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# The serum vitamin D metabolome: What we know and what is still to discover.

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## Abstract

Vitamin D, referring to the two forms, D2 from the diet and D3 primarily derived from phototransformation in the skin, is a prohormone important in human health. The most hormonally active form, 1α,25-dihydroxyvitamin D (1α,25$(OH)_2$D), formed from vitamin D via 25hydroxyvitamin D (25(OH)D), is not only important for regulating calcium metabolism, but has many pleiotropic effects including regulation of the immune system and has anti-cancer properties. The major circulating form of vitamin D is 25(OH)D and both D2 and D3 forms are routinely measured by LC/MS/MS to assess vitamin D status, due to their relatively long half-lives and much higher concentrations compared to 1α,25$(OH)_2$D. Inactivation of both 25(OH)D and 1α, 25$(OH)_2$D is catalyzed by CYP24A1 and 25-hydroxyvitamin D3 3-epimerase. Initial products from these enzymes acting on 25(OH)D3 are 24*R*,25$(OH)_2$D3 and 3-epi-25(OH)D3, respectively, and both of these can also be measured routinely in some clinical laboratories to further document vitamin D status. With advances in LC/MS/MS and its increased availability, and with the help of studies with recombinant vitamin D-metabolizing enzymes, many other vitamin D metabolites have now been detected and in some cases quantitated, in human serum. CYP11A1 which catalyzes the first step in steroidogenesis, has been found to also act on vitamins D3 and D2 hydroxylating both at C20, but with some secondary metabolites produced by subsequent hydroxylations at other positions on the side chain. The major vitamin D3 metabolite, 20*S*-hydroxyvitamin D3 (20*S*(OH)D3), shows biological activity, often similar to 1α,25$(OH)_2$D3 but without calcemic effects. Using standards produced enzymatically by purified CYP11A1 and characterized by NMR, many of these new metabolites have been detected in human serum, with semi-quantitative measurement of 20*S*(OH)D3 indicating it is present at comparable

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Conflict of Interest

The authors declare no conflict of interest.

concentrations to 24*R*,25(OH)$_2$D3 and 3-epi-25(OH)D3. Recently, vitamin D-related hydroxylumisterols derived from lumisterol3, a previtamin D3 photoproduct, have also been measured in human serum and displayed biological activity in initial *in vitro* studies. With the current extensive knowledge on the reactions and pathways of metabolism of vitamin D, especially those catalyzed by CYP24A1, CYP27A1, CYP27B1, CYP3A4 and CYP11A1, it is likely that many other of the resulting hydroxyvitamin D metabolites will be measured in human serum in the future, some contributing to a more detailed understanding of vitamin D status in health and disease.

**Keywords**

Vitamin D; 25-hydroxyvitamin D3; metabolome; LC/MS/MS; cytochrome P450

## 1. Introduction and overview

### 1.1. Scope of review

In this review we report the detection and/or concentrations of many vitamin D metabolites in human serum. Metabolites now measured routinely in serum plus metabolites that have been measured either quantitatively or semi-quantitatively are summarized in Table 1. For consistency, any metabolite concentrations reported in the literature as pg/ml have been converted to nM or pM. As well as listing all the vitamin D metabolites detected in serum that we could find reported in the literature, we also have endeavored to comment on the known or predicted enzymatic reactions producing them and briefly comment on their biological activity. We also predict what other vitamin D metabolites might be detected in the serum in the future based on known or likely pathways of vitamin D metabolism. We have generally used abbreviated secosteroid names, with hydroxy represented by OH and the number of hydroxyl groups shown by subscript (e. g. 24*R*,25(OH)$_2$D3 = 24*R*, 25dihydroxyvitamin D3), and have endeavored to show the stereochemistry, where known.

### 1.2. Vitamin D synthesis and mode of action

Vitamin D3 (Fig. 1) is formed in the skin by the action of ultraviolet B (UVB) radiation on 7dehydrocholesterol (7DHC) (Fig. 2). Only a small proportion of circulating vitamin D3 is derived from the diet. In contrast, the structurally and functionally related vitamin D2 (Fig. 1) is not synthesized by animals but is derived entirely from the diet being produced by the action of UVB radiation on the fungal membrane sterol, ergosterol [1]. In the skin, the initial photoproduct formed from 7DHC is previtamin D3 which undergoes a slow thermal isomerization over several hours to form vitamin D3 (Fig. 2) [2, 3]. With prolonged UVB radiation the majority of the previtamin D undergoes further photochemical reactions, one of which involves the resealing of the B-ring producing lumisterol3, a stereoisomer of 7DHC, and another which involves isomerization of the double bonds in the triene system of previtamin D3 to produce tachysterol3 [2].

Vitamin D3 is a prohormone and is activated by sequential hydroxylations at C25 and C1α to the most hormonally active form, 1α,25-dihydroxyvitamin D3 [1α,25(OH)$_2$D3], by enzymes of the cytochrome P450 (CYP) family. 25-Hydroxylation is primarily catalyzed by

CYP2R1 in the endoplasmic reticulum of the liver with a possible contribution from CYP27A1 present in liver mitochondria [4–9]. The resulting 25-hydroxyvitamin D3 [25(OH)D3] is the major circulating form of vitamin D and as described later is the form generally used to monitor vitamin D status. Circulating 25(OH)D3 is 1α-hydroxylated by CYP27B1 mainly in kidney mitochondria producing 1α,25$(OH)_2$D3 (Fig. 2) which is released into the bloodstream. CYP27B1 is present at lower levels in many other tissues such as the skin and placenta so these tissues therefore have the ability to produce 1α, 25$(OH)_2$D3 locally [10]. Approximately 90% of 25(OH)D3 in the plasma is carried bound to DBP (vitamin D binding protein), a specific transport protein for the major forms of vitamin D present in the circulation, with most of the remainder bound to serum albumin [11, 12]. The DBP concentration in serum is 20-fold higher than the total circulating forms of vitamin D. DPB binds 25(OH)D3 with 10- to 100-fold higher affinity than 1α, 25$(OH)_2$D3. Vitamin D2 metabolites bind slightly less well to DPB than their vitamin D3 counterparts.

In the absence of a supplement, vitamin D2 levels are usually low compared to vitamin D3. Vitamin D2 is activated similarly to vitamin D3, producing 1α,25$(OH)_2$D2 [5, 13]. Vitamins D2 and D3 (Fig. 1), collectively referred to as vitamin D, work primarily by binding to the vitamin D receptor (VDR), a member of the nuclear receptor family. The VDR acts as a transcription factor when bound to 1α,25$(OH)_2$D, regulating many target genes which contain a vitamin D response element in their promoter [6, 14]. Some rapid responses of 1α, 25$(OH)_2$D3 such as the stimulation of intestinal calcium transport, appear to involve VDR located in the plasma membrane and are not mediated by effects on transcription [6, 14, 15]. It has been proposed that 1α,25$(OH)_2$D3-membrane-associated, rapid response steroid-binding protein (1,25D3-MARRS, also known as PDIA3) can act as an alternative membrane bound receptor for 1α,25$(OH)_2$D3 [16, 17]. The actions of 24*R*,25$(OH)_2$D3 are independent of the VDR, indicating that a different receptor is involved [18, 19]. In addition, biologically active vitamin D hydroxyderivatives can act as inverse agonists on the retinoic acid-related orphan receptors (RORs) α and γ [20–22]. It is therefore clear that there is more than one receptor for vitamin D metabolites.

### 1.3. Biological effects of vitamin D

It is well established that 1α,25$(OH)_2$D is the major hormonally active form of vitamin D which plays a fundamental role in body calcium and phosphorous homeostasis, ensuring proper functioning of the skeletomuscular system [1, 6, 23–27]. However, over several decades evidence has accumulated for pleiotropic activities of 1α,25$(OH)_2$D on different cell functions such as proliferation, differentiation, apoptosis, senescence, migration, and protective and reparative mechanisms. These are regulated by different signal transduction pathways initiated predominantly by genomic and nongenomic actions of the VDR in a cell-type and anatomic-location-dependent manner [6, 28–34].

Specifically, 1α,25$(OH)_2$D3 shows anti-inflammatory and immunomodulatory properties (predominantly downregulation of adaptive and upregulation of innate immunity) having both preventive and therapeutic effects in autoimmune and inflammatory disorders [35–37]. It plays an important role in reproduction, pregnancy, placental functions and fetal and child

development [3843]. 1α,25(OH)$_2$D3 is also important in neurodevelopment as well as in the functioning of the adult central and peripheral nervous system [38, 44, 45]. It is involved in the regulation of global metabolic and endocrine homeostasis and the functions of different endocrine organs, as well as in the functioning of the cardiovascular system [2, 6, 29, 46–50]. It also inhibits malignant transformation, tumor progression and has anti-cancer properties on a variety of tumors including those of gastrointestinal, genitourinary, breast, prostate and lung origins [32, 33, 46, 51], as well as melanoma [52, 53]. In the skin, it plays an important role in the formation of the epidermal barrier and hair cycling. It also has photoprotective properties and has a wide variety of ameliorating effects on skin cancer and on proliferative and inflammatory cutaneous diseases [1, 3, 30, 31, 54–59]. The above are only examples of organs and systems regulated by 1α,25(OH)$_2$D3 of which a full description would exceed the scope of this review.

Other vitamin D metabolites besides 1α,25(OH)$_2$D display biological activity and these will be discussed later under each metabolite. One metabolite of note that appears to have a physiological role is 24*R*,25(OH)$_2$D3, the first product in the C24-oxidation pathway of 25(OH)D3 metabolism catalyzed by CYP24A1 (Figs 2 and 3). This metabolite can stimulate growth plate development and bonefracture repair, and protect against osteoarthritis, but these effects do not appear to be mediated by binding to the VDR [18, 19, 60–63]. Very recently it was reported that the effects of 24*R*,25(OH)$_2$D3 are mediated by its binding to the enzyme FAM57B2 on which it acts as an allosteric modulator, stimulating lactosylceramide synthesis which in turn affects cartilage maturation [64].

A number of other active vitamin D metabolites have been reported to be produced by an alternative activation pathway to that for 1α,25(OH)$_2$D, involving CYP11A1 (Fig. 4). 20*S*(OH)D3 is the best characterized product of this pathway. It displays high potency for the inhibition of proliferation and the promotion of differentiation in epidermal cells, and for the regulation of immune cells and fibroblasts (reviewed in [65–67]). These metabolites are also active *in vivo* as further discussed in Sections 2.2.6 and 3.1, but unlike 1α,25(OH)$_2$D3 and 25(OH)D3, pharmacological doses do not raise serum calcium in rodents [68–70].

### 1.4. Vitamin D inactivation and modulation

Vitamin D inactivation is catalyzed by CYP24A1 with initial hydroxylation of both 1α,25(OH)$_2$D3 and 25(OH)D3 occurring primarily at C24 to produce 1α,24,25(OH)$_3$D3 and 24*R*,25(OH)$_2$D3, respectively (Figs 2 and 3). The side chains of both of these initial products are further oxidized by CYP24A1 in the C24-oxidation pathway with the C-C bond between C23 and C24 being cleaved, ultimately producing the C23 acid (reviewed in [5, 71]). A minor pathway in humans, also catalyzed by CYP24A1, involves initial hydroxylation at C23 and is therefore known as the C23-oxidation pathway. CYP24A1 and the C23 and C24 pathways are described in more detail later (Sections 1.6.5, 2.1.4 and 3.2). 25(OH)D3, and to some extent other vitamin D metabolites such as 25(OH)D2, 24*R*,25(OH)$_2$D3 and 1α,25(OH)$_2$D3, can also be acted on by 25-hydroxyvitamin D3–3-epimerase which carries out a 3β → 3α inversion, producing 3-epi-25(OH)D3 in the case of 25(OH)D3 (Fig. 2) [72]. 3-Epi-1α,25(OH)$_2$D3 generally has reduced biological activity compared to 1α,25(OH)$_2$D3 [14, 73–75] and is discussed further in Sections 2.1.3 and 3.6.

Sulfation and glucuronidation may also contribute to vitamin D inactivation and excretion as described in Section 2.2.5.

### 1.5. Liquid chromatography tandem-mass spectrometry of vitamin D

Advances in liquid chromatography tandem-mass spectrometry (LC/MS/MS) systems over recent years [11, 76–79], have led to reports of many new forms of vitamin D3 in human serum. Binding of vitamin D metabolites, particularly 25(OH)D, to DBP makes direct analysis of aqueous (serum) samples difficult [11]. Therefore, extraction of aqueous samples with organic solvents which can also serve to precipitate and remove serum proteins, is commonly used prior to LC/MS/MS. Solid phase extraction is sometimes used for further sample clean up before LC, particularly for quantitative assays.

Over recent years internal standards with 3 to 6 deuterium- or $^{13}$C-labels have become commercially available for 25(OH)D3 and a number of other common vitamin D metabolites. These are are added to serum prior to extraction to correct for recoveries for quantitative analyses [76, 77]. They have the advantage over structurally-different analogs as standards of having identical solubility and chromatographic properties to the unlabeled analyte but can be distinguished by MS due to the increase in mass from the presence of the isotope.

The measurements of specific masses of vitamin D metabolites for quantitation, or as most commonly used, mass transitions in the selected reaction monitoring (SRM) mode, are highly selective for species of vitamin D amongst the numerous lipophilic molecules present in serum extracts [76, 77]. However, the various forms of monohydroxylated vitamins D3 (geometric or stereoisomers) have the same masses and generally show the same transitions, with the loss of water being the most common transition from parent to daughter ion. Thus, mass spectrometry itself does not distinguish between the different monohydroxyvitamin D3 isomers [11, 76, 77]. The same applies to the different dihydroxyvitamin D3 species and also to the different hydroxyvitamins D2. Therefore, chromatographic separation of the various forms of vitamin D present in the serum by LC prior to MS/MS is crucial to the measurement of specific forms of vitamin D.

One of the drawbacks of using mass spectrometry for the analysis of vitamin D metabolites is the poor ionization they display with ESI and APCI due to the lack of readily chargeable groups, which limits the sensitivity of detection [11, 76, 77, 80]. This can be largely overcome by derivatization, most commonly with Cookson-type reagents which react with the diene double bonds in vitamin D under a water-free environment (reviewed in [80]). The disadvantage is an additional step in sample preparation.

### 1.6. Vitamin D metabolizing CYPs and their heterologous expression

**1.6.1. Relevance of CYPs to the vitamin D metabolome—**Strongly contributing to the identification of many new vitamin D metabolites in recent years is the ability to express and purify recombinant CYP enzymes that metabolize vitamin D which became possible following the cloning of their genes. Much important original work on determining the role of CYPs on vitamin D metabolism was done by purifying or partially purifying some of

these CYPs from animal or human tissues, but the extensive older literature on this is beyond the scope of this review. General information on the vitamin D-metabolizing CYPs can be found in recent reviews [5, 13, 14], here we will focus on more recent studies on their metabolism of vitamin D and heterologous expression. The activity of mitochondrial CYPs requires adrenodoxin reductase and adrenodoxin as redox partners whereas microsomal CYPs require NADPH-P450 oxidoreductase [81], so these must either be coexpressed with the CYP or added separately to the expressed CYP for activity assays. Studies with the expressed and purified recombinant enzymes have enabled some of the already established pathways to be confirmed and new pathways to be characterized, as described below. The use of these enzymes in scaled up reactions has permitted sufficient metabolites to be made (typically > 100 nmol) to elucidate their structures by NMR. These enzymatically-synthesized secosteroids have been used as standards to identify the metabolites in serum and tissues, and to confirm that the pathways operate *in vivo*. This has bypassed the requirement for more complex chemical synthesis to confirm the identities of new metabolites.

**1.6.2. CYP27A1—**The human gene encoding mitochondrial CYP27A1 was cloned by Cali and Russell [82] in 1991 and shown to be expressed in the liver and other tissues including muscle and skin [83]. Initial heterologous expression was carried out in COS M6 cells to study its role in the oxidation of the sterol side chain in bile acid synthesis, believed to be its major function [82]. The gene was subsequently expressed in *E. coli* and shown to catalyze the conversion of vitamin D3 to 25(OH)D3 and also to slowly catalyze the 1α- and 27-hydroxylation of vitamin D3 [84]. Its ability to catalyze 25hydroxylation of vitamin D3 was consistent with earlier studies demonstrating this activity for the enzyme partially purified from human liver mitochondria [85]. Later studies with expressed and purified CYP27A1 suggested that the 1α-hydroxylase activity was too low to be of physiological importance [86]. Transfected COS-1 cells have been used to characterize the hydroxylation of vitamin D2 by CYP27A1 (Section 3.4) [87] and bacterially expressed and purified CYP27A1 was used to characterize its metabolism of 20(OH)D3 (Section 2.2.6) and lumisterol3 (Section 4) [88, 89].

**1.6.3. CYP2R1—**It was established that the liver microsomal fraction as well as liver mitochondria (containing CYP27A1) displayed vitamin D 25-hydroxylase activity prior to identification of the enzymes involved [90]. CYP2R1 was cloned by Cheng et al in 2003 [83] and shown to be a microsomal vitamin D3 25-hydroxylase present in liver and many other tissues including brain, testis and skin. The enzyme was expressed in *E. coli* and purified, permitting its crystal structure to be determined [91] and substrate specificity to be examined. This revealed that unlike CYP27A1which cannot hydroxylate vitamin D2 at C25 [87], it acted as a 25-hydroxylase on both vitamins D3 and D2 [92]. Studies on its kinetics compared to CYP27A1 [92, 93] and genetic deficiency indicate that it is the major vitamin D 25-hydroxylase in humans [7–9]. It can also catalyze 25-hydroxylation of 20(OH)D3 [93].

**1.6.4. CYP27B1—**The 25(OH)D3–1α-hydroxylase (CYP27B1) gene was independently cloned by four groups in 1997 [94–97]. CYP27B1 is expressed at highest levels in the kidney, placenta and disease-activated macrophages but is also expressed in many other

tissues at lower levels, suggesting local production of 1α,25(OH)$_2$D3 for intercrine actions [10]. Due to its greater expression level and stability than the human enzyme, mouse CYP27B1 was expressed in *E. coli* and subsequently purified [98–100]. One of these studies indicated that mouse CYP27B1 could not only hydroxylate 25(OH)D3 at C1α but could also slowly carry out this reaction on vitamin D3 [99], however we could not detect any activity with vitamin D as substrate for the mouse enzyme in a membrane-reconstituted system [100]. Human CYP27B1 was expressed in *E. coli* and activity measurements were made using the *E. coli* membrane fraction. Results showed that the enzyme catalyzes the 1α-hydroxylation of 24*R*,25(OH)$_2$D3 with a catalytic efficiency ($k_{cat}/K_m$) of double that for 25(OH)D3 [101]. We succeeded in expressing human CYP27B1 in *E. coli* and partially purifying the labile enzyme [102]. This enabled a detailed study of its substrate specificity to be carried out in a reconstituted membrane environment which revealed that CYP27B1 was highly specific for catalyzing 1α-hydroxylation on a range of endogenously-produced vitamin D metabolites. Catalytic efficiencies for 1α-hydroxylation of 25(OH)D3, 25(OH)D2 and 24*R*,25(OH)$_2$D3 were fairly similar but lower for 24-oxo-25(OH)D3 and 24-oxo-23,25(OH)$_2$D3, later intermediates of the C24-oxidation pathway of vitamin D3 inactivation by CYP24A1 (see Fig. 3). Human CYP27B1 also acted on hydroxyvitamin D products of CYP11A1 action on vitamin D (see Fig. 4) with catalytic efficiencies ranging from undetectable for substrates with a 17α-OH group such as 17α,20*S*(OH)$_2$D3, to efficiencies three times higher than that for 1α-hydroxylation of 25(OH)D3 for 20*S*, 24*R*(OH)$_2$D3 and 20*S*,25(OH)$_2$D3 [103].

**1.6.5. CYP24A1—**The gene encoding rat CYP24A1 was originally cloned by Ohyama et al [104] in 1991 and the expressed enzyme was shown to catalyze the 24-hydroxylation of 25(OH)D3 in transfected COS-7 cells. CYP24A1 is a mitochondrial enzyme expressed highly in the kidney, especially when 1α,25(OH)$_2$D3 levels are elevated, but is also expressed at lower levels in many vitamin D target tissues including bone and intestine [71]. The major steps in the C24-pathway of 1α,25(OH)$_2$D3 oxidation to calcitroic acid were largely elucidated from earlier studies, including those with perfused rat kidneys [105, 106], before heterologous expression of the enzyme became possible. Expression of the rat enzyme in *E. coli* and activity assays using the membrane fraction demonstrated that a single enzyme, CYP24A1, could catalyze all the oxidation steps from 1α,25(OH)$_2$D3 through to 24-oxo1,23*S*,25(OH)$_3$D3 and calcitroic acid (Fig. 3), with similar reactions for conversion of 25(OH)D3 through to 23(OH)- 24,25,26,27-tetranor D3 [107, 108]. Expression of rat CYP24A1 in *E. coli* also resulted in sufficient pure enzyme to determine its crystal structure, but without substrate in the active site [109].

Human cDNA encoding CYP24A1 was isolated in 1993 by Chen et al [110]. Studies with expressed human CYP24A1 in *Sf21* insect cells indicated that the enzyme could catalyze most, if not all, of the steps in the C23 and C24 oxidation pathways of 25(OH)D3 and 1α,25(OH)$_2$D3 metabolism [111]. Sakaki et al [112] also demonstrated that human CYP24A1 in the membrane fraction from *E. coli* cells could catalyze all the steps of both the C23- and C24- oxidation pathways of metabolism of 25(OH)D3 and 1α,25(OH)$_2$D3. These workers reported that the catalytic efficiency for 24hydroxylation of 1α,25(OH)$_2$D3 by CYP24A1 in bacterial membranes was 1.7-fold higher than for 25(OH)D3, however our own studies with

partially purified enzyme in a reconstituted system resembling the inner mitochondrial membrane (described next), showed that the catalytic efficiency was 3-fold higher for 25(OH)D3 than for 1α,25$(OH)_2$D3 [113]. Like human CYP27B1, human CYP24A1 expressed poorly in *E. coli* and was labile. Nevertheless we were able to express and partially purify enough of the enzyme for a full kinetic analysis of each step in a membranere constituted system [113]. The most efficient step in the metabolism of 1α, 25$(OH)_2$D3 by the C24oxidation pathway was cleavage of the side chain of 24-oxo-1α,23*S*, 25$(OH)_3$D3 between C23 and C24 where the initial product was identified as 1α(OH)-23-oxo-tetranorvitamin D3 and not the 1α,23$(OH)_2$tetranorvitamin D3 previously reported in the literature (see Fig. 3) [71]. The least efficient step was the oxidation of 1α,24*R*, 25$(OH)_3$D3 to 24-oxo-1α,25$(OH)_2$D3. All the intermediates except 24-oxo1α, 23,25$(OH)_3$D3 which had a very low $K_m$, had $K_m$ values from 0.41 to 3.7 times that for 1α, 25$(OH)_2$D3, indicating that 1α,25$(OH)_2$D3 and 25(OH)D3 can compete with them for binding to the single active site on the enzyme. Based on this it was predicted that all of these intermediates will accumulate to some degree during conversion of 1α,25$(OH)_2$D3 to calcitroic acid, as seen in enzymatic assays. We observed that the ratio of initial hydroxylation products at C24 to C23 was 4:1, in agreement with the results of Sakaki et al [112], indicating that the C24-oxidation pathway predominates in humans. The rat enzyme almost exclusively catalyzes the C24 oxidation pathway while the C23-oxidation pathway predominates for opossum CYP24A1 [71, 112].

The metabolism of 1α,25$(OH)_2$D2 by human CYP24A1 has been partially elucidated using the expressed enzyme in *E. coli* membranes [114] and is discussed in Section 3.3. Expressed human CYP24A1 can also oxidize CYP11A1-derived secosteroids (see Fig. 4) which is described in Section 2.2.6.

**1.6.6. CYP11A1—**CYP11A1 (also known as cytochrome P450scc), catalyzes the cleavage of the side chain of cholesterol and is the most studied of the mitochondrial CYPs [65, 81]. Its product, pregnenolone is the common precursor of all steroid hormones. CYP11A1 is expressed at high levels in the adrenal cortex, gonads and placenta and at lower levels in several other tissues including the brain, skin and gut [115]. Since it is relatively easy to purify the bovine enzyme from the adrenal cortex, it was well characterized before heterologous expression in *E. coli* was reported, initially for the bovine enzyme [116] and later for the human enzyme [117]. In 2003 Guryev et al [118] showed that the bovine enzyme could hydroxylate the side chain of vitamin D3 at C20 and C22. We extended these observations showing that C23 and C17 were also major sites of hydroxylation, but that no cleavage of the side chain occurred [119–121], leading to the pathway described in Fig. 4. We also confirmed that the recombinant human enzyme and CYP11A1 in mitochondria from the human placenta could catalyze these hydroxylations [122]. The CYP11A1-derived metabolites of vitamin D2 and D3, many of which have been detected in serum and display biological activity, are further described in Sections 2.2.6 and 3.1

## 2. Quantitative and qualitative analysis of vitamin D metabolites in serum

The concentrations of a number of vitamin D metabolites in serum can be measured quantitatively, some routinely in many laboratories and others in only a limited number of

research laboratories, as listed in Table 1 and described below. Others have been measured by assays that are semi-quantitative and many others have been detected, most commonly by LC/MS/MS, but levels not determined. These metabolites are also described in this section.

## 2.1. Vitamin D metabolites that can be routinely measured in serum

**2.1.1. 25(OH)D—**25(OH)D3 is the major circulating form of vitamin D3 in the bloodstream and is commonly used to monitor vitamin D status. It is primarily produced in the liver by the action of CYP2R1 on circulating vitamin D3 (Fig. 2) although other CYP enzymes in the liver including CYP27A1 can also catalyze this reaction [4, 93, 123]. Concentrations are typically in the range of 40–100 nM (Table 2) [124–127]. There has been much debate regarding the serum concentration necessary for good health and the reader is directed to other reviews for this [11, 78, 128–130]. Although generally considered to be an inactive precursor to 1α,25(OH)$_2$D3, some recent evidence suggests that it has biological activity in its own right at high concentrations that may be responsible for vitamin D toxicity [16, 63, 131, 132]. In cultured mouse skin fibroblasts in which C*yp27b1* had been knocked out (preventing conversion of 25(OH)D3 to 1α,25(OH)$_2$D3), 25(OH)D3 (500 nM) regulated many but not all of the same genes regulated by 1α,25(OH)$_2$D3, plus over 100 genes distinct from those regulated by 1α,25(OH)$_2$D3 [63]. 25(OH)D3 adopts a similar pose to 1α, 25(OH)$_2$D3 in the active site of the VDR [131] but has much lower affinity [133]. Recently it was reported that 25(OH)D3 may regulate lipid metabolism independently from binding to the VDR by preventing the activation of sterol regulatory element binding proteins (SREBPs) which are transcription factors that control lipid homeostasis. The direct target of 25(OH)D3 was reported to be the SREBP cleavage-activating protein (SCAP) [134].

25(OH)D2 is derived entirely from dietary vitamin D2. Serum levels of 25(OH)D2 are low and variable depending on the amount of D2 in the diet with wild mushrooms being a major source, or whether a vitamin D2 supplement is taken [1]. In the study by Shah and coworkers [125] of a United Kingdom cohort of 32 healthy individuals, the mean serum 25(OH)D2 concentration was 18 nM compared to 28 nM for 25(OH)D3. The high ratio of D2 to D3 is suggestive of vitamin D2 supplementation in this cohort, as does the high 1α, 25(OH)$_2$D2 concentration (see below). In the other studies listed in Table 2, the 25(OH)D2 concentrations were 4.0 – 9.5% of the 25(OH)D3 concentrations.

**2.1.2. 1α,25(OH)$_2$D—**The major hormonally active form of vitamin D, 1α,25(OH)$_2$D, is reported to be in serum in the range of 50 to 150 pM, as measured by a competitive binding assay following HPLC [135], which is almost 1000 times lower than 25(OH)D3. Using an LC/MS/MS assay which could distinguish between the D2 and D3 forms of 1α,25(OH)$_2$D, 1α,25(OH)$_2$D3 was reported to be in serum at a mean concentration of 28 pM in 32 healthy individuals. 1α,25(OH)$_2$D2 was higher for this cohort at 170 pM, [125], indicative of dietary D2 supplementation (Table 2). 1α,25(OH)$_2$D2 levels were low (below the level of quantitation) in the study of Jenkinson et al [127], with the mean 1α,25(OH)$_2$D3 concentration being more than 4-times higher than that reported by Shah et al [125] and similar to that reported by Duan et al [124] (Table 2). Serum 1α,25(OH)$_2$D3 and 1α, 25(OH)$_2$D2 are produced in the kidney by the action of CYP27B1 on 25(OH)D obtained from the circulation [14]. Many other tissues express CYP27B1 too which can modulate the

local level of this hormone utilizing plasma 25(OH)D as substrate [10, 14, 51]. The biological activities of 1α,25(OH)$_2$D are described earlier in Section 1.3.

**2.1.3. 3-Epi-25(OH)D—**3-Epi-25(OH)D3 and 3-epi-25(OH)D2 have a 3α-hydroxyl group rather than the 3β-hydroxyl group present in 25(OH)D3 and 25(OH)D2, respectively (Fig. 2), and are produced by 25-hydroxyvitamin D3 3-epimerase. This enzyme is present in the endoplasmic reticulum of a range of cells/tissues including liver, bone and skin [72, 136], but not kidney [137]. It is poorly characterized and surprisingly the gene encoding it has not been identified. Measurements of 25(OH)D3–3-epimerase activity in microsomes prepared from osteoblastic UMR-106 cells indicate that it uses NADPH as cofactor for the 3β → 3α epimerization, and can also catalyze the epimerization of 1α,25(OH)$_2$D3 and 24*R*,25(OH)$_2$D3, although at lower rate than for 25(OH)D3. The reaction is reported to be irreversible [72]. With the development of LC/MS/MS methods it is now possible to routinely quantitate 3-epi25(OH)D3 in serum (Table 2). The concentration of 3-epi-25(OH)D3 in serum varies greatly and has been reported to range from below 1% up to 25% of that of 25(OH)D3 with an average of 4.75% in one study [138] and similar values in others [139, 140] (Table 2). Another study showed it is higher in children being 8.7 to 61.1% of the 25(OH)D3 concentration [141]. The 3-epi-25(OH)D2 concentration was measured in the study by Shah and coworkers [125] and the mean was 1.1 nM compared to 6.1 nM for 3-epi-25(OH)D3.

The 1α-hydroxyderivative of 3-epi-25(OH)D3 produced by CYP27B1, 3-epi-1α, 25(OH)$_2$D3 [142], generally displays lower biological activity compared to 1α,25(OH)$_2$D3. It binds to the VDR with 35- to 120-fold lower affinity than 1α,25(OH)$_2$D3 and has markedly reduced ability to stimulate intestinal calcium absorption [73–75]. It also showed reduced ability to stimulate rat *Cyp24a1* gene expression and was less effective in reducing proliferation of HL-60 promyelocytic leukaemia cells and keratinocytes, and in stimulating the differentiation of rat UMR-106 osteosarcoma cells [142]. 3-Epi1α,25(OH)$_2$D3 is metabolized by CYP24A1 in human keratinocytes, but at a slower rate than 1α,25(OH)$_2$D3 [136, 143, 144]. It therefore appears to have greater metabolic stability than 1α,25(OH)$_2$D3, indicating that despite a lower potency and/or efficacy, it may have longer lasting metabolic effects *in vivo*. It should be noted that there are some reported cases where 3-epi-1α, 25(OH)$_2$D3 displays equal or even stronger activity to 1α,25(OH)$_2$D3, such as in the stimulation of apoptosis of HL-60 cells [75], the suppression of parathyroid hormone secretion by cultured parathyroid cells [145] and the stimulation of surfactant synthesis and alveolar septal thinning during perinatal lung maturation [146].

Based on the above, 3-epi-25(OH)D3 is generally considered to be “a less active precursor” than 25(OH)D3 and not reflective of vitamin D status, and hence 25(OH)D3 should be measured separately from 3-epi-25(OH)D3. Additionally, 3-epi-25(OH)D3 displays a higher ionization efficiency than 25(OH)D3 in LC/MS/MS with electrospray ionization, resulting in an overestimation of the contribution of 3-epi-25(OH)D3 in procedures that do not separate the two epimers [147].

**2.1.4. 24R,25(OH)$_2$D3—**24*R*,25(OH)$_2$D3 has been identified as the major dihydroxyvitamin D3 species in serum (see Table 2 and [148]). Its mean concentration

varies considerably between the four studies listed in Table 2. Other studies showed 24*R*,25(OH)$_2$D3 levels within the range listed in Table 2 [149, 150]. A strong correlation between the 24*R*,25(OH)$_2$D3 and 25(OH)D3 concentrations has been seen in a number of studies reflecting that 24*R*,25(OH)$_2$D3 is a primary metabolite of 25(OH)D3 [124, 126, 139, 149–151]. 24*R*,25(OH)$_2$D3 is produced by the action of CYP24A1 (Fig. 2), primarily in the kidney but also in vitamin D target tissues [71]. While 24*R*,25(OH)$_2$D3 represents the first metabolite in the inactivation/removal of 25(OH)D3 by the C24-oxidation pathway catalyzed by CYP24A1 [5, 13, 71, 112, 113], it has biological activity mediated by its binding to FAM57B2 and independent of the VDR, stimulating growth plate development and bone-fracture repair, as described in Section 1.3 [19, 60–64]. The C24 pathways of 25(OH)D3 and 1α,25(OH)$_2$D3 are discussed further in Section 3.2. To our knowledge there are no reports of 24*R*,25(OH)$_2$D2, a product of CYP24A1 action on 25(OH)D2, being present in the serum (see Section 3.3).

**2.1.5. Vitamin D2 and D3—**Quantitative measurements of both vitamin D2 and vitamin D3 in serum were made by LC/MS/MS by Shah and coworkers [125] in the study referred to above for measurement of 25(OH)D2 levels (Table 1). In healthy individuals the mean vitamin D2 and D3 concentrations were 2.3 nM and 7.5 nM, respectively. The vitamin D3 concentration compares well to that of 6.0 nM reported in a separate study by us [152].

## 2.2. Other forms of vitamin D that have been measured quantitatively or qualitatively

**2.2.1. 23,25(OH)$_2$D3—**23*R*,25(OH)$_2$D3 has been measured in human serum with the mean concentration being 0.42 nM [125]. The likely source of this secosteroid is from the action of CYP3A4, a major drug metabolizing cytochrome P450 of the liver, on 25(OH)D3 [153]. A stereoisomer of this, 23*S*,25(OH)$_2$D3, is produced as the first intermediate of the CYP24A1-catalysed C23 oxidation pathway of 25(OH)D3 inactivation [5, 13, 154] and could reasonably be predicted to be present in human serum, being produced in the ratio 1:4 with 24*R*,25(OH)$_2$D3 by human CYP24A1 [112, 113]. It is present in human urine as the glucuronide [155]. It is not clear whether the two C23 epimers were separated under the chromatographic conditions used by Shah and coworkers [125].

**2.2.2. 25,26(OH)$_2$D3—**During the search for the active form of vitamin D3 (1α,25(OH)$_2$D3), one of the first metabolites of 25(OH)D3 identified in plasma was 25,26(OH)$_2$D3. Its concentration was reported to be 1.75 nM using a competitive binding assay [148], but 25,26(OH)$_2$D3 displayed little antirachitic activity and was half as effective as 25(OH)D3 at stimulating intestinal calcium transport [156]. It was originally thought to be a mixture of both C26-hydroxy and C27-hydroxy stereoisomers around the asymmetric C25 carbon (i. e. 25*R*,26(OH)$_2$D3 and 25*S*,26(OH)$_2$D3) [157, 158], but was later reported to be the 25*R*,26(OH)$_2$D3 epimer [159]. 25,26(OH)$_2$D3 was believed to be an intermediate in the C23 oxidation pathway of lactone synthesis catalyzed by CYP24A1, but this was later disproven [160]. It has been reported that 25,26(OH)$_2$D3 can be produced by the action of CYP3A4 on 25(OH)D3 in liver microsomes [153]. It has also been tentatively identified as a minor product of CYP27A1 action on 25(OH)D3 [123, 161]. 25,26(OH)$_2$D3 can undergo 1α-hydroxylation, as demonstrated with chicken kidney homogenates, producing 1α,25,26(OH)$_3$D3 which displays 1 to 10% of the activity of 1α,25(OH)$_2$D3 on intestinal

calcium transport and mobilization of calcium from bone [154]. The 1α-hydroxymetabolite is yet to be detected in human serum. In similar older studies searching for the active form of vitamin D it was reported that 21,25(OH)$_2$D3 is present in the blood of pigs given a high dose of vitamin D3 [156], but whether it is present in human serum, or which enzyme is responsible for its synthesis does not appear to have been addressed.

**2.2.3. 4β,25(OH)$_2$D3 and 4α,25(OH)$_2$D3—**Studies on 25(OH)D3 metabolism by CYP3A4 (and human liver microsomes) have identified 4β,25(OH)$_2$D3 and 4α,25(OH)$_2$D3 as major products [153]. The former was reported to be present in the plasma of 25 healthy adults by LC/MS/MS with a mean concentration of 96 pM, range 5–308 pM, comparable to the mean 1α,25(OH)$_2$D3 concentration (144 pM). Its concentration showed a good correlation with that of 25(OH)D3. The other epimer, 4α,25(OH)$_2$D3, was detected in some but not all of the plasma samples tested. It remains to be determined whether these 4-hydroxy-metabolites of 25(OH)D3 have any biological activity.

**2.2.4. 1β,25(OH)$_2$D3—**Recently it was reported that a metabolite identified as 1β, 25(OH)$_2$D3 based on its chromatographic retention time and its mass spectrum, is present in human serum [162]. However, its identification needs confirmation under a range of HPLC conditions compared to authentic dihydroxyvitamin D3 standards, or preferably by NMR. Its concentration was reported to be 26 pM in healthy subjects (range 7–46 pM), with the 1β/1α ratio ranging from 0.05–0.44. Good correlation of its concentration with that of 25(OH)D3 was observed. Its concentration was similar in patients with kidney failure suggesting that it does not arise from the action of CYP27B1, but rather from an unidentified enzyme acting directly on 25(OH)D3. It has previously been shown to be a poor agonist for the genomic binding site on the VDR but a good antagonist of the non-genomic actions of 1α, 25(OH)$_2$D3 such as the rapid stimulation of calcium transport by the intestine [74, 163, 164]. Its presence causes an added degree of complexity to measurements of serum 1α, 25(OH)$_2$D3 concentrations and thus to their interpretation, with most methods appearing to not distinguish between the two epimers. 1β,25(OH)$_2$D3 can be metabolized by both the C23 and C24 oxidation pathways catalyzed by CYP24A1 [143].

**2.2.5. Conjugated forms of vitamin D—**25-Hydroxyvitamin D3–3-sulfate (25(OH)D3–3-sulfate) has been identified as a major form of vitamin D3 in human serum with the first quantitative measurement on 10 healthy individuals giving a mean serum concentration of 46 nM [165]. The 25(OH)D3–3-sulfate concentration is higher than that of 25(OH)D3 in the fetal circulation, including at birth, where the concentration is approximately 73 nM [166, 167]. A quantitative LC/MS/MS assay for both 25(OH)D3–3-sulfate and 25(OH)D3 in newborn plasma (20 μL) has been reported using DAPTAD derivatization without interference from 3-epi25(OH)D3 [167]. A quantitative assay of four sulfated forms of vitamin D: vitamin D3–3-sulfate, vitamin D2–3-sulfate, 25(OH)D3–3-sulfate and 25(OH)D2–3-sulfate has been recently reported using LC/MS/MS with ESI in the negative ion mode, without sample derivatization [168]. All four sulfated forms were quantitated in human serum, with mean concentrations of vitamin D2–3-sulfate and vitamin D3–3-sulfate being 0.50 and 0.70 nM, respectively, and 25(OH)D2–3-sulfate and 25(OH)D3–3sulfate being 1.5 and 10.4 nM, respectively.

The cytosol from several human tissues including liver and small intestine can catalyze the 3sulfation of 25(OH)D3, with SULT2A1 identified as the major sulfotransferase involved [169]. Other sulfotransferases including SULT2B1a and SULT2B1b can 3-sulfate 7DHC, providing another possible synthetic pathway leading to vitamin D3–3-sulfate and 25(OH)D3–3-sulfate [169]. It has been proposed that 25(OH)D3–3-sulfate is a storage form of 25(OH)D3 [165] but to our knowledge measurements of its half-life in serum are yet to be made. It is likely that its hydrolysis and activation to 1α,25$(OH)_2$D3 are necessary for any biological activity, although no direct measurement of the binding of 25(OH)D3–3-sulfate to the VDR have been made. Vitamin D3–3-sulfate was much less active on bone calcium metabolism when fed to rats compared to vitamin D3 [170]. Since SULT2A1 can act on 1α, 25$(OH)_2$D3, the resulting sulfate ester may also be present in the serum, especially in the fetus.

Recently, 25(OH)D3–3-glucuronide was measured in the serum of six healthy volunteers along with 25(OH)D3–3-sulfate, using DAPTAD sample derivatization. The reported mean concentration of 25(OH)D3–3-glucuronide was 3.4 nM with 25(OH)D3–3-sulfate being 55.6 nM [171]. Vitamin D metabolites are primarily excreted in bile as their glucuronides [155]. The 3-monoglucuronide of vitamin D3 itself has been reported to be present in bile [172] and glucuronides of 23*S*,25$(OH)_2$D3, 24*R*,25$(OH)_2$D3 and 24-oxo,23*S*,25$(OH)_2$D3 have been detected in human urine [155], so these conjugates may also be present at low concentrations in human serum.

**2.2.6. New vitamin D-hydroxyderivatives formed from CYP11A1 action on vitamin D—**Over the last decade it has become clear that CYP11A1, the enzyme that catalyzes the first step in steroid hormone synthesis by hydroxylating then cleaving the side chain of cholesterol to produce pregnenolone, can also hydroxylate the side chains of vitamin D2 and D3 (see Section 1.6.6 and Fig. 4). The two main mono-hydroxylated species produced from D3 are 20*S*(OH)D3 and 22(OH)D3 [118–122]. They are also produced *ex vivo* from vitamin D3 in placenta, adrenal glands, epidermal keratinocytes [122], Caco-2 colon cells [173] and dermal fibroblasts [174], and are detectable in human serum, epidermis and adrenal glands *in vivo* [152]. These secosteroids are active *in vitro* on a range of cell types in culture and act as biased agonists on the VDR, displaying many but not all of the effects of 1α,25$(OH)_2$D3 (reviewed in [52, 67]). They are also active when administered to rodents, reducing the symptoms of skin fibrosis, rheumatoid arthritis and decreasing DNA damage caused by UVB irradiation [175–177]. These novel secosteroids lacking the C1α-hydroxyl group are noncalcemic, while 1α,20*S*$(OH)_2$D3 displays low calcemic activity [68–70, 175]. They also express diverse phenotypic effects dependent on cell type (reviewed in [52, 65, 66]) that are secondary to binding to either the VDR, and/or to RORα and γ on which they act as reverse agonists [20, 21, 67, 178–183].

From semi-quantitative analysis (without deuterated standards to correct for recoveries/matrix effects), the mean serum concentrations of 20*S*(OH)D3 and 22(OH)D3 (from 13 adults) were 2.9 nM and 6.0 nM, respectively [152]. This indicates that these secosteroids are present in serum at comparable concentrations to 3-epi-25(OH)D3 and 24*R*,25$(OH)_2$D3. Fully quantitative measurements using deuterated standards and highly rigorous separation methodologies are therefore urgently required for the accurate measurement of these novel

secosteroids in a large cohort of individuals. It should be noted that because these secosteroids are made by CYP11A1 and require neither CYP2R1 nor CYP27B1 for their synthesis, regulation of their levels may be very different to that for 25(OH)D3 or 1α, $25(OH)_2D3$. While a hormonal role for these hydroxyvitamins can be predicted based on the above serum concentrations and their potencies *in vitro*, this is yet to be established. They are present at relatively high concentrations in the epidermis where they may exert local actions [152].

CYP11A1 produces a number of other hydroxyvitamin D3 derivatives besides 20*S*(OH)D3 and 22(OH)D3. The major pathway for D3 metabolism, as originally determined with purified CYP11A1, is shown in Fig. 4. All the major dihydroxy- and trihydroxy-metabolites shown in Fig. 4 except 17α,20*S*$(OH)_2D3$ (which has not been studied) have been detected in serum by LC/MS/MS, but not quantified (Table 3) [65]. Separation of metabolites was carried out by chromatography on C18 columns with both acetonitrile in water and methanol in water solvent systems. The initial step with an acetonitrile gradient was performed using a 25 cm column which had strong resolving power for these and other secosteroids [113, 121, 184, 185], with the collected fractions then being analyzed by UPLC/MS using a 5 cm column and methanol gradient [152, 173].

Purified CYP27B1 can act on 22(OH)D3, 20*S*(OH)D3, 20*R*,22$(OH)_2D3$ and 20*S*, 23*S*$(OH)_2D3$ hydroxylating them at C1α [103]. To date 1α,20*S*,23*S*$(OH)_3D3$ has been found in human serum (Table 3), epidermis, placenta and the pig adrenal gland. 1α, 20*S*$(OH)_2D3$, while not being detectable in serum, was detected in human epidermis, dermal fibroblasts, placenta and pig adrenal gland, indicating local *in vivo* production [122, 152, 174]. CYP27A1, CYP24A1, CYP2R1 and CYP3A4 can act on 20*S*(OH)D3 producing 20,24$(OH)_2D3$ and/or 20,25$(OH)_2D3$ and/or 20,26$(OH)_2D3$ and all these products have been detected in serum (Table 3). In contrast to 20(OH)D3, they are all very good substrates for C1α hydroxylation by CYP27B1 [103] and their 1α-hydroxyderivatives have been detected in skin [152]. CYP24A1 can also act on 20,23$(OH)_2D3$ producing 20,23,24$(OH)_3D3$ and 20,23,25$(OH)_3D3$ and a number of other products, including one indicative of C23-C24 bond cleavage, but the presence of these metabolites in human serum has not been investigated [185]. Many of the CYP11A1-derived secosteroids listed in Table 3 have potencies, at least on cultured skin cells, as good as or even better than 1α, $25(OH)_2D3$ (reviewed in [21, 67]). While the levels of these metabolites remain to be determined, concentrations in the $10^{-10}$–$10^{-7}$ M range may be physiologically relevant at the systemic level and quantitation of their serum concentrations will be a substantial future challenge for the field of vitamin D metabolomics.

## 3. Other metabolites predicted to be in serum based on known pathways

### 3.1. CYP11A1-derived metabolites

Other CYP11A1-derived metabolites listed in Table 3, not yet detected in serum, may well be present at low concentrations. CYP11A1 can act on vitamin D2 as well as D3, also producing biologically active products. The initial hydroxylation is at C20 producing 20*S*(OH)D2 as the major product, with other products being 17,20$(OH)_2D2$ and 17,20,24$(OH)_3D2$ [180, 186, 187]. 20*S*(OH)D2 is hydroxylated by CYP27B1 producing 1α,

20*S*(OH)$_2$D2 [180]. These secosteroids were produced *exvivo* from D2 in the placenta, adrenal gland, epidermal keratinocytes and colon cells [173], and they are likely to be present in serum, especially in people with high serum vitamin D2 levels. They also appear to be biologically active on normal and malignant skin cells displaying anti-proliferative and pro-differentiation effects [180, 187]. They can act via the VDR [180] and RORα and γ [20, 21].

As well as acting on vitamin D, CYP11A1 also cleaves the side chain of 7DHC to produce 7dehydropregnenolone (7DHP) [118, 188]. It should be noted that 7DHC is a better substrate for CYP11A1 than cholesterol [188] and additional products of its action on 7DHC are 22-hydroxy-7DHC and 20,22-dihydroxy-7DHC [189, 190]. 7DHP can be metabolized by steroidogenic enzymes to several steroidal 5,7-dienes or their derivatives [188–191]. These 5,7-dienes (including their precursors) can be produced in tissues expressing CYP11A1 [189, 190] with some of them being detectable in the human epidermis, serum and pig adrenal glands [152]. It is predicted [188] that these steroidal 5,7-dienes will undergo phototransformation to the corresponding secosteroids after exposure to UVB in the skin, producing vitamin D derivatives with a short (pregnenolone-like) or no (androgenlike) side chain, which could potentially enter the bloodstream [59, 65, 191–193].

These Δ7-steroids and their secosteroidal derivatives display biological activities in normal and malignant cells [194], apparently without influencing calcium metabolism [195]. Specifically, they show *in vitro* anti-proliferative effects against melanoma cells, melanocytes, keratinocytes and fibroblasts as well as anti-fibrotic activities [189, 191, 192, 194, 196, 197]. They also showed moderate anti-leukemic effects [69]. 21(OH)pD (pregnacalciferol) also increased the sensitivity of keratinocytes to reactive oxygen species (ROS) [198] and stimulated the expression of elements of the hypothalamus-pituitary-adrenal axis in keratinocytes [199].

### 3.2. CYP24A1-derived metabolites of vitamin D3

Besides 24*R*,25(OH)$_2$D3, described above, other intermediates of the C24 and C23 oxidation pathways starting from both 25(OH)D3 and 1α,25(OH)$_2$D3 are likely to be present in human serum. The C24 oxidation pathway, starting from 1α,25(OH)$_2$D3 and finishing with the excretory product, calcitroic acid, is shown in Fig. 3 and its elucidation described in Section 1.6.5. The C24 oxidation pathway for 25(OH)D3 is the same except for the lack of the 1α-hydroxyl group.

Intermediates of the 25(OH)D3 oxidation pathway can be acted on by CYP27B1 to convert them into intermediates of the 1α,25(OH)$_2$D3 oxidation pathway [98, 101, 102, 200]. Kinetic studies on human CYP24A1 show that the enzyme releases the intermediates from the active site (as confirmed by the presence of 24*R*,25(OH)$_2$D3 in the serum) and they must compete with substrate and other intermediates for conversion to calcitroic acid [113]. Thus we predict that all intermediates, except the unstable aldehyde (1α-(OH)-23-oxo-24,25,26,27-tetranorvitamin D3), will be present in human serum. In support of this, 1α,24*R*,25(OH)$_3$D3 and 24-oxo-1α,25(OH)$_2$D3 have been detected in the plasma of rats and dogs given a pharmacological dose of 1α,25(OH)$_2$D3 [201, 202]. Another secosteroid, 25,26,27-trinor-23-ene-1α-hydroxyvitamin D3 has been identified as a minor product of

CYP24A1 action on 1α,25(OH)$_2$D3 [203], presumably arising from some cleavage between C24 and C25 rather than C23 and C24.

Approximately 20% of 25(OH)D3 and 1α,25(OH)$_2$D3 are oxidized by the C23 oxidation pathway catalyzed by CYP24A1 (Fig. 3), with initial production of 23*S*,25(OH)$_2$D3 or 1α,23*S*,25(OH)$_3$D3, respectively [112, 113]. These are subsequently hydroxylated at C26 followed by formation of the C23-C26 lactol which is oxidized to the lactone, 1α,25(OH)$_2$D3–23-26-lactone in the case of 1α,25(OH)$_2$D3 (Fig. 3). All these intermediates are also likely to be present at low levels in human serum, with 1α,25(OH)$_2$D3–23-26-lactone having been detected in the serum of rats and dogs administered 1α,25(OH)$_2$D3 [201, 202]. Intermediates of both the C23 and C24 oxidation pathways starting from 25(OH)D3, namely 24*R*,25(OH)$_2$D3, 23*S*,25(OH)$_2$D3 and 24-oxo-23*S*,25(OH)$_2$D3 have been detected as glucuronides in human urine [155].

While both the C24 and C23 oxidation pathways catalyzed by CYP24A1 are believed to be primarily involved in inactivation of 1α,25(OH)$_2$D3 and removal of its precursor 25(OH)D3, some metabolites retain activity. As mentioned earlier (Section 1.3), 24*R*,25(OH)$_2$D3, the first intermediate of 25(OH)D3 catabolism by the C24 oxidation pathway, can stimulate growth plate development and bone-fracture repair, but this does not appear to be mediated by binding to the VDR [18, 19, 60–63]. The activity of 1α,25(OH)$_2$D3 metabolites on intestinal calcium absorption and bone calcium mobilization in the chick declined with successive steps in the C24 oxidation pathway with the third product, 24-oxo-1α,23*S*,25-(OH)$_3$D3, having only a modest effect [204]. However, all C24 oxidation pathway intermediates with a full-length side chain (prior to cleavage to give the C23 aldehyde, 1α(OH)-23-oxo-24,25,26,27-tetranorvitamin D3, Fig. 3), were able to stimulate HL-60 cells to differentiate into monocytes/macrophages with comparable potency and efficacy to 1α,25(OH)$_2$D3 [205]. The last intermediate with an intact side chain, 24-oxo-1α,23*S*,25(OH)$_3$D3, was equipotent to 1α,25(OH)$_2$D3 for inhibiting clonal growth of HL-60 cells in soft agar [205]. It was also equipotent in the suppression of parathyroid hormone secretion by bovine parathyroid cells, despite being 10 times less effective than 1α,25(OH)$_2$D3 in competing with radiolabeled 1α,25(OH)$_2$D3 for binding to the VDR [206]. The final product of the C23 oxidation pathway, 1α,25(OH)$_2$D3–23-26-lactone, retains some ability to stimulate intestinal calcium uptake and bone calcium mobilization in the chick [207]. For 20*S*(OH)D3, CYP24A1 may play an activating role since hydroxylation at C24 or C25 by this enzyme gives products more potent than 20*S*(OH)D3 (and 1α,25(OH)$_2$D3) for the inhibition of colony formation by melanoma cells [208].

### 3.3. CYP24A1-derived metabolites of vitamin D2

Metabolism of 1α,25(OH)$_2$D2 to 1α,24,25(OH)$_3$D2 by bovine kidney homogenates, presumably by CYP24A1, was reported in 1986, with this metabolite showing poor ability to stimulate intestinal calcium transport and bone resorption in rats compared to 1α,25(OH)$_2$D3 [209]. Subsequent studies with bacterially expressed human CYP24A1 demonstrated that it can hydroxylate 1α,25(OH)$_2$D2 at C24, C26 and C28 producing at least 10 metabolites with tentatively identified ones including 1α,24,25,26(OH)$_4$D2 and 1α,24,25,28(OH)$_4$D2 [114]. Some 24-oxo-25,26,27-trinor-1α(OH)D2 was also produced

indicating bond cleavage can occur between C24 and C25, from precursor 1α,24,25(OH)$_3$D2. Perfusion of rat kidneys with 1α,25(OH)$_2$D2 resulted in the production of both 1α,24*R*,25,28(OH)$_4$D2 and 1α,24*S*,25,28(OH)$_4$D2 as the only two major products [210]. However, differences in the metabolism of 1α,25(OH)$_2$D2 by expressed human and rat CYP24A1 have been noted [114] so the rat kidney is not a good model for the human kidney for metabolism of vitamin D2 substrates. The biological activity of 1α,24*S*,25,28(OH)$_4$D2 was tested in rats and found to be low compared to 1α,25(OH)$_2$D3 with respect to intestinal calcium transport and bone calcium resorption [210], so conversion to this product is consistent with an inactivation role for CYP24A1 on 1α,25(OH)$_2$D2. The human keratinocyte cell line, HPK1A-*ras* was reported to convert 1α,25(OH)$_2$D2 to calcitroic acid [211] which is surprising since the presence of the C22-C23 double bond and the C24-methyl group (C28) in 1α,25(OH)$_2$D2 would hinder this from occurring by the pathway elucidated for 1α,25(OH)$_2$D3 metabolism (Fig. 3).

25(OH)D2 has been reported to be metabolized to 24,25(OH)$_2$D2 and 24,25,26(OH)$_3$D2 by perfused rat kidneys [212, 213]. It has also been shown to be metabolized to 24*R*,25,28(OH)$_3$D2 and 24*S*,25,28(OH)$_3$D2 by perfused rat kidneys and rat kidney homogenates with evidence for further metabolism to water-soluble products [214], presumably by CYP24A1. The metabolism of 25(OH)D2 would therefore appear to be similar to that for its 1α-hydroxylated product, described above. However, detailed studies of 25(OH)D2 metabolism by recombinant human CYP24A1 are lacking to date. The presence of the CYP24A1-derived metabolites of 25(OH)D2 and 1α,25(OH)$_2$D2 (described above) in human serum is likely, especially where dietary vitamin D2 supplementation is used.

### 3.4. Vitamin D metabolites produced by CYP27A1

CYP27A1 acts on vitamin D3 producing 25(OH)D3 as the major product but other minor metabolites including (25*R*)-27(OH)D3 and (25*S*)-27(OH)D3 have been tentatively identified in studies with the enzyme expressed in COS-1 monkey kidney cells [87] and *E. coli* [123]. Both C25 epimers of 27(OH)D3 were reported to stimulate the production of intestinal calcium binding protein and to increase serum calcium levels in vitamin D-deficient rats fed a low-calcium diet [215]. These effects appeared to be dependent on 1α-hydroxylation as they were not seen in nephrectomized rats.

CYP27A1 does not hydroxylate vitamin D2 at C25 but rather at C24 and C27 producing 24(OH)D2 and 27(OH)D2 [87]. Human HepG2 cells (which express CYP27A1) can hydroxylate 1α-(OH)D2 at C24 producing 1α,24*S*(OH)$_2$D2 suggesting that the 24*S* isomer might also be produced with vitamin D2 as substrate [87]. The stereochemistry of hydroxylation at C27 has not been addressed for vitamin D2.

24(OH)D2 and 1α,24(OH)$_2$D2 have been detected in rat blood from animals supplemented with vitamin D2 [216]. 24(OH)D2 can by hydroxylated in the 1α-position by rat kidneys (presumably by CYP27B1) as a likely source of 1α,24(OH)$_2$D2 *in vivo* [216]. Similarly, these metabolites were measured in human patients with disorders in calcium homeostasis that were given large doses of vitamin D2 [217]. The chromatographic conditions used did not separate possible 24*R* and 24*S* epimers of 24(OH)D2. 24(OH)D2 concentrations were in the nM range while 1α,24(OH)$_2$D2 levels were in the pM range and generally comparable to

the 1α,25(OH)$_2$D2 concentrations. With more sensitive assays it is likely that these metabolites will be found in the serum of humans with a more physiological vitamin D2 intake. However, 24(OH)D2 was recently reported to be below the limit of quantitation in all serum samples from 53 healthy volunteers with a mean 25(OH)D2 concentration of 4.5 nM [127]. 1α,24(OH)$_2$D2 is biologically active, binding to the VDR and inhibiting skin cell proliferation with comparable potency to 1α,25(OH)$_2$D3, but with a lesser ability to raise serum calcium levels in rats [216, 218, 219].

### 3.5. Vitamin D metabolites produced by CYP3A4

As described in Sections 2.2.1 to 2.2.3, CYP3A4 hydroxylates 25(OH)D3 producing 23*R*, 25(OH)$_2$D3, 4β,25(OH)$_2$D3, 4α,25(OH)$_2$D3 and 25,26(OH)$_2$D3 [153], which have been measured in serum (Table 1). CYP3A4 hydroxylates 1α,25(OH)$_2$D3 at C23 and C24, producing the two C23 epimers, 1,23*R*,25(OH)$_3$D3 and 1,23*S*,25(OH)$_3$D3, as well as 1,24*S*, 25(OH)$_3$D3 [220, 221]. The latter C24*S* epimer is the opposite configuration to that made by CYP24A1, 1,24*R*,25(OH)$_3$D3 (Fig. 3). These metabolites are yet to be detected in serum. CYP3A4 can also hydroxylate 20*S*(OH)D3 producing 20*S*,24(OH)$_2$D3 and 20*S*,25(OH)$_2$D3 (Table 3) [222].

CYP3A4 acts as an alternative 25-hydroxylase to CYP2R1 on vitamin D2 producing 25(OH)D2, but unlike CYP2R1 it cannot hydroxylate vitamin D3 at C25 [223, 224]. CYP3A4 also converts vitamin D2 to 24(OH)D2 [223]. This product and its CYP27B1 metabolite, 1α,24(OH)$_2$D2, have been detected in the serum of patients treated with a large daily dose of vitamin D2 [217]. The stereochemistry at C24 has not been determined.

### 3.6. 3-Epimers of vitamin D

As noted in Section 2.1.3, 25-hydroxyvitamin D 3-epimerase converts 25(OH)D3 to 3-epi-25(OH)D3 and 25(OH)D2 to 3-epi-25(OH)D2, and both of these epimers are present in human serum.

Microsomes containing the epimerase have also been reported to act on 1α,25(OH)2D3 and 24*R*,25(OH)$_2$D3 producing the 3α-epimers [72]. In addition, 3-epi-1α,25(OH)$_2$D2 has been detected in rat plasma following the administration of a pharmacological dose of 1α, 25(OH)2D2, indicating the reaction occurs *in vivo* [202]. There is an urgent need to purify the epimerase and identify the gene encoding it to more fully elucidate its regulation and influence on the vitamin D metabolome. It is not yet known if it can act on 20*S*(OH)D3 or its hydroxyderivatives, or other vitamin D metabolites mentioned above. Because both CYP27B1 and CYP24A1 can act on 3-epi-25(OH)D3 [136, 142–144], a small fraction of all the intermediates of the C23- and C24-oxidation pathways catalyzed by CYP24A1 could be present in human serum as the 3α-epimers, too. The 25-hydroxyvitamin D 3epimerase thus has the potential to essentially double the complexity of the vitamin D metabolome!

## 4. Vitamin D-related metabolites in serum

Prolonged exposure of skin to UV radiation causes photoconversion of previtamin D3 to lumisterol3 (L3) and tachysterol3 (Fig. 2). Tachysterol3 also absorbs UV radiation which converts it to L3 via previtamin D3 so that L3 predominates in skin after extended exposure

to UVB [2, 3]. The conversion of previtamin D3 to L3 was thought to provide a protective mechanism to prevent vitamin D3 intoxication, with L3 believed to be an inactive end product [1]. Recently we reported that L3 can be hydroxylated by CYP11A1 which is present at low concentrations in skin and high concentrations in the adrenal cortex [184, 225]. Major products were identified as 20-(OH)L3, 22-(OH)L3, 24(OH)L3 and 20,22-$(OH)_2$L3, with a small amount of the C20-C22 cleavage product, pregnalumisterol (pL) [184, 225]. We observed that L3 and some of its CYP11A1-derived products are present in serum (Table 1) [225]. The mean concentration of L3 (51 nM) in 13 samples analyzed was 10 times higher than that of vitamin D3 (5.0 nM) and similar to the serum 7DHC concentration [225]. 20(OH)L3, 22-(OH)L3, 20,22-$(OH)_2$L3 and pL were also measured in serum with the 20-(OH)L3 concentration being highest at 25.2 nM, more than half the mean 25(OH)D3 concentrations listed in Table 2. All these hydroxylumisterols were also detected in skin [225] which contains a low CYP11A1 concentration [115, 188]. However, since L3 is present in the bloodstream, the major source of these hydroxylumisterols in the serum may be the adrenal cortex and to a lesser degree the gonads, where concentrations of CYP11A1 are high.

The CYP11A1-derived hydroxylumisterols display potent biological activity *in vitro*, with many effects overlapping with those of 1α,25$(OH)_2$D3. For example, they inhibit SKMEL 188 melanoma cell colony formation in soft agar with $EC_{50}$ values in the $10^{-9}$ to $10^{-10}$ M concentration range [225]. They also inhibit keratinocyte proliferation [225]. Analysis of a panel of genes expressed in epidermal keratinocytes showed that they stimulated the expression of many genes including differentiation markers, genes encoding anti-oxidative enzymes and stress response genes [225]. Chemically synthesized 1α,25$(OH)_2$L3, while not identified as a natural product, is an excellent agonist for the non-genomic pocket of the VDR (A-pocket) and has photoprotective effects mediated by increased expression of DNA repair enzymes [57]. Docking studies predict that the CYP11A1-derived hydroxylumisterols also bind to this site better than to the genomic site suggesting that this binding may mediate their activity [225]. There is also evidence from molecular modeling, ligand binding and cell based reporter assays that the hydroxylumisterols can also act via the nuclear receptors, ROR α and γ, as inverse agonists [225].

Most recently we have shown that CYP27A1, primarily a cholesterol 27-hydroxylase involved in bile acid synthesis in liver, can hydroxylate L3 with high catalytic efficiency, comparable to that of its best known natural substrate, 5β-cholestane-3α,7α,12α-triol [89]. The three major products were 25(OH)L3, (25*R*)-27-(OH)L3 and (25*S*)-27-(OH)L3. All three displayed biological activity, inhibiting melanoma cell colony formation, similarly to that seen for the CYP11A1-derived hydroxylumisterols. Given the relatively high concentration of L3 in serum, noted above, and the high concentration of CYP27A1 in liver mitochondria [83, 85], it is highly likely that these new metabolites will be present in serum.

Metabolism of the other previtamin D3 over-radiation product, tachysterol3, has not been investigated in detail yet but based on the L3 data it is likely to be present in the serum and possibly metabolized by CYP11A1 or other vitamin D-metabolizing CYPs. In preliminary studies in our laboratories (unpublished), incubations of tachysterol3 with pig adrenal glands and liver extracts led to the generation of species corresponding to mono- and

dihydroxytachysterols by LC/MS, however the lack of chemical standards has, as yet, prevented their full identification.

## 5. An extended array of vitamin D metabolites is of clinical use

Vitamin D status is routinely assessed by measuring serum 25(OH)D3 levels as this is relatively easy to measure and has a long half-life in the bloodstream, in the order of 2–3 weeks [129]. 1α,25$(OH)_2$D3 on the other hand is more difficult to measure, being present in serum at a concentration almost 1000 fold lower than that of 25(OH)D3. Also, it has a much shorter half-life, in the order of 4 h, and thus does not necessarily reflect long term vitamin D status. 3-Epi-25(OH)D3 is now routinely measured separately from 25(OH)D3 as it is generally regarded as a “less active precursor” as described in Section 2.1.3. The ratio of 3-epi-25(OH)D3 to total circulating vitamin D3 shows promise for the detection of disease states such as rheumatoid arthritis, type 1 diabetes and Alzheimer’s disease [125]. The measurement of 24*R*,25$(OH)_2$D levels along with that of 25(OH)D3 has been reported to provide additional useful information on vitamin D status and has led to the determination of the serum 24*R*,25$(OH)_2$D3 to 25(OH)D3 ratio, referred to as the vitamin D metabolite ratio (VMR) [139, 149, 151, 226, 227]. It is predictive of the response of 25(OH)D3 to vitamin D supplementation. The rationale for its use is that the 24*R*,25$(OH)_2$D3 concentration provides information on the activity of 1α,25$(OH)_2$D3 on the VDR since 1α,25$(OH)_2$D3 strongly stimulates the expression of *CYP24A1*, which encodes the enzyme responsible for converting 25(OH)D3 into 24*R*,25$(OH)_2$D3. Both the VMR and higher 24*R*,25$(OH)_2$D3, but not the 25(OH)D3 concentration, were found to correlate with a decreased incidence of hip fractures in elderly adults [226].

A number of genetic disorders in vitamin D metabolism resulting from mutations in *CYP* genes encoding vitamin D hydroxylases have been described (reviewed in [228]). Measuring a range of vitamin D metabolites may assist in the initial identification of possible polymorphisms and genetic deficiencies, especially where the mutation does not cause a severe disease or an obvious phenotype. For example, normal 25(OH)D3 and decreased 1α,25$(OH)_2$D3 concentrations may be indicative of a partial CYP27B1 deficiency while low 24*R*,25$(OH)_2$D3 with normal 25(OH)D3 and elevated 1α,25$(OH)_2$D3 levels may indicate CYP24A1 deficiency [228].

The relatively high concentrations of 25(OH)D3–3-sulfate in serum, particularly in infants (section 2.2.5) suggests that this metabolite should be measured to fully assess vitamin D status. Its concentrations in adults are comparable to those of 3-epi-25(OH)D3, but unlike 3-epi-25(OH)D3 it may be physiologically active in terms of being able to be converted to 1α,25$(OH)_2$D3. However, how rapidly it can be converted to 25(OH)D3 to “buffer” 25(OH)D3 concentrations remains to be established.

Currently there is insufficient information on the physiological importance of some of the newly identified vitamin D metabolites such as 20*S*(OH)D3, 22(OH)D3 and 20,23$(OH)_2$D3, which result from the action of CYP11A1 on vitamin D3 (section 2.2.6), to evaluate their contribution to vitamin D sufficiency for non-skeletal actions. Given that they show beneficial effects without calcemic activity in cell culture and animal experiments, a detailed

study of how their concentrations vary with sun exposure, vitamin D supplementation and disease states is required. Finally, the discovery of L3 in the serum, and more importantly its side-chain hydroxyderivatives at relatively high concentrations, suggests that these might be important metabolites to measure in the future given that they display potent biological activity on cultured cells, with some effects similar to those displayed by 1α,25(OH)$_2$D3. Since they are derived from previtamin D3 and not vitamin D3 or D2 (Fig. 2), UV radiation but not dietary supplements of these vitamins, will elevate their levels.

## 6. Concluding remarks

The ability to separate complex mixtures of hydroxyvitamin D compounds by HPLC and to detect small quantities by mass spectrometry has resulted in a substantial expansion of the vitamin D metabolome in recent years. Since mass spectrometry is not particularly good at distinguishing between different monohydroxyvitamin D3 species or between different dihydroxyvitamin D3 species, with many geometric and stereoisomers displaying similar ions, identifications are commonly based on LC retention times. The same applies to the different monohydroxy and dihydroxyvitamin D2 species. It is therefore very important that a full range of standards is available to compare retention times with any newly discovered metabolite in the serum, and that a range of chromatographic conditions be used in the separation and identification. Deuterated or $^{13}$C-labelled secosteroids are required as standards for quantitation by mass spectrometry and their chemical synthesis can represent a major endeavor or expense. Enzymatic production of these secosteroids using recombinant vitamin D-metabolizing CYPs is a good option for small scale synthesis of these standards.

It is likely that with further improvements in sample preparation, derivatization and mass spectrometry, more vitamin D metabolites will be added to the vitamin D metabolome. Detailed analysis of the catalytic specificities and substrate profiles of recombinant CYPs has helped to identify potential new members of the vitamin D metabolome, and chemical and enzymatic synthesis has already enabled the biological activities of some to be assessed, as described in this review.

Many of the more recently discovered metabolites of vitamin D in the serum, including those produced by CYP11A1 such as 20*S*(OH)D3 and 22(OH)D3, require a more thorough investigation of their physiological importance. *In vitro* experiments indicate high potency, in many cases comparable to 1α,25(OH)$_2$D3, so serum concentrations in the low nM range may be enough for an important physiological role. Furthermore, some of these hydroxyderivatives appear to be biased agonists on the VDR, exerting full biological activity with respect to their actions on some genes regulated via the VDR, but with little effect on others. For example, 20*S*(OH)D3 inhibits cell proliferation and promotes keratinocyte differentiation with a potency similar to 1α,25(OH)$_2$D3, but has no effect on calcium levels and only a minimal effect on *CYP24A1* expression [66, 67]. Lastly, the finding that several hydroxyvitamin D3 compounds and the newly discovered hydroxylumisterols can act on the RORs α and γ [20, 225], the reported involvement of the 1,25D3-MARRS in some of the actions of 1α,25(OH)$_2$D3 [16, 17], plus the VDR-independent actions of 24*R*,25(OH)$_2$D3, indicate that receptors besides the VDR need to be considered when assessing the relationship between vitamin D physiology and the serum vitamin D metabolome.

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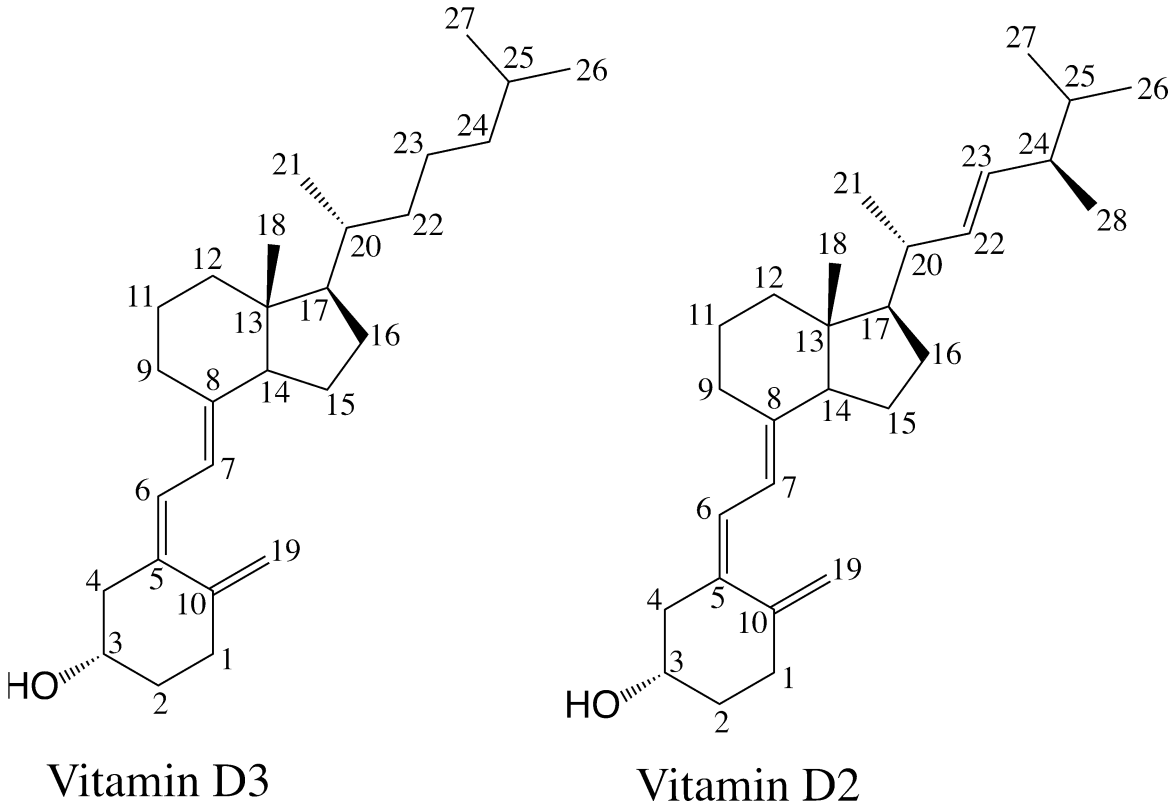


**Fig 1.**
The structures of vitamins D3 and D2 with carbon numbering.

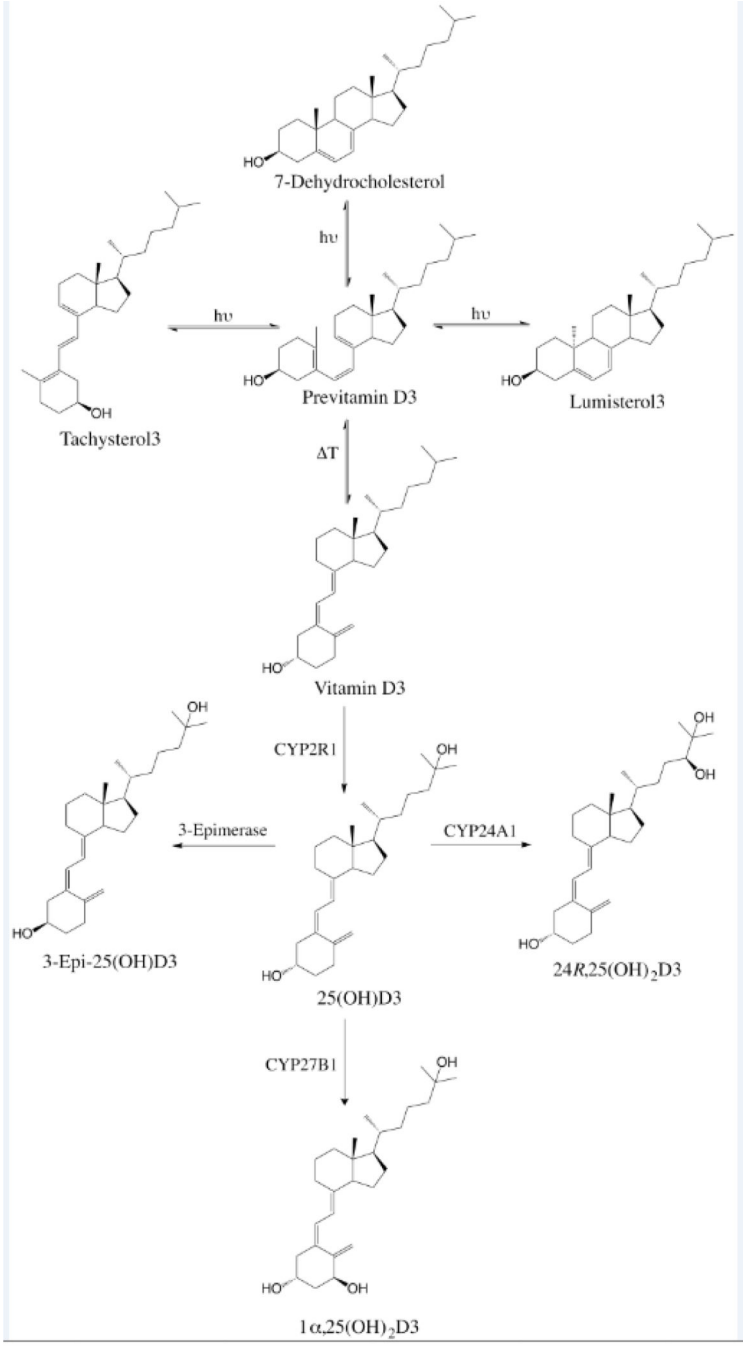


**Fig. 2.**

Major classical pathways for the synthesis, activation and inactivation of vitamin D3. Note that vitamin D2 is activated by CYP2R1 and CYP27B1 as shown for 25(OH)D3, can undergo a similar epimerization producing 3-epi-25(OH)D2 and can also be hydroxylated at C24 by CYP24A1.

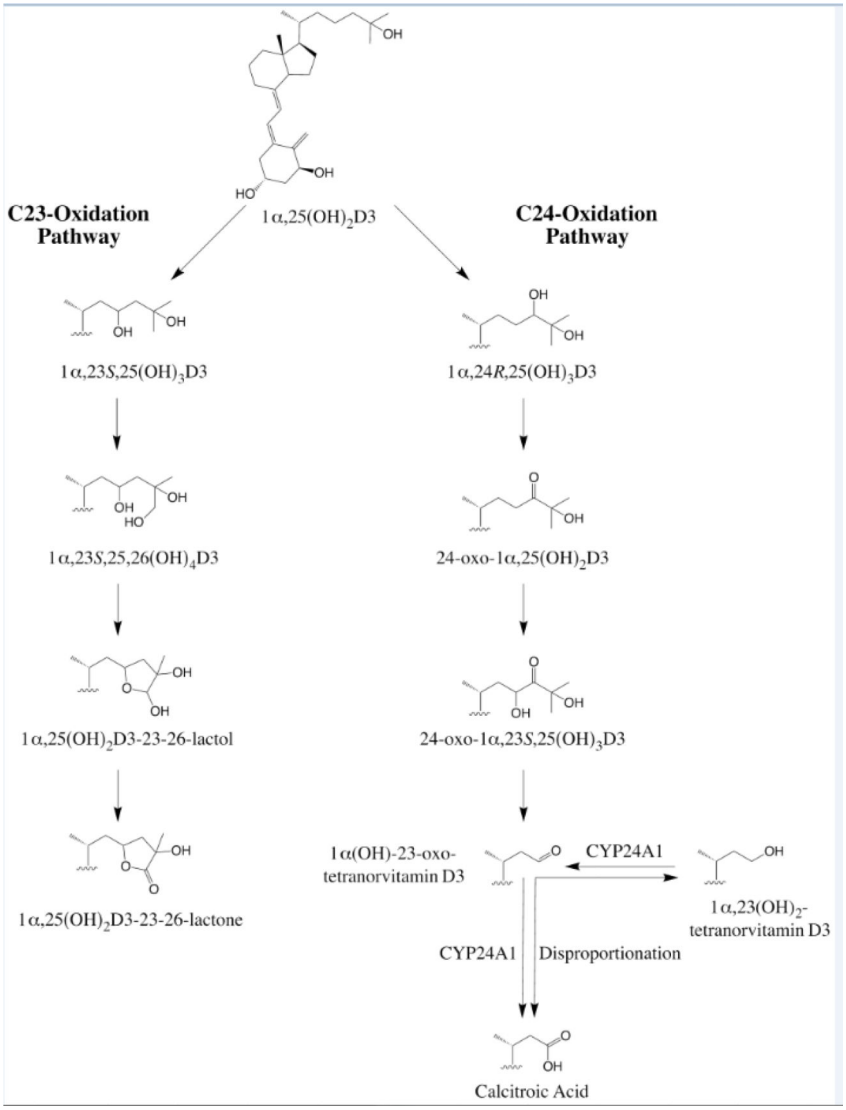


**Fig. 3.**

The C23 and C24 oxidation pathways of 1α,25(OH)$_2$D3 metabolism by CYP24A1. A nonenzymatic product of the pathway, 1α,23-dihydroxy-24,25,26,27-tetranorvitamin D3, results from disproportionation of the C23 (oxo) aldehyde (2 × C23 aldehyde → 1 × C23 alcohol + 1 × C23 carboxylic acid [113]. 25(OH)D3 undergoes similar C23 and C24 oxidation pathways to those shown for 1α,25(OH)$_2$D3.

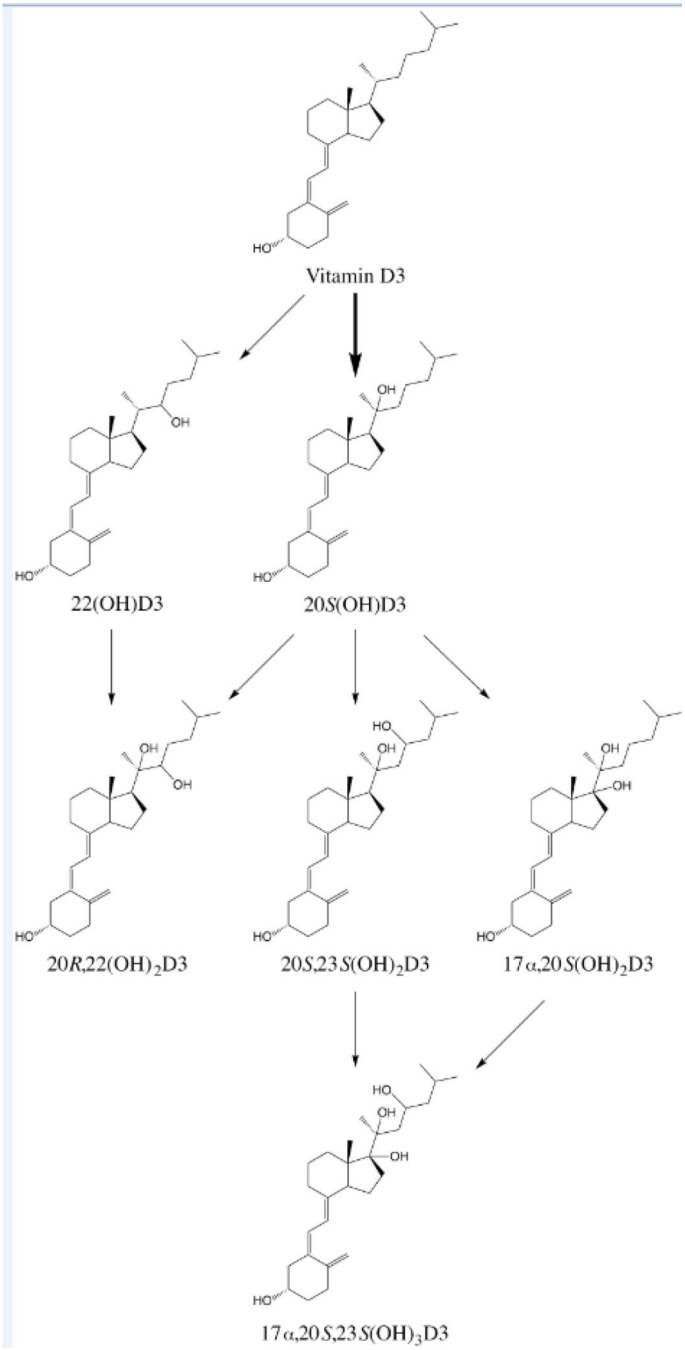


**Fig. 4.**

Pathways for the metabolism of vitamin D3 by CYP11A1. The bold arrow indicates the major pathway producing 20*S*(OH)D3. Further metabolism of the products of this pathway by other CYPs is shown in Table 3.

**Table 1.**

Summary of major vitamin D metabolites measured in human serum. Routine, refers to the quantitative measurement of multiple samples in multiple laboratories. Semi-quantitative refers to LC/MS/MS procedures that did not include an isotopically-labelled standard for quantitation. Additional metabolites derived from the action of CYP11A1 that have been detected but not quantified in human serum are listed in Table 3. See text for references to the measurement of each metabolite.

| Metabolite type | Metabolite | Measurement type |
|---|---|---|
| parent vitamin D | vitamin D3 | routine |
| | vitamin D2 | routine |
| monohydroxyvitamin D | 25(OH)D3 | routine |
| | 25(OH)D2 | routine |
| | 3-epi-25(OH)D3 | routine |
| | 3-epi-25(OH)D2 | quantitative |
| | 20*S*(OH)D3 | semi-quantitative |
| | 22(OH)D3 | semi-quantitative |
| dihydroxyvitamin D | 1α,25(OH)2D3 | routine |
| | 1α,25(OH)2D2 | routine |
| | 1β,25$(OH)_2$D3 | quantitative |
| | 23*S*,25$(OH)_2$D3 | quantitative |
| | 24*R*,25$(OH)_2$D3 | routine |
| | 25,26$(OH)_2$D3 | quantitative |
| | 4α,25(OH)2D3 | quantitative |
| | 4β,25$(OH)_2$D3 | quantitative |
| vitamin D conjugates | vitamin D3–3-sulfate | quantitative |
| | vitamin D2–3-sulfate | quantitative |
| | 25(OH)D3–3-sulfate | quantitative |
| | 25(OH)D2–3-sulfate | quantitative |
| | 25(OH)D3–3-glucuronide | quantitative |
| Lumisterol3 (L3) | L3 | semi-quantitative |
| | 20(OH)L3 | semi-quantitative |
| | 22(OH)L3 | semi-quantitative |
| | 20,22$(OH)_2$L3 | semi-quantitative |
| | pregnalumisterol (pL) | semi-quantitative |

**Table 2.**

Mean concentrations of the most commonly measured vitamin D hydroxymetabolites in human serum from four studies. NM, not measured; BLQ, below limit of quantitation.

| 25(OH)D3 (nM) | 25(OH)- D2 (nM) | 3-Epi-25(OH)D3 (nM) | 24*R*,25-$(OH)_2$D3 (nM) | 1α,25-$(OH)_2$D3 (nM) | 1α,25-$(OH)_2$D2 (nM) | Sample size | Reference |
|---|---|---|---|---|---|---|---|
| 27.7 | 17.9 | 6.1 | 0.92 | 0.027 | 0.17 | 32 | [125] |
| 42.6 | 1.7 | 1.5 | 3.7 | NM | NM | 156 | [126] |
| 39.3 | 4.5 | 4.75 | 6.0 | 0.115 | BLQ | 42–116 | [127] |
| 54.9 | 3.4 | NM | 14.0 | 0.100 | NM | 22 | [124] |

**Table 3.**

Metabolites produced by CYP11A1 and further metabolites produced from the action of other CYPs. The stereochemistry of the primary CYP11A1-derived products, where known, is given. Where metabolites have not been detected is serum, their detection in either human skin or the porcine adrenal gland is indicated, as reported in [152]. ND, not determined.

| CYP11A1 metabolite | Further metabolite | CYPs involved | Site of detection | References |
|---|---|---|---|---|
| 20*S*(OH)D3 | | | human serum | [118–120, 122] |
| | 1,20(OH)$_2$D3 | 27B1 | human skin | [103, 122] |
| | 20,24(OH)$_2$D3 * | 3A4 or 24A1 | human serum | [185, 208, 222] |
| | 20,25(OH)$_2$D3 | 2R1, 3A4, 24A1 or 27A1 | human serum | [88, 93, 185, 222] |
| | 20,26(OH)$_2$D3 | 27A1 | human serum | [88] |
| | 1,20,24(OH)$_3$D3 | 3A4 or 24A1 and 27B1 | porcine adrenal gland | [103] |
| | 1,20,25(OH)$_3$D3 | 2R1, 3A4, 24A1 or 27A1 and 27B1 | porcine adrenal gland | [103] |
| | 1,20,26(OH)$_3$D3 | 27A1 and 27B1 | porcine adrenal gland | [103] |
| 22(OH)D3 | | | human serum | [121, 122] |
| | 1,22(OH)$_2$D3 | 27B1 (low activity) | ND | [103] |
| 20*R*,22(OH)$_2$D3 | | | human serum | [121, 122] |
| | 1,20,22(OH)$_3$D3 | 27B1 (low activity) | ND | [103] |
| 20*S*,23*S*(OH)$_2$D3 | | | human serum | [120–122, 229] |
| | 1,20,23(OH)$_3$D3 | 27B1 | human serum | [103] |
| | 20,23,24(OH)$_3$D3 | 24A1 | ND | [185] |
| | 20,23,25(OH)$_3$D3 | 24A1 | ND | [185] |
| 17α,20*S*(OH)$_2$D3 | | | ND | [120, 121] |
| 17α,20*S*,23*S*(OH)$_3$D3 | | | human serum | [120–122] |

* Both 24*R* and 24*S* epimers of 20,24(OH)2D3 have been identified as products of CYP24A1 and CYP3A4 action on 20*S*(OH)D3.