken up by the transport proteins cluster of differentiation 36 (CD36), Nieman Pick C1 like protein 1 (NPC1L1) and scavenger receptor B1 (SCARB1). High concentrations of vitamin D also enter the enterocyte by passive diffusion. Parts of the sterols are subsequently effluxed back into the lumen by SCARB1 and the heterodimeric transporters adenosine triphosphate binding cassette (ABC) G5/G8. Cholesterol is esterified by acyl-CoA cholesterol acyltransferase (ACAT) and embedded in high density lipoproteins (HDL) via ABCA1. Cholesterol and vitamin D are incorporated into chylomicrons, secreted into the lymph, and then transported to the liver. There, HDL and chylomicron-remnants are absorbed via SCARB1 and the low-density lipoprotein-receptor (LDLR), respectively. Then, cholesterol is transformed into bile acids by CYP7A1 and CYP27A1. ABCB11 and ABCG5/G8 facilitate the secretion of bile acids and sterols, respectively, into the bile, from which the sterols (re-)enter the enterohepatic circulation. Vitamin D is hydroxylated to 25(OH)D by cytochrome P450 (CYP) 2R1, CYP27A1 or CYP3A4. 25(OH)D is then bound to the vitamin D binding protein (DBP) and transported to the kidneys via the blood. Hepatic ergosterol is transformed to brassicasterol by the 7-dehydrocholesterol reductase (DHCR7). Hypothetically, ergosterol and/or brassicasterol competitively inhibit the intestinal and hepatic efflux of sterols through ABCG5/G8 and/or interfere in the synthesis of bile acids by altering the composition of the dietary mixed micelles or through inhibition of CYP7A1 (dashed green lines) (He et al., 2020; Ravid et al., 2008; Reboul, 2015; Repa and Mangelsdorf, 2002; Shefer et al., 1998).

4.5 Differences in the biological activities of vitamin D_2 and vitamin D_3

Due to the predominant occurrence of ergosterol in WGO, a UVB-exposure leads mainly to the formation of vitamin D_2 (**Study 1**). To elucidate, whether the lower efficiency of vitamin D_2 from UVB-exposed WGO in **Study 1** compared to the vitamin D_3-supplemented oil was based on differences in the metabolic routes of the D-vitamers, further studies with initially vitamin D-depleted mice were conducted. Surprisingly, the total 25(OH)D concentration was 1.9-times higher in mice fed a diet containing 25 µg of deuterated vitamin D_2 per kg (102 ± 18 nmol/L) than in mice fed equal amounts of vitamin D_3 (54.5 ± 11.3 nmol/L) (**Study 4**). These data present a sharp contrast with data from human intervention studies, in which vitamin D_3 is considered to be much more efficient in raising total 25(OH)D levels than vitamin D_2 (reviewed in Tripkovic et al., 2012).

The 25-hydroxylation of vitamin D is facilitated through hepatic CYP-enzymes in rodents and humans: the mitochondrial CYP27A1 and the microsomal CYP2R1, CYP3A4 and CYP2J2 (Aiba et al., 2006; Jones et al., 2014a). The hydroxylation of vitamin D differs between vitamin D_2 and vitamin D_3. The main hepatic hydroxylase CYP2R1, hydroxylates both vitamin D_2 and vitamin D_3 (Shinkyo et al., 2004; Zhu et al., 2013), while CYP27A1 only 25-hydroxylates vitamin D_3 (Guo et al., 1993) and CYP3A4 vitamin D_2 (Gupta et al., 2004) (**Figure 5B**). CYP2J2 was first found in rats (in rats referred to as CYP2J3) (Yamasaki et al., 2004) and shows different preferences towards vitamin D_2 and vitamin D_3. Human CYP2J2 seems to prefer vitamin D_2 over vitamin D_3, while rat CYP2J3 shows a higher activity towards vitamin D_3 (Aiba et al., 2006). As the extrahepatic CYP2J2 is mainly expressed in the cardiovascular system (Xu et al., 2013), it plays a minor role in vitamin D metabolism. Hence, suggesting differences in the hepatic hydroxylation of vitamin D_2 and vitamin D_3, the mRNA and protein expressions of CYP27A1, CYP2R1 and CYP3A4 in the livers of mice in **Study 4** were analyzed, but found no differences between the groups of mice. Additionally, murine hepatoma cells cultivated with vitamin D_2 or vitamin D_3, preferred to hydroxylase vitamin D_3 over vitamin D_2 (**Study 4**), indicating that the differences in 25(OH)D levels observed in the mice were not based on differences in hepatic hydroxylation.

In circulation, 85 % of the 25(OH)D is bound to DBP, 15 % is bound to albumin and approximately <0.1 % is non-protein bound (so-called "*free 25(OH)D*") (Bikle and Schwartz, 2019; Bouillon et al., 2020). In **Study 4**, the concentration of free 25(OH)D was higher in the vitamin D_2, than in the vitamin D_3-receiving mice, confirming previous data by Chun et al. (Chun et al., 2016). Higher concentrations of free 25(OH)D under vitamin D_2 supplementation, are based on a lesser affinity of 25(OH)D_2 to DBP than of

25(OH)D_3 (Hollis, 1984). This is also the reason for the reported lower plasma half-life of 25(OH)D_2 (Jones et al., 2014b). Binding of 25(OH)D to DBP is also crucial for the subsequent megalin-facilitated uptake of 25(OH)D into the kidney cells and the hydroxylation by the CYP27B1 (Nykjaer et al., 1999). In contrast to the 25(OH)D levels, the concentration of the bioactive vitamin D hormone 1,25$(OH)_2$D was lower with a vitamin D_2 than with a vitamin D_3 supplementation (**Study 4**). Thus, the low formation of 1,25$(OH)_2D_2$ was presumably due to higher concentrations of free 25(OH)D_2 and, therefore, an increased loss and an impaired endocytosis of 25(OH)D_2 in the kidney. This was confirmed by the renal mRNA abundances of megalin (encoded by the *LRP2* gene) and CYP27B1, which were numerically lower in mice fed vitamin D_2 than in mice fed vitamin D_3 (**Study 4**). Nevertheless, it has to be mentioned that the concentrations of the 1,25$(OH)_2$D is lower by the factor of 1000 than the concentrations of 25(OH)D and therefore does not explain the differences in 25(OH)D concentrations. In recent years, it has been suggested that the "*free hormone hypothesis*", which states that the non-protein bound fractions of hormones generally bound to transport proteins can enter the cell and exert physiological effects, is also applicable for vitamin D (Bikle and Schwartz, 2019; Bouillon et al., 2020; Chun et al., 2014). This is corroborated by the finding that DBP-null mice maintain normal bone and calcium homeostasis (Safadi et al., 1999; Zella et al., 2008) and that 25(OH)D seems to be more bioavailable in immune cells in absence of DBP (Chun et al., 2010). Besides, many extra-renal tissues also contain CYP27B1, indicating that they are capable to produce 1,25$(OH)_2$D independently (Bikle, 2009; Bikle, 2021; Bouillon and Bikle, 2019) Hence, free 25(OH)D might play a pivotal role in extra-renal effects of vitamin D (Chun et al., 2014).

Since there is evidence that non-hydroxylated vitamin D in circulation reflects the body storages better than the circulating 25(OH)D concentrations (Best et al., 2021; Kiourtzidis et al., 2020a), we analyzed the vitamin D levels in circulation and in tissue storages. In **Study 1** and **Study 4**, the concentration of total vitamin D in circulation was markedly lower than the 25(OH)D levels, but was higher in mice fed vitamin D_3 than in mice fed vitamin D_2. Analysis of body storages revealed higher total vitamin D concentrations in intestinal mucosa, liver, kidney and retroperitoneal adipose tissue in vitamin D_3 than in vitamin D_2 fed mice (**Study 4**). The current findings are in accordance to previous data that also show higher values in the circulating vitamin D concentrations under a vitamin D_3 than a vitamin D_2 supplementation in humans (Jakobsen et al., 2017) and rats (Horst et al., 1982). Studies on vitamin D in tissues of humans are limited (Best et al., 2021; Piccolo et al., 2013) and so far, there is only one human intervention study, investigating vitamin D body storages after supplementation with vitamin D_2 or vitamin D_3 (Heaney et al., 2011). In line with the present data, Heaney and coworkers

found higher adipose tissue storages after a bolus dosage of vitamin D_3 than of vitamin D_2 (Heaney et al., 2011). In 2013, Lipkie et al. re-analyzed organs from rats receiving vitamin D_2 from UVB-treated yeasts (previously published in 2011 by Hohman et al.) (Lipkie et al., 2013). Interestingly, they found lower vitamin D concentrations in liver and fat tissue of vitamin D_3-fed rats than in their counterparts, fed comparable amounts of vitamin D_2 from yeast. In **Study 1**, vitamin D_2 from the UVB-exposed WGO also led to remarkably higher hepatic vitamin D concentrations, suggesting a D-vitamer independent impact of the UVB-treatment on vitamin D metabolism e.g. via the formation of photoisomers (see **chapter 4.2**).

Based on the fecal excretion of vitamin D_3 and vitamin D_2 in **Study 4**, the absorption of the D-vitamers does not differ. This confirms previous data from the group of Reboul, which showed that vitamin D_3 and vitamin D_2 are absorbed equally in colorectal adenocarcinoma (CaCo2)-cells (Reboul et al., 2011). After intestinal absorption, vitamin D enters the circulation via the lymph at the basolateral site of the enterocyte (**Figure 5**). It has been suggested, that 25(OH)D_3 is preferably transported via DBP, since both can be found in the same density-fraction in ultracentrifuged venous blood samples of healthy volunteers (Haddad et al., 1993). In contrast, 25(OH)D_2 can be found in greater quantities in the lipoprotein-fractions. Studies with ruminants confirmed that 25(OH)D_3 is more readily transported in the protein-fraction (containing DBP), 25(OH)D_2 in the high-density lipoprotein (HDL)-containing lipoprotein-fraction, and both are transported via chylomicrons (Hymøller and Jensen, 2016; Hymøller and Jensen, 2017). The authors concluded, that the differences in the transport may explain, why vitamin D_3 is physiologically more efficient than vitamin D_2 in dairy cows.

Most species like cows (Hymøller and Jensen, 2011; Sommerfeldt et al., 1983), birds (Horst et al., 1982; Hoy et al., 1988), cats (Morris, 2002), pigs (Horst et al., 1982), and monkeys (Marx et al., 1989) maintain higher 25(OH)D levels, while receiving vitamin D_3 instead of vitamin D_2. In general, humans also show different preferences toward the vitamin D forms. The 25(OH)D concentrations increase approximately 1.3-times more efficiently with vitamin D_3 than with vitamin D_2 supplementation (Armas et al., 2004; Jakobsen et al., 2017; Lehmann et al., 2013; Trang et al., 1998). Interestingly, rodents like rats prefer vitamin D_2 over vitamin D_3, as they show 2.5-times higher concentrations of 25(OH)D after oral dosages of equal amounts of vitamin D_2 and vitamin D_3 (Horst et al., 1982). In **Study 4**, we showed for the first time, that mice also prefer vitamin D_2 for hydroxylation, showing 1.9-times higher 25(OH)D concentrations in vitamin D_2-fed, than in vitamin D_3-fed mice. Therefore, it is tempting to speculate that mice react differentially to vitamin D_2 than humans. Although mice are generally able to endogenously synthesize vitamin D_3 in the skin (Irving et al., 2019), they live nocturnally (Wang et al.,

2020), and therefore are not sufficiently exposed to sunlight. Hence, mice have to ingest vitamin D with their diets. Since they are omnivorous, they eat a variety of plant materials (e.g. seeds or grains) and animal-derived foods (e.g. insects), supplying for both, vitamin D_2 and vitamin D_3 (National Research Council (U.S.), 1995; Oonincx et al., 2018). This suggests a superior adaption to vitamin D_2 in mice than in humans. Whether this adaption was reached through a more efficient hepatic hydroxylation of vitamin D_2 compared to vitamin D_3 was investigated as part of **Study 4**. Here, incubation of murine and human hepatoma cells with vitamin D_2 resulted in a significant lower formation of 25(OH)D than an incubation with vitamin D_3. However, this effect was much more pronounced in the human than in the murine cells. The hydroxylation ratio (% 25(OH)D of vitamin D) for vitamin D_3 and vitamin D_2 were in human hepatoma cells 4.7 ± 0.4 % and 0.12 ± 0.05 %, respectively, and in murine hepatoma cells 0.34 ± 0.02 % and 0.07 ± 0.01 %, respectively (**Study 4**). First, these findings indicate that human liver cells generally have a greater 25-hydroxylation activity than murine cells in culture. Second, human liver cells 25-hydroxylate vitamin D_3 more efficiently than vitamin D_2. Third, although also preferring vitamin D_3 as a substrate for 25-hydroxylation, murine liver cells are also well adapted to vitamin D_2. Ultimately, mice and humans react differentially to vitamin D_2 and vitamin D_3. Thus, data obtained from rodents are probably not completely transferable to humans.

4.6 Limitations and strength of the studies

In general, there are certain limitations in human intervention studies regarding vitamin D. First, as vitamin D can be produced endogenously in the skin by exposure to the sun, it is crucial to elucidate sole influences of dietary vitamin D during the winter, when the endogen synthesis is low (O'Neill et al., 2016). Therefore, a major limitation of **Study 2** was the beginning of the 6-week intervention in late winter (February 2018) in Jena (Germany, 51 °N), resulting in a beginning endogenous vitamin D synthesis in the final weeks of the intervention in April. This would explain the increase in 25(OH)D_3 in the groups receiving either no oil or untreated WGO. Second, the uncertainty of (1) the individual responses of the participants to a vitamin D supplementation (Carlberg and Haq, 2018) and (2) of the vitamin D body storages (Kiourtzidis et al., 2020a; Martinaityte et al., 2017) might have required a higher number of participating subjects in **Study 2**. Finally, the lack of a standardized background diet and the unequal distribution of the gender in the control group in comparison with the other groups (males, 3; females,11) is another limitation of **Study 2**. This might be the reason for the differences in the food

intakes assessed by food frequency protocols, although the vitamin D intakes in the background diets were ultimately equal between the groups.

Animal studies are generally considered to be good models of the basic functions and metabolism of nutrients and vitamins (Ross, 2012). However, data obtained from mice studies may not be transferred to humans without restriction (Musther et al., 2014). Therefore, we conducted a study which aimed to compare the vitamin D data from mice with those obtained from humans. Thus the availability from data from mice studies (**Study 1**, **Study 3, Study 4**), cell culture studies with murine and human hepatoma cells (**Study 3, Study 4**) and a randomized human intervention trial (**Study 2**) is a strength of this thesis. Another strength is the analysis of the vitamin D metabolites via LC-MS/MS. The LC-MS/MS analysis of vitamin D has first been reported in 1991 (Watson et al., 1991) and is nowadays considered as the gold standard in vitamin D measurement (Carter, 2009; La Hunty et al., 2010; Zerwekh, 2008). In contrast to e.g. analysis via radio or enzyme immunoassay, analysis via LC-MS/MS can distinguish between 25(OH)D_2 and 25(OH)D_3. Other advantages of the LC-MS/MS analysis are the possibilities to measure other vitamin D metabolites (e.g. vitamin D_2 and vitamin D_3) simultaneously and in other tissues than simply serum or plasma. Finally, a strength of **Study 4** is the use of deuterated compounds, which allows the accurate tracking of orally-ingested D-vitamers in the body.

4.7 Conclusions

The aim of this thesis was to elucidate, whether plant oils are potential sources of vitamin D and if they can contribute to improve the vitamin D status of the population. Therefore, the following questions have been answered:

(1) Are edible plant oils potential sources of vitamin D or vitamin D precursors?
Vitamin D_2 and vitamin D_3 were not quantifiable in the selected plant oils. However, edible plant oils contain considerable amounts of the precursors of vitamin D_2 and vitamin D_3. WGO in particular was identified to have high concentrations of both, ergosterol and 7-DHC.

(2) Is the UVB-treatment of plant oils a successful strategy to enhance the vitamin D concentration?
Treatment with UVB-light was shown to be a successful strategy to increase the concentrations of predominantly vitamin D_2 in plant oils. The levels of the concentrations achieved depend on the initial concentrations of vitamin D precursors, the duration of the treatment, the intensity of the UVB-emitting lamp and

the thickness of the exposed oil-layer. Additionally, vitamin D concentrations increase upon storage in the dark and moderate thermal treatment whilst cooking, presumably through the transformation of previtamin D in vitamin D. Oxidative markers were not negatively affected by the UVB-treatment, but UVB-exposed WGO was characterized by a slightly lesser taste than non-treated WGO. When used as a sole source of plant oils, a 10-min UVB-exposed WGO would supply 50 – 150 % of the recommendations for the daily vitamin D intake given by the nutrition societies of the German speaking countries.

(3) Is vitamin D from UVB-exposed plant oils bioavailable?

UVB-exposed WGO is not considered to be a potential source of vitamin D for humans, since vitamin D_2 from UVB-exposed WGO was able to increase the total 25(OH)D concentrations in mice, but not in humans. Dietary intake of UVB-exposed WGO was able to improve the vitamin D status in vitamin D-depleted mice significantly, but to a lesser extent than control mice fed a vitamin D_3 supplemented WGO. In contrast, UVB-exposed WGO did increase the 25(OH)D_2 concentrations of healthy males and females with insufficient vitamin D levels, but without increasing the concentrations of total 25(OH)D. This was due to a substantial decrease of the 25(OH)D_3 concentrations in participants which received the UVB-exposed WGO daily. The reason for the decline in 25(OH)D_3 remains elusive, but is presumably based on a reduced formation of 25(OH)D_3 through vitamin D_2 by hepatic hydroxylases and/or an increased catabolism of 25(OH)D_3 by catabolic enzymes, such as CYP24A1 or CYP3A4.

(4) Is ergosterol a modulator of the vitamin D metabolism?

Some plant oils, especially WGO, contain high amounts of ergosterol. Ergosterol was shown to modify the vitamin D metabolism in mice, since high-ergosterol fed mice showed higher concentrations of non-hydroxylated vitamin D_3 in serum and tissues. The higher vitamin D_3 concentrations were not due to a stimulated activation or degradation of vitamin D_3. However, ergosterol could modify the vitamin D metabolism by increasing the intestinal absorption either through a competitive inhibition of the sterol-effluxing ABCG5/G8 transporters or a modulation of the bile acid composition of DMM.

(5) Is there a difference in the metabolic routes of vitamin D_2 and vitamin D_3?

Data from a mice study and cell culture studies with murine and human hepatoma cells revealed differences of the metabolic routes between vitamin D_2 and vitamin D_3. In mice, which were fed either vitamin D_2 or vitamin D_3, those that received vitamin D_2 developed higher 25(OH)D concentrations than those fed vitamin D_3. In

contrast, the non-hydroxylated vitamin D and 1,25$(OH)_2$D concentrations were lower in the vitamin D_2 than in the vitamin D_3-fed mice. The latter can be explained by the lower affinity of 25$(OH)D_2$ to DBP, the resulting higher abundance of free 25$(OH)D_2$ and the presumably lowered endocytosis in the kidney cells, where hydroxylation at C1 is facilitated by CYP27B1. The differences in 25(OH)D concentrations in mice in response to vitamin D_2 and vitamin D_3 cannot be explained by (1) differences in the hepatic hydroxylation, since murine hepatoma cells in culture hydroxylated vitamin D_3 more efficiently than vitamin D_2 and (2) differences in the intestinal absorption, since fecal loss was equal in all groups of mice. It is suggested, that vitamin D_2 and vitamin D_3 are metabolized differentially between mice and humans. Data from cell culture studies showed that hepatoma cells generally prefer vitamin D_3 over vitamin D_2. Nevertheless, in murine hepatoma cells the difference between the 25$(OH)D_2$ and 25$(OH)D_3$ concentrations was less pronounced than in human hepatoma cells.

5 Summary

To combat vitamin D deficiency, alternative strategies are needed. Hence, the current thesis examined the potential of UVB-exposed plant oils to improve vitamin D status. First, analysis of eight selected plant oils intended for the use in human nutrition (avocado, linseed, olive, pumpkinseed, rapeseed, soya, sunflower oil and wheat germ oil (WGO)) revealed high concentrations of the vitamin D precursors, ergosterol and 7-dehydrocholesterol (7-DHC). In particular, WGO was identified to have high concentrations of the vitamin D precursors. Short-term exposure to ultraviolet light type B (UVB) was able to vastly increase the vitamin D concentrations of WGO. Here, predominantly ergosterol was transformed to vitamin D_2. Second, the efficiency of UVB-exposed WGO in improving vitamin D status, assessed by the serum/plasma concentration of 25-hydroxyvitamin D (25(OH)D), was tested in mice and in a randomized-controlled human intervention study. Here, vitamin D_2 from UVB-exposed WGO increased the 25(OH)D levels of vitamin D-depleted mice efficiently, but to a lesser extent than WGO supplemented with vitamin D_3. In humans, UVB-exposed WGO was not able to improve the vitamin D status, since the increase in 25(OH)D_2 was accompanied by a decrease in 25(OH)D_3. Decreases in 25(OH)D_3 were only observable in the participants, who received the UVB-exposed WGO and not in the control groups receiving either no oil or untreated WGO. The metabolic pathway for the decline of 25(OH)D_3 in response to vitamin D_2 remains elusive, but is suggested to be due to a vitamin D_2 induced expression of catabolic cytochrome P450 (CYP)-enzymes, which then catabolize 25(OH)D_3 more efficiently than 25(OH)D_2.

To test whether high concentrations of ergosterol in the plant oils may affect the vitamin D metabolism, further studies were conducted. Compared to their non-treated counterparts, high ergosterol-fed mice showed statistically significant higher concentrations of non-hydroxylated vitamin D_3 in tissues. The higher vitamin D_3 concentration were not based on differences in the hepatic hydroxylation or the renal activation or degradation, since the concentrations of 25(OH)D_3, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) and 24,25-dihydroxyvitamin D_3 were similar between all groups. In addition, the mRNA abundances of intestinal transporters were not altered through an oral intake of ergosterol and therefore not indicative of a differing intestinal absorption. Finally, concentrations of liver lipids were similar between all groups, suggesting that the differences in the vitamin D concentrations were independent from differences in the lipid composition.

As the UVB-exposure of plant oils mainly resulted in the formation of vitamin D_2, studies comparing the metabolic routes of vitamin D_2 and vitamin D_3 were conducted. In contrast to data obtained from human studies, mice fed iso-amounts of deuterated D-vitamers showed nearly doubled 25(OH)D concentrations when fed vitamin D_2 instead of vitamin D_3. In

addition, the concentration of $1,25(OH)_2D_3$ was lower in these mice. This is presumably due to a higher concentration of non-vitamin D protein (DBP)-bound, “free” 25(OH)D, since binding DBP is crucial for renal uptake and activation of 25(OH)D. In contrast to the 25(OH)D data, extensive analysis of vitamin D stored in tissues showed also lowered concentrations of non-hydroxylated vitamin D in mice fed vitamin D_2 than in mice fed vitamin D_3. Protein- and mRNA-expressions were not indicative for differences in the vitamin D hydroxylation between the two groups of mice. Furthermore, hepatoma cell culture studies proved the higher 25(OH)D concentrations, as seen in the vitamin D_2-receiving mice, to be independent of any hepatic processes. Differences in the metabolic routes of vitamin D_2 or vitamin D_3 between mice and humans were also addressed. Although, the difference in the hydroxylation ratio of the D-vitamers was more pronounced in human hepatoma cells than in murine hepatoma cells, both cell lines hydroxylated vitamin D_3 more efficiently than vitamin D_2.

In conclusion, UVB-treatment is a successful strategy to enhance the vitamin D concentrations of plant oils, by stimulating the transformation of vitamin D precursors to vitamin D, predominately from ergosterol to vitamin D_2. UVB-exposed WGO was able to increase the 25(OH)D concentrations of vitamin D-depleted mice, however, failed to improve the vitamin D status of vitamin D-deficient but otherwise healthy humans. The ability of vitamin D_2 compared to vitamin D_3 in raising 25(OH)D levels is still somewhat controversial. However, the high concentration of ergosterol in plant oils might be beneficial, as ergosterol was shown to increase the vitamin D_3 concentrations in tissues.

6 Zusammenfassung

Um einer unzureichenden Versorgung der Bevölkerung mit Vitamin D entgegenzuwirken, werden lebensmittelbasierte Strategien zur Verbesserung des Vitamin D Status gesucht. Die vorgelegte Arbeit hat daher das Potential von UVB-bestrahlten Pflanzenölen zur Verbesserung des Vitamin D Status untersucht. Die Analytik von acht ausgewählten Pflanzenölen (Avocado-, Lein-, Oliven-, Kürbiskern-, Raps-, Soja-, Sonnenblumenöl und Weizenkeimöl (WGO)) zeigte, dass Pflanzenöle hohe Gehalte an den Vitamin D Vorstufen Ergosterol und 7-Dehydrocholesterol aufweisen. WGO zeigte besonders hohe Gehalte an Vitamin D Vorstufen, insbesondere an Ergosterol, welches unter Einwirkung von Ultraviolettem (UV)-Licht Typ B (UVB) zu Vitamin D_2 umgewandelt werden konnten. Die Bioverfügbarkeit von Vitamin D aus UVB-exponiertem WGO wurde zunächst in einer Studie an Vitamin-D-depletierten Mäusen und später in einer randomisiert-kontrollierten Interventionsstudie an Vitamin D-defizienten Probanden getestet. Hierbei erhöhte Vitamin D_2 aus UVB-exponiertem WGO den 25-Hydroxyvitamin D-Spiegel (25(OH)D) von Mäusen effizient, aber in geringerem Maße als Vitamin D_3-angereichertes WGO. Beim Menschen war UVB-exponiertes WGO nicht in der Lage den Vitamin-D-Status zu verbessern, da die beobachteten höheren Werte an 25(OH)D_2 von einer signifikanten Reduktion der 25(OH)D_3 Konzentrationen begleitet wurde. Ein Absinken der 25(OH)D_3 war nur bei den Probanden zu beobachten, die UVB-exponiertes WGO konsumierten, nicht aber bei den Kontrollgruppen, die entweder kein Öl oder unbehandeltes WGO erhielten. Bisher ist nicht geklärt, welcher Stoffwechselweg für die Reduktion der 25(OH)D_3 Konzentrationen verantwortlich ist, möglich wäre jedoch eine durch Vitamin D_2 induzierte Expression Vitamin D-abbauender Cytochrom P450 (CYP)-Enzyme, welche dann 25(OH)D_3 effizienter katabolisieren als 25(OH)D_2.

Um zu prüfen, ob die hohen Konzentrationen an Ergosterol in den Pflanzenölen den Vitamin-D-Stoffwechsel beeinflussen können, wurden weitere Studien durchgeführt. Mäuse, welche hohe Konzentrationen an Ergosterol über das Futter erhielten, wiesen dabei signifikant höhere Gewebekonzentrationen an Vitamin D_3 auf als unbehandelte Kontrolltiere. Die höheren Vitamin D_3-Konzentrationen beruhten nicht auf Unterschieden in der hepatischen Hydroxylierung und der renalen Aktivierung oder Inaktivierung von Vitamin D, da es keine Unterschiede in den Konzentrationen an 25(OH)D_3, 1,25-Dihydroxyvitamin D (1,25$(OH)_2D_3$) oder 24,25-Dihydroxyvitamin D_3 zwischen den Gruppen gab. Analysen der mRNA-Expressionen intestinaler Transporter oder der hepatischen Lipidkonzentration deuten ebenfalls nicht auf Unterschiede in der intestinalen Absorption oder Lipidzusammensetzung hin.

Da die UVB-Bestrahlung von Pflanzenölen hauptsächlich zur Bildung von Vitamin D_2 führte, wurden die Stoffwechselwege von Vitamin D_2 und Vitamin D_3 im Rahmen weiterer Studien an Mäusen und Hepatoma-Zellen verglichen. Dabei zeigten Mäuse die mit Vitamin D_2 gefüttert wurden fast doppelt so hohe 25(OH)D-Konzentrationen, als Mäuse, welche identische Mengen an Vitamin D_3 erhielten. Im Gegensatz dazu zeigten Vitamin D_2-gefütterte Mäuse jedoch geringere Gehalte an bioaktivem $1,25(OH)_2D$, welches auf den erhöhten Gehalt an nicht Protein-gebundenem, „freiem“ 25(OH)D und der damit verbundenen verminderten renalen Aufnahme und anschließenden 1α-Hydroxylierung von 25(OH)D zurückzuführen ist. Im Gegensatz zu den 25(OH)D-Konzentrationen, zeigten umfassende Analysen der Vitamin D-Gewebespeicher geringere Konzentrationen an nicht-hydroxyliertem Vitamin D bei Vitamin D_2-gefütterten als bei Vitamin D_3-gefüttereten Mäusen. Protein- und mRNA-Expressions-Analysen deuten dabei nicht auf Unterschiede auf translationaler oder transkriptioneller Ebene zwischen den Gruppen hin. Weiterführende Zellkulturstudien zeigten zudem, dass die beobachteten höheren 25(OH)D-Konzentrationen von Vitamin D_2-gefütterten Mäusen unabhängig von hepatischen Prozessen sind. Stoffwechselunterschiede zwischen murinen und humanen Hepatoma-Zelllinien bei Inkubation mit Vitamin D_2 oder Vitamin D_3 wurden ebenfalls untersucht. Obwohl hier der Unterschied in der Hydroxylierungsrate der D-Vitamere beim Menschen ausgeprägter war als bei der Maus, wurde bei beiden Zelllinien vorzugsweise hydroxyliertes Vitamin D_3 anstelle von Vitamin D_2 gebildet.

Zusammenfassend lässt sich sagen, dass die UVB-Exposition von Pflanzenölen eine erfolgreiche Strategie darstellt, um die Vitamin-D-Konzentration dieser Öle zu erhöhen, indem die Umwandlung von Vitamin-D-Vorläufern in Vitamin D, vor allem von Ergosterol in Vitamin D_2, stimuliert wird. UVB-exponiertes WGO erhöhte die 25(OH)D-Konzentration von Vitamin D-depletierten Mäusen, konnte jedoch den Vitamin-D-Status von Vitamin D-defizienten, aber ansonsten gesunden Probanden nicht verbessern. Obwohl die Wirksamkeit von Vitamin D_2 im Vergleich zu Vitamin D_3 noch umstritten ist, könnten die hohen Ergosterol-Konzentrationen in Pflanzenölen von Vorteil sein, da Ergosterol nachweislich die Gewebe Konzentrationen an Vitamin D_3 im murinen Organismus erhöht.

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Supplementary Table S1 Responses of circulating 25(OH)D in human intervention studies with daily consumption of UVB-exposed non-animal food sources.

Reference	Study groups	VitD, µg/d[1]	n	Dur, wk[2]	Total 25(OH)D, nmol/L			Δ25(OH)D_2, nmol/L	Δ25(OH)D_3, nmol/L
					Baseline	End	Δ		
Urbain et al., 2011;	+UVB mushrooms + placebo	100	8	4	28.7 ± 10.0	48.2 ± 8.7	+19.5 ± 9.4	+46.0 ±12.5	- 9.5 ± 6.8
	-UVB mushrooms + vitamin D_2	100	8		34.0 ± 11.0	51.5 ± 7.7	+17.5 ± 9.8	+44.2 ± 6.7	-18.0 ± 4.5
	-UVB mushrooms + placebo	0.2	9		38.7 ± 14.2	24.5 ± 7.2	- 14.2 ± 12.3	- 0.2 ± 0.7	- 4.0 ± 6.0
Mehrotra et al., 2014	+UVB mushrooms (low) + placebo	12.5	8		42.6	46.5	+ 3.9	+ 5.4	- 1.5
	+UVB mushrooms (high) + placebo	65	12		42.3	53.7	+11.4	+16.4	-5.0
	-UVB mushrooms + vitamin D_3 (low)	30	8		40.0	59.7	+19.7	-0.3	+20.0
	-UVB mushrooms + vitamin D_3 (high)	183	8		46.7	95.9	+ 49.2	-2.0	+51.2
Stepien et al., 2013	+UVB mushrooms	15	21	4	49.0 ± 19.0	36.8 ± 16.6	-12.2 ± 17.9	+ 5.0 ± 5.1	-17.2 ± 17.6
	-UVB mushrooms	0	21		39.8 ± 12.7	30.6 ± 15.1	- 9.2 ± 20.6	+ 0.9 ± 1.9	-10.1 ± 14.7
	vitamin D_3	15	22		47.8 ± 17.2	57.3 ± 17.7	+ 9.5 ± 14.1	- 0.3 ± 1.8	+ 9.8 ± 17.1
	placebo	0	22		54.9 ± 21.1	41.7 ± 20.1	-13.2 ± 17.5	+ 2.5 ± 4.5	-15.7 ± 19.1
Shanely et al., 2014	+UVB mushrooms	15	17	6	62.2 ± 12.9	69.0 ± 12.6	+ 6.7 ± 12.8	+23.4 ± 8.5	-16.5 ± 11.4
	placebo	0	16		64.5 ± 20.1	62.0 ± 20.7	- 2.5 ± 20.4	-0.02 ± 1.50	- 2.5 ± 10.4
Stephensen et al., 2012	+UVB mushrooms (low) + placebo	8.8	9	6	74 ± 24	nr	- 6.5 ± 11.1	+13.8 ± 7.3	-10.4 ± 6.4
	+UVB mushrooms (high) + placebo	17.1	9		85 ± 40	nr	- 7.3 ± 8.6	+12.7 ± 3.9	-20.6 ± 15.4
	-UVB mushrooms + vitamin D_2	28.2	7		71 ± 16	nr	- 7.3 ± 8.6	+32.8 ± 3.8	-29.5 ± 18.3
	-UVB mushrooms + placebo	0.8	10		102 ± 56	nr	+ 2.6 ± 10.4	+ 1.2 ± 3.9	- 3.9 ± 16.3
Nieman et al., 2013	+UVB mushrooms	95	13	6	91.5 ± 15.3	93.5 ± 17.1	+ 2.0 ± 16.3	+20.3 ± 4.9	-18.7 ± 5.7
	-UVB mushrooms	0	15		102 ± 20.3	96.5 ± 17.4	- 5.2 ± 19.0	+ 0.2 ± 3.0	- 5.3 ± 2.7

25(OH)D values are reported as means ± SD (if possible). [1] Vitamin D through the intervention. [2] Duration of the interventions. 25(OH)D, 25-hydroxyvitamin D; nr, not reported; ± 0, unchanged. Modified from Cashman et al. (2016b).

Supplementary Table S1 (continued) Responses of circulating 25(OH)D in human intervention studies with daily consumption of UVB-exposed non-animal food sources.

Reference	Study groups	VitD, µg/d[1]	n	Dur, wk[2]	Total 25(OH)D, nmol/L			Δ25(OH)D_2, nmol/L	Δ25(OH)D_3, nmol/L
					Baseline	End	Δ		
Study 2	+UVB wheat germ oil	23.7	16	6	35.2 ± 9.5	35.1 ± 7.2	- 0.13 ± 11.2	- 9.40 ± 8.48	+ 9.27 ± 3.9
	-UVB wheat germ oil	<LOQ	16		33.6 ± 11.6	43.0 ± 15.7	+9.43 ± 6.09	± 0	+ 9.43 ± 6.09
	No oil	-	14		38.0 ± 10.2	47.6 ± 10.5	+9.55 ± 6.84	± 0	+ 9.55 ± 6.84
Itkonen et al., 2016	+UVB yeast bread + placebo	26.3	9	8	64.4 ± 15.1	nr	± 0	+ 6.4	± 0
	-UVB yeast bread + vitamin D_2	24.4	9		63.5 ± 11.3	nr	+ 9.6	+ 31.3	- 21.7
	-UVB yeast bread + vitamin D_3	25.0	8		66.6 ± 14.8	nr	+ 17.0	± 0	+18.5
	-UVB yeast bread + placebo	<LOQ	7		66.2 ± 18.6	nr	± 0	± 0	± 0

25(OH)D values are reported as means ± SD (if possible). [1] Vitamin D through the intervention. [2] Duration of the interventions. 25(OH)D, 25-hydroxyvitamin D; n.r., not reported; ± 0, unchanged. Modified from Cashman et al. (2016b).

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Declaration on oath

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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