# Modifiers of absorption, tissue distribution and activation of vitamin D: the role of NPC1L1, SR-B1, CD36, and ABC-G5/G8 transporters

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# List of abbreviations

| | |
|---|---|
| $1,24,25(OH)_3D$ | 1α,24,25-Trihydroxyvitamin D |
| $1,25(OH)_2D$ | 1α,25-Dihydroxyvitamin D, calcitriol, hormonally active vitamin D |
| $24,25(OH)_2D$ | 24,25-Dihydroxyvitamin D |
| 25(OH)D | 25-Hydroxyvitamin D, calcidiol |
| $25(OH)D_3$-$d_3$ | Triple-deuterated 25-hydroxyvitamin $D_3$ |
| 7-DHC | 7-Dehydrocholesterol |
| ABC | ATP-binding cassette |
| AI | Adequate intake |
| ALAT | Alanine aminotransferase |
| ASAT | Aspartate aminotransferase |
| BGP | Bone gla protein |
| BLT-1 | Block lipid transport-1 |
| CaBP9k | Calbindin-D9k |
| CD36 | Cluster determinant 36 |
| CYP | Cytochrome P450 |
| D-A-CH | Deutsche Gesellschaft für Ernährung, Österreichische Gesellschaft für Ernährung, Schweizerische Gesellschaft für Ernährung |
| DBP | Vitamin D binding protein |
| ESCEO | European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis |
| ESPGHAN | European Society for Paediatric Gastroenterology, Hepatology, and Nutrition |
| FGF23 | Fibroblast growth factor 23 |
| FOXO1 | Forkhead box O1 |
| HDL | High-density lipoprotein |
| IOM | Institute of Medicine |
| KO | Knockout |
| LC-MS/MS | Liquid chromatography-tandem mass spectrometry |
| LDL | Low-density lipoprotein |
| LRP5 | Low-density lipoprotein receptor-related protein 5 |
| LXR | Liver X receptor |
| memVDR | Membrane VDR |

| | |
|---|---|
| NAFLD | Nonalcoholic fatty liver disease |
| NPC1L1 | Niemann-Pick C1-like protein 1 |
| PPAR | Peroxisome proliferator-activated receptor |
| PTH | Parathyroid hormone |
| RANKL | Receptor activator of nuclear factor-kappaB ligand |
| RDA | Recommended dietary allowance |
| RI | Recommended intake |
| RKI | Robert Koch Institute |
| RNI | Recommended nutrient intake |
| RXR | Retinoid X receptor |
| SCARB1 | Scavenger receptor class B member 1 |
| SI | Safe intake |
| SNPs | Single nucleotide polymorphisms |
| SPP1 | Secreted phosphoprotein 1 |
| SR-B1 | Scavenger receptor class B type 1 |
| SREBP | Sterol regulatory element-binding protein |
| TRPV6 | Transient receptor potential vanilloid subfamily member 6 |
| UV | Ultraviolet |
| UVB | Ultraviolet B light |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D response element |
| Vitamin $D_3$-$d_3$ | Triple-deuterated vitamin $D_3$ |
| VLDL | Very low-density lipoprotein |
| WT | Wild-type |

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# 1 Introduction

## 1.1 Prevalence of vitamin D insufficiency in the population

Vitamin D deficiency and insufficiency have been acknowledged as a global health problem (Holick 2017; Roth et al. 2018). It is estimated that more than one billion people worldwide are not sufficiently provided with vitamin D (Holick 2017). Vitamin D is a group of fat-soluble compounds that belong to the secosteroids, i.e., steroids with a ring cleavage. Vitamin D can be produced endogenously in the skin by exposure to sunlight (Norman 2008). Alternatively, it is ingested by foods. The clinical outcome of vitamin D deficiency is rickets in children and osteomalacia in adults, bone diseases which are characterized by soft or fragile bones, respectively. Systematic reviews and meta-analyses of epidemiological studies show that vitamin D deficiency is associated with a great number of other diseases such as cancer (Zhang et al. 2015; Zhao et al. 2019; Hossain et al. 2019), respiratory infections (Jat 2017; Martineau et al. 2019; Maretzke et al. 2020), and autoimmune diseases such as multiple sclerosis, and diabetes mellitus type 1 (Wang et al. 2015; Gregoriou et al. 2017; McLaughlin et al. 2018; Maretzke et al. 2020).

Currently there is an ongoing debate regarding the optimal circulating level of 25-hydroxyvitamin D (25(OH)D), which is measured to assess the vitamin D status of an individual (Zerwekh 2008; Cashman et al. 2017). However, there is a good agreement among health authorities that vitamin D deficiency is defined as serum levels of 25(OH)D below 25-30 nmol/L (Table 1). This threshold was set with respect to the elevated risk of rickets and osteomalacia. The Institute of Medicine (IOM) suggests that serum 25(OH)D levels below 30 nmol/L are indicative for vitamin D deficiency and levels between 30 and 50 nmol/L are suggestive for vitamin D insufficiency. Individuals with serum 25(OH)D levels of at least 50 nmol/L are sufficiently provided with vitamin D (Institute of Medicine 2011). However, the Endocrine Society of the USA considers 25(OH)D levels of less than 50 nmol/L to be insufficient and levels above 75 nmol/L as adequate (Holick et al. 2011). It is noteworthy that the thresholds recommended from the Endocrine Society target patients at risk for vitamin D deficiency (such as patients with malabsorption syndromes or chronic kidney disease), in contrast to the IOM whose guidelines are set up for the general healthy population. At serum 25(OH)D levels above 125 nmol/L, adverse effects such as hypercalcemia/hypercalciuria and nephrolithiasis are likely to occur (Institute of Medicine 2011; Rizzoli et al. 2013). Although vitamin D intoxication is a rare phenomenon some case reports described accidental

vitamin D intoxication, in individuals showing serum 25(OH)D levels higher than 375 nmol/L (Chiricone et al. 2003; Klontz and Acheson 2007; De Vincentis et al. 2021). As summarised in Table 1, many health authorities suggest circulating levels of 25(OH)D above 50 nmol/L as an indicator of sufficient vitamin D status and optimal bone health. According to data from the Robert Koch Institute (RKI) more than half of the adults (18-79 years old) in the German population have serum 25(OH)D levels below 50 nmol/L (Hintzpeter et al. 2008a; Rabenberg et al. 2015). Moreover, 15.6% of men and 17.0% of women in Germany had serum 25(OH)D levels below 25 nmol/L (Hintzpeter et al. 2008a) and 30.8% and 29.7% below 30 nmol/L, respectively (Rabenberg et al. 2015). The same institute conducted a similar study on 10,015 children and adolescents, aged 1-17 years old. The authors concluded that vitamin D deficiency afflicts a high proportion of children and adolescents living in Germany and demonstrated that 7.1% of boys and girls from 1-2 years old and 17.7% of boys and 16.8% of girls from 3-17 years old had severe to moderate vitamin D deficiency with serum 25(OH)D levels below 25 nmol/L (Hintzpeter et al. 2008b). It is noteworthy that the measured levels of 25(OH)D also depend on the assay applied. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay is considered the gold standard due to improved sensitivity, accuracy, and reproducibility in comparison to other methods for the measurement of vitamin D metabolites such as enzyme-linked immunoassays and chemiluminescent immunoassays (El-Khoury et al. 2011; Volmer et al. 2015; Müller and Volmer 2015; Wan et al. 2017). In 2018, the original 25(OH)D values of the RKI studies have been corrected retrospectively (Rabenberg et al. 2018). After correction, approximately 15.0% of adults (18-79 years old) and 12.5% of children (1-17 years old) had serum 25(OH)D levels below 30 nmol/L, demonstrating a lower prevalence of vitamin D deficiency in the German population than before the correction (Rabenberg et al. 2018). The health issue of vitamin D deficiency occurs even in countries with southern latitude, i.e., Greece, where the skin is exposed to the sun for more months in a year. A cross-sectional epidemiological study on 2,386 children from 9-13 years old demonstrated that 5.2% and 52.5% of Greek children had serum 25(OH)D levels below 30 and 50 nmol/L, respectively (Manios et al. 2017). A study across Europe, which included 55,844 European individuals, demonstrated that irrespective of age, ethnicity and latitude, 13.0% of Europeans had serum 25(OH)D levels below 30 nmol/L and 40.4% below 50 nmol/L on average in the year (Cashman et al. 2016). A cross-sectional study, which measured plasma 25(OH)D levels of 2,100 adults (35-65 years old) from Germany, demonstrated that 59.9% of the measurements were below 50 nmol/L on a yearly

average, whereas the percentage was increased to 82.2% in winter (Kühn et al. 2014), emphasizing the increase of vitamin D deficient people at times of low sun exposure.

**Table 1.** Vitamin D deficiency and sufficiency thresholds defined by the levels of circulating 25(OH)D as recommended by various authorities and societies.

| Authority/Society | Deficiency [nmol/L] | Sufficiency [nmol/L] | Reference |
|---|---|---|---|
| Institute of Medicine | <30 | ≥50 | Institute of Medicine 2011 |
| European Food Safety Authority | <30 | ≥50 | European Food Safety Authority 2016 |
| ESPGHAN | <25 | >50 | Braegger et al. 2013 |
| D-A-CH Nutrition Societies | <30 | ≥50 | D-A-CH 2018 |
| Endocrine Society | <50 | >75 | Holick et al. 2011 |
| ESCEO | <25 | 50-75* <br> ≥75† | Rizzoli et al. 2013 |
| The Society for Adolescent Health and Medicine | <50‡ | 75-125‡ | The Society for Adolescent Health and Medicine 2013 |

25(OH)D, 25-hydroxyvitamin D; D-A-CH, Deutsche Gesellschaft für Ernährung, Österreichische Gesellschaft für Ernährung, Schweizerische Gesellschaft für Ernährung; ESCEO, European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis; ESPGHAN, European Society for Paediatric Gastroenterology, Hepatology, and Nutrition.
* Circulating 25(OH)D at the population level.
† A desirable level of circulating 25(OH)D for the elderly, who are at high risk for fractures.
‡ Recommended level of circulating 25(OH)D during adolescence.
To convert nmol/L to ng/mL multiply by 0.4.

Sun exposure represents the main source (80-90%) of vitamin D in the body (Herrmann et al. 2017; Sassi et al. 2018). However, many factors (i.e., time of day, season, latitude, altitude, use of sun protection products, clothing, lifestyle, skin pigmentation, age, etc.) influence the cutaneous photosynthesis of vitamin D (Hossein-nezhad and Holick 2013) and, subsequently, the endogenous production of vitamin D, especially in times of low sun exposure. In cases of an inadequate sun exposure, vitamin D must be supplied via the diet or supplements. The recommended vitamin D intake in different countries is summarized in Table 2. The German Nutrition Society recommends a daily vitamin D intake of 20 µg in times of lacking endogenous synthesis (German Nutrition Society 2012). The European Food Safety Authority sets an adequate intake for vitamin D at 15 µg/day (European Food Safety

Authority 2016). In North America, the recommended dietary allowance of vitamin D for children, adolescents and adults 1 to 70 years of age was set at 15 µg/day and for adults older than 70 years of age was set at 20 µg/day (Institute of Medicine 2011). However, adults in Germany consume on average 2-4 µg of vitamin D per day (Max Rubner-Institut 2008), which results the consequence from the low availability of significant food sources of vitamin D.

**Table 2.** Recommended vitamin D intake in different countries. The endogenous synthesis of vitamin D is assumed missing or minimal.

| **Country** | **Infants (<1 year) [µg/day]** | **Children & Adolescents [µg/day]** | **Adults [µg/day]** | **Reference** |
|---|---|---|---|---|
| USA<br>Canada | $10^{AI}$ | $15^{RDA}$ | $15^{RDA}$ (≤70 years)<br>$20^{RDA}$ (>70 years) | Institute of Medicine 2011 |
| European Union | $10^{AI}$ | $15^{AI}$ | $15^{AI}$ | European Food Safety Authority 2016 |
| Germany<br>Austria<br>Switzerland | $10^{AI}$ | $20^{AI}$ | $20^{AI}$ | German Nutrition Society 2012<br>D-A-CH 2018 |
| Nordic countries | $10^{RI}$ | $10^{RI}$ | $10^{RI}$ (<75 years)<br>$20^{RI}$ (≥75 years) | Nordic Council of Ministers 2014 |
| UK | $8.5\text{-}10^{SI}$ | $10^{RNI}$ | $10^{RNI}$ | Scientific Advisory Committee on Nutrition and Health 2016 |
| Australia<br>New Zealand | $5^{AI}$ | $5^{AI}$ | $5^{AI}$ (≤50 years)<br>$10^{AI}$ (51-70 years)<br>$15^{AI}$ (>70 years) | National Health and Medical Research Council. Australian Government Department of Health and Ageing. New Zealand Ministry of Health. 2006 |

AI, adequate intake; D-A-CH, Deutsche Gesellschaft für Ernährung, Österreichische Gesellschaft für Ernährung, Schweizerische Gesellschaft für Ernährung; RDA, recommended dietary allowance; RI, recommended intake; RNI, recommended nutrient intake; SI, safe intake.
To convert µg to IU multiply by 40.

Only 10-20% of vitamin D in the body derives from the diet and supplements (Herrmann et al. 2017; Sassi et al. 2018). The two main isoforms of vitamin D are vitamin $D_3$ (cholecalciferol) mainly from animal origin and vitamin $D_2$ (ergocalciferol) which is present in plants and mushrooms (Heaney 2008; Jäpelt and Jakobsen 2013). There are only a few natural sources of vitamin D. The highest amounts were found in fatty fish, such as salmon, mackerel, tuna, and sardines (Lehmann et al. 2015). Other sources of vitamin $D_3$ are eggs, meat, and dairy products. Mushrooms are rich in vitamin $D_2$ (O'Mahony et al. 2011). In plants and fungi, e.g., edible mushrooms, the synthesis of vitamin $D_2$ is initiated by the exposure of ergosterol, also referred to as provitamin $D_2$, to ultraviolet (UV) radiation. Subsequently, ergosterol is converted to previtamin $D_2$, which is unstable and thermally isomerizes to vitamin $D_2$ or absorbs UV radiation and isomerizes to lumisterol$_2$, or tachysterol$_2$ (Kalaras et al. 2012). Notably, vitamin $D_3$ has been identified in some flowering plants (Boland et al. 2003). Vitamin $D_3$ is the main metabolite that exists in the food, however, 25(OH)$D_3$, vitamin $D_2$ and 25(OH)$D_2$ also exist in the food (Mattila et al. 1995a, 1995b; Clausen et al. 2003; Jakobsen and Saxholt 2009). Because most foods contain none or only small quantities of vitamin D, strategies were developed to produce vitamin D fortified foods, e.g., dairy products, cereals, beverages, etc. (Maurya et al. 2020).

In addition to environmental and dietary factors, the circulating levels of 25(OH)D are also affected by genetic factors. There are several single nucleotide polymorphisms (SNPs) that influence the vitamin D metabolism. Two independent meta-analyses of genome-wide association studies have reported SNPs in or near genes that encode proteins crucial for vitamin D synthesis, transportation and degradation (Ahn et al. 2010; Wang et al. 2010). According to data from both studies, the strongest genome-wide associations with circulating 25(OH)D levels were observed in the following genes: *GC*, that encodes for vitamin D binding protein (DBP) (rs2282679); *DHCR7*, that encodes for 7-dehydrocholesterol reductase (rs12785878); and *CYP2R1*, which encodes for vitamin D 25-hydroxylase (rs10741657). In the population, the frequency of rs2282679, rs12785878, and rs10741657 SNPs is 29%, 23%, and 40%, respectively (Wang et al. 2010). Additionally, Wang et al. (2010) have further identified a SNP (rs6013897) in the *CYP24A1* gene coding for 25(OH)D 24-hydroxylase.

## 1.2 Metabolism of vitamin D

In animals, including humans, the production of vitamin $D_3$ occurs during sun exposure of the skin (Figure 1). 7-Dehydrocholesterol (7-DHC), which is also known as provitamin $D_3$, is an intermediate in the cholesterol synthesis pathway (Sharpe et al. 2014) and the precursor

of vitamin $D_3$ (Prabhu et al. 2016). Photosynthesis of vitamin $D_3$ in the skin is initiated when 7-DHC absorbs ultraviolet B light (UVB, 290-315 nm) to break the B-ring and form previtamin $D_3$ (Holick et al. 1981; MacLaughlin et al. 1982; Holick 2004a). Previtamin $D_3$ is thermodynamically unstable and immediately undergoes thermally induced isomerization to vitamin $D_3$ or isomerizes by the excessive UVB radiation to lumisterol$_3$, or tachysterol$_3$ (Holick et al. 1981; MacLaughlin et al. 1982; Holick 2004a). Vitamin $D_3$ is also sensitive to UVB radiation and is, thereby, converted to inactive photoproducts (suprasterol 1 and 2 and 5,6-trans-vitamin $D_3$) (Webb et al. 1989; Holick 2007). The photoconversion of both previtamin $D_3$ and vitamin $D_3$ to inactive photoproducts indicates that the body protects itself from vitamin D intoxication during prolonged exposure to sunlight (Holick 2004b).

Vitamin $D_3$ synthesized in the skin is drawn into the circulation by the DBP, as shown in Figure 1. Dietary absorbed vitamin $D_3$ and vitamin $D_2$ are embodied in chylomicrons and enter the circulation, where they bind to DBP and lipoproteins, which transport vitamin D to the liver for further processing (Hossein-nezhad and Holick 2013) or adipose tissues for storage (Blum et al. 2008) (Figure 1). Assays using rat plasma DBP showed that vitamin $D_3$ metabolites have a greater affinity for DBP than do vitamin $D_2$ metabolites (Hollis 1984). Several studies in humans demonstrated that vitamin $D_2$ increases less effectively the systemic 25(OH)D than vitamin $D_3$ (Armas et al. 2004; Heaney et al. 2011; Lehmann et al. 2013; Itkonen et al. 2016; Tripkovic et al. 2017). Additionally, a systematic review and meta-analysis of randomized controlled trials, which compared the effectiveness of vitamin $D_3$ and vitamin $D_2$ in raising serum 25(OH)D, showed that vitamin $D_3$ is significantly more effective than vitamin $D_2$ (Tripkovic et al. 2012). However, other studies conducted in humans demonstrated that vitamin $D_2$ is as effective as vitamin $D_3$ in raising and maintaining the circulating 25(OH)D levels (Holick et al. 2008; Biancuzzo et al. 2010, 2013). It is noteworthy that both vitamin D isoforms are effective in the prevention and treatment of rickets and osteomalacia (Munns et al. 2016; Bouillon et al. 2016).

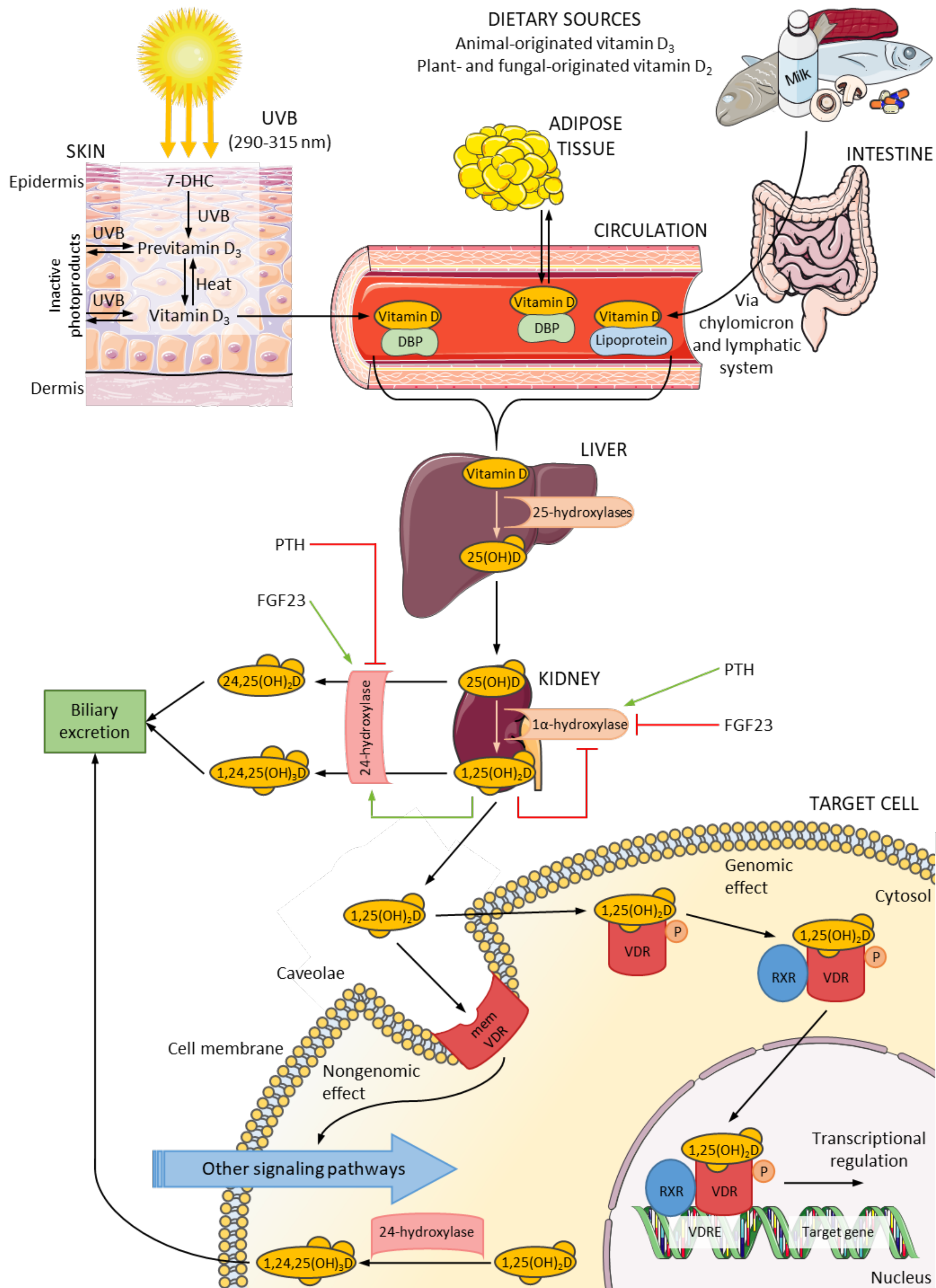


**Figure 1. Photosynthesis and metabolism of vitamin D.** This figure was adapted and modified according to (Holick et al. 1981; Holick 2007; Deeb et al. 2007; Keane et al. 2017; Herrmann et al. 2017; Jeon and Shin 2018) and created with images adapted and modified from Servier Medical Art by Servier licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/; https://creativecommons.org/licenses/by/3.0/legalcode). 1,24,25(OH)$_3$D,

1α,24,25-trihydroxyvitamin D; 1,25$(OH)_2$D, 1α,25-dihydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; 7-DHC, 7-dehydrocholesterol; DBP, vitamin D binding protein; FGF23, fibroblast growth factor 23; memVDR, membrane VDR; P, phosphorylation; PTH, parathyroid hormone; RXR, retinoid X receptor; UVB, ultraviolet B; VDR, vitamin D receptor; VDRE, vitamin D response element.

The chemical structures of vitamin $D_3$ and vitamin $D_2$ are similar, thus both isoforms undergo the same metabolism in the body. However, the subsequent activation of these vitamin D isoforms may have some differences because of the different affinities for the enzymes (Holmberg et al. 1986). For the activation of vitamin D, two hydroxylation steps are necessary (Figure 1). The first hydroxylation of vitamin D occurs in the liver, where vitamin D 25-hydroxylases add a hydroxyl group at carbon 25 producing the major circulating form of vitamin D, 25(OH)D (calcidiol) (Jeon and Shin 2018). Primarily, this hydroxylation is performed by the cytochrome P450 (CYP) enzyme CYP2R1 (Cheng et al. 2004), which was first identified by Cheng and co-workers in microsomal mouse liver (Cheng et al. 2003). CYP27A1 is a mitochondrial enzyme, which 25-hydroxylates vitamin $D_3$ but not vitamin $D_2$, unlike CYP2R1, which 25-hydroxylates both vitamin $D_3$ and vitamin $D_2$ (Guo et al. 1993; Sawada et al. 2000; Bikle 2014). Other CYP enzymes such as CYP3A4 and CYP2J2 have been reported to have 25-hydroxylase activity too (Bikle 2014). The hydroxylation of vitamin D to 25(OH)D in the liver is suggested to be not closely regulated and depends on the concentration of the substrate (Bhattacharyya and DeLuca 1973; Zerwekh 2008).

25(OH)D formed in the liver is transported by the DBP to the kidneys, where 25(OH)D undergoes a second hydroxylation at α-position of carbon 1 by the mitochondrial 25(OH)D 1α-hydroxylase producing the hormonally active vitamin D metabolite, 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D, calcitriol) (Jeon and Shin 2018). CYP27B1 is the only enzyme recognized with 25(OH)D 1α-hydroxylase activity (Bikle 2014). The activated vitamin D metabolite binds to DBP, which transports this hormone to the target tissues, e.g., bone, intestine (Haussler et al. 2013; Jeon and Shin 2018), where vitamin D regulates calcium and phosphorus homeostasis (Heaney 2008). Even though kidneys are the main source of circulating 1,25$(OH)_2$D (Fraser and Kodicek 1970), many extrarenal tissues such as bone, placenta, epithelium such as keratinocytes and prostate, endocrine glands such as parathyroid gland, testes and ovary and immune cells such as macrophages, monocytes, T and B cells, express CYP27B1 and produce 1,25$(OH)_2$D with an autocrine and paracrine functions (Bikle et al. 2018). Unlike the hepatic production of 25(OH)D, the renal production of 1,25$(OH)_2$D is tightly regulated by parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and 1,25$(OH)_2$D itself (Figure 1). PTH is a stimulator whereas FGF23 and 1,25$(OH)_2$D are

inhibitors of the renal 1,25(OH)$_2$D synthesis (Bikle 2014; Christakos et al. 2016). However, the regulation of the extrarenal production of 1,25(OH)$_2$D differs from that of the renal (Bikle et al. 2018).

In the target tissues, 1,25(OH)$_2$D regulates the transcription of the genes in both genomic and nongenomic manner by binding to the vitamin D receptor (VDR) (Figure 1). The VDR is a member of the nuclear receptor family and behaves as a ligand-activated transcription factor (McDonnell et al. 1987; Baker et al. 1988; Kamei et al. 1995). In the genomic pathway, the interaction of 1,25(OH)$_2$D with cytosolic VDR leads to the phosphorylation of VDR. The 1,25(OH)$_2$D-VDR complex binds to retinoid X receptor (RXR) in cytosol forming a heterodimeric complex, which moves to nucleus and binds to the vitamin D response element (VDRE) of target genes and thus activates or represses the mRNA expression of genes (Jeon and Shin 2018). Table 3 shows the target genes of 1,25(OH)$_2$D such as *PTH*, *FGF23* and *klotho*, which are involved in calcium and phosphorus homeostasis, as well as *CYP27B1* and *CYP24A1*, which play a significant role in the metabolism of vitamin D. In the nongenomic pathway, which typically responses faster than the genomic (Norman 2006), 1,25(OH)$_2$D binds to membrane VDR (memVDR) inducing changes in other signaling pathways and thus influences indirectly the transcriptional rate of the target genes (Jeon and Shin 2018). It is speculated that the rapid actions of 1,25(OH)$_2$D activate the mitogen-activated protein kinase–extracellular signal-regulated kinase 1 and 2 cascade, which may engage in cross-talk with the genomic pathway to modulate gene expression (Lösel and Wehling 2003; Deeb et al. 2007). Of note, it is estimated that the hormonally active vitamin D regulates directly or indirectly about 2000 genes (Hossein-nezhad and Holick 2013; Hossein-nezhad et al. 2013) and consequently it is important not only for bone health but also for other biological functions, e.g., in the immune system (Gil et al. 2018; Umar et al. 2018). Concerning bone health, the major function of 1,25(OH)$_2$D is the stimulation of intestinal calcium absorption. 1,25(OH)$_2$D induces the expression of transient receptor potential vanilloid subfamily member 6 (*TRPV6*), a gene that encodes a calcium channel, which is localized at the apical membrane of enterocytes and transports calcium from the intestinal lumen into the enterocytes (Meyer et al. 2006; Haussler et al. 2013). Another gene regulated by 1,25(OH)$_2$D is calbindin-D9k (*CaBP9k*) which catalyses the transcellular calcium transport (Brehier and Thomasset 1990; Haussler et al. 2013). In the basolateral membrane, plasma membrane calcium ATPase 1b and 2c are responsible for the efflux of calcium into the circulation (Haussler et al. 2013). In case of an inadequate dietary calcium intake, both 1,25(OH)$_2$D and PTH induce the expression of receptor activator of nuclear

factor-kappaB ligand (*RANKL*) in osteoblasts to stimulate bone resorption and maintain calcium homeostasis (Kim et al. 2006).

**Table 3.** 1,25$(OH)_2$D-regulated genes.

| Gene | Transcriptional regulation | Function of the encoded protein | Reference |
|---|---|---|---|
| *PTH* | repression | Calcium and phosphorus homeostasis | Demay et al. 1992<br>Liu et al. 1996 |
| *FGF23* | activation | Calcium and phosphorus homeostasis | Kolek et al. 2005<br>Haussler et al. 2012 |
| *klotho* | activation | Calcium and phosphorus homeostasis | Forster et al. 2011 |
| *RANKL* | activation | Bone resorption | Kim et al. 2006 |
| *BGP* | activation | Bone metabolism | Terpening et al. 1991 |
| *SPP1* | activation | Bone metabolism | Noda et al. 1990 |
| *LRP5* | activation | Bone anabolism | Fretz et al. 2006 |
| *TRPV6* | activation | Intestinal calcium absorption | Meyer et al. 2006 |
| *CaBP9k* | activation | Intestinal calcium absorption | Brehier and Thomasset 1990 |
| *CYP27B1* | repression | Vitamin D activation | Murayama et al. 1999 |
| *CYP24A1* | activation | Vitamin D degradation | Zou et al. 1997 |

1,25$(OH)_2$D, 1α,25-dihydroxyvitamin D; BGP, bone gla protein; CaBP9k, calbindin-D9k; CYP24A1, cytochrome P450 family 24 subfamily A member 1; CYP27B1, cytochrome P450 family 27 subfamily B member 1; FGF23, fibroblast growth factor 23; LRP5, low-density lipoprotein receptor-related protein 5; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor-kappaB ligand; SPP1, secreted phosphoprotein 1; TRPV6, transient receptor potential vanilloid subfamily member 6.

1,25$(OH)_2$D and 25(OH)D can also be degraded to less active metabolites (Figure 1). The degradation included the hydroxylation of carbon 24 by the mitochondrial 25(OH)D 24-hydroxylase in the kidneys and target tissues (Jones et al. 2014; Jeon and Shin 2018). CYP24A1, which is a multifunctional enzyme (Beckman et al. 1996), has 24-hydroxylase activity and catalyzes the conversion of 25(OH)D to 24,25-dihydroxyvitamin D (24,25$(OH)_2$D) and the conversion of 1,25$(OH)_2$D to 1α,24,25-trihydroxyvitamin D (1,24,25$(OH)_3$D). The renal regulation of 24-hydroxylase is the reciprocal of that of 1α-hydroxylase (Figure 1). PTH increases the degradation of 24-hydroxylase whereas FGF23

increases the expression of 24-hydroxylase. Moreover, 1,25$(OH)_2$D enhances its own inactivation by inducing the expression of 24-hydroxylase (Bikle 2014). 24-Hydroxylation is followed by several oxidation reactions producing compounds, which are excreted primarily through the bile into feces and marginally through urine (DeLuca 1976; Gil et al. 2018).

## 1.3 Biomarkers of vitamin D

In clinical practice, the level of circulating 25(OH)D has widely been accepted as a good biomarker for the assessment of vitamin D status (Zerwekh 2008; Cashman et al. 2017). A reason supporting the suitability of 25(OH)D as a biomarker of vitamin D status is the long half-life of circulating 25(OH)D, which is approximately 2-3 weeks (Batchelor and Compston 1983; Jones et al. 2014; Herrmann et al. 2017). Therefore, 25(OH)D is the most abundant circulating form of vitamin D and it is suggested that 25(OH)D reflects the vitamin D stores in the body gained from both diet and sun exposure of the skin. Furthermore, it is suggested that hepatic production of 25(OH)D is not under strict regulation and is dependent on the concentration of vitamin D (Bhattacharyya and DeLuca 1973; Zerwekh 2008). A randomized controlled trial with patients suffering from hip fracture and vitamin D insufficiency (25(OH)D below 50 nmol/L) demonstrated that after oral supplementation of 1000 IU of vitamin D per day for three months, all patients achieved serum 25(OH)D levels above 50 nmol/L (Glendenning et al. 2009). Another randomized controlled trial, which was conducted during the winter months, showed that daily consumption of 20 μg (800 IU) vitamin $D_3$ for three months improved 25$(OH)D_3$ levels to at least 50 nmol/L (Lehmann et al. 2016). Additionally, a cross-sectional analysis of dietary, lifestyle, and genetic determinants of vitamin D status found a strong seasonal fluctuation in plasma 25(OH)D levels (Kühn et al. 2014). The aforementioned studies show that circulating 25(OH)D levels display a significant response to dietary supplements, as well as to sun exposure.

In contrast to 25(OH)D, the circulating levels of 1,25$(OH)_2$D have been regarded as an unsuitable biomarker of vitamin D status because of its short half-life of approximately 4 hours (Gray et al. 1978; Brandi et al. 2002; Herrmann et al. 2017) and the strong regulation by the renal 1α-hydroxylase (Christakos et al. 2016). PTH and FGF23, which are the main regulators of 1α-hydroxylase and subsequently of 1,25$(OH)_2$D synthesis, ensure the homeostasis of 1,25$(OH)_2$D (Christakos et al. 2016). Individuals with vitamin D deficiency often develop secondary hyperparathyroidism, a medical condition of the parathyroid glands characterized by excessive PTH secretion, thereby resulting in normal or even elevated circulating levels of 1,25$(OH)_2$D (Arya et al. 2004). From an analytical aspect, the circulating

levels of 1,25$(OH)_2$D are three orders of magnitude lower than 25(OH)D, thus, the measurement of the bioactive vitamin D metabolite is a challenging task (El-Khoury et al. 2011). However, its measurement has been considered helpful in the diagnosis of several inherited or acquired disorders of vitamin D metabolism, such as chronic kidney disease (Holick 2009; Herrmann et al. 2017), because these disorders are related to alterations in circulating levels of 1,25$(OH)_2$D (Holick 2009).

While the circulating 25(OH)D levels have currently been accepted as a good biomarker of vitamin D status, the levels of circulating PTH have been suggested as a functional indicator of vitamin D status (Prentice et al. 2008) mainly because increased PTH secretion represents a risk factor for osteoporosis, which is a skeletal disorder characterized by low bone mass that leads to an increased risk of fracture (Mazzuoli et al. 1998). Additionally, it is demonstrated that vitamin D supplementation can lower the increased PTH levels in the circulation (Malabanan et al. 1998). The information regarding the use of PTH as functional marker of vitamin D status is scarce.

Recently, 24,25$(OH)_2$D, which is the first metabolite in the vitamin D catabolic pathway, has obtained attention as a potential vitamin D status biomarker. More precisely, the circulating levels of 24,25$(OH)_2$D could be a useful indicator of vitamin D catabolism (Tang et al. 2017). The half-life of 24,25$(OH)_2$D is approximately 1 week and its circulating levels depend on the availability of 25(OH)D and the expression of 24-hydroxylase (Jongen et al. 1989; Herrmann et al. 2017). Additionally, a cross-sectional study found a strong correlation (Pearson r range, 0.64-0.88) between the circulating levels of 25(OH)D and 24,25$(OH)_2$D (de Boer et al. 2014). A subsequent randomized controlled trial also found a strong correlation ($r = 0.908$) between 25$(OH)D_3$ and 24,25$(OH)_2D_3$ and demonstrated that the magnitude of 24,25$(OH)_2D_3$ increase is similar to that of 25$(OH)D_3$ after supplementation of 20 µg vitamin $D_3$ per day (Lehmann et al. 2016). Accordingly, a few researchers suggested calculating the ratio of both compounds to assess vitamin D status (Kaufmann et al. 2014; Cashman et al. 2015). The 24,25$(OH)_2$D:25(OH)D ratio appears to be predictive of 25(OH)D response to vitamin D supplementation (Wagner et al. 2011).

Apart from hydroxylated vitamin D metabolites, nonhydroxylated vitamin D is discussed as an alternative biomarker to evaluate vitamin D status. The half-life of vitamin D is approximately 24 hours; therefore, its circulating concertation depends on the most recent dietary intake and sun exposure (Clemens et al. 1982; Zerwekh 2008). Moreover, it is hypothesized that serum vitamin D could be a better biomarker than 25(OH)D (Jorde and Grimnes 2018). This hypothesis was based on the argument that vitamin D-dependent cells

likely prefer nonhydroxylated vitamin D instead of 25(OH)D and 1,25$(OH)_2$D. As many cells are equipped with the necessary hydroxylases, they can activate intracellular vitamin D for their own needs.

Recently, an analytical method has been developed that quantitates vitamin D metabolites from dried blood spots. This approach to evaluating vitamin D status is less invasive and could be applicable in population-based vitamin D studies (Zakaria et al. 2020). Interestingly, a recent clinical trial demonstrated that vitamin D measured in dried blood spots does not differ significantly from vitamin D measured in plasma (Nybo et al. 2021).

Although 25(OH)D has been commonly used as a biomarker to assess vitamin D metabolism, data from the literature suggest that 25(OH)D is not really suitable to reflect the amounts of stored vitamin D in the body.

## 1.4 Intestinal absorption and tissue distribution of vitamin D

The synthesis, activation, degradation, and functions of vitamin D are well investigated. However, significantly less data are available regarding the intestinal absorption and tissue uptake of vitamin D. Both hydroxylated and nonhydroxylated vitamin D metabolites can generally be absorbed (Maislos and Shany 1987; Leichtmann et al. 1991). It is assumed that vitamin D follows the same fate as lipids in the upper gastrointestinal tract (Borel 2003). The absorption of vitamin D occurs in the small intestine. In experiments on rats and mice, optimal vitamin D absorption was observed in both jejunum and ileum (Hollander et al. 1978; Goncalves et al. 2015). Clinical trials and experiments conducted with Caco-2 cells demonstrated that the intestinal absorption of vitamin $D_3$ and vitamin $D_2$ is equivalent (Armas et al. 2004; Biancuzzo et al. 2010; Reboul et al. 2011). It was also shown that vitamin $D_3$ absorption is highly dependent on the presence of biliary salts (Maislos and Shany 1987), which together with lipids form mixed micelles, thus, increasing the solubility of vitamin D (Thompson et al. 1969).

Vitamin D can interact with many other food components that affect its bioavailability. Several studies demonstrated that patients suffering from intestinal fat malabsorption syndromes malabsorbed also vitamin D (Lo et al. 1985; Lark et al. 2001; Farraye et al. 2011). Furthermore, clinical trials indicated that the absorption of dietary vitamin $D_3$ is greater when the meal contains fat (Dawson-Hughes et al. 2015; Raimundo et al. 2015). Both cholesterol and phytosterols could compete with vitamin D for its intestinal absorption. Vitamin $D_3$ absorption was significantly decreased when Caco-2 cells were incubated with these sterols

(Reboul et al. 2011; Goncalves et al. 2011). In Caco-2 cells, long-chain fatty acids and pinoresinol from olive oil significantly reduce the uptake of vitamin $D_3$ (Goncalves et al. 2013, 2016). Other fat-soluble vitamins can also affect the vitamin D intestinal absorption. Studies using Caco-2 cells showed that vitamins A and E significantly decreased the uptake of vitamin $D_3$ (Reboul et al. 2011; Goncalves et al. 2015).

Initially, it was assumed that the absorption of vitamin D occurs exclusively by passive diffusion (Hollander and Truscott 1976; Hollander et al. 1978; Hollander 1981). Nowadays, there is evidence mainly from *in vitro* and *ex vivo* studies that lipid transporters (i.e., Niemann-Pick C1-like protein 1 (NPC1L1), scavenger receptor class B type 1 (SR-B1), cluster determinant 36 (CD36), or ATP-binding cassette (ABC) transporters) may be involved in the absorption of vitamin D (Reboul et al. 2011). Precisely, it was showed that ezetimibe (NPC1L1 inhibitor) and block lipid transport-1 (BLT-1, SR-B1 inhibitor) significantly decreased the cellular uptake of vitamin $D_3$ in Caco-2 cells by approximately 30% and 50%, respectively (Reboul et al. 2011). Moreover, human embryonic kidney cell transfection with human NPC1L1, SR-B1, or CD36 led to a significant increase of vitamin $D_3$ uptake and this increase was significantly impaired by the corresponding inhibitors of these transporters (Reboul et al. 2011). The effect of ezetimibe, BLT-1, and sulfo-*N*-succinimidyl oleate (CD36 inhibitor) on vitamin $D_3$ uptake was confirmed *ex vivo* using mouse intestinal explants (Reboul et al. 2011).

As mentioned above, some evidence indicates the involvement of lipid transporters in the intestinal absorption of vitamin D. A transporter of interest is NPC1L1, a protein with 13 transmembrane domains (Davies et al. 2000; Altmann et al. 2004; Wang et al. 2009). Five of the 13 transmembrane domains constitute a sterol-sensing domain (Wang et al. 2009; Huang et al. 2020). NPC1L1 also contains 7 small intracellular domains and 3 extracellular domains, i.e., N-terminal domain, middle extracellular domain, and C-terminal extracellular domain (Wang et al. 2009; Huang et al. 2020). The N-terminal domain captures cholesterol from intestinal micelles and seems to be essential for NPC1L1-mediated cholesterol uptake (Zhang et al. 2011; Huang et al. 2020). A loop in the middle extracellular domain of NPC1L1 appears to be important for ezetimibe binding (Weinglass et al. 2008). Recently, it was shown that all three extracellular domains form a pocket in which ezetimibe binds (Huang et al. 2020). Ezetimibe is a clinically used cholesterol-lowering pharmaceutical that specifically binds to NPC1L1 transporter (Lipka 2003; Garcia-Calvo et al. 2005; Temel et al. 2007). After oral administration, ezetimibe is rapidly absorbed, extensively glucuronidated and recycled by the enterohepatic circulation to its target sites (Kosoglou et al. 2005; Garcia-Calvo et al. 2005). In

humans, both intestinal and hepatic NPC1L1 appear to be targets of ezetimibe (Temel et al. 2007). Concerning the inhibition of cholesterol uptake, ezetimibe-glucuronide is at least as potent as ezetimibe (van Heek et al. 2000; Wang et al. 2009). Ezetimibe inhibits the function of NPC1L1 by blocking the passage of cholesterol from the N-terminal domain to the sterol-sensing domain and therefore the internalization of NPC1L1 (Huang et al. 2020). In humans, NPC1L1 is primarily expressed along the entire small intestine (the highest levels were found in the jejunum) and in the liver (Altmann et al. 2004; Davies et al. 2005) and is localized at the apical membrane of enterocytes (Altmann et al. 2004) and the canalicular membrane of hepatocytes (Yu et al. 2006), respectively. Data on the regulation of *NPC1L1* expression are inconsistent. As reviewed, cholesterol may regulate the expression of *NPC1L1* through sterol regulatory element-binding protein 2 (SREBP-2) (Yu 2008; Davis and Altmann 2009). Moreover, *NPC1L1* has been shown to be regulated by a variety of other transcriptional factors, including liver X receptor (LXR), RXR, peroxisome proliferator-activated receptor (PPAR) α, and PPARδ (Yu 2008; Davis and Altmann 2009). Polymorphisms in the human *NPC1L1* gene have been found to influence the absorption of sterols and the plasma levels of low-density lipoprotein (LDL) cholesterol (Davis and Altmann 2009; Wang et al. 2011). NPC1L1 has been characterized as a critical protein for the intestinal absorption of cholesterol (Figure 2). Mice deficient in NPC1L1 and wild-type (WT) mice treated with ezetimibe displayed a similar and significant reduction (approximately 70%) in absorbed cholesterol (Altmann et al. 2004). Randomized controlled trials on patients with primary hypercholesterolemia demonstrated that ezetimibe significantly reduced plasma LDL cholesterol levels after 12 weeks of treatment (Bays et al. 2001; Knopp et al. 2003). Further, ezetimibe treatment of patients with primary hypercholesterolemia indicated that 10 mg ezetimibe per day for 12 weeks did not alter the serum levels of fat-soluble vitamins (Knopp et al. 2003). Moreover, a randomized controlled trial that included healthy volunteers found no effect of ezetimibe (10 mg/day) on the mean change in serum 25(OH)D after a single oral dose of 50,000 IU vitamin $D_3$ (Silva et al. 2015). Apart from cholesterol, NPC1L1 also mediates the intestinal absorption of phytosterols (Davis et al. 2004). In the liver, NPC1L1 has been suggested to facilitate the reuptake of cholesterol from bile back into hepatocytes limiting the excessive biliary cholesterol loss (Temel et al. 2007). Besides sterols, NPC1L1 may interact with other fat-soluble compounds. It was shown using Caco-2 cells and *in situ* rat perfusions that NPC1L1 is involved in the intestinal absorption of vitamin E (Narushima et al. 2008; Abuasal et al. 2010).

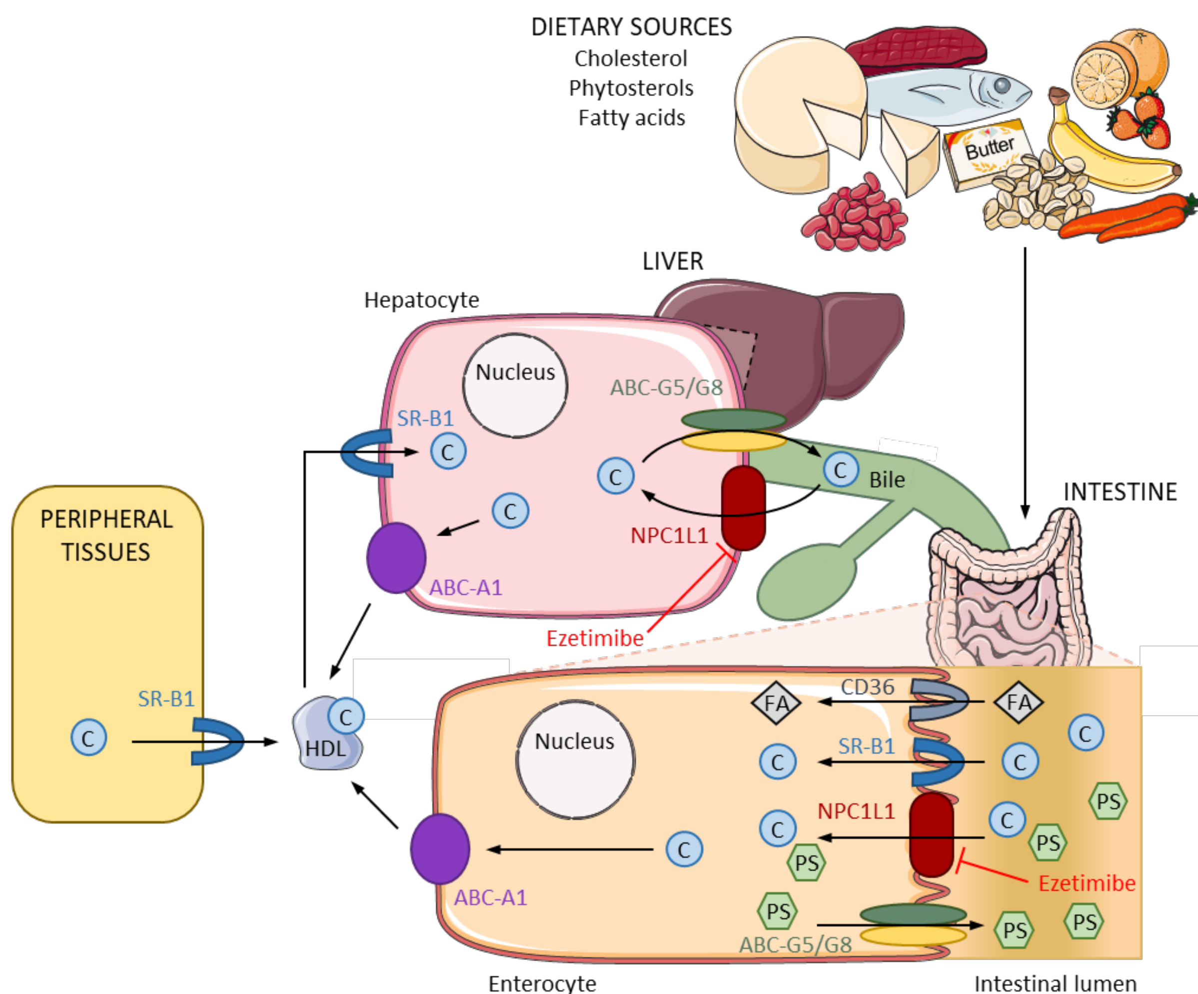


**Figure 2. Physiological role of NPC1L1, SR-B1, CD36, ABC-G5/G8, and ABC-A1.** ABC-A1, which is a member of the ABC transporters, is expressed in various tissues and localized at the basolateral membrane of the cells (Timmins et al. 2005; Brunham et al. 2006). In the intestine and liver, ABC-A1 is responsible for HDL production, and thus, it can play a role in the basolateral efflux of cellular phospholipids, cholesterol, and phytosterols (Field et al. 2004; Timmins et al. 2005; Brunham et al. 2006). This figure was adapted and modified according to (Brewer and Santamarina-Fojo 2003; Rhainds and Brissette 2004; Davis and Altmann 2009; Jia et al. 2011; Abumrad and Davidson 2012; Pepino et al. 2014) and created with images adapted and modified from Servier Medical Art by Servier licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/; https://creativecommons.org/licenses/by/3.0/legalcode). ABC-A1, ATP-binding cassette subfamily A member 1; ABC-G5/G8, ATP-binding cassette subfamily G member 5/subfamily G member 8; C, cholesterol; CD36, cluster determinant 36; FA, fatty acids; HDL, high-density lipoprotein; NPC1L1, Niemann-Pick C1-like protein 1; PS, phytosterols; SR-B1, scavenger receptor class B type 1.

Another lipid transporter that could be important for vitamin D uptake is SR-B1, a multiligand receptor (Valacchi et al. 2011) with a high affinity for high-density lipoprotein (HDL) (Acton et al. 1996). SR-B1 has two small intracellular domains and two transmembrane domains separated by a large extracellular domain (Calvo and Vega 1993). It is highly expressed in hepatocytes and steroidogenic cells (Acton et al. 1996; Landschulz et al. 1996; Murao et al. 1997; Cao et al. 1997). In lower concentrations, SR-B1 is expressed in

several other cells, including macrophages (Buechler et al. 1999), adipocytes (Murao et al. 1997), and endothelial cells (Yeh et al. 2002), as well as in the apical membrane of enterocytes (Lobo et al. 2001). In humans, SR-B1 is located in the whole intestine, in the duodenum as well as in the rectum (Lobo et al. 2001). The regulation of the scavenger receptor class B member 1 (*SCARB1*) gene that encodes for the SR-B1 protein has binding sites for many transcription factors, such as SREBP-1, which upregulates the expression of *SCARB1* in response to altered intracellular sterol levels (Lopez and McLean 1999; Shen et al. 2018). Other transcription factors that positively regulate the expression of the *SCARB1* gene are steroidogenic factor 1, prolactin regulatory element-binding protein, LXR/RXR, and PPARα, whereas Yin Yang 1 transcription factor and microRNAs regulate it negatively (Shen et al. 2018). In humans, polymorphisms on the *SCARB1* gene are associated with elevated plasma HDL cholesterol levels (Shen et al. 2018). It was shown that SR-B1 primarily mediates selective HDL-derived cholesterol uptake in the liver (Acton et al. 1996) (Figure 2). Subsequent studies confirmed the physiological role of SR-B1 in cholesterol homeostasis. These studies demonstrated that mice deficient in SR-B1 had increased levels of plasma cholesterol, which resulted from the decreased hepatic uptake of cholesterol (Rigotti et al. 1997; Varban et al. 1998). Also, SR-B1 facilitates the efflux of cholesterol from peripheral tissues back into the liver. In the intestine, SR-B1 has been shown to facilitate the absorption of dietary cholesterol (Hauser et al. 1998; Bietrix et al. 2006). Aside from HDL, SR-B1 can bind and transport other ligands, including vitamin E (Mardones et al. 2002; Reboul et al. 2006) and carotenoids (During et al. 2005).

A second scavenger receptor protein that might be relevant in the uptake and tissue distribution of vitamin D is CD36. Like SR-B1, CD36 can interact with a wide range of ligands and has two small intracellular domains, two transmembrane domains, and a large extracellular domain (Neculai et al. 2013; Pepino et al. 2014). CD36 is expressed in numerous cell types, including enterocytes, myocytes, adipocytes, and immune cells but not in hepatocytes (Abumrad et al. 1993; Pepino et al. 2014). *CD36* gene expression is modulated by several transcription factors, including PPARs, LXR, pregnane X receptor, CCAAT/enhancer-binding protein, signal transducer and activator of transcription 3, and forkhead box O1 (FOXO1) (Wang and Li 2019). Noncoding RNAs are also involved in the regulation of CD36 mRNA levels (Wang and Li 2019). *CD36* gene polymorphisms have been associated with alterations in plasma lipid levels (Love-Gregory and Abumrad 2011). The broad expression of CD36 is accompanied by a similarly wide range of functions relating to lipid homeostasis and immune responses. Concerning lipid homeostasis, CD36 has a high affinity for fatty acids and

facilitates fatty acid uptake into enterocytes (Chen et al. 2001), cardiomyocytes (Habets et al. 2007), and adipocytes (Abumrad and Goldberg 2016). Studies on CD36-deficient mice demonstrated that the uptake of fatty acid analogs was reduced in the heart (50-80%), skeletal muscle (40-75%), and adipose tissue (60-70%) compared to WT mice (Coburn et al. 2000). Additionally, human CD36 deficiency was accompanied by abnormally elevated levels of lipids and lipoproteins in the blood (Yamashita et al. 2007). In the human duodenum and jejunum, CD36 is colocalized with SR-B1 in the apical membrane of enterocytes (Lobo et al. 2001) (Figure 2). CD36 can also mediate the intestinal absorption of cholesterol (Werder et al. 2001; Nassir et al. 2007) and β-carotene (van Bennekum et al. 2005). Enterocytes isolated from mice deficient in CD36, compared to WT counterparts, exhibited a 60% reduction in cholesterol uptake (Nassir et al. 2007).

Finally, the superfamily of ABC transporters could be relevant in vitamin D metabolism. In particular, ABC-G5 and ABC-G8, which are predicted to have six transmembrane domains and an intracellular nucleotide-binding domain (Berge et al. 2000; Lee et al. 2016), form a heterodimeric complex (ABC-G5/G8) (Graf et al. 2003). This complex is localized in the apical membranes of enterocytes, hepatocytes, and gallbladder epithelial cells (Berge et al. 2000; Graf et al. 2003; Klett et al. 2004a). *ABC-G5* and *ABC-G8* genes are arranged in a head-to-head (i.e., 3´ to 5´ and 5´ to 3´) configuration and separated by 374 base pairs (Berge et al. 2000). Due to this conformation, it is assumed that both genes have a bidirectional promoter and common regulatory elements. Both *ABC-G5* and *ABC-G8* genes are regulated by several transcriptional factors, including hepatocyte nuclear factor 4 alpha, LXRα, LXRβ, liver receptor homolog-1, and FOXO1 (Zein et al. 2019). Polymorphisms in these genes are related to gallstone formation and dyslipidemia (Chen et al. 2008; Kuo et al. 2008; von Kampen et al. 2013; Zein et al. 2019). ABC-G5/G8 limits the accumulation of phytosterols and, to a minor extent, of cholesterol (Figure 2). In the intestine, ABC-G5/G8 catalyses the sterol export from the enterocytes back into the lumen (Yu et al. 2002b; Plösch et al. 2004) and, in the liver, promotes the secretion of sterols into bile (Yu et al. 2002b, 2002a; Graf et al. 2003; Klett et al. 2004b). Disruption of ABC-G5 and ABC-G8 in mice resulted in a 2- to 3-fold increase in the absorption of dietary phytosterols and extremely low levels of biliary cholesterol (Yu et al. 2002a). In humans, mutations in either ABC-G5 or ABC-G8 cause sitosterolemia, a rare autosomal recessive disorder characterized by the accumulation of sterols (Berge et al. 2000). No data are available on the transport of vitamin D by ABC-G5/G8.

# 2 Hypothesis and objectives of this study

Currently, circulating 25(OH)D is considered the most reliable biomarker for defining vitamin D status (Zerwekh 2008; Cashman et al. 2017). However, the measurement of other vitamin D metabolites besides 25(OH)D was discussed in the *First International Conference on Controversies in Vitamin D* (Sempos et al. 2018). Recently, it was hypothesized that circulating nonhydroxylated vitamin D might be a better biomarker of vitamin D status, questioning the reliability of 25(OH)D as a vitamin D status biomarker (Jorde and Grimnes 2018). Additionally, limited data are available regarding the suitability of 25(OH)D as an indicator of body vitamin D stores and the mobilization of the stored vitamin D. It is also not exactly known how sensitively other metabolites other than 25(OH)D, such as vitamin D or $24,25(OH)_2D$, change after oral administration of vitamin D. Concerning the best and most sensitive biomarker reflecting vitamin D stores in the body, changes in vitamin D consumption, and mobilization of stored vitamin D, the following questions were addressed:

**I. Which parameter best indicates the stored levels of vitamin D in the body?**

As mentioned above, circulating 25(OH)D is currently used to evaluate the vitamin D status. However, whether it may also reflect the stored vitamin D in the body remains to be elucidated. To this end, WT mice (C57BL/6N) were used to elucidate the concentrations of vitamin D metabolites such as vitamin $D_3$, $25(OH)D_3$, and $24,25(OH)_2D_3$ in plasma, liver, and adipose tissues in response to increasing dietary vitamin $D_3$ doses and assess the most suitable vitamin D metabolite in the plasma reflecting the stored vitamin D. Moreover, the increase of vitamin $D_3$ in erythrocytes to increasing doses of dietary vitamin $D_3$ was examined (**Paper 1**).

**II. How respond the vitamin D metabolites to dietary changes of vitamin D?**

The second mouse study in **Paper 1** aimed to assess the response of vitamin D metabolites in plasma and liver to changes in the amounts of dietary vitamin $D_3$. In this study, the response of different vitamin D metabolites to changes in orally administered vitamin $D_3$ was investigated.

**III. Are there tissue-specific differences in the mobilization of vitamin D?**

Vitamin D is mainly stored in liver and adipose tissue. However, only a few data exist, which investigated the contribution of adipose tissue to release vitamin D into the circulation. WT mice were fed a vitamin D-free diet to investigate and compare the rate of vitamin D mobilization from liver and adipose tissues (**Paper 1**).

Another subject addressed in the present thesis concerns the intestinal absorption and tissue distribution of vitamin D. As outlined in **section 1.4**, lipid transporters (i.e., NPC1L1, SR-B1, CD36, and ABC-G5/G8) may be involved in vitamin D intestinal absorption. However, most of the studies concerning the protein-mediated absorption of vitamin D were either carried out on cells or on *ex vivo* models. In this regard, the current thesis using *in vivo* models attempted to address the following question:

**IV. What is the role of NPC1L1, SR-B1, CD36, and ABC-G5/G8 transporters in the uptake, tissue distribution, and activation of vitamin D?**

In a first study, the vitamin D absorption via NPC1L1 was investigated by using ezetimibe as a specific NPC1L1 inhibitor (**Paper 2**). In this regard, WT mice were fed daily with labeled vitamin $D_3$ and ezetimibe for six weeks. The role of SR-B1, CD36, and ABC-G5/G8 transporters for vitamin D uptake, tissue distribution, and activation is largely unknown. In a next study, the role of these transporters in vitamin D metabolism was investigated by use of SR-B1, CD36, or ABC-G5/G8 knockout (KO) mice in comparison to WT mice (**Paper 3**).

# 3 Publications

## 3.1 Paper 1: Markers indicating body vitamin D stores and responses of liver and adipose tissues to changes in vitamin D intake in male mice

**Kiourtzidis M.**, Kühn J., Brandsch C., Baur A.-C., Wensch-Dorendorf M., and Stangl G.I. (2020). Markers indicating body vitamin D stores and responses of liver and adipose tissues to changes in vitamin D intake in male mice. Nutrients *12*, 1391. https://doi.org/10.3390/nu12051391.

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# Markers Indicating Body Vitamin D Stores and Responses of Liver and Adipose Tissues to Changes in Vitamin D Intake in Male Mice

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**Abstract:** Circulating 25-hydroxyvitamin D (25(OH)D) is regarded as the most reliable biomarker of vitamin D status. However, limited data exist concerning the suitability of 25(OH)D as an indicator of body vitamin D stores and the ability of adipose tissue to mobilize vitamin D. In the first study, in which male mice received different vitamin $D_3$ doses for three weeks, we found strong linear response relationships between vitamin $D_3$ intake and levels of vitamin $D_3$ in the plasma ($p < 0.001$), liver ($p < 0.001$) and adipose tissues ($p < 0.001$), and strong positive correlations between plasma and tissue stores of vitamin $D_3$ ($p < 0.001$). Plasma levels of 25(OH)$D_3$ and 24,25-dihydroxyvitamin $D_3$ (24,25(OH)$_2D_3$) showed weak or no correlations with tissue vitamin $D_3$ stores. Data from a second study demonstrate a strong and rapid response of plasma 25(OH)$D_3$ in vitamin $D_3$-treated mice with a low vitamin D status. Additionally, mice fed a vitamin D-free diet showed a strong and rapid decline in vitamin $D_3$ in the liver, whereas the decline in different adipose tissues was distinctly lower than that in the liver. To conclude, tissue stores of vitamin $D_3$ were best reflected by plasma vitamin $D_3$. In contrast to the liver, adipose tissues responded less sensitively to an absence of vitamin D intake.

**Keywords:** 25(OH)D; adipose tissue; liver; mice; vitamin D

## 1. Introduction

The research interest in vitamin D has increased significantly due to the high prevalence of vitamin D insufficiency worldwide [1] and the multiple functions of vitamin D besides mineral metabolism, e.g., in the immune system [2,3]. Thus, numerous epidemiological studies have been conducted linking vitamin D to health outcomes. The concentration of 25-hydroxyvitamin D (25(OH)D), which is analyzed in serum, plasma or dried blood spots by different measures, has widely been accepted as a good biomarker of vitamin D status in adults and infants [4,5]. The suitability of 25(OH)D as a vitamin D status biomarker is based on the finding that 25(OH)D has a long-term half-life of at least 2 weeks in plasma, thereby reflecting vitamin D stores in the body [6,7]. Additionally, it is assumed that the synthesis of 25(OH)D in the liver is not strictly regulated and depends on the available quantity of vitamin D [5]. In contrast to 25(OH)D, bioactive 1$\alpha$,25-dihydroxyvitamin D (1,25(OH)$_2$D) has been considered an inappropriate biomarker of vitamin D status because its synthesis and activity are tightly regulated by the parathyroid hormone and fibroblast growth factor 23 [8]. Both hormones ensure constant levels of plasma 1,25(OH)$_2$D for a wide range of vitamin D concentrations in the body. 24,25-Dihydroxyvitamin D (24,25(OH)$_2$D), which is formed from 25(OH)D and 1,25(OH)$_2$D by the

multicatalytic enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) [9] and destined for excretion, is usually not used as a biomarker of vitamin D status. The measurement of 24,25(OH)$_2$D, for example, has been recommended for the diagnosis of patients with mutations in CYP24A1 [10].

However, a few scientists recommend assessing the ratio of 25(OH)D to 24,25(OH)$_2$D to evaluate vitamin D status [11,12]. Currently, few data are available regarding the suitability of nonhydroxylated vitamin D as a status marker. In the *First International Conference on Controversies in Vitamin D*, the measurement of potential vitamin D biomarkers other than 25(OH)D was discussed [13]. Recently, the reliability of 25(OH)D as a biomarker of vitamin D status has been critically discussed. In 2018, Jorde and Grimnes hypothesized that nonhydroxylated vitamin D in serum could be a more useful parameter for assessing vitamin D status [14]. The authors argued that vitamin D-dependent cells possibly prefer circulating vitamin D, not 25(OH)D or 1,25(OH)$_2$D, to synthesize bioactive vitamin D metabolites for their own needs.

Additionally, data from human intervention studies indicate that the activity of the 25-hydroxylases that catalyze the synthesis of 25(OH)D from vitamin D is regulated because the increase in circulating 25(OH)D following vitamin D treatment is higher in individuals with low baseline levels of 25(OH)D than in those with high 25(OH)D levels [15,16]. Other data, which indicate that circulating levels of 25(OH)D do not necessarily reflect vitamin D stores in the body, come from mouse studies. Mice that were fed diets containing 7-dehydrocholesterol or ergosterol showed significantly increased vitamin D stores in the liver and kidney, while having unchanged serum concentrations of 25(OH)D [17,18]. Additionally, mice that received ezetimibe, an inhibitor of the sterol transporter Niemann-Pick C1-like 1 protein, had markedly reduced vitamin D stores in the liver, kidney, adipose tissues and muscle, while the circulating concentrations of 25(OH)D had increased [19].

Due to many open issues concerning the best and most sensitive biomarker reflecting tissue vitamin D stores and changes in vitamin D consumption, we conducted two mouse studies. The first study addressed response relationships between vitamin D$_3$ intake, tissue levels of vitamin D$_3$ and circulating concentrations of different vitamin D$_3$ metabolites. The second study aimed to ascertain the response of plasma and tissue concentrations of vitamin D metabolites to changes in the oral vitamin D$_3$ supply in mice with a low, adequate or high vitamin D status. Additionally, we studied the rate of mobilization of vitamin D$_3$ from the liver and different types of adipose tissues in response to an absent vitamin D intake.

## 2. Materials and Methods

### 2.1. Mouse Studies

In total, two studies using male wild-type mice (C57BL/6N; Charles River, Sulzfeld, Germany) were conducted. The experimental procedures were approved by the local ethics committee (Martin Luther University Halle-Wittenberg, Germany; approval numbers: H1-4/T1-18, H1-4/T1-19; date of approval: 17 July 2018, 1 March 2019, respectively). All experimental procedures followed the guidelines for the care and handling of laboratory animals according to the US National Research Council [20]. Mice were housed in a room controlled for temperature (22 ± 2 °C), light (12-h light, 12-h dark cycle, lamps emitting no ultraviolet irradiation) and relative humidity (50–60%) and had free access to food and water. The basal diets used in these studies consisted of (per kg): 288 g starch, 200 g sucrose, 200 g casein, 175 g coconut fat, 60 g of a vitamin and mineral mixture, 50 g cellulose, 25 g soybean oil and 2 g of DL-methionine. Except for vitamin D$_3$, vitamins and minerals were added to the diet according to the recommendation of the National Research Council [21].

The first experiment was conducted as a dose-response study to elucidate concentrations of vitamin D metabolites in plasma, the liver and adipose tissues (mesenteric, retroperitoneal and subcutaneous) in response to increasing doses of dietary vitamin D$_3$ and to assess the most suitable plasma marker indicating tissue stores of vitamin D$_3$. Therefore, 30 five-week-old mice with an initial body weight of 19.6 ± 0.6 g were randomly assigned to 10 groups ($n$ = 3), receiving 5 µg, 10 µg, 15 µg, 20 µg, 25 µg,

30 µg, 35 µg, 40 µg, 45 µg or 50 µg of vitamin $D_3$ per kg of diet for three weeks. The dietary vitamin D concentrations chosen for the study included levels below and above the presumed need for vitamin D.

The second study aimed to assess the extent and rate of changes in the plasma and tissue vitamin D metabolites in response to switching the vitamin $D_3$ supply from low to adequate and from high to adequate. Therefore, 72 five-week-old mice (initial body weight: 18.4 ± 2.0 g) were randomly assigned to 3 groups ($n$ = 24) and fed diets with either low (5 µg/kg), adequate (25 µg/kg) or high (50 µg/kg) concentrations of vitamin $D_3$ for four weeks to induce different statuses of vitamin $D_3$. Then, all mice received a diet containing 25 µg/kg of vitamin $D_3$. Thus, the study included three intervention groups: a low vitamin $D_3$ status group that received a vitamin $D_3$-adequate diet (5→25 $D_3$), a high vitamin $D_3$ status group that received a vitamin $D_3$-adequate diet (50→25 $D_3$) and a control group that was fed the vitamin $D_3$-adequate diet (25→25 $D_3$) over the whole experimental period. Six mice from each group were analyzed for plasma and liver concentrations of vitamin D metabolites at the baseline (after the 4-week treatment with 5, 25 and 50 µg/kg vitamin $D_3$, respectively) and 7, 14 and 21 days after switching the dietary vitamin $D_3$ concentrations.

Additionally, to elucidate the response of circulating vitamin D metabolites and vitamin D stores in liver and adipose tissues to an absent vitamin D supply, 24 five-week-old mice (initial body weight: 17.4 ± 1.8 g), which received a vitamin $D_3$-adequate diet (25 µg/kg) for 3 weeks, were placed on a vitamin D-free diet (0 µg/kg). Over a two-day interval, three mice each were analyzed for vitamin D metabolites in the plasma, liver, and mesenteric, retroperitoneal and subcutaneous adipose tissues over a period of 14 days.

### 2.2. *Blood and Tissue Sampling*

Prior to sampling, each mouse was food deprived for 4 h, anesthetized with diethyl ether, decapitated and exsanguinated. Blood was used for the analyses of vitamin D metabolites in whole blood, the erythrocytes and the plasma. The liver and mesenteric, retroperitoneal and subcutaneous adipose tissues were harvested, immediately snap-frozen in liquid nitrogen and subsequently stored at −80 °C until the analysis of the vitamin D metabolites.

To quantify vitamin D metabolites in the erythrocytes, blood was collected in heparinized tubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 2000 × $g$ for 10 min at 20 °C. The obtained plasma was removed and stored for further analyses. The erythrocyte fraction was washed three times with ice-cold isotonic sodium chloride solution and then centrifuged (2000× $g$, 10 min, 20 °C). To ensure that the erythrocytes contained no adherent vitamin D, the supernatant obtained after each washing step was analyzed for vitamin D metabolites. After the second washing cycle, all vitamin D metabolites in the supernatant were below the limit of quantitation (LOQ, vitamin $D_3$: 0.1 nmol/L, 25(OH)$D_3$: 0.8 nmol/L, 24,25$(OH)_2D_3$: 5.1 nmol/L).

### 2.3. *Analysis of Vitamin D Metabolites*

Vitamin $D_3$, 25(OH)$D_3$ and 24,25$(OH)_2D_3$ were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Blood and tissue samples were prepared as previously described [18,19]. A mixture of 7-fold deuterated vitamin $D_3$ (Toronto Research Chemicals Inc., Toronto, ON, Canada) and 6-fold deuterated 25(OH)$D_3$ (Chemaphor Chemical Services, Ottawa, ON, Canada) was used as an internal standard. All samples were subjected to HPLC (1260 Infinity Series, Agilent Technologies, Waldbronn, Germany) coupled to a tandem mass spectrometer (QTRAP 5500, SCIEX, Darmstadt, Germany). The HPLC conditions have been described elsewhere [19]. For quantification of vitamin $D_3$ and 25(OH)$D_3$, a Hypersil ODS C18 column (120 A, 5 µm, 150 × 2.0 $mm^2$, VDS Optilab, Berlin, Germany) was used, and for quantification of 24,25$(OH)_2D_3$, a Poroshell C18 column (120 A, 2.7 µm, 50 × 4.6 $mm^2$, Agilent Technologies) was used.

Ionization was achieved by positive electrospray, and data were recorded in multiple reaction monitoring mode with the following transitions: vitamin $D_3$ 560 > 298, 7-fold deuterated vitamin $D_3$ 567 > 298, 25(OH)$D_3$ 576 > 298, 24,25$(OH)_2D_3$ 592 > 298, and 6-fold deuterated 25(OH)$D_3$

582 > 298. Metabolites were present as adducts of 4-phenyl-1,2,4-triazoline-3,5-dione (Sigma-Aldrich, Steinheim, Germany).

The intraday precisions of the analytical method for vitamin $D_3$, 25(OH)$D_3$ and 24,25(OH)$_2$$D_3$ were 5.15%, 2.07% and 12.9%, respectively, determined in pooled plasma samples.

The circulating concentration of 1,25(OH)$_2$D was analyzed by a commercial enzyme-linked immunoassay (Immunodiagnostic Systems, Frankfurt am Main, Germany) following the procedure given by the manufacturer with modifications [17].

### 2.4. Statistical Analysis

Statistical analysis was performed using the SAS 9.4 software package (SAS Institute Inc., Cary, NC, USA). Least-squares means (LSM) were estimated using the MIXED procedure, and the differences in LSM were tested for significance. LSM were considered to be significantly different at $p < 0.05$. Regression models (M1, M1a) were fitted using PROC REG and PROC NLIN. Depending on the experimental design and data structure, one of the following models was used for the measured traits:

$$y_{ij} = a + b \cdot x_{ij} + e_{ij} \quad \text{(M1)}$$

$$y_{ij} = a + b \cdot x_{ij} + c \cdot (x_{ij} \cdot x_{ij}) + e_{ij}, \text{ for } x_{ij} < x_0 \text{ and } y_{ij} = plateau + e_{ij}, \text{ for } x_{ij} \geq x_0 \quad \text{(M1a)}$$

$$y_{ijk} = \mu + diet_i + time_j + (diet \times time)_{ij} + e_{ijk} \quad \text{(M2)}$$

$$y_{ij} = \mu + time_i + e_{ij} \quad \text{(M3)}$$

Regression models (M1, M1a) were used to model the traits of the dose-response study (Section 2.1) depending on vitamin $D_3$ doses ranging from 5 to 50 µg/kg of diet with 5 µg/kg increments and 3 mice per dose. The M1 model used a linear regression, and M1a used a curvilinear-plateau model [22]. Model M2 was used to analyze the diet and time effects, including the 2-way interactions for the second study described in Section 2.1. Model M3 was used to analyze the time effect for the last study described in Section 2.1.

The factors diet and time as well as their interaction were considered fixed effects in the models. The F-test was used to assess differences in fixed effects levels ($p < 0.05$). Finally, the Tukey adjusted *t*-test (for main effects with more than 2 levels) or a *t*-test (main effects with 2 levels and interactions) was used to assess pairwise differences ($p < 0.05$). To assess correlations between vitamin D metabolites in plasma and stores of vitamin D in tissues, Pearson correlation coefficients were calculated using the CORR procedure.

## 3. Results

### 3.1. First Mouse Study

All mice included in this study gained body weight during the 3-week treatment without showing any differences in the final body weights between the 10 groups of mice (mean body weight 23.6 ± 0.9 g, $n = 30$). First, we investigated the response of plasma vitamin $D_3$ metabolites to increasing doses of dietary vitamin $D_3$. The data show a linear increase in the plasma concentration of vitamin $D_3$ in response to feeding increasing doses of vitamin $D_3$, ranging from 5 to 50 µg/kg (Figure 1A). In contrast, plasma concentrations of 25(OH)$D_3$ and 24,25(OH)$_2$$D_3$ showed a disproportionally strong increase in the low-dose ranges of dietary vitamin $D_3$ and a plateau-like response near the presumed need for vitamin $D_3$ (Figure 1B,C).

Compared to the plasma vitamin $D_3$ responses, the vitamin $D_3$ stores in liver, mesenteric, retroperitoneal and subcutaneous adipose tissues showed a linear dose-response to increasing doses of vitamin $D_3$ (Figure 2A–D). The concentrations of 25(OH)$D_3$ and 24,25(OH)$_2$$D_3$ in all groups and tissues analyzed were below the LOQ (25(OH)$D_3$: liver: 2.5 pmol/g, mesenteric adipose tissue: 2.8 pmol/g, retroperitoneal adipose tissue: 3.3 pmol/g, subcutaneous adipose tissue: 2.8 pmol/g; 24,25(OH)$_2$$D_3$:

liver: 5.0 pmol/g, mesenteric adipose tissue: 7.6 pmol/g, retroperitoneal adipose tissue: 8.9 pmol/g, subcutaneous adipose tissue: 7.4 pmol/g).

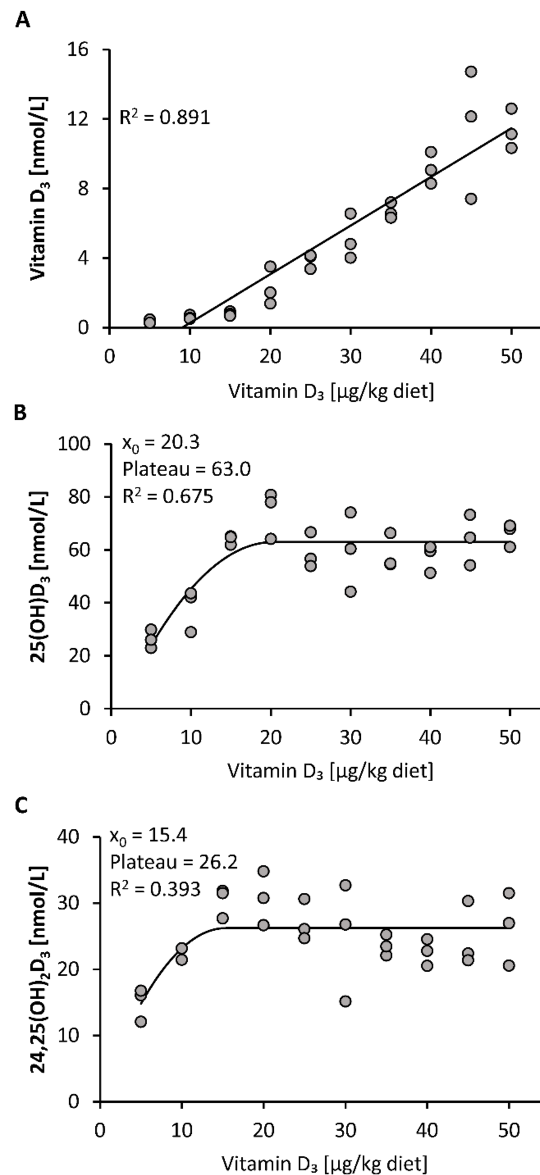


**Figure 1.** Plasma concentrations of vitamin $D_3$ (**A**), 25(OH)$D_3$ (**B**) and 24,25(OH)$_2D_3$ (**C**) in mice that were fed 10 different doses of dietary vitamin $D_3$ for three weeks ($n$ = 3). The regression line is linear for vitamin $D_3$ (**A**) and curvilinear-plateau for 25(OH)$D_3$ (**B**) and 24,25(OH)$_2D_3$ (**C**). 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin $D_3$; $R^2$, squared correlation; $x_0$, the x value at which the curve merges into the plateau.

As blood spot samples are also used for vitamin D status analysis, we compared the response of vitamin $D_3$ in whole blood and isolated blood cells to feeding different doses of dietary vitamin $D_3$. Figure 3 illustrates that the proportion of vitamin $D_3$ in the blood cells (mainly erythrocytes) was distinctly lower than that in whole blood, indicating that most of the circulating vitamin $D_3$ was allocated to plasma, and that erythrocytes may not serve as a vitamin D store. The concentrations of 25(OH)$D_3$ and 24,25(OH)$_2D_3$ in erythrocytes were below the LOQ (25(OH)$D_3$: 7.9 nmol/L; 24,25(OH)$_2D_3$: 9.6 nmol/L) in each group of mice.

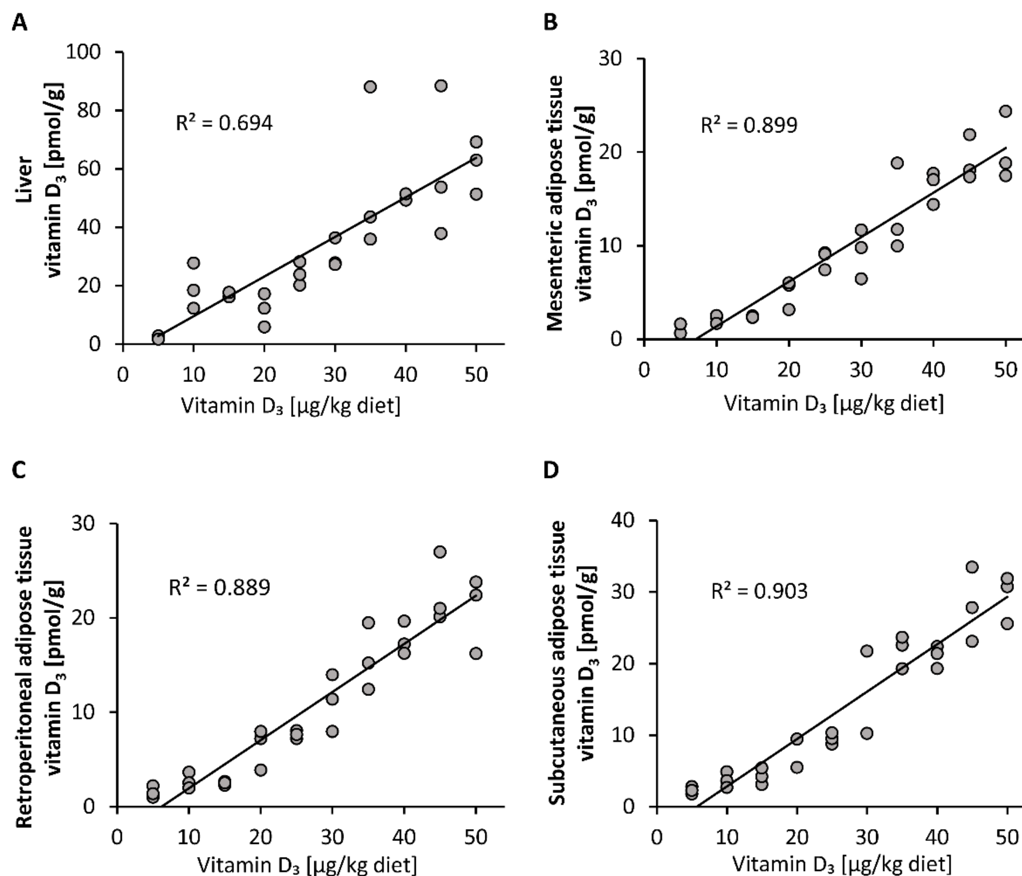


**Figure 2.** Concentrations of vitamin $D_3$ in the liver (**A**) and mesenteric (**B**), retroperitoneal (**C**) and subcutaneous (**D**) adipose tissues of mice that were fed 10 different doses of dietary vitamin $D_3$ for three weeks ($n = 3$). The regression line is linear for vitamin $D_3$ in the analyzed tissues. $R^2$, squared correlation.

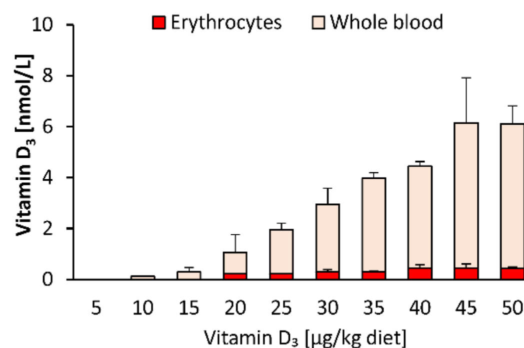


**Figure 3.** Concentrations of vitamin $D_3$ in erythrocytes and whole blood of mice that were fed 10 different doses of dietary vitamin $D_3$ for three weeks ($n = 3$). Data are presented as the means ± SD.

To elucidate the most suitable plasma vitamin D metabolite that may serve as an indicator of tissue vitamin D stores, we looked for correlations between circulating levels of different vitamin $D_3$ metabolites and tissue stores of vitamin $D_3$. Analyses revealed that levels of plasma vitamin $D_3$ showed a strong correlation with vitamin $D_3$ stores in liver, mesenteric, retroperitoneal and subcutaneous adipose tissues ($p < 0.001$, Figure 4A,D,G,J). However, the plasma concentration of 25(OH)$D_3$ showed only weak correlations with vitamin $D_3$ stores in liver, retroperitoneal and subcutaneous adipose tissues ($p < 0.05$, Figure 4B,H,K) and no correlation with vitamin $D_3$ in mesenteric adipose tissue ($p > 0.05$, Figure 4E). Plasma levels of 24,25(OH)$_2$$D_3$ did not correlate with vitamin $D_3$ stores in any tissues analyzed ($p > 0.05$, Figure 4C,F,I,L).

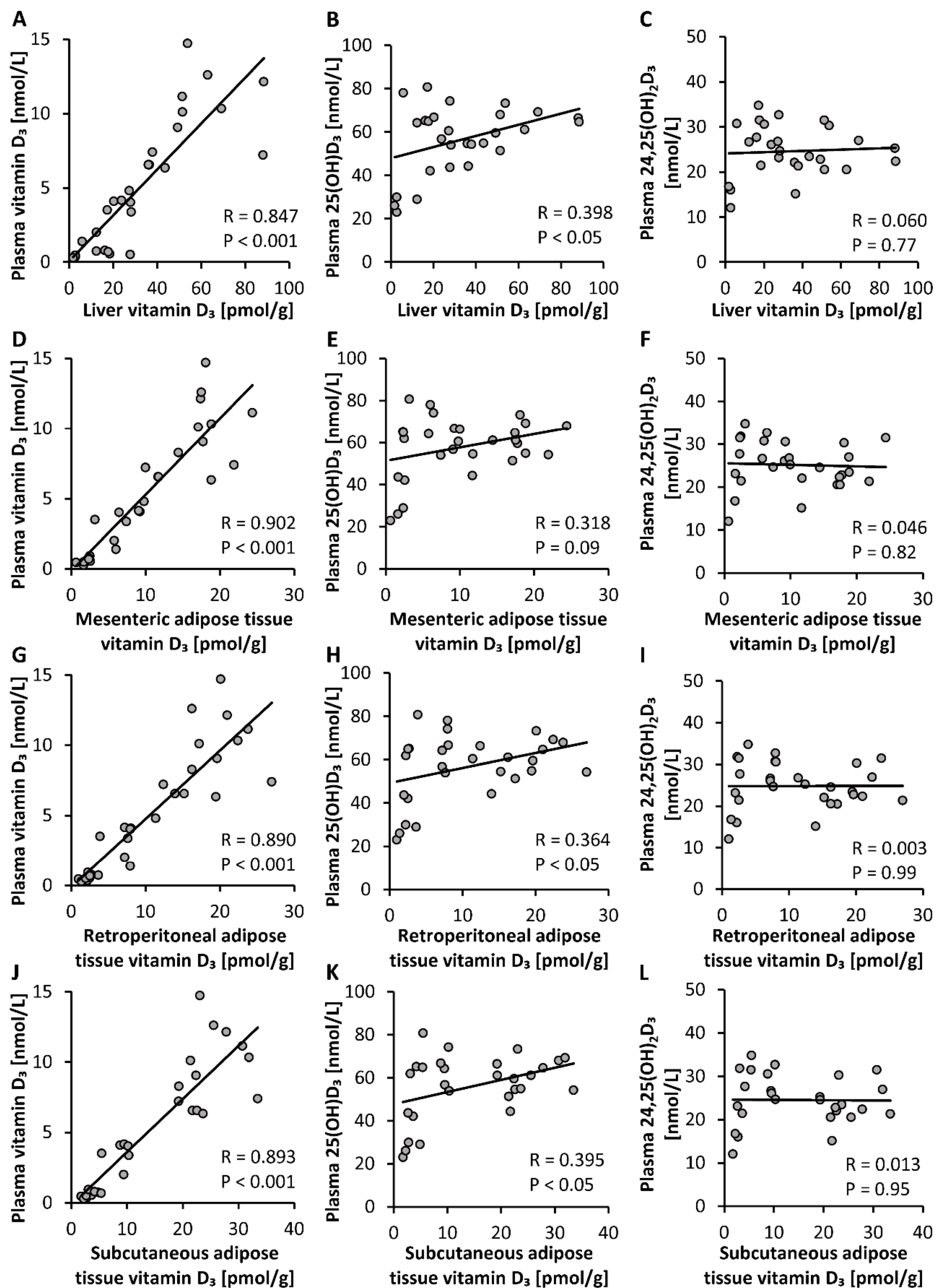


**Figure 4.** Correlations between concentrations of plasma vitamin $D_3$ metabolites and vitamin $D_3$ stores in the liver (**A**–**C**) and mesenteric (**D**–**F**), retroperitoneal (**G**–**I**) and subcutaneous (**J**–**L**) adipose tissues of mice that were fed diets containing 5 µg, 10 µg, 15 µg, 20 µg, 25 µg, 30 µg, 35 µg, 40 µg, 45 µg or 50 µg vitamin $D_3$ per kg of diet for three weeks ($n = 3$). 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin $D_3$; R, correlation coefficient.

### 3.2. Second Mouse Study

First, this study aimed to elucidate the response of vitamin D metabolites in plasma to adequate amounts of dietary vitamin $D_3$ in mice having a low, adequate or high vitamin D status that was induced by a 4-week treatment with diets containing 5 µg/kg (low), 25 µg/kg (adequate) and 50 µg/kg (high) of vitamin $D_3$. The final body weights of the mice were not affected by the different treatments (group 5→25 $D_3$: 28.6 ± 2.3 g, group 25→25 $D_3$: 30.3 ± 1.7 g, group 50→25 $D_3$: 27.0 ± 2.1 g). Figure 5 illustrates that the plasma concentrations of 25(OH)$D_3$ and 24,25(OH)$_2D_3$ markedly and rapidly increased in mice with a low vitamin D status that were treated with an adequate dose of vitamin $D_3$ (Figure 5B,D), while the concentration of vitamin $D_3$ in the plasma and liver showed a moderate increase (Figure 5A,E). In contrast, the level of vitamin $D_3$ in the plasma and liver declined rapidly and strongly when mice with a high vitamin D status were fed an adequate dose of vitamin $D_3$ (Figure 5A,E), whereas the plasma concentration of 25(OH)$D_3$ slightly increased (Figure 5B) and that of 24,25(OH)$_2D_3$ remained unchanged (Figure 5D). The concentration of plasma 1,25(OH)$_2$D did not show significant differences between the treatments (Figure 5C). The vitamin D metabolites in the plasma and liver of control mice with an adequate vitamin D status that received an adequate dose of vitamin $D_3$ over the experimental period remained largely unaltered (Figure 5A–E).

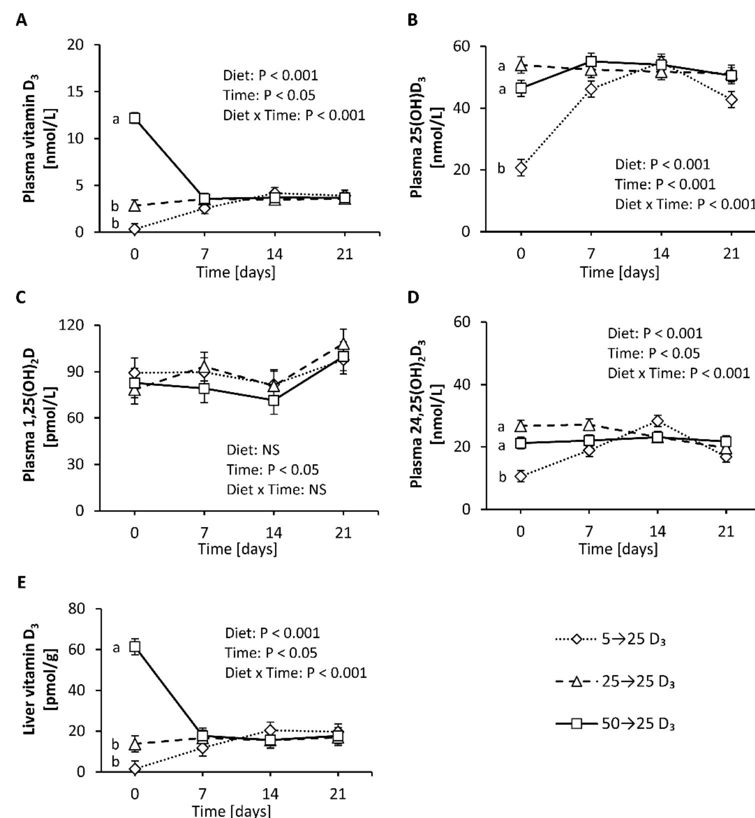


**Figure 5.** Concentrations of vitamin $D_3$ (**A**), 25(OH)$D_3$ (**B**), 1,25(OH)$_2$D (**C**) and 24,25(OH)$_2D_3$ (**D**) in plasma and vitamin $D_3$ in the liver (**E**) of mice with a low, adequate or high vitamin D status (induced by feeding diets with 5 µg/kg, 25 µg/kg, and 50 µg/kg vitamin $D_3$, respectively, for four weeks) that received diets with 25 µg/kg vitamin $D_3$ for three weeks. Six mice per group were used for the analysis of plasma and liver vitamin D metabolites at baseline (day 0) and 7, 14 and 21 days each. Data are presented as least-squares means (LSM) ± standard error of LSM ($n$ = 6). Different letters indicate significant differences between the groups at a given time point. 5→25 $D_3$, low vitamin $D_3$ status group that received a vitamin $D_3$-adequate diet; 50→25 $D_3$, high vitamin $D_3$ status group that received a vitamin $D_3$-adequate diet; 25→25 $D_3$, control group that was fed a vitamin $D_3$-adequate diet over the whole experimental period; 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; 1,25(OH)$_2$D, 1α,25-dihydroxyvitamin D; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin $D_3$; NS, not significant.

Finally, we analyzed the rate of decline in circulating and tissue levels of vitamin $D_3$ over two-day intervals in mice with an adequate vitamin D status that were placed on a vitamin D-free diet. The findings demonstrate that the level of vitamin $D_3$ in the plasma and liver declined strongly and rapidly within 2 to 4 days after feeding the vitamin D-free diet (Figure 6A,C), while the plasma concentration of 25(OH)$D_3$ showed a more moderate decline after subjecting mice to a vitamin D-free diet (Figure 6B). In contrast, when looking at the vitamin $D_3$ levels in the adipose tissues, we found small and more linear reductions over time in the mesenteric, retroperitoneal and subcutaneous adipose tissues (Figure 6D–F).

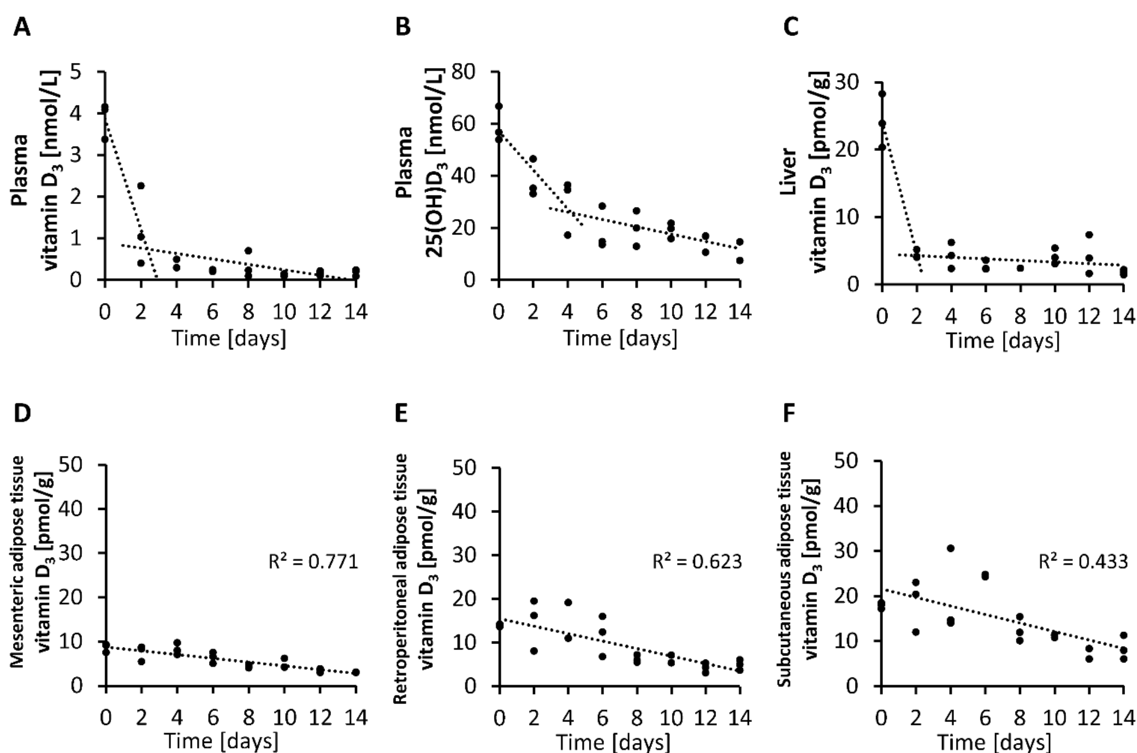


**Figure 6.** Concentrations of vitamin $D_3$ (**A**) and 25(OH)$D_3$ (**B**) in plasma and vitamin $D_3$ in the liver (**C**) and mesenteric (**D**), retroperitoneal (**E**) and subcutaneous (**F**) adipose tissues measured over two-day intervals from mice that were placed on a vitamin D-free diet (0 µg/kg). Day 0 represents the baseline levels of mice that were fed 25 µg/kg vitamin $D_3$ with their diet ($n$ = 3). 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; $R^2$, squared correlation.

## 4. Discussion

It is a consensus that circulating 25(OH)D is the most representative measure for vitamin D status [23–25]. 25(OH)D is primarily used for the diagnosis of vitamin D deficiency and to evaluate associations between vitamin D status and disease morbidity and mortality in epidemiological studies. The question, however, arose whether circulating 25(OH)D may also reflect the quantity of stored vitamin D. This is important with respect to the fact that stored vitamin D, particularly in the liver and adipose tissue, has been suggested to serve as an important source of vitamin D that can be mobilized in times of absent endogenous vitamin D synthesis or low vitamin D intake to counteract vitamin D insufficiency [26,27]. The contribution of stored vitamin D to improving vitamin D status became evident in a prospective, double-blind cohort study that was published in 2017 [28]. In this human study, large subcutaneous adipose tissue stores of vitamin D were associated with a reduced decline in serum 25(OH)D concentrations in the following year. This finding suggests the importance of stored vitamin D in reducing the risk of vitamin D insufficiency. Having a good serum biomarker that reliably indicates vitamin D stores would, therefore, be important for public health and clinical practice. As it

is difficult to assess the liver and adipose tissue stores of vitamin D in humans, we conducted two studies in mice.

The important findings of the first study were that the plasma, liver and adipose tissue concentrations of vitamin $D_3$ increased linearly with increasing doses of orally administered vitamin $D_3$, and that the circulating vitamin $D_3$ strongly correlated with the vitamin $D_3$ stores in the liver and all adipose tissues analyzed (R between 0.85 and 0.90). However, current data also show that blood cells, in particular erythrocytes, may not serve as important vitamin D stores. In contrast to vitamin $D_3$, circulating 25(OH)$D_3$ showed only a weak correlation (R between 0.36 and 0.40), and 24,25$(OH)_2D_3$ showed no correlation with vitamin $D_3$ in the liver and adipose tissues. Therefore, it seems that neither vitamin D metabolite is an ideal parameter reflecting tissue stores of vitamin D in the body.

The weak correlation between circulating 25(OH)$D_3$ and the tissue stores of vitamin $D_3$ is attributable to the fact that the response of 25(OH)$D_3$ to increasing doses of vitamin $D_3$ does not follow a linear relationship. Current data from a dose-relationship study in mice and several findings from human studies (e.g., [29,30]) show a disproportional increase in circulating 25(OH)D following increasing vitamin D doses. In general, dose-response curves of nutrient status markers normally follow a quadratic or curvilinear function and reach a transient plateau, or flattening of the curve, when the nutrient need is covered. Thus, the observed curvilinear-plateau response of 25(OH)$D_3$ to increasing vitamin $D_3$ doses indicates regulation and makes 25(OH)D a sensitive biomarker of low and adequate vitamin D status. Gallagher et al. assume that the curvilinear plateau of 25(OH)D is a result of an increased degradation of 25(OH)D to 24,25$(OH)_2$D [31]. However, as we also observed a plateau in 24,25$(OH)_2D_3$ despite increasing vitamin $D_3$ doses, we assume that 25(OH)D is not primarily regulated via its degradation to 24,25$(OH)_2$D but rather by hepatic synthesis. This contradicts, at least in part, the common view that the production of 25(OH)D is not significantly regulated and primarily depends on the availability of vitamin D [5]. Another interesting finding of the current study was the observation that 25(OH)$D_3$ showed no decline when switching mice from a vitamin $D_3$-rich diet to a vitamin $D_3$-adequate diet. Based on the findings that circulating levels of 25(OH)D do not linearly respond to vitamin D intake and do not necessarily reflect tissue stores of vitamin D, the significance and reliability of analyzed plasma 25(OH)D for classifying vitamin D deficiency/inadequacy is limited. The measurement of plasma vitamin D could provide additional and more reliable information concerning vitamin D uptake and the body's stores of vitamin D than 25(OH)D. Thus, plasma vitamin D could serve as a clinically relevant parameter to estimate the body's stores of vitamin D in humans. The clinical implementation of plasma vitamin D analysis could be used to assess depleted vitamin D stores or to avoid an unnecessary use of vitamin D supplements. However, it must be noted that data on human tissue stores of vitamin D and the ability of mobilization are currently insufficient. Further studies are necessary to elucidate cut-off values of vitamin D that indicate adequate or insufficient tissue storages of vitamin D.

To elucidate the suitability of liver and adipose tissues to mobilize vitamin D in times of an absent vitamin D supply, we investigated the decline in vitamin $D_3$ in the tissues of mice consuming no vitamin D. Here, we found a rapid and strong reduction in vitamin $D_3$ in the liver that corresponded to the decline in vitamin $D_3$ in plasma. However, in contrast to plasma vitamin $D_3$, 25(OH)$D_3$ showed a less rapid decline to a vitamin D-free diet, which is suggested to be a result of stimulated hepatic synthesis of 25(OH)D and possibly by the longer half-life of 25(OH)D compared to vitamin D [6,7]. In contrast to that in the liver, vitamin $D_3$ in adipose tissues showed a weak and continuous reduction after the consumption of a vitamin D-free diet. The data suggest that adipose tissues can principally release vitamin D, but in contrast to the liver, this mobilization is lower and less rapid. This is an interesting finding because the vitamin $D_3$ levels in the adipose tissues increased to a similar extent as the vitamin $D_3$ levels in the liver in response to the feeding of increasing doses of vitamin $D_3$.

The observation that adipose tissues may contribute to maintaining circulating vitamin D and 25(OH)D to only a minor extent was also shown in a study conducted with obese patients who underwent a gastric bypass [32]. The authors of this study concluded that vitamin D in adipose tissue

does not significantly contribute to circulating 25(OH)D, although the individuals included in that study showed a marked loss of body fat [32].

The role of adipose tissues in vitamin D metabolism is a fundamental question because of the high prevalence of overweight and obesity worldwide [33]. In the first study, we observed a linear increase in vitamin $D_3$ in the liver and adipose tissues with increasing doses of dietary vitamin $D_3$. Interestingly, the storage of vitamin $D_3$ in adipose tissues has already been started when feeding low doses of vitamin $D_3$ that are noticeably below the requirement. If vitamin D is trapped in adipose tissue and not released, if necessary, the question will arise as to why adipose tissue has this great potential to store vitamin D, although it appears not to take part significantly in combating vitamin D deficiency. However, the current data assume that vitamin D in adipose tissues is not completely trapped and can be released, although to a significantly lesser extent and much more slowly than vitamin D in the liver. It is possible that the decline in vitamin D in adipose tissues is merely a consequence of adipose tissue turnover associated with the release of vitamin D that can be used for 25(OH)D synthesis.

To conclude, vitamin D status is currently assessed by the measurement of only one vitamin D metabolite: 25(OH)D. Here, we found that tissue stores of vitamin $D_3$ were best reflected by circulating vitamin D, not by 25(OH)D. Importantly, we observed that adipose tissues can release vitamin D as their vitamin D concentrations decrease continuously in cases of an absent vitamin D consumption, but in contrast to the liver this mobilization was comparatively very small. The measurement of plasma vitamin D can be a valuable tool to estimate the body's vitamin D store, and help to prevent a pending decline in vitamin D status or to avoid an unnecessary use of vitamin D supplements.

**Author Contributions:** Conceptualization, M.K., J.K., C.B., A.-C.B. and G.I.S.; Formal Analysis, M.W.-D.; Investigation, M.K. and C.B.; Writing—Original Draft Preparation, M.K., J.K. and G.I.S.; Writing—Review and Editing, M.K., J.K., C.B. and G.I.S.; Visualization, M.K. and C.B.; Supervision, G.I.S.; Project Administration, G.I.S.; Funding Acquisition, G.I.S. All authors have read and agreed to the published version of the manuscript.

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## Abbreviations

| | |
|---|---|
| 25(OH)D | 25-hydroxyvitamin D |
| $1,25(OH)_2D$ | $1\alpha$,25-dihydroxyvitamin D |
| $24,25(OH)_2D$ | 24,25-dihydroxyvitamin D |
| CYP24A1 | 25-hydroxyvitamin D-24-hydroxylase |
| LOQ | limit of quantitation |
| LSM | least-squares means |

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## 3.2 Paper 2: Inhibition of Niemann-Pick C1-like protein 1 by ezetimibe reduces uptake of deuterium-labeled vitamin D in mice

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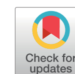
# Inhibition of Niemann-Pick C1-like protein 1 by ezetimibe reduces uptake of deuterium-labeled vitamin D in mice



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ABSTRACT

For a long time, orally ingested vitamin D was assumed to enter the body exclusively via simple passive diffusion. Recent data from *in vitro* experiments have described Niemann-Pick C1-like protein 1 (Npc1l1) as an important sterol transporter for vitamin D absorption. However, short-term applications of ezetimibe, which inhibits Npc1l1, were not associated with reduced vitamin D uptake in animals and humans. The current study aimed to elucidate the effect of long-term inhibition of Npc1l1 by ezetimibe on the uptake and storage of orally administered triple deuterated vitamin $D_3$ (vitamin $D_3$-$d_3$).

Therefore, 30 male wild-type mice were randomly assigned into three groups and received diets with 25 µg/kg of vitamin $D_3$-$d_3$ that contained 0 (control group), 50 or 100 mg/kg ezetimibe for six weeks. Mice fed diets with 50 or 100 mg/kg ezetimibe had lower circulating levels of cholesterol than control mice (-12 %, -15 %, $P < 0.01$). In contrast, the concentrations of 7-dehydrocholesterol in serum ($P < 0.001$) and liver ($P < 0.05$) were higher in mice treated with ezetimibe than in control mice, indicating an increased sterol synthesis to compensate for cholesterol reduction. Long-term application of ezetimibe significantly reduced the concentrations of vitamin $D_3$-$d_3$ in the serum and tissues of mice. The magnitude of vitamin $D_3$ reduction was comparable between the two ezetimibe groups. In comparison to the control group, mice treated with ezetimibe had lower concentrations of deuterated vitamin $D_3$ compared with the control group in serum (62 %, $P < 0.001$), liver (79 %, $P < 0.001$), kidney (54 %, $P < 0.001$), adipose tissues (55 %, $P < 0.001$) and muscle (41 %, $P < 0.001$). Surprisingly, the serum concentration of deuterated 25-hydroxyvitamin $D_3$ was higher in the group fed 100 mg/kg ezetimibe than in the control group ($P < 0.05$). The protein expression of the vitamin D hydroxylases Cyp2r1, Cyp27a1, Cyp3a11, Cyp24a1 and Cyp2j3 in liver and Cyp27b1 and Cyp24a1 in kidney remained largely unaffected by ezetimibe. To conclude, Npc1l1 appears to be crucial for the uptake of orally ingested vitamin D because long-term inhibition of Npc1l1 by ezetimibe strongly reduced the levels of deuterium-labeled vitamin D in the body; the observed rise in deuterated 25-hydroxyvitamin $D_3$ in serum of these mice can not be explained by the expression levels of the key enzymes involved in vitamin D hydroxylation.

## 1. Introduction

Research interest in vitamin D has increased largely because vitamin D not only regulates calcium and phosphate homeostasis, and in turn bone health [1], but is also important for the immune system and inflammatory processes [2]. Although vitamin D can be synthesized endogenously in skin after sun exposure, many people worldwide are affected by vitamin D deficiency or insufficiency [3]. To meet the need for vitamin D in times of low sun exposure, vitamin D must be supplied via foods or supplements.

*Abbreviations:* $1,25(OH)_2D$, 1α,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; $25(OH)D_3$-$d_3$, triple deuterated 25-hydroxyvitamin $D_3$; 7-DHC, 7-dehydrocholesterol; ANOVA, one-way analysis of variance; Npc1l1, Niemann-Pick C1-like protein 1; PTAD, 4-phenyl-1,2,4-triazolin-3,5-dione; PTH, parathyroid hormone; vitamin $D_3$-$d_3$, triple deuterated vitamin $D_3$

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For many years, orally ingested vitamin D was assumed to enter the body exclusively via simple passive diffusion [4–6]. In 2011, Niemann-Pick C1-like protein 1 (Npc1l1) was suggested to be an important apical transporter of vitamin D in human colorectal tumor cells [7]. In that study, the cellular uptake of vitamin D was reduced by approximately 30 % when CaCo-2 cells were incubated with ezetimibe, a specific inhibitor of Npc1l1. Ezetimibe belongs to a new class of cholesterol-lowering pharmaceuticals that acts at the brush border of the small intestine and selectively impedes the uptake of cholesterol into the enterocytes by inhibiting the cholesterol transporter Npc1l1 [8]. In contrast, the same group found no effect on vitamin D absorption when mice were treated for a few hours with intraperitoneally administered ezetimibe [7]. No effect of ezetimibe on vitamin D absorption was also found by Heek et al. (2001), who treated rats with ezetimibe one hour prior to the administration of vitamin D [9]. Data from a randomized, controlled trial that included 51 young, healthy individuals who received either ezetimibe or a placebo and a single dose of 50,000 IU vitamin D showed that the circulating concentrations of 25-hydroxyvitamin D (25(OH)D), which is the primary biomarker of the vitamin D status, were comparable between the ezetimibe and the placebo group [10].

Thus, in contrast to the *in vitro* data, findings from *in vivo* studies are not indicative of an important role of Npc1l1 in vitamin D uptake. However, it must be noted that the relevant *in vivo* studies in this field used either a single dose of ezetimibe or a single dose of vitamin D and were conducted over short periods. The effect of long-term inhibition of Npc1l1 by ezetimibe on vitamin D uptake and storage is largely unknown.

In the current study, we aimed to elucidate the role of Npc1l1 in the uptake of vitamin D. Thus, mice were fed two different concentrations of ezetimibe in combination with labeled vitamin $D_3$ (triple deuterated vitamin $D_3$, vitamin $D_3$-$d_3$) over a period of six weeks. The main goals of our study were to elucidate the impact of ezetimibe on (i) the uptake and storage of orally administered vitamin $D_3$-$d_3$ in the body, (ii) the hydroxylation of vitamin $D_3$-$d_3$ to triple deuterated 25-hydroxyvitamin $D_3$ (25(OH)$D_3$-$d_3$), and (iii) the protein expression of P450 enzymes catalyzing hydroxylation of vitamin D, such as Cyp2r1, Cyp27a1, Cyp3a4, Cyp24a1, Cyp27b1 and Cyp2j3. The latter goals result from the fact that ezetimibe can inhibit Cyp3a4 [11], an enzyme that has been reported to accelerate the degradation of 25(OH)D and 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D) [12–14]. Additionally, ezetimibe has been described as a pharmaceutical that can inhibit Cyp2c8 [11]. Cyp2c8 is an enzyme that is involved in the metabolism of numerous drugs [15] and endogenous compounds, such as arachidonic acid [16] and all-*trans*-retinoic acid [17]. Thus, the possible effects of ezetimibe on vitamin D status can either be attributed to the inhibition of Npc1l1 or to the modulation of vitamin D hydroxylation and catabolism.

## 2. Materials and methods

### 2.1. Animals and treatments

All mice included in the study were housed individually in a room controlled for temperature (22 ± 2 °C), light (12-h light, 12-h dark cycle, lamps emitted no ultraviolet irradiation) and relative humidity (50–60 %). The experimental procedure followed the established guidelines for the care and handling of laboratory animals and was approved by the local council of Saxony-Anhalt (Landesverwaltungsamt, Halle (Saale), Germany, approval number: 42505-2-1397 MLUG).

Thirty male 6-week-old wild-type mice (C57BL/6 N; Charles River, Sulzfeld, Germany), with an initial body weight of 22.0 ± 1.7 g, were randomly assigned to three groups of 10 animals each and were fed diets with 25 μg/kg vitamin $D_3$-$d_3$ (Sigma-Aldrich, Steinheim, Germany) that contained 0 (control group), 50 or 100 mg/kg ezetimibe (Cayman Chemical, Ann Arbor, USA) [18] for six weeks. The use of labeled vitamin $D_3$ allows an estimation of the amount of vitamin D that entered the body. The diet concentration of vitamin $D_3$-$d_3$ corresponds to the recommended amount of vitamin D for growing mice [19]. To avoid differences in food intake and in consequent differences in the intake of ezetimibe and vitamin $D_3$-$d_3$, each mouse received 2 g diet per day, which corresponded to a 20 % reduction of the *ad libitum* food intake. The basal diet contained (per kg) 288 g starch, 200 g sucrose, 200 g casein, 175 g coconut oil, 60 g of a vitamin and mineral mixture, 50 g cellulose, 25 g soybean oil, and 2 g of DL-methionine. Vitamins and minerals were added to the diet according to the recommendation of the National Research Council [19]. Vitamin D was added to the diet in the form of vitamin $D_3$-$d_3$. All mice had free access to water.

### 2.2. Sample collection

After six weeks of treatment, mice were food deprived for 4 h, anaesthetized with diethyl ether and decapitated. Blood was collected into microtubes (Serum Z, Sarstedt, Nümbrecht, Germany) to obtain serum for analyses of cholesterol and vitamin D metabolites. Liver, kidney, heart, subcutaneous and retroperitoneal adipose tissues, gastrocnemius muscle and the rest of the body (without the intestinal adipose tissue, brain, testicles, intestine and the tissues mentioned above) were harvested to analyze concentrations of labeled vitamin D metabolites. Liver and kidney were additionally used for protein expression analysis of hydroxylation enzymes. The rest of the body was homogenized for quantification of labeled vitamin D metabolites. All samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C until analyses.

### 2.3. Analysis of 7-dehydrocholesterol, vitamin D and 25(OH)D

The concentrations of 7-dehydrocholesterol (7-DHC), vitamin $D_3$, vitamin $D_3$-$d_3$, 25(OH)$D_3$ and 25(OH)$D_3$-$d_3$ were measured by liquid chromatography-tandem mass spectrometry (LC–MS/MS). To quantify their concentrations in serum, tissues, and rest of the body, sixfold deuterated 7-DHC (Chemaphor Chemical Services, Ottawa, Canada), sevenfold deuterated vitamin $D_3$ (Toronto Research Chemicals Inc., Toronto, Canada), and sixfold deuterated 25(OH)$D_3$ (Chemaphor Chemical Services) were added to the samples as internal standards. Subsequently, samples were saponified with potassium hydroxide, extracted with n-hexane and washed with ultrapure water. After evaporation of the solvents, serum samples were immediately derivatized with 4-phenyl-1,2,4-triazolin-3,5-dione (PTAD, Sigma-Aldrich) [20], whereas samples of tissues and the rest of the body were solved in n-hexane/isopropanol (99/1, v/v) and fractionated by normal-phase HPLC (1100 Series, Agilent Technologies, Waldbronn, Germany) as described elsewhere [21,22]. Collected fractions of 7-DHC, vitamin $D_3$, and 25(OH)$D_3$ in tissues and the rest of the body were dried and subsequently derivatized with PTAD [20]. After derivatization, all samples were dissolved in methanol and 10 mM ammonium formate (4/1, v/v), and analyzed by HPLC (1260 Infinity Series, Agilent Technologies) coupled to an electrospray ionization tandem mass spectrometer (QTRAP 5500, SCIEX, Darmstadt, Germany).

Two different HPLC columns were used for chromatographic separation prior to mass spectrometric analyses. If not otherwise stated, the HPLC system was equipped with a Hypersil ODS C18 column (120 A, 5 μm, 150 × 2.0 $mm^2$, VDS Optilab, Berlin, Germany) and set to a column temperature of 40 °C and a flow rate of 576 μL/min. The mobile phases consisted of (A) acetonitrile and (B) a mixture of acetonitrile/water (1/1, v/v) with 5 mM ammonium formate and 0.1% formic acid. The following gradient was used: 0.0–3.1 min, 85.0% B; 4.0 min, 83.5% B; 5.0 min, 65.0% B; 8.0 min, 40.0% B; 18.0 min, 23.5% B; 20.0–23.0 min, 0.0% B; 24.0 min, 95.0% B; and 25.0–30.0 min, 85.0% B. In order to achieve an improved chromatographic separation of unlabeled vitamin $D_3$ in retroperitoneal adipose tissue, gastrocnemius muscle and the rest of the body, we used an HPLC system equipped with

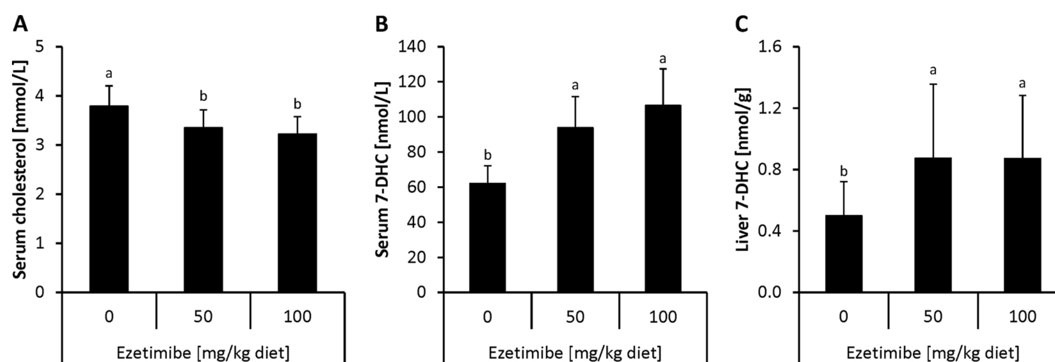



**Fig. 1.** Cholesterol concentrations in serum (A) and 7-DHC concentrations in serum (B) and liver (C) after ezetimibe treatment. Mice were fed diets with 25 µg/kg triple deuterated vitamin $D_3$ that contained 0 (control group), 50 or 100 mg/kg ezetimibe for six weeks. Data are presented as the mean ± SD, n = 10. [a, b] indicate significantly different means between groups ($P < 0.05$, multiple group comparison). 7-DHC, 7-dehydrocholesterol.

a Kinetex C18 column (100 A, 2.6 µm, 100 × 2.1 $mm^2$, phenomenex, Torrance, USA); the column temperature was set to 20 °C. The following flow rate was used: 0.0–3.5 min, 225 µL/min; 10.0–25.0 min, 200 µL/min; and 28.0–30.0 min, 225 µL/min. The mobile phases were the same as previously described. The following gradient was used: 0.0–2.1 min, 100.0% B; 3.5 min, 30.0% B; 10.0 min, 20.0% B; 13.0–25.0 min, 0.0% B; and 28.0–30.0 min, 100.0% B.

Data were recorded in positive mode by multiple reaction monitoring with the following transitions (metabolites were present as adducts of PTAD): vitamin $D_3$ 560 > 298, vitamin $D_3$-$d_3$ 563 > 301, sevenfold deuterated vitamin $D_3$ 567 > 279, 25(OH)$D_3$ 576 > 298, 25(OH)$D_3$-$d_3$ 579 > 301, sixfold deuterated 25(OH)$D_3$ 582 > 298, 7-DHC 560 > 365, and sixfold deuterated 7-DHC 566 > 371. The concentrations of 7-DHC, vitamin $D_3$, vitamin $D_3$-$d_3$, 25(OH)$D_3$ and 25(OH)$D_3$-$d_3$ in the samples were calculated on the basis of calibration curves using the peak area ratios of metabolites to internal standard compounds.

### 2.4. Analysis of serum cholesterol, 1,25(OH)$_2$D and parathyroid hormone

Cholesterol in serum samples was quantified by using an enzymatic reagent kit (Diagnostic Systems, Holzheim, Germany). The serum concentration of 1,25(OH)$_2$D was analyzed by a commercial enzyme-linked immunoassay (Immunodiagnostic Systems, Frankfurt am Main, Germany) and parathyroid hormone (PTH) was measured by a two-site enzyme-linked immunosorbent assay (Immutopics, San Clemente, USA). All analyses were performed by following the procedures given by the manufacturers.

### 2.5. Western blot analysis of hydroxylases

Protein expression of cytochrome P450 enzymes in liver (Cyp2r1, Cyp27a1, Cyp3a11, Cyp24a1 and Cyp2j3) and kidney (Cyp27b1 and Cyp24a1), which are involved in hydroxylation of vitamin D, was analyzed by western blotting. Therefore, liver and kidney samples were homogenized in buffer by using an MM 400 (Retsch, Haan, Germany). The homogenates were centrifuged, and the supernatant was used for western blotting. The protein concentration was determined by a bicinchoninic acid protein assay.

Twenty micrograms of protein lysates were separated on an SDS-PAGE gel. Afterwards, proteins were transferred onto nitrocellulose by semi-dry blotting. Actin or glyceraldehyde-3-phosphate dehydrogenase (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-Cyp2r1 (ab80101, Abcam), anti-Cyp27a1 (ab126785, Abcam), anti-Cyp24a1 (ab175976, Abcam), anti-Cyp27b1 (ab206655, Abcam), anti-Cyp2j3 (BYT-ORB459169-100, Biozol, Eching, Germany) and anti-Cyp3a11 (ab197053, Abcam). Primary antibodies were detected using HRP-conjugated secondary antibodies (anti-rabbit IgG, (#7074, New England Biolabs), anti-mouse IgG (#7076, New England Biolabs) or anti-biotin IgG (#7075, New England Biolabs) using ECL Prime western blotting detection reagent (GE Healthcare, Munich, Germany). The density of each specific band was measured using a computer-assisted imaging analysis system (G:BOX, Gene Tools, Syngene, Cambridge, UK).

### 2.6. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS version 22.0 (IBM, Armonk, USA). All data were subjected to the Shapiro-Wilk normality test. For normally distributed parameters, the three study groups were compared by one-way analysis of variance (ANOVA). When ANOVA revealed significant differences, data with equally distributed variances were compared with the Tukey-HSD post hoc test. The Games-Howell test was used for data with unequally distributed variances. In cases of non-normally distributed parameters, data were subjected to the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test revealed significant differences, groups were compared by the Mann-Whitney U test for post hoc comparisons. A Bonferroni correction for multiple testing was used. Data were considered significantly different at $P < 0.05$.

## 3. Results

### 3.1. Effect of ezetimibe on body weight, cholesterol and 7-DHC

Final body weights of mice fed 0, 50 or 100 mg ezetimibe per kg diet did not differ (0 mg/kg ezetimibe: 23.7 ± 2.1 g, 50 mg/kg ezetimibe: 23.8 ± 2.0 g, 100 mg/kg ezetimibe: 23.7 ± 1.8 g). To elucidate the effectiveness of ezetimibe in reducing cholesterol uptake, we analyzed the serum concentration of cholesterol. Here, we found that both groups of ezetimibe-treated mice had lower serum cholesterol concentrations than control mice ($P < 0.01$, Fig. 1A). The reduction in circulating cholesterol was not different between the two groups that were treated with 50 and 100 mg ezetimibe per kg diet. Analysis of 7-DHC, an endogenous precursor of cholesterol, revealed that ezetimibe-treated mice had higher concentrations of 7-DHC in serum ($P < 0.001$) and liver ($P < 0.05$) than control mice. The serum and liver concentration of 7-DHC did not differ significantly between the ezetimibe groups (Fig. 1B,C).

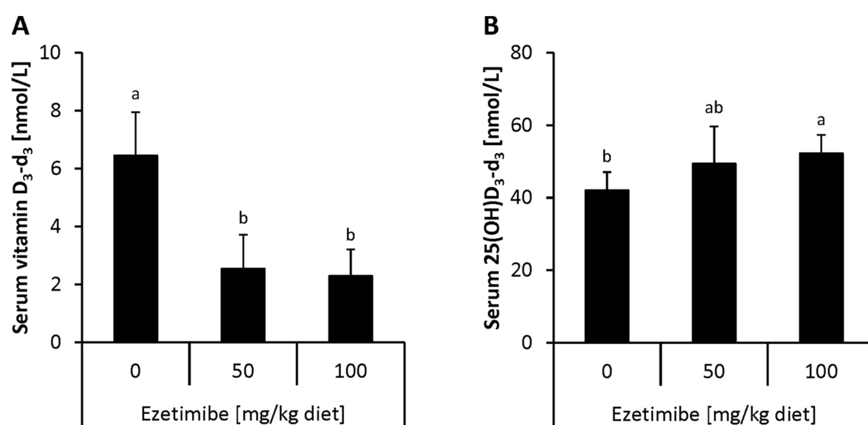



**Fig. 2.** Vitamin $D_3$-$d_3$ (A) and 25(OH)$D_3$-$d_3$ (B) concentrations in serum after ezetimibe treatment. Mice were fed diets with 25 μg/kg triple deuterated vitamin $D_3$ that contained 0 (control group), 50 or 100 mg/kg ezetimibe for six weeks. Data are presented as the mean ± SD, n = 10. [a, b] indicate significantly different means between groups (P < 0.05, multiple group comparison). 25(OH) $D_3$-$d_3$, triple deuterated 25-hydroxyvitamin $D_3$; vitamin $D_3$-$d_3$, triple deuterated vitamin $D_3$.

### 3.2. *Effect of ezetimibe on vitamin $D_3$-$d_3$, 25(OH)$D_3$-$d_3$, 1,25(OH)$_2$D and PTH in serum and tissues*

To elucidate the impact of ezetimibe on the uptake of deuterated vitamin D, we quantified the concentrations of vitamin $D_3$-$d_3$ and 25(OH)$D_3$-$d_3$ in the serum, liver, kidney, heart, subcutaneous and retroperitoneal adipose tissues, and gastrocnemius muscle and the rest of the body. Mice fed diets with 50 or 100 mg/kg ezetimibe had markedly lower concentrations of non-hydroxylated vitamin $D_3$-$d_3$ in serum (P < 0.001, Fig. 2A), liver, kidney, heart, subcutaneous and retroperitoneal fat, gastrocnemius muscle (P < 0.001, Fig. 3) and the rest of the body (P < 0.001, Fig. 4) than control mice, indicating a reduced uptake of vitamin D owing to ezetimibe treatment. The maximum reduction of vitamin $D_3$-$d_3$ was already reached in the group fed 50 mg/kg ezetimibe. Concentrations of unlabeled vitamin $D_3$ in serum, liver and heart of all mice were below the limit of quantitation (0.9 nmol/L, 0.3 pmol/g, 0.6 pmol/g, respectively). Concentrations of unlabeled vitamin $D_3$ in other tissues were extremely low and did not differ between the groups (kidney: 0 mg/kg ezetimibe: 0.4 ± 0.2 pmol/g, 50 mg/kg ezetimibe: 0.4 ± 0.3 pmol/g, 100 mg/kg ezetimibe: 0.5 ± 0.3 pmol/g, subcutaneous adipose tissue: 0 mg/kg ezetimibe: 1.6 ± 0.5 pmol/g, 50 mg/kg ezetimibe: 1.8 ± 0.8 pmol/g, 100 mg/kg ezetimibe: 1.7 ± 0.9 pmol/g, retroperitoneal adipose tissue: 0 mg/kg ezetimibe: 1.2 ± 0.3 pmol/g, 50 mg/kg ezetimibe: 1.4 ± 0.6 pmol/g, 100 mg/kg ezetimibe: 1.4 ± 0.8 pmol/g, gastrocnemius muscle: 0 mg/kg

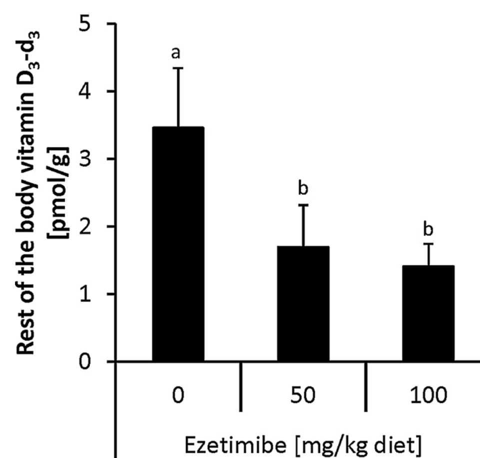



**Fig. 4.** Vitamin $D_3$-$d_3$ concentrations in the rest of the homogenized body (without the liver, kidney, heart, subcutaneous, retroperitoneal and intestinal adipose tissues, gastrocnemius muscle, brain, testicles and intestine) after ezetimibe treatment. Mice were fed diets with 25 μg/kg triple deuterated vitamin $D_3$ that contained 0 (control group), 50 or 100 mg/kg ezetimibe for six weeks. Data are presented as the mean ± SD, n = 10. [a, b] indicate significantly different means between groups (P < 0.05, multiple group comparison). Vitamin $D_3$-$d_3$, triple deuterated vitamin $D_3$.

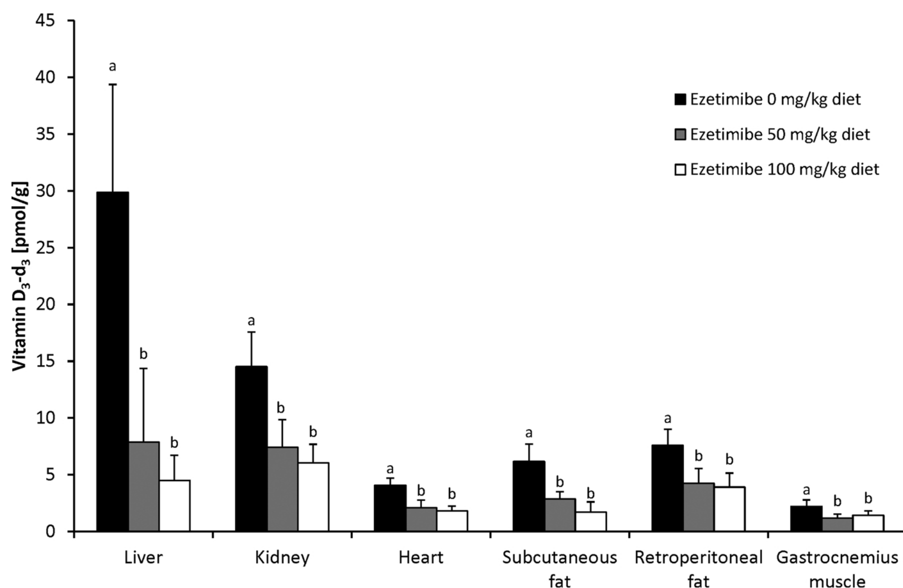



**Fig. 3.** Vitamin $D_3$-$d_3$ concentrations in tissues after ezetimibe treatment. Mice were fed diets with 25 μg/kg triple deuterated vitamin $D_3$ that contained 0 (control group), 50 or 100 mg/kg ezetimibe for six weeks. Data are presented as the mean ± SD, n = 10. [a, b] indicate significantly different means between groups (P < 0.05, multiple group comparison). Vitamin $D_3$-$d_3$, triple deuterated vitamin $D_3$.

ezetimibe: 2.4 ± 2.7 pmol/g, 50 mg/kg ezetimibe: 2.2 ± 2.6 pmol/g, 100 mg/kg ezetimibe: 3.1 ± 4.5 pmol/g, rest of the body: 0 mg/kg ezetimibe: 0.2 ± 0.1 pmol/g, 50 mg/kg ezetimibe: 0.2 ± 0.1 pmol/g, 100 mg/kg ezetimibe: 0.2 ± 0.1 pmol/g).

In contrast to vitamin $D_3$-$d_3$, the concentration of 25(OH)$D_3$-$d_3$ in serum was significantly higher in mice fed diets with 100 mg/kg ezetimibe than in the control group ($P < 0.05$, Fig. 2B). The group that received 50 mg/kg ezetimibe showed slightly increased serum concentrations of 25(OH)$D_3$-$d_3$, which were not different from those of the other groups. The concentrations of 25(OH)$D_3$-$d_3$ in the tissues and the rest of the body were below the limit of quantitation (liver: 2.4 pmol/g, kidney: 3.9 pmol/g, heart: 3.5 pmol/g, subcutaneous adipose tissue: 5.6 pmol/g, retroperitoneal adipose tissue: 3.6 pmol/g, gastrocnemius muscle: 5.7 pmol/g, rest of the body: 0.3 pmol/g) in all groups of mice. The concentrations of 25(OH)$D_3$ in serum were also below the limit of quantitation (5.3 nmol/L). The serum concentrations of 1,25(OH)$_2$D (0 mg/kg ezetimibe: 78.8 ± 20.7 pmol/L, 50 mg/kg ezetimibe: 60.0 ± 21.1 pmol/L, 100 mg/kg ezetimibe: 63.5 ± 19.2 pmol/L, P = 0.107) and PTH (0 mg/kg ezetimibe: 3.0 ± 1.1 pmol/L, 50 mg/kg ezetimibe: 3.3 ± 0.9 pmol/L, 100 mg/kg ezetimibe: 3.3 ± 0.9 pmol/L, P = 0.77) did not significantly differ between the three groups of mice.

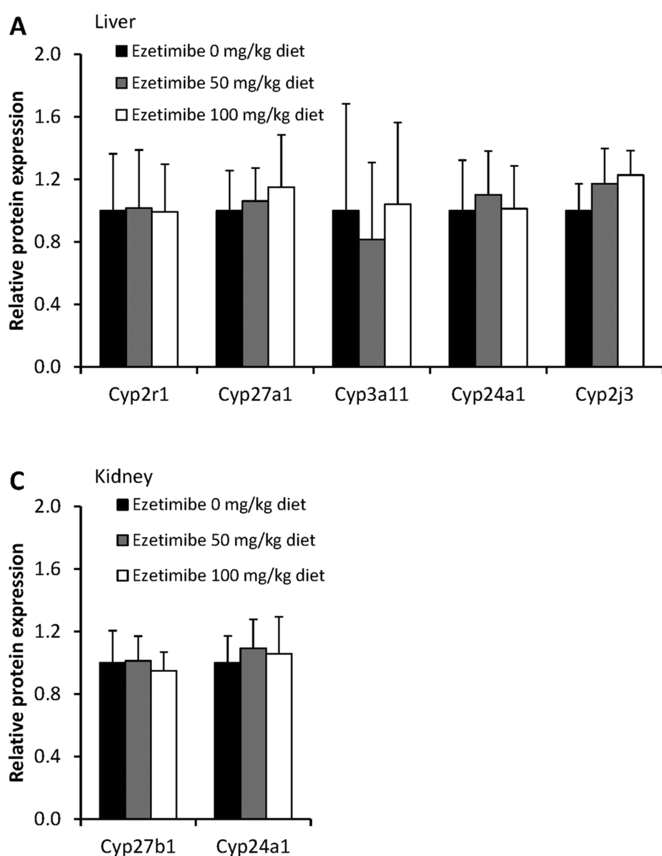
### 3.3. Effect of ezetimibe on the protein expression of hydroxylases in liver and kidney

To elucidate the impact of ezetimibe on Cyp450 enzymes, which are involved in the hydroxylation of vitamin D, we analyzed the expression levels of Cyp2r1, Cyp27a1, Cyp3a11 (the mouse homolog of the human Cyp3a4), Cyp24a1 and Cyp2j3 in livers and Cyp27b1 and Cyp24a1 in kidneys of mice by western blotting. Data show that the hepatic expression of Cyp2r1, Cyp27a1, and Cyp3a11 which are involved in the synthesis of 25(OH)D from vitamin D, did not differ between the three groups of mice (Fig. 5A,B). However, there was a tendency towards higher expression of Cyp2j3 in the livers of mice treated with ezetimibe compared to control mice (P = 0.052). The expression of hepatic and renal Cyp24a1, which catalyzes the catabolism of vitamin D, and renal Cyp27b1, was not influenced by ezetimibe treatment (Fig. 5A-D).

## 4. Discussion

The current study aimed to elucidate the role of Npc1l1 in the uptake of dietary vitamin D by using ezetimibe as a pharmaceutical inhibitor of Npc1l1. We showed that ezetimibe effectively inhibited Npc1l1 and, in turn, cholesterol absorption because serum concentrations of cholesterol in mice fed ezetimibe were lower and concentrations of 7-DHC in serum and liver were higher than in control mice. Cholesterol balance is achieved both by synthesis in the body and by absorption in the gastrointestinal tract. Furthermore, 7-DHC is an intermediate in the synthesis of cholesterol from lanosterol and is characteristic of stimulated cholesterol synthesis [23], which typically increases in cases of reduced cholesterol absorption [24]. However, current data further indicate that stimulated cholesterol synthesis could not completely compensate for a reduction in serum cholesterol.

The data demonstrated that ezetimibe-treated mice had markedly lower concentrations of orally administered deuterium-labeled vitamin $D_3$ (vitamin $D_3$-$d_3$) in all tissues analyzed. As we did not find any significant differences in the serum and tissue concentrations of vitamin $D_3$-$d_3$ between mice fed 50 and 100 mg/kg ezetimibe, it can be assumed that the maximum effect of ezetimibe to inhibit vitamin D uptake was already reached at a dose of 50 mg ezetimibe per kg diet. The lowest levels of vitamin $D_3$-$d_3$ in ezetimibe-treated mice were observed in livers (approximately -80%), followed by serum and the subcutaneous adipose tissue, which had -63% lower vitamin $D_3$-$d_3$ compared the control. The decline in the concentration of vitamin $D_3$-$d_3$ in other tissues in mice treated with ezetimibe ranged between 40% and 55%.



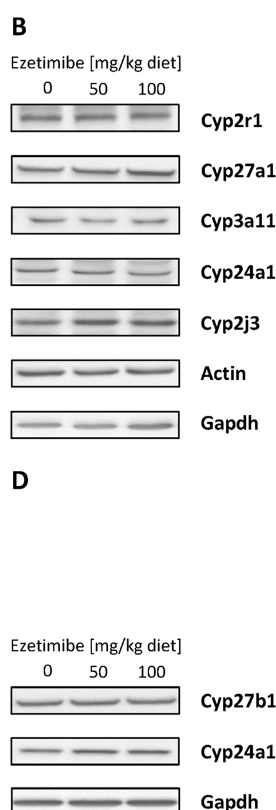




**Fig. 5.** Western blot analysis of the relative hydroxylase expression levels after ezetimibe treatment. Relative protein expression of Cyp2r1, Cyp27a1, Cyp3a11, Cyp24a1 and Cyp2j3 in liver (A) and Cyp27b1 and Cyp24a1 in kidney (C). (B, D) Representative western blot images. Mice were fed diets with 25 µg/kg triple deuterated vitamin $D_3$ that contained 0 (control group), 50 or 100 mg/kg ezetimibe for six weeks. Data are presented as the mean ± SD, n = 10.

Based on these findings, we assume that Npc1l1 plays an important role in the absorption of dietary vitamin D. Thus, our results confirmed the findings of Reboul and coworkers, who showed that Npc1l1 is important for the uptake of vitamin $D_3$ in *in vitro* models of enterocytes [7].

In addition to its function in the intestine, Npc1l1 has been reported to mediate the reuptake of cholesterol from bile to hepatocytes and to be a negative regulator of Niemann-Pick C2 [25], which is necessary for biliary cholesterol secretion [26]. Thus, Npc1l1 in the liver appears to counteract the loss of cholesterol via bile. Because the primary excretion route of vitamin $D_3$ is bile [27], it can be assumed that ezetimibe-mediated inhibition of Npc1l1 could have caused not only an inhibition of intestinal vitamin D absorption but also a reduced reuptake of vitamin D from bile into liver. Davies et al. (2005) reported significant differences in the mRNA expression of Npc1l1 between humans and mice. In humans, Npc1l1 is highly expressed in the livers, whereas in mice, Npc1l1 is predominantly expressed in the intestine [28]. Thus, it is likely that the site of action of ezetimibe on vitamin D status differs between humans and mice. Recently, published data showed that ezetimibe not only inhibits Npc1l1 but also stimulates the ATP binding cassette transporters G5/G8, which stimulate cholesterol excretion in the intestine and bile [29,30]. Therefore, ezetimibe in our study might have reduced vitamin D uptake by an additional stimulation of vitamin D excretion. Despite the low tissue levels of vitamin D in mice treated with ezetimibe, serum concentrations of $1,25(OH)_2D$, the bioactive form of vitamin D, the renal expression of Cyp27b1, which catalyzes the formation of $1,25(OH)_2D$, and PTH, a sensitive biomarker of deranged calcium homeostasis in vitamin D deficiency, were not changed by ezetimibe. Therefore, it can be concluded that mice treated for six weeks with ezetimibe did not develop functional vitamin D deficiency.

Surprisingly, we found higher concentrations of serum $25(OH)D_3$-$d_3$ in mice treated with 100 mg ezetimibe per kg diet, although the concentrations of vitamin $D_3$-$d_3$ in serum and tissues of these mice were extremely low. Generally 25(OH)D is the primary biomarker of vitamin D status in humans and mice [3,31]. However, only a few human studies that were conducted with ezetimibe have analyzed the serum concentration of 25(OH)D. A previous study that included patients suffering from primary hypercholesterolemia found no effects of ezetimibe (10 mg per day) on 25(OH)D serum levels of these patients after 12 weeks of treatment [32]. Another study with hypercholesterolemic patients showed that a daily medication composed of 10 mg simvastatin and 10 mg ezetimibe resulted in a 37% increase in serum 25(OH)D concentration [33]. However, analysis revealed that simvastatin was responsible for the increase in 25(OH)D because monotherapy with simvastatin was associated with a major increase in 25(OH)D [33]. However, both human intervention studies did not clearly indicate a role of ezetimibe in modulating vitamin D status and the concentration of 25(OH)D. To elucidate whether ezetimibe treatment can alter the expression of hydroxylases, that catalyze the formation and degradation of 25(OH)D, we analyzed Cyp2r1, Cyp27a1, Cyp3a11, Cyp2j3 and Cyp24a1 in liver and also Cyp24a1 in kidney, but found no significant differences between the three groups of mice. We observed only a marginal increase in the expression of Cyp2j3, which has been reported to have vitamin D 25-hydroxylase activity [34], in mice treated with ezetimibe. Overall, it has to be concluded that ezetimibe does not largely affect the expression of key enzymes, involved in 25(OH)D synthesis and catabolism. The results further indicate that the higher serum levels of deuterated 25(OH)D in the ezetimibe-treated mice compared to control mice were not caused by an increased expression of vitamin D hydroxylases. To ascertain if mice treated with ezetimibe had in turn lower concentrations of unlabeled 25(OH)D, we analyzed the unlabeled vitamin D metabolites in serum and the tissues of all mice. However, we found only extremely low concentrations of these metabolites in all groups of mice. A possible explanation for the finding that ezetimibe-treated mice had higher serum levels of 25(OH)D despite low concentrations of vitamin D in their tissues is an increased activity of enzymes involved in the conversion of vitamin D to 25(OH)D to prevent a drop in 25(OH)D levels. Nevertheless, we are convinced that a long-term treatment with ezetimibe will also lead to reduction of 25(OH)D and the bioactive $1,25(OH)_2D$.

## 5. Conclusions

To conclude, the data demonstrated that ezetimibe treatment largely reduces the serum and tissue concentrations of orally administered deuterium-labeled vitamin $D_3$. Based on these findings, we assumed that the cholesterol transporter Npc1l1 plays a crucial role in the uptake of dietary vitamin D. In long-term therapies with ezetimibe, patients who depend on oral vitamin D supplementation should be regularly analyzed for their vitamin D status.

## Author contributions

JK and GIS conceived and designed the mouse experiments. MK performed the mouse experiments. MK, JK, ACB and FH analyzed the data. AS performed western blot analysis. MK, AS and GIS wrote the manuscript. All authors reviewed the manuscript.

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## Declaration of competing interest

None.

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## 3.3 Paper 3: Vitamin D status of mice deficient in scavenger receptor class B type 1, cluster determinant 36 and ATP-binding cassette proteins G5/G8

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# Vitamin D Status of Mice Deficient in Scavenger Receptor Class B Type 1, Cluster Determinant 36 and ATP-Binding Cassette Proteins G5/G8

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**Abstract:** Classical lipid transporters are suggested to modulate cellular vitamin D uptake. This study investigated the vitamin D levels in serum and tissues of mice deficient in SR-B1 (Srb1$^{-/-}$), CD36 (Cd36$^{-/-}$) and ABC-G5/G8 (Abcg5/g8$^{-/-}$) and compared them with corresponding wild-type (WT) mice. All mice received triple-deuterated vitamin $D_3$ (vitamin $D_3$-$d_3$) for six weeks. All knockout mice vs. WT mice showed specific alterations in their vitamin D concentrations. Srb1$^{-/-}$ mice had higher levels of vitamin $D_3$-$d_3$ in the serum, adipose tissue, kidney and heart, whereas liver levels of vitamin $D_3$-$d_3$ remained unaffected. Additionally, Srb1$^{-/-}$ mice had lower levels of deuterated 25-hydroxyvitamin $D_3$ (25(OH)$D_3$-$d_3$) in the serum, liver and kidney compared to WT mice. In contrast, Cd36$^{-/-}$ and WT mice did not differ in the serum and tissue levels of vitamin $D_3$-$d_3$, but Cd36$^{-/-}$ vs. WT mice were characterized by lower levels of 25(OH)$D_3$-$d_3$ in the serum, liver and kidney. Finally, Abcg5/g8$^{-/-}$ mice tended to have higher levels of vitamin $D_3$-$d_3$ in the serum and liver. Major alterations in Abcg5/g8$^{-/-}$ mice were notably higher levels of 25(OH)$D_3$-$d_3$ in the serum and kidney, accompanied by a higher hepatic mRNA abundance of *Cyp27a1* hydroxylase. To conclude, the current data emphasize the significant role of lipid transporters in the uptake, tissue distribution and activation of vitamin D.

**Keywords:** ATP-binding cassette transporters G5/G8; cluster determinant 36; scavenger receptor class B type 1; vitamin D; mice

## 1. Introduction

Most vitamin D in the body is synthesized from cutaneous 7-dehydrocholesterol (7-DHC) via ultraviolet B (UVB) light exposure. Due to seasonal fluctuations in the intensity of UVB radiation or situations that limit the endogenous synthesis of vitamin D, many individuals are at high risk of developing vitamin D deficiency. Thus, several health authorities have established guidelines on vitamin D intake to prevent or treat vitamin D deficiency [1–5]. The regular intake of a vitamin D supplement is a common and efficient strategy to improve vitamin D status. However, despite the widespread use of vitamin D supplements, there is insufficient knowledge on the intestinal absorption and cellular uptake of orally ingested vitamin D. In vitro and ex vivo data from Reboul and coworkers indicate a role of cholesterol transporters in vitamin D uptake [6].

Recently, we showed that the sterol transporter Niemann-Pick C1-like protein 1 (NPC1L1) plays a crucial role in vitamin D uptake and storage because mice treated with the NPC1L1 inhibitor ezetimibe showed significantly reduced concentrations of vitamin D in the liver, adipose tissue, the kidney

and the heart [7]. However, the contribution of other lipid transporters, such as scavenger receptor class B type 1 (SR-B1), cluster determinant 36 (CD36) and ATP-binding cassette transporters G5 and G8 (ABC-G5/G8), for the uptake of ingested vitamin D are still uncertain.

A putative membrane receptor for vitamin D uptake is SR-B1. It normally functions as a high-density lipoprotein (HDL) receptor and is pivotal for the uptake of cholesterol from peripheral tissues back into the liver. However, SR-B1 is expressed not only in the liver but also in many different types of cells, such as enterocytes, macrophages, endothelial cells, and adipocytes (reviewed by Shen et al. [8]). Previous data show that SR-B1 is involved in the uptake of tocopherols and carotenoids [9–11]. Reboul et al. further demonstrated that mice overexpressing intestinal SR-B1 had markedly higher intestinal uptake of vitamin D than wild-type (WT) mice [6]. The role of SR-B1 in tissue storage and the status of vitamin D is currently not known.

A second transporter, which might be important for the uptake and tissue distribution of vitamin D, is CD36. CD36 has a significant role in lipid homeostasis, as it is involved in fatty acid uptake in enterocytes [12], cardiomyocytes [13,14], and adipocytes [14] but not in hepatocytes [14]. In 2011, Reboul et al. demonstrated that transfection of human embryonic kidney (HEK) cells with human CD36 significantly increased the uptake of vitamin D [6].

Finally, the ABC transporter G family could be relevant in vitamin D metabolism. ABC-G5 and ABC-G8 form a heterodimeric complex located in the brush-border membrane of enterocytes, the canalicular membranes of hepatocytes and the membranes of epithelial cells in the gallbladder [15]. They are responsible for the efflux of nonesterified sterols, such as plant sterols and, to a minor extent, cholesterol, from enterocytes back into the intestinal lumen (for review, see Zein et al. [16]). In the liver, ABC-G5/G8 facilitates the excretion of sterols into bile [17]. Mutations in either of these two genes causes sitosterolemia, which is characterized by increased intestinal absorption and decreased biliary secretion of plant sterols [18]. On the other hand, plant sterols can stimulate the intestinal expression of ABC-G5/G8 [19]. A possible role of ABC-G5/G8 in vitamin D absorption was described by Goncalves et al., who showed that phytosterols can impair vitamin D intestinal absorption in vitro and in mice [20]. In contrast, a meta-analysis of human trials performed in 2003 showed no changes in circulating vitamin D after the consumption of plant sterols and stanol esters [21]. Thus, the significance of ABC-G5/G8 in vitamin D metabolism remains obscure.

Because data concerning the roles of SR-B1, CD36 and ABC subtype G transporters for vitamin D uptake, tissue distribution and activation are scarce, we conducted studies with mice that were deficient in SR-B1, CD36 or ABC-G5/G8. This is also relevant as polymorphisms of these transporters and their impact on lipid metabolism have been described in humans [22–24]. To elucidate the fate of orally administered vitamin D in these mouse models, we used standardized diets with triple-deuterated vitamin $D_3$ (vitamin $D_3$-$d_3$).

## 2. Materials and Methods

### 2.1. Animals and Feeding

The experimental design of this study was planned and performed in consideration of animal welfare standards according to the US National Research Council (NRC) [25]. It was approved by the animal welfare authority of Saxony-Anhalt (Landesverwaltungsamt Halle (Saale), Germany, approval number: 42502-2-1461 MLU, date of approval: 19 April 2018). Three different knockout mouse models and two corresponding WT mouse models were included in the study. Each group consisted of 5–7 male mice. Sample size was calculated using G*Power software (version 3.1.9.6) to determine statistically significant differences in the tissue levels of vitamin $D_3$-$d_3$. Recently published data on the importance of the NPC1L1 transporter for the uptake and tissue distribution of vitamin D in mice treated with ezetimibe [7] were used as a basis for sample size calculation. Using an estimated effect size of 3,76 (Cohen's d), a significance level of 5% and a power of 0.95, a minimum of 5 mice per group was considered to be a sufficient sample size. Homozygous Srb1 knockout ($Srb1^{-/-}$)

mice were obtained by mating mice heterozygous for Srb1 generated by Rigotti et al. [26] ($Srb1^{+/-}$, B6;129S2-*Scarb1*$^{tm1Kri}$/J, #003379, The Jackson Laboratory, Bar Harbor, ME, USA). Cd36 knockout ($Cd36^{-/-}$) mice (B6.129S1-*Cd36*$^{tm1Mfe}$/J) were supplied by the Jackson Laboratory (#019006). Male WT C57BL/6J mice served as the appropriate control for $Srb1^{-/-}$ and $Cd36^{-/-}$ mice and were also purchased from the Jackson Laboratory (#000664). Abcg5/g8 double knockout ($Abcg5/g8^{-/-}$) mice (B6;129S6-Del(17Abcg5-Abcg8)1Hobb/J) were bred in our facility by mating homozygous male $Abcg5/g8^{-/-}$ mice with heterozygous females ($Abcg5/g8^{+/-}$). The breeding pairs were obtained from the Jackson Laboratory (#004670). Homozygous $Abcg5/g8^{+/+}$ mice were used as the corresponding WT control.

All mice were housed individually in Macrolon cages in a climatic room (22 ± 2 °C, 50–60% humidity), with a 12/12 h light-dark cycle. To avoid the endogenous synthesis of vitamin D, we used lamps that did not emit UV light.

Before starting the study, all mice received a diet with 25 µg (1000 IU) of vitamin $D_3$/kg for one week to standardize their vitamin D status. Subsequently, mice aged 6 to 8 weeks were fed a standardized diet containing 25 µg/kg (1000 IU/kg) vitamin $D_3$-$d_3$ (Sigma-Aldrich, Steinheim, Germany) as the only source of vitamin D for six weeks. This feeding period has been shown to be long enough to induce distinct changes in vitamin D metabolism [7]. The use of deuterated vitamin D ensures a closer tracking of ingested vitamin D and its metabolic route in the body. To ensure that all mice are supplied with the same amount of vitamin $D_3$-$d_3$, the diet was restricted to 2.4 g per day. The supplied amount of diet corresponds to 80% of the *ad libitum* food intake. Since $Srb1^{-/-}$ mice did not fully ingest their provided food, their mean food intake was 2.2 ± 0.2 g/d. The concentration of vitamin $D_3$-$d_3$ in the diet met the recommendations for vitamin D in growing mice [27]. The experimental diet (per kg) consisted of 288 g starch, 200 g sucrose, 200 g casein, 175 g coconut fat, 25 g soybean oil, 50 g cellulose, 60 g of a mineral-vitamin-mixture that contains, except vitamin D, quantities of vitamins and minerals according to the NRC [27]) and 2 g DL-methionine. All mice had free access to water.

### 2.2. Sample Collection

After six weeks of feeding the vitamin $D_3$-$d_3$-containing diets, mice were anaesthetized and decapitated after 4 h of fasting. Blood was collected in serum tubes (Sarstedt, Nümbrecht, Germany) for analyses of cholesterol, triglycerides, transaminases and vitamin D metabolites. As we recently demonstrated that liver, retroperitoneal adipose tissue, kidney and heart are the major tissues showing substantial quantities of vitamin D [7], we decided to use these tissues to analyze the concentrations of deuterium-labeled vitamin $D_3$ metabolites. The liver was additionally used for analyses of cholesterol and triglycerides. The mRNA abundance of genes involved in vitamin D hydroxylation was analyzed in the liver and kidney. After snap freezing the tissues in liquid nitrogen, they were stored together with the serum samples at −80 °C until analysis.

### 2.3. Analysis of Cholesterol and Triglycerides

Cholesterol and triglycerides were quantified in serum and liver samples with the Diagnostic Systems assay (Holzheim, Germany). Preparation of the liver samples was done as described elsewhere [28].

### 2.4. Analysis of Transaminases

As biomarkers of hepatic injury, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were measured in serum using a commercial photometric assay (Diagnostic Systems).

### 2.5. Analysis of 7-Dehydrocholesterol and Vitamin D Metabolites

The concentrations of 7-DHC, vitamin $D_3$-$d_3$, and triple-deuterated 25-hydroxyvitamin $D_3$ (25(OH)$D_3$-$d_3$) were quantified in serum and tissues by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Sample preparation and quantification of the sterols were performed

as described recently [7]. Mass transitions were (as adducts of 4-phenyl-1,2,4-triazoline-3,5-dione; Sigma-Aldrich): 7-DHC 560 > 365, 7-fold deuterated 7-DHC 567 > 372, vitamin $D_3$-$d_3$ 563 > 301, 7-fold deuterated vitamin $D_3$ 567 > 298, 25(OH)$D_3$-$d_3$ 579 > 301, and 6-fold deuterated 25(OH)$D_3$ 582 > 298. The limit of quantification, which was defined as a signal-to-noise ratio above 10, for 25(OH)$D_3$-$d_3$ was 3.0 pmol/g in adipose tissue and 3.2 pmol/g in the heart. Concentrations of the other vitamin D metabolites in serum or tissues were present in reliably measurable ranges.

The serum concentration of 1$\alpha$,25-dihydroxyvitamin D (1,25(OH)$_2$D) was analyzed by a commercial ELISA (Immunodiagnostic Systems, Frankfurt am Main, Germany). The protocol was given by the manufacturer.

### *2.6. Analysis of Relative mRNA Abundance*

The relative mRNA abundance of genes involved in vitamin D hydroxylation was analyzed in the liver and kidney using real-time RT-PCR. The complete procedure, including trizol-based extraction of total RNA, cDNA synthesis and a detailed protocol of the RT-PCR was previously described [29]. The modified ΔΔCT method of Pfaffl was used for the calculation of the relative mRNA concentration [30]. For normalization, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, XM_001473623, purchased from Eurofins Genomics, Ebersberg, Germany), ribosomal protein lateral stalk subunit P0 (*Rplp0*, NM_007475, Eurofins Genomics) and hypoxanthine phosphoribosyltransferase (*Hprt*, NM_013556.2, Sigma-Aldrich) were used as reference genes. Primer pairs for sterol 27-hydroxylase (*Cyp27a1*, NM_024264), vitamin D 25-hydroxylase (*Cyp2r1*, NM_177382) and 25-hydroxyvitamin D 1α-hydroxylase (*Cyp27b1*, NM_010009.2) were purchased from Sigma-Aldrich (www.kicqstart-primers-sigmaaldrich.com).

### *2.7. Statistical Analysis*

All data are presented as the means ± standard deviations (SD). The SPSS version 25.0 (IBM, Armonk, NY, USA) was used for statistical data treatment. Because data were normally distributed (Shapiro–Wilk test), each group of knockout mice was compared with the corresponding group of WT mice by using Student's *t*-test. $p < 0.05$ was considered significantly different.

## 3. Results

### *3.1. Body Weight, Lipids and Serum Transaminases*

The final body weights and weight gain of Cd36$^{-/-}$ mice and Abcg5/g8$^{-/-}$ mice did not differ from those of their corresponding WT mice; however, the final body weights of Srb1$^{-/-}$ mice were slightly lower than those of the corresponding WT mice, which was attributed to the moderately lower food intake in this group ($p < 0.05$; Table 1). No differences in the absolute and relative liver weights were observed between the Srb1$^{-/-}$ or Cd36$^{-/-}$ mice and their corresponding WT counterparts, whereas higher absolute and relative liver weights were observed in Abcg5/g8$^{-/-}$ mice than in the corresponding WT mice (Table 1). Serum levels of ASAT and ALAT did not differ between knockout mice and the corresponding WT mice (Table 1), assuming that the higher liver weights of Abcg5/g8$^{-/-}$ mice are not indicative of liver injury.

To elucidate the impact of SR-B1, CD36 and ABC-G5/G8 deficiency on lipid status, we analyzed serum concentrations of triglycerides, cholesterol and 7-DHC and liver levels of triglycerides and cholesterol. Srb1$^{-/-}$ mice had significantly higher concentrations of triglycerides ($p < 0.01$), cholesterol ($p < 0.001$) and 7-DHC ($p < 0.001$) in serum than WT mice, whereas the levels of triglycerides and cholesterol in the liver did not differ between the two groups of mice (Table 1).

**Table 1.** Food intake, final body weights, circulating lipids, serum transaminases and lipid levels in liver of SR-B1-, CD36- and ABC-G5/G8-deficient mice compared to the corresponding WT mice.

| | WT | $Srb1^{-/-}$ | $Cd36^{-/-}$ | WT vs. $Srb1^{-/-}$ *p* Value | WT vs. $Cd36^{-/-}$ *p* Value | WT | $Abcg5/g8^{-/-}$ | WT vs. $Abcg5/g8^{-/-}$ *p* Value |
|---|---|---|---|---|---|---|---|---|
| Food intake (g/d) | 2.4 ± 0.0 | 2.2 ± 0.2 | 2.4 ± 0.0 | 0.018 | 0.549 | 2.4 ± 0.0 | 2.4 ± 0.0 | 0.186 |
| Body weight gain (g) | 4.8 ± 1.5 | 3.1 ± 1.1 | 3.9 ± 0.9 | 0.073 | 0.264 | 3.2 ± 1.1 | 4.1 ± 1.3 | 0.287 |
| Final body weight (g) * | 26.6 ± 1.6 | 24.0 ± 1.9 | 26.8 ± 1.8 | 0.043 | 0.869 | 28.6 ± 2.6 | 28.3 ± 1.8 | 0.808 |
| **Serum** | | | | | | | | |
| Triglycerides (mmol/L) | 0.92 ± 0.06 | 1.34 ± 0.25 | 0.73 ± 0.06 | 0.005 | 0.001 | 0.88 ± 0.30 | 2.20 ± 0.38 | <0.001 |
| Cholesterol (mmol/L) | 3.67 ± 0.51 | 10.73 ± 0.99 | 4.85 ± 0.46 | <0.001 | 0.004 | 3.37 ± 1.30 | 6.02 ± 0.58 | 0.001 |
| 7-DHC (nmol/L) | 136 ± 12 | 973 ± 250 | 180 ± 25 | <0.001 | 0.005 | 174 ± 54 | 236 ± 19 | 0.041 |
| ASAT (U/L) | 222 ± 41 | 232 ± 49 | 217 ± 77 | 0.734 | 0.900 | 283 ± 30 | 226 ± 99 | 0.222 |
| ALAT (U/L) | 28.7 ± 6.8 | 40.5 ± 14.1 | 35.6 ± 9.7 | 0.120 | 0.222 | 37.8 ± 14.6 | 46.0 ± 6.3 | 0.516 |
| **Liver** | | | | | | | | |
| Absolute weight (g) | 0.91 ± 0.05 | 0.83 ± 0.08 | 1.04 ± 0.13 | 0.078 | 0.051 | 0.87 ± 0.18 | 1.23 ± 0.11 | 0.003 |
| Relative weight (g/100 g body weight) | 4.06 ± 0.41 | 3.85 ± 0.23 | 4.40 ± 0.39 | 0.339 | 0.210 | 3.16 ± 0.49 | 4.83 ± 0.46 | <0.001 |
| Triglycerides (mg/g) | 60.8 ± 12.1 | 65.2 ± 44.9 | 91.9 ± 36.7 | 0.837 | 0.102 | 140.4 ± 60.9 | 128.9 ± 41.1 | 0.730 |
| Cholesterol (mg/g) | 3.79 ± 0.54 | 3.63 ± 0.83 | 2.89 ± 0.47 | 0.737 | 0.027 | 4.76 ± 1.27 | 3.38 ± 0.85 | 0.067 |

* Body weight range of age-adjusted WT mice on standard chow: 26.9–33.0 g (data provided by the Jackson Laboratory, https://www.jax.org/strain/000664). Data are presented as the means ± SD, $n$ = 5–7. All mice were fed diets with 25 µg/kg triple-deuterated vitamin $D_3$ (vitamin $D_3$-$d_3$) for six weeks. Each group of knockout mice was compared by their corresponding wild-type (WT) mice by Student's *t* test. 7-DHC, 7-dehydrocholesterol; $Abcg5/g8^{-/-}$, ATP-binding cassette transporters G5/G8 knockout mice; ALAT, alanine transaminase; ASAT, aspartate transaminase; $Cd36^{-/-}$, cluster determinant 36 knockout mice; $Srb1^{-/-}$, scavenger receptor class B type 1 knockout mice.

In contrast, $Cd36^{-/-}$ mice were characterized by lower serum levels of triglycerides ($p = 0.001$) and higher serum levels of cholesterol ($p < 0.01$) and 7-DHC ($p < 0.01$) than WT mice. Compared to WT mice, liver levels of cholesterol were lower in $Cd36^{-/-}$ mice than in WT mice ($p < 0.05$); triglyceride levels in the livers of $Cd36^{-/-}$ mice were 1.5-fold higher than those of the WT mice, although this difference did not reach significance level (Table 1).

Finally, the serum concentrations of triglycerides, cholesterol and 7-DHC in $Abcg5/g8^{-/-}$ mice were distinctly higher than those in the corresponding WT mice ($p < 0.001$, $p = 0.001$, $p < 0.05$), whereas the liver levels of cholesterol tended to be lower in $Abcg5/g8^{-/-}$ mice than in WT mice ($p = 0.067$; Table 1). Liver triglycerides did not differ between these two groups (Table 1).

### *3.2. Concentrations of Deuterium-Labeled Vitamin $D_3$ in Serum and Tissues*

To investigate the impacts of SR-B1, CD36 and ABC-G5/G8 on vitamin D tissue distribution and serum vitamin D levels, we first quantified the concentrations of deuterium-labeled vitamin $D_3$ in the serum, liver, retroperitoneal adipose tissue, kidney and heart of $Srb1^{-/-}$, $Cd36^{-/-}$ and $Abcg5/g8^{-/-}$ mice and compared them with those of the corresponding WT mice.

Here, we found markedly higher concentrations of vitamin $D_3$-$d_3$ in the serum ($p < 0.001$; Figure 1A), retroperitoneal adipose tissue ($p < 0.001$), kidney ($p < 0.001$) and heart ($p < 0.01$) of $Srb1^{-/-}$ mice than in those of WT mice, whereas the liver concentration of vitamin $D_3$-$d_3$ did not differ between these two groups of mice (Figure 2A). In contrast, no significant differences in the vitamin $D_3$-$d_3$ concentrations in the serum (Figure 1A), liver, retroperitoneal adipose tissue, kidney or heart (Figure 2A) were observed between the $Cd36^{-/-}$ and WT mice.

$Abcg5/g8^{-/-}$ mice in comparison to WT mice tended to have higher concentrations of vitamin $D_3$-$d_3$ in the serum ($p = 0.056$; Figure 1B) and liver ($p = 0.054$; Figure 2B), whereas no differences in vitamin $D_3$-$d_3$ were found in the retroperitoneal adipose tissue, kidney or heart (Figure 2B).

### *3.3. Serum and Tissue Levels of Hydroxylated Vitamin $D_3$ Metabolites and mRNA Abundance of Hepatic and Renal Hydroxylases*

To elucidate the impacts of SR-B1, CD36 and ABC-G5/G8 deficiency on hydroxylated vitamin $D_3$ metabolites, we analyzed 25(OH)$D_3$-$d_3$ in serum and tissues and the circulating levels of 1,25$(OH)_2$D, the bioactive form of vitamin D.

In comparison to WT mice, $Srb1^{-/-}$ mice had slightly lower levels of 25(OH)$D_3$-$d_3$ in serum ($p = 0.083$; Figure 1C) and significantly lower levels of 25(OH)$D_3$-$d_3$ in the liver ($p < 0.05$) and kidney ($p = 0.010$; Figure 2C). In $Cd36^{-/-}$ mice, the 25(OH)$D_3$-$d_3$ concentrations in the serum, liver and kidney were also significantly lower than those in the WT mice ($p < 0.001$, $p < 0.05$, $p < 0.05$, respectively; Figures 1C and 2C). Finally, $Abcg5/g8^{-/-}$ mice, compared to WT mice, were characterized by higher concentrations of 25(OH)$D_3$-$d_3$ in the serum ($p < 0.01$) and kidney ($p < 0.05$) but not in the liver (Figures 1D and 2D). The serum concentrations of 1,25$(OH)_2$D were not differentially influenced by the genotype (Figure 1E,F).

To ascertain whether hepatic enzymes involved in the conversion of vitamin D to 25(OH)D were influenced by the genotype, we analyzed the mRNA abundances of *Cyp27a1* and *Cyp2r1*, which are crucial for 25(OH)D synthesis. The data show that the relative mRNA expression levels of both genes in the liver were not significantly affected by SR-B1 and CD36 because $Srb1^{-/-}$ mice and $Cd36^{-/-}$ mice did not show differences in the mRNA abundance of *Cyp27a1* and *Cyp2r1* from that of the WT mice (Figure 3A). In contrast, $Abcg5/g8^{-/-}$ mice were characterized by a moderately higher mRNA abundance of *Cyp27a1* in their livers than the corresponding WT mice ($p < 0.05$), whereas the mRNA abundance of *Cyp2r1* was not different from that of WT mice (Figure 3B). Besides hepatic hydroxylases, we analyzed the mRNA abundance of the renal *Cyp27b1*, the key enzyme for the synthesis of 1,25$(OH)_2$D. Analysis revealed that both $Srb1^{-/-}$ mice ($p < 0.001$) and $Cd36^{-/-}$ mice ($p < 0.05$) had significantly higher *Cyp27b1* mRNA abundance in their kidneys than the corresponding WT controls (Figure 3A). In contrast, mRNA abundance of renal *Cyp27b1* was not affected in $Abcg5/g8^{-/-}$ mice (Figure 3B).

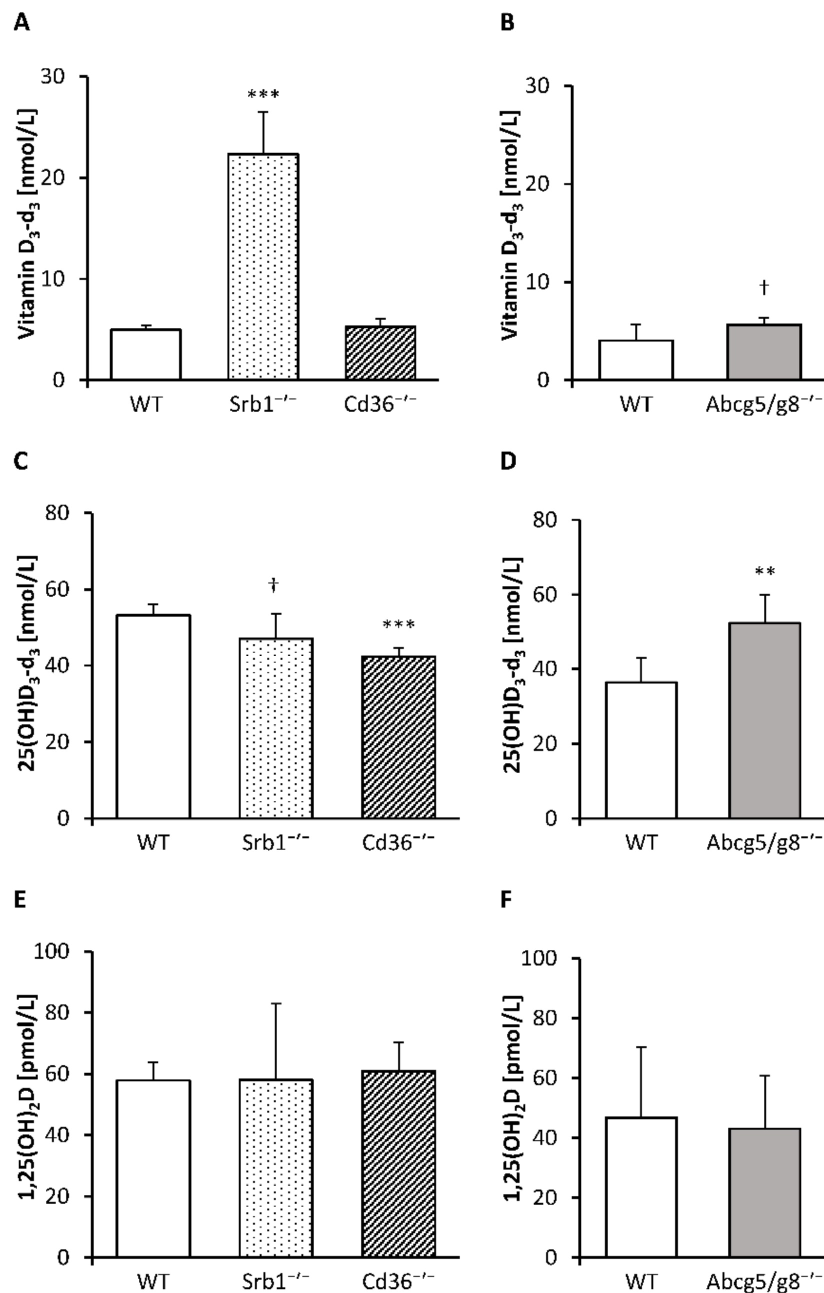


**Figure 1.** Serum concentrations of vitamin $D_3$-$d_3$ in $Srb1^{-/-}$ and $Cd36^{-/-}$ mice versus corresponding wild-type (WT) mice (**A**) and $Abcg5/g8^{-/-}$ mice versus corresponding WT mice (**B**). Serum concentrations of 25(OH)$D_3$-$d_3$ in $Srb1^{-/-}$ and $Cd36^{-/-}$ mice versus corresponding WT mice (**C**) and $Abcg5/g8^{-/-}$ mice versus corresponding WT mice (**D**). Serum concentrations of 1,25$(OH)_2$D in $Srb1^{-/-}$ and $Cd36^{-/-}$ mice versus corresponding WT mice (**E**) and $Abcg5/g8^{-/-}$ mice versus corresponding WT mice (**F**). All mice were fed diets containing 25 µg/kg triple-deuterated vitamin $D_3$ (vitamin $D_3$-$d_3$) for six weeks. Data are presented as the means ± SD, $n$ = 5–7; *** $p < 0.001$, ** $p < 0.01$, $^{\dagger}$ $p < 0.1$ (compared to the corresponding WT mice, Student's $t$ test). 1,25$(OH)_2$D, 1α,25-dihydroxyvitamin D; 25(OH)$D_3$-$d_3$, triple-deuterated 25-hydroxyvitamin $D_3$; $Abcg5/g8^{-/-}$, ATP-binding cassette transporters G5/G8 knockout mice; $Cd36^{-/-}$, cluster determinant 36 knockout mice; $Srb1^{-/-}$, scavenger receptor class B type 1 knockout mice.

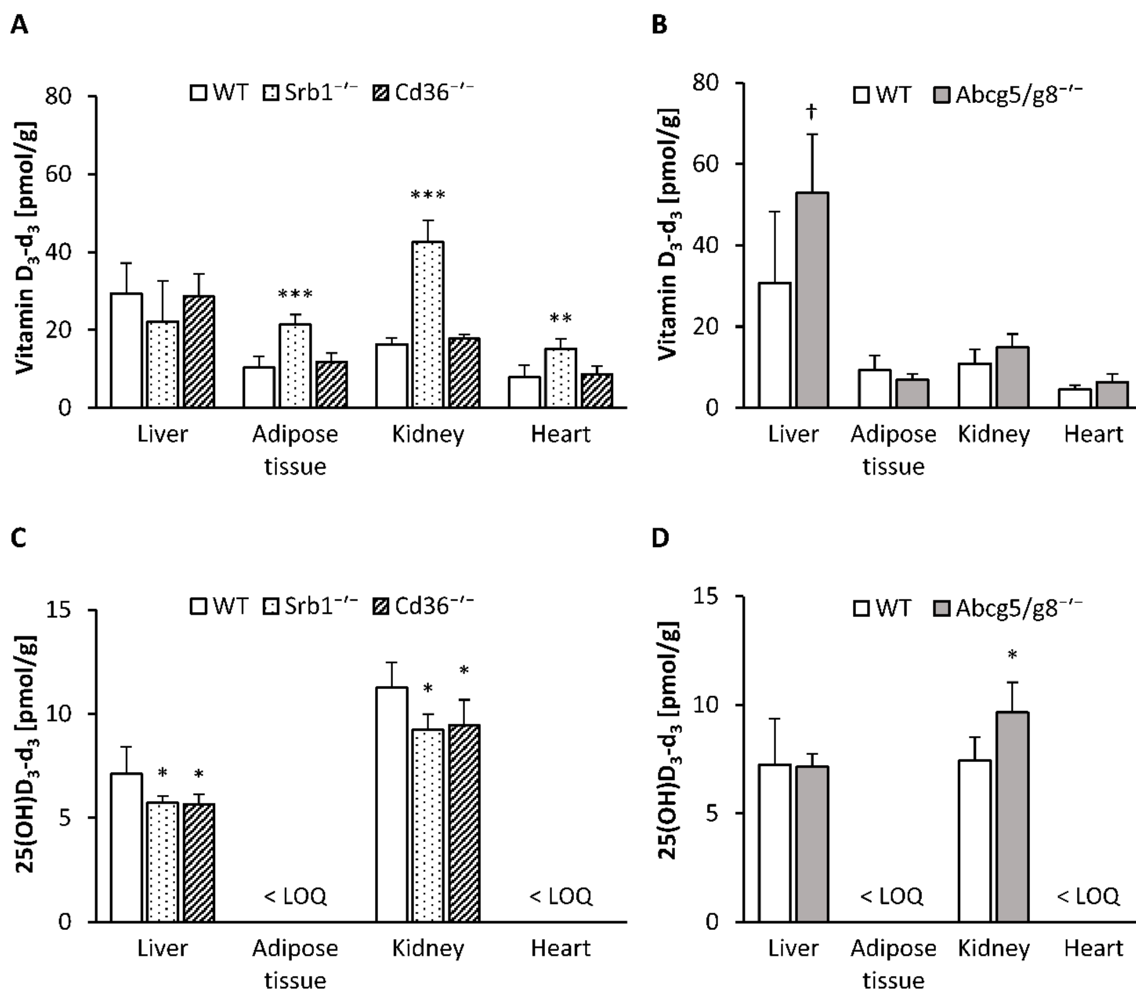


**Figure 2.** Concentrations of vitamin $D_3$-$d_3$ in the liver, retroperitoneal adipose tissue, kidney and heart of $Srb1^{-/-}$ and $Cd36^{-/-}$ mice versus corresponding wild-type (WT) mice (**A**), and $Abcg5/g8^{-/-}$ mice versus corresponding WT mice (**B**). Concentrations of 25(OH)$D_3$-$d_3$ in the liver, retroperitoneal adipose tissue, kidney and heart of $Srb1^{-/-}$ and $Cd36^{-/-}$ mice versus corresponding WT mice (**C**), and $Abcg5/g8^{-/-}$ mice versus corresponding WT mice (**D**). All mice were fed diets containing 25 µg/kg triple-deuterated vitamin $D_3$ (vitamin $D_3$-$d_3$) for six weeks. Data are presented as the means ± SD, $n$ = 5–7; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, † $p < 0.1$ (compared to the corresponding WT mice, Student's $t$ test). The limit of quantification (LOQ) of 25(OH)$D_3$-$d_3$ was 3.0 pmol/g for the retroperitoneal adipose tissue and 3.2 pmol/g for the heart. 25(OH)$D_3$-$d_3$, triple-deuterated 25-hydroxyvitamin $D_3$; $Abcg5/g8^{-/-}$, ATP-binding cassette transporters G5/G8 knockout mice; $Cd36^{-/-}$, cluster determinant 36 knockout mice; $Srb1^{-/-}$, scavenger receptor class B type 1 knockout mice.

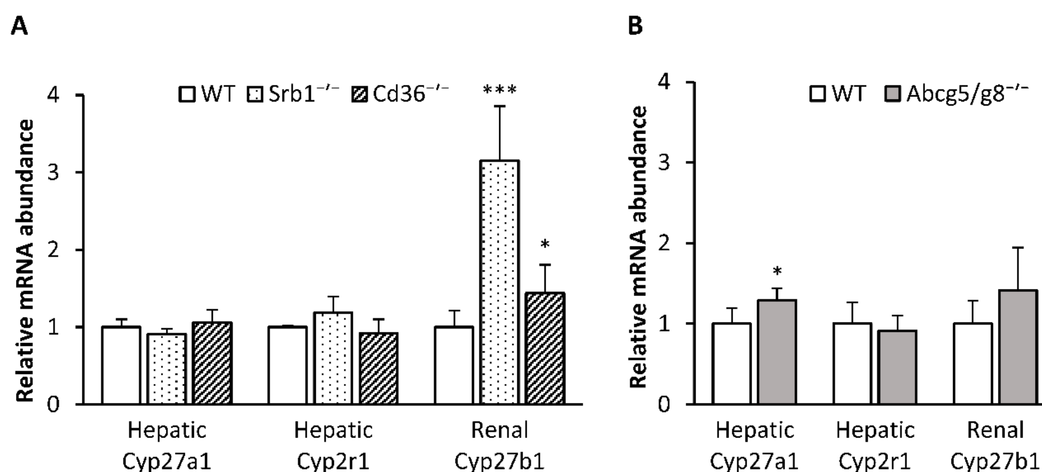


**Figure 3.** Relative mRNA abundance of hydroxylases in the liver and kidney of Srb1$^{-/-}$ and Cd36$^{-/-}$ mice versus corresponding wild-type (WT) mice (**A**), and Abcg5/g8$^{-/-}$ mice versus corresponding WT mice (**B**). All mice were fed diets containing 25 µg/kg triple-deuterated vitamin $D_3$ (vitamin $D_3$-$d_3$) for six weeks. Data are presented as the means ± SD, $n$ = 5–7; *** $p < 0.001$, * $p < 0.05$ (compared to the corresponding WT mice, Student's $t$ test). Abcg5/g8$^{-/-}$, ATP-binding cassette transporters G5/G8 knockout mice; Cd36$^{-/-}$, cluster determinant 36 knockout mice; Cyp27a1, sterol 27-hydroxylase; Cyp27b1, 25-hydroxyvitamin D 1α-hydroxylase; Cyp2r1, vitamin D 25-hydroxylase; Srb1$^{-/-}$, scavenger receptor class B type 1 knockout mice.

## 4. Discussion

The current study aimed to investigate the levels of vitamin D metabolites in tissues and serum of mice deficient in SR-B1, CD36 and ABC-G5/G8. These proteins are primarily involved in the transport of lipids. However, some data, mainly from in vitro studies, have indicated that these transporters could also play an important role in the uptake and tissue distribution of fat-soluble vitamins, including vitamin D. To our knowledge, this is the first study that investigated the impact of these transporters in mice with global SR-B1, CD36 or ABC-G5/G8 knockouts. To track the metabolic route of vitamin D given orally, we used diets with deuterium-labeled vitamin D and controlled the amount of ingested food to avoid pronounced differences in the intake of this vitamin.

The first transporter that we focused on was SR-B1. The high serum levels of lipids, in particular cholesterol, in Srb1$^{-/-}$ mice vs. WT mice fits well with the commonly known function of SR-B1 as an HDL receptor that primarily mediates the uptake of HDL lipids, in particular cholesterol, from circulation into the liver [26,31,32]. The impaired uptake of peripheral cholesterol into liver became further evident by the extremely high serum concentration of 7-DHC in the Srb1$^{-/-}$ mice, which indicates a stimulated cholesterol synthesis as a regulatory measure to counteract a decline in cellular cholesterol levels [33,34]. Main alterations in the vitamin D metabolism of Srb1$^{-/-}$ mice in comparison to the WT mice were a pronounced accumulation of vitamin $D_3$-$d_3$ (approximately fivefold) and a slight reduction of 25(OH)$D_3$-$d_3$ in serum. These findings indicate that SR-B1 is not only crucial for the hepatic uptake of HDL cholesterol but is also very important for the uptake of vitamin D from circulation into the liver to synthesize 25(OH)D, the primary biomarker of vitamin D status. We further assume a significant role of HDL for vitamin D transport, because the observed accumulation of serum vitamin $D_3$-$d_3$ in the Srb1$^{-/-}$ mice paralleled the accumulation of circulating cholesterol. The high vitamin $D_3$-$d_3$ levels in tissues other than the liver in the Srb1$^{-/-}$ mice can be attributed to the high availability of vitamin $D_3$-$d_3$ in circulation or the impaired reverse transport of peripheral vitamin D into the liver. It is important to note that the diminished uptake of vitamin D in the livers of Srb1$^{-/-}$ mice was not associated with an increase in the mRNA expression of the hepatic vitamin D hydroxylases *Cyp27a1* and *Cyp2r1*. Data indicate that the reduced availability of 25(OH)$D_3$-$d_3$ in the Srb1$^{-/-}$ mice had caused an increase in the mRNA expression of the renal *Cyp27b1* to prevent a decline in bioactive 1,25(OH)$_2$D.

To sum up, the current data indicate an important function of SR-B1 for the uptake of circulating vitamin D into liver. However, the findings were not indicative of a significant importance of SR-B1 for intestinal vitamin D absorption, which has previously been observed in the proximal intestinal fragments of mice overexpressing SR-B1 [6].

The second transporter that we analyzed was CD36. The role of CD36 in lipid metabolism is currently a subject of controversial discussion. It is suggested that CD36 has multiple functions in the lipid metabolism. Thus, the overall importance of CD36 for lipid metabolism can be best investigated by use of a global CD36 knockout model, CD36 is assumed to facilitate the cellular uptake of fatty acids [35,36], which may explain the high expression of CD36 in tissues with pronounced fatty acid metabolism, such as adipose tissue and the heart [37]. It appears to be also involved in hepatic cholesterol uptake [38] and biliary cholesterol transport [39]. The increased serum cholesterol and reduced levels of liver cholesterol which we observed in the $Cd36^{-/-}$ mice are consistent with data obtained from $Cd36^{-/-}$ mice [38] and emphasize the impaired hepatic sterol metabolism in these mice. Other studies demonstrated that CD36 is primarily necessary to form chylomicrons [40] and regulate the output of very low-density lipoproteins (VLDL) [41]. The latter function of CD36 might explain why $Cd36^{-/-}$ mice used in the current study had, on average, 51% higher triglyceride levels in their livers and 21% lower serum triglycerides than the corresponding WT mice. These findings fit well to data which showed that CD36 deletion exacerbated the steatosis in ob/ob mice by impairing the hepatic triglyceride secretion via VLDL [42]. Interestingly, we observed that $Cd36^{-/-}$ mice showed significantly lower 25(OH)$D_3$-$d_3$ levels in the serum than WT mice. It should be noted that vitamin D binding protein, which functions as a vitamin D carrier, was found to be present in VLDL [43]. Thus, we assume that the lower serum and kidney levels of 25(OH)$D_3$-$d_3$ in the $Cd36^{-/-}$ mice could have been caused by a reduced transport of 25(OH)D from liver via VLDL into the circulation. Surprisingly, we found not only lower levels of 25(OH)$D_3$-$d_3$ in serum, but also in the livers of $Cd36^{-/-}$ mice which are not indicative of an impaired transfer of this vitamin D metabolite into the circulation. Interestingly, low serum levels of 25(OH)D have also been observed in patients suffering from nonalcoholic liver diseases compared to those of healthy subjects and in mice developing fatty livers in response to a high-fat diet compared to those fed a standard chow diet [44]. Thus, we may speculate that the accumulation of liver lipids in the $Cd36^{-/-}$ mice could have hampered the 25(OH)D synthesis, although the mRNA abundance of the vitamin D hydroxylases in the livers are not indicative of an affected expression of these enzymes. Comparable to the findings in the $Srb1^{-/-}$ mice, the reduced 25(OH)D levels in the $Cd36^{-/-}$ mice were associated with an increased mRNA expression of renal *Cyp27b1* mRNA to prevent a decline in 1,25$(OH)_2$D.

Finally, we investigated the impact of ABC-G5/G8 on the levels of vitamin D metabolites in serum and tissues. The ABC-G5/G8 transporters are known to exert two common functions: the reverse intestinal transport of sterols [16] and the hepatic excretion of sterols into bile [17,45]. The significantly higher levels of serum lipids found in the $Abcg5/g8^{-/-}$ mice of the current study correspond to data on polymorphic variants of ABC-G5/G8 in humans that have been associated with elevated levels of circulating cholesterol and triglycerides [46,47]. The present findings show that $Abcg5/g8^{-/-}$ mice had higher serum and liver levels of vitamin $D_3$-$d_3$ and increased serum and renal levels of 25(OH)$D_3$-$d_3$ compared to WT mice. The improved vitamin D status of the $Abcg5/g8^{-/-}$ mice support the hypothesis that ABC-G5/G8 can also function as reverse vitamin D transporters. Thus, we speculate that the increased serum and liver levels of vitamin $D_3$-$d_3$ in $Abcg5/g8^{-/-}$ mice were attributed to a higher absorption rate and reduced excretion of vitamin $D_3$-$d_3$ into bile. We further assume that the increase in 25(OH)$D_3$-$d_3$ was caused not only by higher levels of available vitamin $D_3$-$d_3$ but also by an increase in the expression of hepatic vitamin D hydroxylases because $Abcg5/g8^{-/-}$ mice showed a higher mRNA abundance of *Cyp27a1* in their livers than WT mice. In this context, it should be mentioned that $Abcg5/g8^{-/-}$ mice had higher liver weights than WT mice, despite having unchanged liver lipids. Increased liver weights were also observed in previous studies using $Abcg5/g8^{-/-}$ mice that were fed a diet with 0.2% plant sterols [48] or $Abcg5^{-/-}$ mice that were fed a commercial chow diet [49].

The authors of the first study concluded that plant sterol accumulation in Abcg5/g8$^{-/-}$ mice could have caused toxic liver effects. Whether traces of naturally occurring phytosterols in the dietary components that were used for preparation of the experimental diets had caused the increase in the liver weights of the Abcg5/g8$^{-/-}$ mice in the current study remain uncertain, but data are not indicative of any injury in the livers of Abcg5/g8$^{-/-}$ mice as they did not show increased serum transaminases ASAT and ALAT compared to the corresponding controls.

In contrast to Srb1$^{-/-}$ and Cd36$^{-/-}$ mice, the expression of renal *Cyp27b1* was not affected in the Abcg5/g8$^{-/-}$ mice, indicating an improved vitamin D status in these animals. Based on the current findings that suggest a role of ABC-G5/G8 for reverse intestinal vitamin D transport, it should be kept in mind that any stimulation of ABC-G5/G8 transport may probably impair vitamin D status. Phytosterols are known stimulators of the intestinal expression and activity of ABC-G5/G8 [19]. The assumption that ABC-G5/G8 can deteriorate vitamin D status is corroborated by previous data that showed a reduced vitamin $D_3$ bioavailability in mice treated with phytosterols [20].

Finally, we would like to point out some limitations of this study. First, we included only male but not female mice in this study. Although sex-specific differences are, so far, not described for SR-B1 and ABC-G5/G8, data indicate that there are sex-specific differences in the expression of CD36 [50]. Thus, future studies investigating the role of CD36 for vitamin D metabolism should include males and females. A second limitation was the restriction of food intake. Although data show that all mice gained weight during the experimental period, their final body weights were slightly lower than those of age-adjusted observed from age-adjusted WT mice which were fed a standard chow diet *ad libitum*. Vitamin $D_3$-$d_3$ supplementation via oral gavage might be an option to avoid diet-related impact on lipid metabolism in these mouse models. We would also like to emphasize that the findings only apply to oral not endogenously synthesized vitamin D.

## 5. Conclusions

To conclude, the current data highlight the role of these transporters in the uptake, tissue distribution and activation of vitamin D. The data suggest that SR-B1 is important for the uptake of ingested vitamin D into the liver for the synthesis of 25(OH)D. Mice deficient in CD36 were characterized by moderately reduced 25(OH)D levels, but otherwise unchanged levels of vitamin D metabolites. It is tempting to speculate that Cd36$^{-/-}$ mice showed an impaired hepatic synthesis of 25(OH)D and transport into the circulation. Finally, the sterol exporters ABC-G5/G8 appear to be also involved in reverse vitamin D transport. This would explain the higher vitamin D concentrations in serum and tissues that we found in the Abcg5/g8$^{-/-}$ mice compared to WT mice.

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## Abbreviations

1,25$(OH)_2$D, 1α,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; 25$(OH)D_3$-$d_3$, triple-deuterated 25-hydroxyvitamin $D_3$; 7-DHC, 7-dehydrocholesterol; ABC-G5/G8, ATP-binding cassette transporters G5/G8; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CD36, cluster determinant 36; Cyp27a1, sterol 27-hydroxylase; Cyp27b1, 25-hydroxyvitamin D 1α-hydroxylase; Cyp2r1, vitamin D 25-hydroxylase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; Hprt, hypoxanthine phosphoribosyltransferase; NPC1L1, Niemann-Pick C1-like protein 1; Rplp0, ribosomal protein lateral stalk

subunit P0; SR-B1, scavenger receptor class B type 1; vitamin $D_3$-$d_3$, triple-deuterated vitamin $D_3$; VLDL, very low-density lipoproteins; WT, wild-type.

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# 4 Discussion

## 4.1 Evaluation of biomarkers indicating changes in vitamin D status

The circulating level of 25(OH)D is considered to be the most appropriate biomarker reflecting the actual vitamin D status. Individuals are normally classified as vitamin D deficient, insufficient, or sufficient on the basis of their 25(OH)D levels, although the cut-off values of 25(OH)D that define deficient, insufficient, or sufficient vitamin D status are still a controversial issue. Current data show that the concentrations of 25(OH)D in plasma do not reflect the change of a high vitamin D status to an adequate and that the circulating concentrations of vitamin D, and not 25(OH)D, best reflect the stored vitamin D in the tissues. Cross-sectional epidemiological studies demonstrated that adipose tissue is a storage reservoir for vitamin D (Blum et al. 2008; Didriksen et al. 2015). The stored vitamin D, particularly in the liver and adipose tissue, constitutes an important vitamin D source, which is suggested to be mobilized in times of low dietary vitamin D intake and absent endogenous vitamin D production (Heaney et al. 2003; Holick 2007). However, the clinical relevance of stored vitamin D in adipose tissue compared to liver is uncertain. First evidence, revealing the contribution of vitamin D stored in adipose tissue to improve vitamin D status, was demonstrated in a prospective, double-blind cohort study (Martinaityte et al. 2017). In this study, large amounts of stored vitamin D in subcutaneous adipose tissue were associated with a reduced decline in circulating levels of 25(OH)D in the following year. Therefore, stored vitamin D could be of value to prevent and counteract vitamin D insufficiency. A systematic review of randomized controlled trials corroborated that circulating 25(OH)D is a robust and reliable marker of vitamin D status (Seamans and Cashman 2009). However, all studies included in this review did not investigate the effectiveness of circulating vitamin D as a marker of vitamin D status. Mouse studies, which indicate that plasma concentrations of 25(OH)D do not necessarily reflect the stored vitamin D in the body, demonstrated that dietary 7-DHC or ergosterol significantly increased vitamin D stores in the liver and kidney, but the circulating concentrations of 25(OH)D remained unaffected (Kühn et al. 2016; Baur et al. 2019). Additionally, current studies have shown that mice treated with ezetimibe demonstrated significantly reduced vitamin D stores in the body, while the circulating 25(OH)D levels increased.

A plethora of studies that investigated the dose-response to vitamin D supplementation monitored only circulating levels of 25(OH)D (Cashman et al. 2008; Garland et al. 2011; Gallagher et al. 2012, 2013, 2014; Ng et al. 2014; Ekwaru et al. 2014; Smith et al. 2016). Data

on the dose-response relationship between vitamin D supplementation and other vitamin D metabolites, besides 25(OH)D, in plasma and tissues are scarce. **Paper 1** investigated the response of circulating vitamin $D_3$, 25(OH)$D_3$, and 24,25(OH)$_2$$D_3$ and stored vitamin $D_3$ in the liver and adipose tissue to increasing doses of dietary vitamin $D_3$. The results revealed that the levels of vitamin $D_3$ in plasma, liver, mesenteric, retroperitoneal, and subcutaneous adipose tissues increased linearly with increasing doses of dietary vitamin $D_3$. In contrast, the levels of hydroxylated vitamin $D_3$ metabolites (25(OH)$D_3$ and 24,25(OH)$_2$$D_3$) in plasma follow a curvilinear-plateau model. This finding is in agreement with the results from human studies, which also showed a disproportional increase of 25(OH)D in serum to increasing vitamin D supplementation (Cashman et al. 2008; Ekwaru et al. 2014). In response to increasing doses of dietary administered vitamin $D_3$, the circulating vitamin $D_3$ increased greater than 25(OH)$D_3$ and 24,25(OH)$_2$$D_3$ (30,5-, 2,5-, and 1,8-fold increase, respectively). Interestingly, the response of 25(OH)$D_3$ and 24,25(OH)$_2$$D_3$ to changes in supplemented vitamin $D_3$ was comparable and highly correlated (de Boer et al. 2014; Lehmann et al. 2016). Normally, dose-response curves of nutrient status markers follow a quadratic or curvilinear model and reach an impermanent plateau when the need for a nutrient is covered. Current data also found that the response of 25(OH)$D_3$ to the increasing doses of vitamin $D_3$ follows a curvilinear-plateau model and indicates a regulation of 25(OH)D. In a randomized controlled trial, which was conducted on healthy postmenopausal women with vitamin D insufficiency, it was suggested that the curvilinear-plateau of 25(OH)D is attributable to increased degradation of 25(OH)D to 24,25(OH)$_2$D (Gallagher et al. 2012). However, the current study demonstrated that the response of 24,25(OH)$_2$$D_3$ to increasing vitamin $D_3$ doses also follows a curvilinear-plateau model suggesting that 25(OH)D is regulated rather by hepatic synthesis and not by its degradation. This partly contradicts the common aspect that the synthesis of 25(OH)D primarily depends on the concentration of vitamin D and is not tightly regulated (Bhattacharyya and DeLuca 1973; Zerwekh 2008).

Another important finding in **Paper 1** was that the levels of plasma vitamin $D_3$ correlated strongly with the stored vitamin $D_3$ in the liver and the mesenteric, retroperitoneal, and subcutaneous adipose tissues. Contrary to vitamin $D_3$, the levels of 25(OH)$D_3$ in plasma were only weakly correlated with vitamin $D_3$ in the liver and the analyzed adipose tissues. Moreover, the levels of 24,25(OH)$_2$$D_3$ in plasma showed no correlation with the stored vitamin $D_3$ in the analyzed tissues. These results indicate that the circulating vitamin D, but not 25(OH)D and 24,25(OH)$_2$D, reflects the vitamin D stored in the tissues. Few human studies examined the vitamin D stores in the liver and adipose tissues because these tissues

are difficult to acquire. A cross-sectional study, which was conducted on obese individuals who were scheduled to undergo gastric bypass, demonstrated a moderately strong positive correlation between serum vitamin $D_3$ and subcutaneous adipose tissue vitamin $D_3$ (Blum et al. 2008). An observational study on obese patients who underwent gastric bypass found no significant correlation between vitamin D levels in subcutaneous adipose tissue and serum 25(OH)D (Pramyothin et al. 2011). The observed weak correlation of circulating $25(OH)D_3$ with the stored vitamin $D_3$ in tissues was attributed to the nonlinear response of $25(OH)D_3$ to increasing vitamin $D_3$ doses. Taken together, it seems that neither circulating 25(OH)D nor $24,25(OH)_2D$ could be used to evaluate the tissue stores of vitamin D and that the circulating vitamin D best reflects the vitamin D stores.

Because the response of circulating 25(OH)D to vitamin D supplementation also depends on the baseline levels of serum 25(OH)D (Didriksen et al. 2013), the second mouse study in **Paper 1** investigated the response of vitamin D metabolites in plasma and liver when changing from a high or a low vitamin D status to an adequate. Interestingly, circulating $25(OH)D_3$ did not decline when the vitamin $D_3$-rich diet of the mice was changed to a vitamin $D_3$-adequate diet. When the vitamin $D_3$-poor diet was switched to a vitamin $D_3$-adequate diet, the levels of $25(OH)D_3$ in the plasma increased. The observation that the circulating levels of 25(OH)D do not necessarily reflect the stored vitamin D in the tissues and the grade of vitamin D adequacy limit the use of circulating 25(OH)D to evaluate the actual vitamin D status. In contrast, vitamin $D_3$ in plasma declined when the vitamin $D_3$-rich diet was changed to a vitamin $D_3$-adequate diet. Thus, the measurement of circulating vitamin D could provide additional information regarding the tissue stores of vitamin D. In clinical practice, the additional analysis of circulating vitamin D could be relevant to evaluate body stores of vitamin D in humans. Circulating $24,25(OH)_2D_3$ responded similarly with $25(OH)D_3$ to changes in vitamin D status as these metabolites are strongly correlated (de Boer et al. 2014; Lehmann et al. 2016). Similarly, the response of plasma vitamin $D_3$ to changes in vitamin D status was comparable to liver vitamin $D_3$ because of their strong correlation. However, the circulating levels of $1,25(OH)_2D$ did not change to different dietary vitamin $D_3$ doses, which confirms previous data showing that $1,25(OH)_2D$ is an inappropriate marker of vitamin D status (Arya et al. 2004; Holick 2009; Herrmann et al. 2017).

In addition to plasma or serum, whole blood spot samples are also used to evaluate vitamin D status (Zakaria et al. 2020). However, data on vitamin D in erythrocytes are scare. Thus, the current study also examined the increase of vitamin $D_3$ in the whole blood and erythrocytes, respectively, to increasing doses of dietary vitamin $D_3$. Unexpectedly, only

small amounts of circulating vitamin $D_3$ were found in erythrocytes. Thus, it is assumed that erythrocytes may not serve as an important storage reservoir for vitamin D, although erythrocytes are discussed as a valuable tool to estimate vitamin E status (Sotomayor et al. 2019).

Because data on the ability of tissues to mobilize stored vitamin D are scarce, a mouse study was conducted that aimed to compare the vitamin D mobilization from liver and various adipose tissues in mice put on a vitamin D-free diet (**Paper 1**). Data demonstrated that the levels of vitamin $D_3$ in the liver declined strongly and rapidly after vitamin D depletion. The reduction of vitamin $D_3$ in the liver was associated with a strong decline of circulating vitamin $D_3$, while the reduction of plasma levels of 25(OH)$D_3$ was less pronounced. It is tempting to speculate that the released vitamin D was rapidly hydroxylated to 25(OH)D. It is further possible that the longer half-life of 25(OH)D in comparison to vitamin D (Batchelor and Compston 1983; Zerwekh 2008; Jones et al. 2014; Herrmann et al. 2017) explains the different responses of both vitamin D metabolites to vitamin D depletion. In contrast to the liver, mesenteric, retroperitoneal, and subcutaneous adipose tissues demonstrated a weak, but continuous decline in the levels of vitamin $D_3$. This finding supports the idea that adipose tissues can generally mobilize vitamin D. However, this mobilization is lower and slower when compared to that of the liver. This is consistent with the finding from a study conducted on minipigs, which were daily exposed to UV radiation (Burild et al. 2015). The authors of this study found that vitamin $D_3$ metabolites in adipose tissue declined slowly after the cessation of UV exposure, which assumes that adipose tissue may not serve as rapidly availably vitamin D storage. Similar results were found by Pramyothin and co-workers who found that the 25(OH)D levels did not alter significantly in obese patients who underwent gastric bypass and a marked body fat loss (Pramyothin et al. 2011).

To conclude, the current study showed that the circulating vitamin D better reflects the amount of stored vitamin D in the tissues than 25(OH)D, the classical biomarker of vitamin D status (Zerwekh 2008; Cashman et al. 2017). Furthermore, data illustrated that adipose tissue could release vitamin D in times of absent vitamin D consumption, but contrary to the liver, this mobilization was lower and less rapid. Thus, the data from the current thesis suggest that the analysis of circulating vitamin D could provide valuable information regarding vitamin D status of an individual in addition to the circulating 25(OH)D.

## 4.2 Role of lipid transporters in the absorption and tissue distribution of vitamin D

Insights from several studies that used *in vitro* models or animals suggest that lipid transporters can interact with fat-soluble micronutrients such as vitamins and carotenoids (Mardones et al. 2002; van Bennekum et al. 2005; During et al. 2005; Reboul et al. 2006; Narushima et al. 2008; Abuasal et al. 2010). Cholesterol and vitamin D display similar chemical structures; thus, it was speculated that these organic molecules could share common absorption pathways. In 2011 data from *in vitro* and *ex vivo* studies indicate that classical lipid transporters such as NPC1L1, SR-B1, CD36, and ABC-G5/G8 might be involved in vitamin D absorption (Reboul et al. 2011). Data presented in the current thesis indicate a significant role of NPC1L1, SR-B1, CD36, and ABC-G5/G8 transporters in the uptake, tissue distribution, and activation of vitamin D. To follow the metabolic pathway of orally administered vitamin D, mice were fed diets that contained deuterium-labeled vitamin $D_3$ (triple-deuterated vitamin $D_3$, vitamin $D_3$-$d_3$). Moreover, to avoid pronounced differences in food intake and consequently, in vitamin $D_3$-$d_3$, the amount of administered food was controlled.

Among all lipid transporters potentially relevant to vitamin D absorption, NPC1L1 has been described as a critical protein for the intestinal absorption of cholesterol (Altmann et al. 2004). The aim of **Paper 2** was to elucidate the role of NPC1L1 in the uptake of orally administered vitamin D. For this purpose, NPC1L1 was pharmacologically inhibited by ezetimibe, which was given through the diet. Data demonstrated that ezetimibe efficaciously inhibited NPC1L1, and therefore the absorption of cholesterol, as ezetimibe-treated mice had lower levels of cholesterol in serum and higher levels of 7-DHC in serum and liver compared with the control mice. In general, cholesterol is synthesized in the body and is absorbed from the diet in the gastrointestinal tract. 7-DHC, which is a precursor of cholesterol, characterizes a stimulated cholesterol synthesis (Sharpe et al. 2014).

The current findings show that the levels of orally ingested vitamin $D_3$-$d_3$ were markedly lower in the serum and tissues of mice treated with ezetimibe than in control mice. Among all the tissues analyzed, the strongest effect was observed in the liver, in which vitamin $D_3$-$d_3$ levels were approximately 80% lower in the group fed ezetimibe than in the control group. The decline of the vitamin $D_3$-$d_3$ levels in each tissue of ezetimibe-treated mice was higher than 40%. The next strongest effect was observed in serum and subcutaneous adipose tissue, which had 63% lower concentrations of vitamin $D_3$-$d_3$ in ezetimibe-treated

mice. Vitamin $D_3$-$d_3$ concentrations in the kidney, heart, retroperitoneal adipose tissue, and gastrocnemius muscle were 54%, 52%, 46%, and 41% lower after ezetimibe treatment, respectively. These findings suggest an important role of NPC1L1 in the intestinal absorption of vitamin D. Hence, the data presented in the current thesis confirmed the findings of Reboul and co-workers, who demonstrated that the cholesterol transporter NPC1L1 plays an important role in the uptake of vitamin $D_3$ using *in vitro* and *ex vivo* models (Reboul et al. 2011). It is noteworthy that two different doses of ezetimibe were used to inhibit NPC1L1; however, no significant differences in the serum and tissue levels of vitamin $D_3$-$d_3$ were observed between mice treated with 50 or 100 mg/kg ezetimibe, suggesting that the maximum effect of ezetimibe was already achieved by the oral dose of 50 mg/kg ezetimibe.

Studies conducted on transgenic mice that express human NPC1L1 in the liver have reported that NPC1L1, besides its function in the intestine, may promote the reabsorption of cholesterol from bile back into the liver (Temel et al. 2007). Furthermore, *in vitro* studies using gene transfer systems have demonstrated that hepatic NPC1L1 may indirectly control the secretion of cholesterol into bile by downregulating Niemann-Pick C2 (Yamanashi et al. 2012), which is a stimulator of ABC-G5/G8-mediated biliary cholesterol efflux (Yamanashi et al. 2011). Therefore, it seems that NPC1L1 in the liver acts against the biliary loss of cholesterol. As vitamin D is excreted mainly via bile (DeLuca 1976; Gil et al. 2018), it is tempting to speculate that the inhibition of NPC1L1 by ezetimibe not only impaired the intestinal absorption of vitamin D but also deteriorated the reabsorption of vitamin D from the bile into the liver. In addition to its action on NPC1L1, ezetimibe has been reported to stimulate ABC-G5/G8 transporter, which is involved in the excretion of cholesterol into the intestinal lumen and bile (Jakulj et al. 2010; Nakano et al. 2016), and which has been shown by the current studies to be also involved in the vitamin D export.

As ezetimibe-treated mice had low vitamin D levels, parameters such as serum $1,25(OH)_2D$ and PTH, as well as renal Cyp27b1, were analyzed to investigate whether these mice developed a functional vitamin D deficiency. PTH has been reported to be a sensitive early biochemical indicator of deranged calcium homeostasis in vitamin D deficiency (Ashby et al. 1989). Analyses revealed that ezetimibe did not alter any of these parameters. These findings assume that the ezetimibe treatment of mice for six weeks had only lowered the levels of stored vitamin D but not hormones involved in calcium regulation.

A surprising result was that the concentrations of triple-deuterated 25-hydroxyvitamin $D_3$ ($25(OH)D_3$-$d_3$) in serum of mice treated with 100 mg/kg ezetimibe were higher than those in control mice, despite their markedly reduced concentrations of vitamin $D_3$-$d_3$ in serum and

tissues. To explain the increased circulating 25(OH)$D_3$-$d_3$ levels of mice treated with 100 mg/kg ezetimibe, possible ezetimibe-induced changes in the expression of hydroxylases responsible for formation and degradation of 25(OH)D were examined. It is well known that ezetimibe can inhibit CYP enzymes such as CYP3A4 and CYP2C8 (Kosoglou et al. 2005). CYP3A4 is an enzyme that catalyses the degradation of 25(OH)D and 1,25$(OH)_2$D (Gupta et al. 2004, 2005; Xu et al. 2006), whereas CYP2C8 has been reported to be involved in the metabolism of several drugs and endogenous compounds (Totah and Rettie 2005). However, there are no data on the effect of ezetimibe on hydroxylases related to vitamin D metabolism. In this regard, hepatic Cyp2r1, Cyp27a1, Cyp3a11 (the mouse homolog of the human CYP3A4), Cyp2j3 (the mouse homolog of the human CYP2J2) and Cyp24a1 and also renal Cyp24a1 of all mice were analyzed. Cyp2r1, Cyp27a1, and Cyp2j3 enzymes have been described to have vitamin D 25-hydroxylase activity, whereas Cyp24a1 has been reported to have 24-hydroxylase activity (Sawada et al. 2000; Cheng et al. 2003, 2004; Zhu and DeLuca 2012; Bikle 2014). However, analysis revealed that none of the analyzed enzymes were significantly affected by ezetimibe. Only hepatic Cyp2j3 of ezetimibe-treated mice showed a tendency towards higher expression. Thus, the protein expression of hydroxylases does not provide a plausible explanation for the finding that the circulating 25(OH)$D_3$-$d_3$ concentration of ezetimibe-treated mice was moderately higher than that of the controls. A possible explanation for the increased circulating 25(OH)D levels of ezetimibe-treated mice despite their low levels of vitamin D in tissues is the stimulated activity of vitamin D hydroxylases to prevent a decline in 25(OH)D levels. Overall, this is consistent with the results demonstrated in **Paper 1**, which also show that 25(OH)D is not a good marker to indicate vitamin D stores.

Although circulating 25(OH)D is used to estimate vitamin D status (Zerwekh 2008; Cashman et al. 2017), only a small number of human studies, which were conducted with ezetimibe, have investigated the circulating levels of 25(OH)D. Randomized controlled trials conducted with hypercholesterolemic patients (Knopp et al. 2003) and healthy volunteers (Silva et al. 2015) did not observe any effect of ezetimibe on circulating 25(OH)D levels. Another study that included patients suffering from primary hypercholesterolemia demonstrated that treatment with simvastatin/ezetimibe 10/10 mg increased the circulating levels of 25(OH)D by 37% (Liberopoulos et al. 2013). However, this increase was attributable to simvastatin because simvastatin monotherapy resulted in a greater increase of the circulating 25(OH)D levels compared to combined therapy with simvastatin/ezetimibe (Liberopoulos et al. 2013). The aforementioned studies were not clearly indicative of a role of ezetimibe in modulating the circulating 25(OH)D concentrations and vitamin D status.

Patients treated with ezetimibe and receiving vitamin D supplements should be checked frequently for their vitamin D status.

Besides NPC1L1, other lipid transporters have also been suggested to play a significant role in the uptake and tissue distribution of vitamin D (Reboul et al. 2011). In this regard, the aim of **Paper 3** was to evaluate the serum and tissue levels of vitamin D metabolites in mice deficient in SR-B1, CD36, and ABC-G5/G8.

The first lipid transporter examined in **Paper 3** was SR-B1, which functions as hepatic HDL transporter (Acton et al. 1996). Interestingly, SR-B1-deficient mice had markedly higher levels of vitamin $D_3$-$d_3$ in serum and tissues — except liver — but lower serum and tissue concentrations of 25(OH)$D_3$-$d_3$ than the corresponding WT mice. Thus, the accumulation of vitamin $D_3$-$d_3$ in serum and the moderate decrease of circulating 25(OH)$D_3$-$d_3$ in SR-B1-deficient mice suggest that SR-B1 is important for the uptake of circulating vitamin D into the liver to synthesize 25(OH)D. As the accumulation of circulating vitamin $D_3$-$d_3$ in SR-B1-deficient mice was comparable to the accumulation of circulating cholesterol, it can also be assumed that HDL plays an important role in the transport of vitamin D. The high concentration of vitamin $D_3$-$d_3$ in tissues except liver of SR-B1-deficient mice is probably caused by the availability of abundant vitamin D in serum. As the liver is the main organ that 25-hydroxylates vitamin D, the reduced 25(OH)$D_3$-$d_3$ levels in the liver and kidney of SR-B1-deficient mice can be attributed to the impaired uptake of circulating vitamin $D_3$-$d_3$ and consequently to the decreased 25(OH)$D_3$-$d_3$ synthesis. To investigate whether the SR-B1 genotype of mice had affected the key enzymes involved in the activation of vitamin D, the mRNA abundance of hepatic Cyp2r1 and Cyp27a1, as well as renal Cyp27b1 were analyzed. Analysis revealed that the low levels of 25(OH)$D_3$-$d_3$ in SR-B1-deficient mice was accompanied by the elevated mRNA expression of the renal Cyp27b1, which is important for the 1,25$(OH)_2$D synthesis, to counteract a drop in the levels of the bioactive 1,25$(OH)_2$D; however, the mRNA expression of hepatic Cyp2r1 and Cyp27a1, which are pivotal for the 25(OH)D synthesis, remained unaffected. Collectively, the data are indicative of a significant function of SR-B1 for the metabolism of vitamin D because SR-B1 seems to facilitate the uptake of vitamin D from the circulation into the liver. However, the data do not indicate a significant involvement of SR-B1 in the intestinal absorption of vitamin D as it was previously proposed (Reboul et al. 2011).

The results also revealed that SR-B1-deficient mice had higher lipid levels in serum compared to WT mice. This finding match those noticed in previous studies that also found increased levels of plasma lipids, particularly cholesterol, in mice deficient in SR-B1 (Rigotti

et al. 1997; Varban et al. 1998). These observations further support the main function of SR-B1 as an HDL receptor that facilitates the uptake of circulating HDL-derived lipids, particularly cholesterol, into the liver (Acton et al. 1996; Rigotti et al. 1997; Varban et al. 1998). Moreover, mice deficient in SR-B1 demonstrated extremely high levels of 7-DHC in serum offering additional evidence about the reduced uptake of peripheral cholesterol in the liver. The increased 7-DHC levels are indicative of stimulated cholesterol synthesis that attempts to counterbalance a drop in cholesterol levels (Prabhu et al. 2014; Sharpe et al. 2014).

The next lipid transporter that was investigated was CD36, which is assumed to have multiple functions in lipid homeostasis. The data demonstrated that mice deficient in CD36 had significantly lower levels of $25(OH)D_3$-$d_3$ in the serum when compared to the corresponding WT mice. CD36 regulates the output of very low-density lipoproteins (VLDL) (Pepino et al. 2014), which has been shown to function as a carrier of DBP that transports vitamin D metabolites (Speeckaert et al. 2010). Thus, it is tempting to speculate that the decreased concentration of $25(OH)D_3$-$d_3$ in the serum and kidney of CD36-deficient mice was caused by a deteriorated transfer of hepatic 25(OH)D through VLDL into the circulation. However, the hampered transport of 25(OH)D into the circulation may not explain the finding of reduced concentration of $25(OH)D_3$-$d_3$ in the liver of CD36-deficient mice. It is also possible that the fat accumulation in the liver of these mice could have contributed to the reduced 25(OH)D synthesis, although the mRNA abundance of hepatic Cyp2r1 and Cyp27a1 remained unchanged. Data obtained from individuals with nonalcoholic fatty liver disease (NAFLD) and mice fed a high-fat diet showed reduced serum concentrations of 25(OH)D compared to healthy individuals and mice fed with standard chow diet, respectively (Zhang et al. 2020). Moreover, a strong association between decreased serum 25(OH)D concentrations and NAFLD has been demonstrated (Barchetta et al. 2011). A meta-analysis of cross-sectional and case-control studies revealed that patients with NAFLD had lower serum 25(OH)D concentrations and were 26% more likely to be vitamin D deficient (Eliades et al. 2013). Similar to the mRNA abundance of renal Cyp27b1 of SR-B1-deficient mice, the expression of renal Cyp27b1 mRNA of mice with CD36 deficiency was increased as a response to the decreased $25(OH)D_3$-$d_3$ levels to counteract a drop in the levels of $1,25(OH)_2D$.

Further, the results presented in **Paper 3** revealed that cholesterol levels of mice deficient in CD36 were higher in the serum and lower in the liver compared to the corresponding WT mice, emphasizing the aggravated hepatic sterol metabolism in CD36-

deficient mice. These findings are in agreement with those observed in previous studies that also found increased circulating cholesterol levels in mice with CD36 deficiency (Febbraio et al. 1999; Yue et al. 2010; Brundert et al. 2011). This increase was mainly attributed to elevated HDL cholesterol. Another previous study showed that mice deficient in SR-B1 had also elevated levels of circulating HDL cholesterol compared to WT mice (Brundert et al. 2005). It seems that both SR-B1 and CD36 play an important role in the tissue distribution of HDL cholesterol. However, a comparison between these proteins indicated that SR-B1 is the major protein involved in HDL metabolism and in HDL-mediated cholesterol transport because the rise of circulating HDL cholesterol was 96% in SR-B1-deficient mice compared to WT mice, whereas the rise of the same parameter in CD36-deficient mice was only 37% (Brundert et al. 2005, 2011). This observation is consistent with the data from **Paper 3** that also showed greater increase in serum cholesterol levels of mice deficient in SR-B1 than in CD36. In addition to HDL, CD36 has been reported to modulate other lipoproteins such as chylomicrons (Nauli et al. 2006) and VLDL (Pepino et al. 2014). Mice deficient in CD36 demonstrated a 60% suppression of VLDL output when compared to WT counterparts (Nassir et al. 2013). In agreement with the *in vivo* data, the secretion of VLDL was decreased in incubated liver slices from CD36-deficient mice *in vitro* (Nassir et al. 2013). Furthermore, CD36 deficiency exacerbated the steatosis in *ob/ob* mice by worsening the hepatic triglyceride secretion via VLDL (Nassir et al. 2013). These data indicate a role of CD36 as a regulator of VLDL secretion. This function of CD36 could possibly explain the increased levels of triglycerides in the liver and the decreased serum triglycerides in CD36-deficient mice observed in **Paper 3**.

All the aforementioned lipid transporters (NPC1L1, SR-B1, and CD36) that are examined in the current studies have been shown to be important for the cellular uptake of vitamin D. However, it was hypothesized that ABC-G5/G8 could reduce the cellular uptake of vitamin D, because it is known as efflux transporter of sterols in the gut (Yu et al. 2002b; Plösch et al. 2004) and the bile (Yu et al. 2002b, 2002a; Graf et al. 2003; Klett et al. 2004b). It was found that mice deficient in ABC-G5/G8 were characterized by an improved vitamin D status, as these mice had elevated levels of vitamin $D_3$-$d_3$ in serum and liver as well as elevated levels of 25(OH)$D_3$-$d_3$ in serum and kidney compared to WT counterparts. These findings support the assumption that the heterodimeric complex ABC-G5/G8 can not only facilitate the export of cholesterol and phytosterols but also the reverse vitamin D transport. Thus, the improved vitamin D status of ABC-G5/G8-deficient mice is probably caused by the increased absorption rate and decreased excretion of vitamin $D_3$-$d_3$ into bile in these mice.

Since ABC-G5/G8-deficient mice had also a higher mRNA abundance of hepatic Cyp27a1, it is speculated that the increased 25(OH)$D_3$-$d_3$ concentrations were not only caused by a higher intestinal uptake of vitamin $D_3$-$d_3$ but also by a stimulated synthesis of 25(OH)$D_3$-$d_3$ in the liver.

It was also found that ABC-G5/G8-deficient mice were characterized by higher levels of lipids in serum when compared with WT mice. This finding is analogous with the results from previous human studies that found a relation between the polymorphisms in the *ABC-G5/G8* genes and the raised levels of triglycerides and cholesterol in the circulation (Acalovschi et al. 2006; Chen et al. 2008). Of interest is the fact that the livers of ABC-G5/G8-deficient mice weighed more than the livers of the WT counterparts, despite not having differences in the levels of lipids in their livers. A previous study demonstrated that ABC-G5/G8-deficient mice fed with high-phytosterol diet had higher liver mass than the WT mice (McDaniel et al. 2013). The authors of this study concluded that the accumulation of phytosterols in mice deficient in ABC-G5/G8 could cause toxicity and liver damage, as they found elevated levels of alanine aminotransferase (ALAT) in the serum of these mice. However, current analysis of serum aspartate aminotransferase (ASAT) and ALAT are not indicative of an injury in the livers of ABC-G5/G8-deficient mice. In view of the aforementioned remarks, it is tempting to speculate that traces of phytosterols that naturally occur in the dietary ingredients of the experimental diets could have caused the higher liver weights of ABC-G5/G8-deficient mice compared to corresponding WT mice. Ongoing studies are necessary to elucidate the role of ABC-G5/G8 for the uptake of potentially toxic sterols and their impact on liver metabolism. As data are indicative of the important role of ABC-G5/G8 in vitamin D export, it is assumed that the vitamin D status could possibly be impaired by any endogenous and exogenous stimulation of the ABC-G5/G8 heterodimeric transporter. Phytosterols have been reported to act as stimulators of the intestinal expression of ABC-G5/G8 (Brufau et al. 2011). Moreover, a previous study conducted in mice that were fed with phytosterols demonstrated that intestinal absorption of vitamin D can be worsened by phytosterols (Goncalves et al. 2011); thus, this finding supports the speculation that ABC-G5/G8 can impair vitamin D status. Ezetimibe has been also mentioned to stimulate ABC-G5/G8 transporter (Jakulj et al. 2010; Nakano et al. 2016). This would be of interest for individuals, who are treated with ezetimibe and are at high risk of vitamin D deficiency, e.g., the elderly. Besides its function in the intestine, ABC-G5/G8 facilitates the excretion of sterols from the liver into bile (Yu et al. 2002b, 2002a; Graf et al. 2003; Klett et al. 2004b).

Thus, it is tempting to hypothesize that ABC-G5/G8 also promotes the hepatic secretion of vitamin D into bile.

Overall, data presented in **Paper 2** and **Paper 3** corroborate the hypothesis that lipid transporters are mainly involved in the intestinal absorption and cellular uptake of vitamin D.

## 4.3 Strengths and limitations of the studies

The current data obtained from the studies have several strengths. Firstly, vitamin D metabolites were measured via LC-MS/MS, which is considered the current gold standard for quantitative analysis (El-Khoury et al. 2011; Volmer et al. 2015; Müller and Volmer 2015). In addition, the use of LC-MS/MS allows the parallel and precise measurement of multiple vitamin D metabolites (Alonso et al. 2023). Secondly, the studies described in **Paper 2** and **Paper 3** were conducted with deuterium-labeled vitamin $D_3$. The use of labeled vitamin D ensured a closer tracking of orally administered vitamin D and its metabolic pathway in the body. Finally, all studies were conducted with standardized diets that could be accurately characterized.

The current data obtained from the studies also have three main limitations. Firstly, the studies described in **Paper 1**, **Paper 2** and **Paper 3** were conducted only with male mice. Female mice were not used to reduce the numbers of animals in the conducted studies. According to the knowledge available so far, no gender-specific differences are reported for NPC1L1, SR-B1, and ABC-G5/G8 lipid transporters. However, previous data indicate gender-specific differences in CD36 expression (Ståhlberg et al. 2004). Thus, the results obtained from the CD36 KO mice and the corresponding WT mice are not necessarily transferable to female mice. Secondly, another limitation is the feeding regime used in the studies with KO mice (**Paper 3**). The food intake of all mice was reduced by 20% of the *ad libitum* food intake. Although, all mice gained weight during the intervention study, the age-adjusted WT mice gained moderately more weight than SR-B1 KO mice. Therefore, it is possible that some of the effects on vitamin D metabolism observed in the SR-B1 KO mice are partly caused by the differences in food intake or weight gain. To avoid a restriction of food intake and consequently diet-related effects in future studies, vitamin $D_3$-$d_3$ might alternatively be supplemented by oral gavage. The final limitation is referred to the translation of the data obtained from mice to humans. Mice are the most regularly used mammalian model in biomedical research because of its small size, short lifespan and reproductive cycle as well as the extensive information related to its anatomy, biology, physiology, and genetics

(Hickman et al. 2017). These animals have been used in a variety of biological and nutritional studies, because their anatomy and physiology have many similarities to those of humans (Perlman 2016). Regarding vitamin D metabolism, many similarities but also some differences between mice and humans are reported (Marcinkowska 2020). In humans, several studies found that the supplementation of vitamin $D_3$ is more effective in raising the circulating levels of 25(OH)D than the supplementation of vitamin $D_2$ (Armas et al. 2004; Heaney et al. 2011; Tripkovic et al. 2012, 2017; Lehmann et al. 2013; Itkonen et al. 2016). In contrast, data from mice indicate that vitamin $D_2$ is more effective in raising 25(OH)D than vitamin $D_3$, supposing a different response of mice and humans to vitamin $D_3$ and vitamin $D_2$ (Baur et al. 2020). Furthermore, it is reported that the tissue pattern of NPC1L1 expression has significant differences between mice and humans. In humans, the highest expression of NPC1L1 is observed in the liver, whereas in mice, NPC1L1 is mainly expressed in the intestine (Davies et al. 2005; Betters and Yu 2010). These differences should be taken into account when data obtained from mice are extrapolated to humans.

## 4.4 Conclusions

The current thesis includes three research articles that have been previously published in peer-reviewed international journals. The main questions addressed in this thesis, could be answered as followed:

**I. Which parameter best indicates the stored levels of vitamin D in the body?**

Data obtained from mouse studies show that the circulating vitamin D is highly correlated with the tissue stores of vitamin D. In contrast, the classical vitamin D status marker 25(OH)D did not show this clear correlation to the tissue concentrations of vitamin D and is only partly suitable to provide information concerning the vitamin D stores in the body. Besides 25(OH)D, the measure of circulating vitamin D would provide additional information concerning the vitamin D status. Data also indicate that erythrocytes may not serve as significant vitamin D storage cells.

**II. How respond the vitamin D metabolites to dietary changes of vitamin D?**

Data show that various vitamin D metabolites respond differently to dietary changes in vitamin D. Plasma concentrations of 25(OH)D and 24,25(OH)$_2$D increased when switching from a poor vitamin $D_3$ diet to a vitamin $D_3$-adequate diet, but remained unchanged when switching from a vitamin $D_3$-rich to a vitamin $D_3$-adequate diet. In contrast, the concentration of vitamin D in plasma

declines when switching the vitamin $D_3$-rich diet to a vitamin $D_3$-adequate diet. This finding supports the additional analysis of circulating vitamin D in clinical practice. As expected, the plasma concentration of 1,25(OH)$_2$D did not change after feeding diets with different quantities of vitamin D.

**III. Are there tissue-specific differences in the mobilization of vitamin D?**

The stored vitamin D, particularly in the liver and adipose tissues, can be mobilized in times of absent dietary vitamin D. The mobilization of vitamin D from adipose tissues was markedly lower and weaker than the vitamin D mobilization from the liver.

**IV. What is the role of NPC1L1, SR-B1, CD36, and ABC-G5/G8 transporters in the uptake, tissue distribution, and activation of vitamin D?**

Data indicate a significant role of lipid transporters NPC1L1, SR-B1, CD36, and ABC-G5/G8 for the uptake, tissue distribution, and activation of vitamin D (Figure 3). The pharmacological NPC1L1 inhibition by ezetimibe demonstrated an important role of NPC1L1 in the intestinal absorption of vitamin D since date show a significant reduction of vitamin D in serum and tissues in mice fed an adequate dose of vitamin D, while the circulating concentration of 25(OH)D showed no decline. Moreover, it is tempting to speculate that NPC1L1 plays a role in the reabsorption of vitamin D from the bile into the liver. In contrast to NPC1L1, SR-B1 appears to play an important role in the uptake of serum vitamin D into the liver, in which it is activated to 25(OH)D. However, mice deficient in CD36 were characterized by decreased 25(OH)D concentrations, while the concentrations of vitamin D in serum and tissues remained unchanged. It is tempting to speculate that CD36 KO mice demonstrated a worsened hepatic 25(OH)D synthesis and transport into the circulation. Lastly, findings on the current thesis showed for the first time that ABC-G5/G8 lipid exporter could facilitate the reverse vitamin D transport. This would explain the higher concentrations of vitamin D in serum and tissues of ABC-G5/G8-deficient mice.

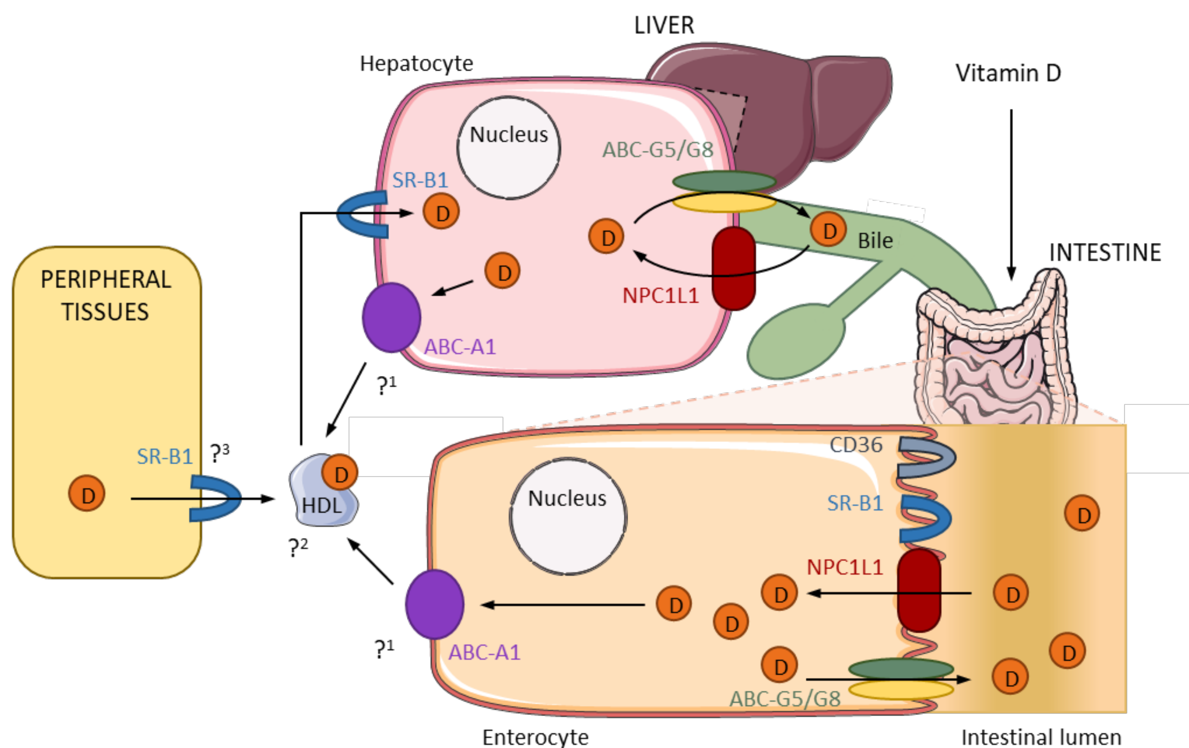


**Figure 3. The role of NPC1L1, SR-B1, CD36, and ABC-G5/G8 transporters in the uptake, tissue distribution, and activation of vitamin D.** ? = putative transporter/pathway that needs further investigation. ?[1] It has been reported that ABC-A1 can play a role in the basolateral efflux of cellular sterols, e.g., cholesterol (Field et al. 2004; Brunham et al. 2006). Future studies should investigate the role of ABC-A1 in the basolateral efflux of vitamin D. ?[2] In the current thesis, it has been speculated that HDL could be important in the vitamin D transport; however, this notion should be examined in future studies. ?[3] As SR-B1 seems to be a crucial modifier of vitamin D metabolism, its putative function as transporter of vitamin D from the periphery should be a research point in future studies. This figure was created with images adapted and modified from Servier Medical Art by Servier licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/; https://creativecommons.org/licenses/by/3.0/legalcode). ABC-A1, ATP-binding cassette subfamily A member 1; ABC-G5/G8, ATP-binding cassette subfamily G member 5/subfamily G member 8; CD36, cluster determinant 36; D, vitamin D; HDL, high-density lipoprotein; NPC1L1, Niemann-Pick C1-like protein 1; SR-B1, scavenger receptor class B type 1.

# 5 Summary

Vitamin D deficiency has been acknowledged as a global health problem that bothers one billion people worldwide. Due to the high prevalence of vitamin D deficiency and the multiple diseases associated with it, research interest in vitamin D has increased notably. Besides its function as a regulator of calcium and phosphorus homeostasis, and in turn of bone health, vitamin D is also important for other biological functions, e.g., in the immune system. Health authorities have established guidelines on vitamin D intake to deal with vitamin D deficiency. A common strategy to improve vitamin D status of the population is the regular intake of vitamin D supplements. The German Nutrition Society recommends an intake of 20 µg vitamin D per day; however, the actual vitamin D intake in Germany is 2-4 µg per day as most foods naturally contain very low amounts of vitamin D. It is widely accepted that the circulating concentration of 25-hydroxyvitamin D (25(OH)D) is a good biomarker for the assessment of vitamin D status.

Although the circulating concentration of 25(OH)D has been commonly used as a biomarker to assess the vitamin D status of an individual, data from the literature suggest that 25(OH)D is not really suitable to reflect the amounts of stored vitamin D in the body. Additionally, limited data are available regarding the mobilization of the stored vitamin D and the response of other metabolites other than 25(OH)D, such as vitamin D or 24,25-dihydroxyvitamin D (24,25(OH)$_2$D), to changes in the amounts of dietary vitamin D. To this end, mouse studies were conducted that aimed to shed light on these issues. Data demonstrated that the concentrations of circulating vitamin D were strongly correlated with the stored vitamin D in the tissues, whereas 25(OH)D concentrations were only weakly correlated with the vitamin D stores in the body suggesting that the circulating vitamin D, and not 25(OH)D, best reflects the vitamin D stored in the tissues. The concentrations of circulating 24,25(OH)$_2$D showed no correlation with the stored vitamin D in the tissues. Interestingly, only small amounts of circulating vitamin D were found in erythrocytes indicating that erythrocytes may not serve as an important storage reservoir for vitamin D. Vitamin D is mainly stored in the liver and adipose tissues. Data that compared the vitamin D mobilization from the liver and various adipose tissues demonstrated that the mobilization of vitamin D from adipose tissues was markedly lower and weaker than the vitamin D mobilization from the liver suggesting a tissue-specific mobilization of vitamin D. Moreover, the concentrations of circulating 25(OH)D and 24,25(OH)$_2$D increased when switching from a poor vitamin D$_3$ diet to a vitamin D$_3$-adequate diet, but remained unchanged when switching

from a vitamin $D_3$-rich to a vitamin $D_3$-adequate diet, whereas the concentrations of circulating vitamin D declined when switching the vitamin $D_3$-rich diet to a vitamin $D_3$-adequate diet. Besides 25(OH)D, the measurement of circulating vitamin D would provide additional information concerning the vitamin D status.

The synthesis, activation, degradation, and functions of vitamin D are well investigated. However, significantly less data are available regarding the intestinal absorption and tissue uptake of vitamin D. Classical lipid transporters are suggested to modulate the uptake of vitamin D. Transporters of interest are Niemann-Pick C1-like protein 1 (NPC1L1), scavenger receptor class B type 1 (SR-B1), cluster determinant 36 (CD36), and ATP-binding cassette subfamily G member 5/subfamily G member 8 (ABC-G5/G8). NPC1L1 mediates the intestinal absorption of cholesterol and phytosterols, and facilitates the reuptake of cholesterol from bile back into hepatocytes. Primarily, SR-B1 mediates selective high-density lipoprotein-derived cholesterol uptake into the liver, and in the intestine, facilitates the absorption of cholesterol. CD36 facilitates fatty acid uptake into enterocytes, cardiomyocytes, and adipocytes. Also, CD36 can mediate the intestinal absorption of cholesterol. ABC-G5/G8 limits the accumulation of phytosterols and, to a minor extent, of cholesterol. To investigate the role of these lipid transporters in terms of intestinal absorption and tissue uptake of vitamin D, *in vivo* models were employed. Data indicated a significant role of NPC1L1, SR-B1, CD36, and ABC-G5/G8 transporters in the uptake, tissue distribution, and activation of vitamin D. The inhibition of NPC1L1 by ezetimibe, a cholesterol-lowering pharmaceutical, resulted in a significant decrease of vitamin D in circulation and tissues suggesting an important role of NPC1L1 in the intestinal absorption of vitamin D. It is tempting to speculate that NPC1L1 also plays a role in the reabsorption of vitamin D from the bile into the liver. SR-B1 seems to be important for the uptake of circulating vitamin D into the liver since data showed an accumulation of vitamin D in the circulation and a moderate decrease of circulating 25(OH)D in SR-B1-deficient mice. However, data were not indicative of a role of SR-B1 in the intestinal absorption of vitamin D. Mice deficient in CD36 were characterized by decreased 25(OH)D concentrations and unchanged concentrations of vitamin D. It is tempting to speculate that CD36-deficient mice demonstrated an impaired hepatic 25(OH)D synthesis and transport into the circulation. The heterodimeric complex ABC-G5/G8 seems to play a role in the reverse vitamin D transport explaining the improved vitamin D status of ABC-G5/G8-deficient mice.

# 6 Zusammenfassung

Vitamin D-Mangel gilt als globales Gesundheitsproblem, das eine Milliarde Menschen weltweit betrifft. Aufgrund der hohen Prävalenz von Vitamin D-Mangel und den damit verbundenen vielfältigen Erkrankungen ist das Forschungsinteresse an Vitamin D deutlich gestiegen. Neben seiner Funktion als Regulator der Kalzium- und Phosphorhomöostase und demzufolge der Knochengesundheit, ist Vitamin D auch für andere biologische Funktionen wichtig, z.B. im Immunsystem. Gesundheitsbehörden haben Richtlinien zur Vitamin D-Zufuhr festgelegt, um einem Vitamin D-Mangel vorzubeugen. Eine gängige Strategie zur Verbesserung des Vitamin D-Status der Bevölkerung ist die regelmäßige Einnahme von Vitamin D-Ergänzungsmitteln. Die Deutsche Gesellschaft für Ernährung empfiehlt eine Zufuhr von 20 µg Vitamin D pro Tag. Die tatsächliche Vitamin D-Zufuhr in Deutschland liegt jedoch bei 2-4 µg pro Tag, da die meisten Lebensmittel natürlicherweise nur sehr geringe Mengen an Vitamin D enthalten. Es ist allgemein anerkannt, dass die zirkulierende Konzentration von 25-Hydroxyvitamin D (25(OH)D) ein guter Biomarker für die Beurteilung des Vitamin D-Status ist.

Obwohl die zirkulierende Konzentration von 25(OH)D häufig als Biomarker zur Beurteilung des Vitamin D-Status einer Person verwendet wird, deuten Daten aus der Literatur darauf hin, dass 25(OH)D nicht wirklich geeignet ist, die Menge an gespeichertem Vitamin D im Körper widerzuspiegeln. Darüber hinaus liegen nur begrenzte Daten zur Mobilisierung des gespeicherten Vitamin D und Veränderung anderer Metaboliten, außer 25(OH)D, wie z.B. Vitamin D oder 24,25-Dihydroxyvitamin D (24,25(OH)$_2$D), auf Änderungen der Vitamin D-Menge in der Nahrung vor. Zu diesem Zweck wurden Mausstudien durchgeführt, die diese Fragen klären sollten. Die Daten zeigten, dass die Konzentrationen von zirkulierendem Vitamin D stark mit dem im Gewebe gespeicherten Vitamin D korrelierten, wohingegen die 25(OH)D-Konzentration nur schwach mit den Vitamin D-Speichern im Körper assoziiert war, was darauf hindeutet, dass das zirkulierendes Vitamin D und nicht 25(OH)D am besten das im Gewebe gespeicherte Vitamin D widerspiegelt. Die Konzentrationen von zirkulierendem 24,25(OH)$_2$D zeigten keine Korrelation mit dem im Gewebe gespeicherten Vitamin D. Interessanterweise wurden nur geringe Mengen an zirkulierendem Vitamin D in Erythrozyten gefunden, was darauf hindeutet, dass Erythrozyten möglicherweise nicht als wichtiges Speicherreservoir für Vitamin D dienen. Vitamin D wird hauptsächlich in der Leber und im Fettgewebe gespeichert. Daten, die die Vitamin D-Mobilisierung aus der Leber und verschiedenen

Fettgeweben verglichen, zeigten, dass die Mobilisierung von Vitamin D aus Fettgewebe deutlich geringer und schwächer war als die Vitamin D-Mobilisierung aus der Leber, was auf eine gewebespezifische Mobilisierung von Vitamin D schließen lässt. Außerdem stiegen die Konzentrationen von zirkulierendem 25(OH)D und 24,25(OH)$_2$D beim Wechsel von einer Vitamin D$_3$-armen Ernährung zu einer Vitamin D$_3$-adäquaten Ernährung an, blieben jedoch unverändert, wenn von einer Vitamin D$_3$-reichen zu einer Vitamin D$_3$-adäquaten Ernährung umgestellt wurde. Darüber hinaus nahmen die Konzentrationen des zirkulierenden Vitamin D ab, wenn von der Vitamin D$_3$-reichen Ernährung auf eine Vitamin D$_3$-adäquate Ernährung umgestellt wurde. Damit lässt sich schlussfolgern, dass die Messung des zirkulierenden Vitamin D neben der Analyse von 25(OH)D zusätzliche Informationen zum Vitamin D-Status liefern könnte.

Synthese, Aktivierung, Abbau und Funktionen von Vitamin D sind gut erforscht. Es liegen jedoch deutlich kaum Daten zur Darmabsorption und Gewebeaufnahme von Vitamin D vor. Es wird vermutet, dass klassische Lipidtransporter die Aufnahme von Vitamin D modulieren. Diesbezüglich relevante Transporter sind Niemann-Pick C1-Like Protein 1 (NPC1L1), Scavenger Receptor Class B Type 1 (SR-B1), Cluster Determinant 36 (CD36) und ATP-Binding Cassette Subfamily G Member 5/Subfamily G Member 8 (ABC-G5/G8). NPC1L1 vermittelt die intestinale Absorption von Cholesterin und Phytosterinen und erleichtert die Wiederaufnahme von Cholesterin aus der Galle zurück in die Hepatozyten. SR-B1 vermittelt hauptsächlich die selektive Aufnahme von High-Density-Lipoprotein-gespeichertem Cholesterin in die Leber und erleichtert im Darm die Absorption von Cholesterin. CD36 fördert schließlich die Aufnahme von Fettsäuren in Enterozyten, Kardiomyozyten und Adipozyten. Außerdem kann CD36 die intestinale Aufnahme von Cholesterin vermitteln. ABC-G5/G8 begrenzt die Akkumulation von Phytosterinen und in geringem Maße auch von Cholesterin. Um die Rolle dieser Lipidtransporter im Hinblick auf die intestinale Absorption und Gewebeaufnahme von Vitamin D zu untersuchen, wurden *in vivo*-Mausmodelle eingesetzt. Die Daten deuteten auf eine signifikante Rolle der NPC1L1-, SR-B1-, CD36- und ABC-G5/G8-Transporter bei der Aufnahme, Gewebeverteilung und Aktivierung von Vitamin D hin. Die Hemmung von NPC1L1 durch Ezetimib, ein cholesterinsenkendes Arzneimittel, führte zu einem signifikanten Rückgang von Vitamin D im Kreislauf und im Gewebe, was auf eine wichtige Rolle von NPC1L1 bei der intestinalen Absorption von Vitamin D schließen lässt. Es ist zu vermuten, dass NPC1L1 auch eine Rolle bei der Rückresorption von Vitamin D aus der Galle in die Leber spielt. SR-B1 scheint für die Aufnahme von zirkulierendem Vitamin D in die Leber wichtig zu sein, da Daten eine

Akkumulation von Vitamin D im Kreislauf und einen moderaten Rückgang von zirkulierendem 25(OH)D bei SR-B1-defizienten Mäusen zeigten. Die Daten deuteten jedoch nicht auf eine Rolle von SR-B1 bei der intestinalen Absorption von Vitamin D hin. CD36-defiziente Mäuse waren durch verringerte 25(OH)D-Konzentrationen, jedoch unveränderte Vitamin D-Konzentrationen charakterisiert. Die Daten lassen darauf schließen, dass CD36-defiziente Mäuse eine beeinträchtigte hepatische 25(OH)D-Synthese und einen beeinträchtigten Transport von 25(OH)D in den Blutkreislauf aufweisen. Der heterodimere Komplex ABC-G5/G8 scheint beim reversen Vitamin D-Transport eine Rolle zu spielen, was den verbesserten Vitamin D-Status von ABC-G5/G8-defizienten Mäusen erklärt.

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## Declaration under Oath / Eidesstattliche Erklärung

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.

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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