Review

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Assessing vitamin D metabolism – four decades of experience

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Abstract: One hundred years ago, the role of vitamin D for bone mineralization and the prevention of rickets was discovered. Vitamin D comprises a group of over 50 metabolites with multiple functions that go far beyond calcium homeostasis and bone mineralization. Approximately 50 years ago, first methods for the measurement of 25-hydroxyvitamin D (25(OH)D) in human blood were developed. Over the years, different analytical principals were employed including competitive protein binding assays, high-performance liquid chromatography, various immunoassay and mass spectrometric formats. Until the recent standardization of serum 25(OH)D measurement, agreement between methods was unsatisfactory. Since then, comparability has improved, but substantial variability between methods remains. With the advent of liquid chromatography tandem mass spectrometry (LC-MS/MS), the accurate determination of 25(OH)D and other metabolites, such as 24,25(OH)2D, becomes increasingly accessible for clinical laboratories. Easy access to 25(OH)D testing has triggered extensive clinical research showing that large parts of the population are vitamin D deficient. The variable response of vitamin D deficient individuals to supplementation indicates that assessing patients' vitamin D stores by measuring 25(OH)D provides limited insight into the metabolic situation. Meanwhile, first evidence has emerged suggesting that the simultaneous measurement of 25(OH)D, 24,25(OH)2D and other metabolites allows a dynamic evaluation of patients' vitamin D status on metabolic principals. This may help to identify patients with functional vitamin D deficiency from those without. It can be expected that research into the assessment vitamin D status will continue for another 50 years and that this will help rationalizing our approach in clinical practice.

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Historical aspects

One-hundred years ago, McCollum et al. introduced the term vitamin D into the scientific literature as an antirachitic substance that is distinct from vitamin A [1]. Rickets, a bone disease that was first mentioned in the 17th century in England, is characterized by impaired bone mineralization, disturbed bone growth and skeletal deformations. In adults, the condition is known as osteomalacia. Observations that rickets could be successfully treated by the use of cod-liver oil and sunlight-exposure sparked discussions whether the disease was caused by the deficiency of a nutritional compound or an environmental factor [2]. The mechanistic relationship between sunlight exposure and nutrition was finally unravelled by Steenbock and Black in 1924, when they showed that the irradiation of selected food items increased their vitamin D activity [3]. This invention led to the establishment of the Wisconsin Alumni Research Foundation (WARF), an organization that pioneered modern food fortification with a patent for the irradiation of yeast added to milk to increase vitamin D content. A few years later, Adolf Windaus identified the chemical structure of vitamin D_3 and its precursor 7-dehydrocholesterol, which earned him the Nobel Prize in 1928 [4]. These milestones of scientific history paved the way for widespread food fortification in the following decades, which led to a drastic reduction of rickets and osteomalacia. While some countries, such as Canada, adopted mandatory vitamin D fortification of milk, the UK ended milk powder and margarine fortification with vitamin D in 1953 after a series of cases with hypercalcaemia that were believed to reflect vitamin D toxicity [5]. Consequently, rickets and osteomalacia are still highly prevalent in the UK [6]. In a study amongst pregnant women, 36% were found to be deficient [6]. Although food fortification drastically reduced rickets and osteomalacia soon after its introduction, vitamin D remained a research topic of great interest until today.

Biochemistry

Synthesis of vitamin D

The term vitamin D does not refer to a single compound, but to a group of over 50 chemically related metabolites with more or less anti-rachitic activity [7–10]. Chemically, they are secosteroids with an open B-ring that forms when UV-B irradiation cleaves the bond between the carbon atoms C9 and C10 of the precursor molecule 7-dehydrocholesterol. Due to the lipophilic nature of secosteroids they circulate bound to vitamin D binding protein (VDBP), albumin and lipoproteins. Skin-derived cholecalciferol (vitamin D_3) and food-derived ergocalciferol (vitamin D2) are the two main forms of vitamin D with vitamin D_2 having an additional double bond between C22 and C23 and a methyl group on C24 [11]. Under physiologic circumstances, food-derived vitamin D can contribute 10–20% of the total vitamin D supply of humans [12–14]. Good sources of vitamin D are fatty fish, liver oil, and egg yolk [12–14]. Higher amounts of vitamin D can be obtained from fortified foods, such as milk and margarine, or vitamin supplements [15].

Vitamin D_2 and D_3 are further metabolized by the same enzymes and form similar metabolites. For each metabolite, the vitamin D_2 derived fraction is rather small and cannot be measured with standard techniques including liquid chromatography tandem mass spectrometry (LC-MS/MS) methods that are commonly used in medical laboratories. The exception is 25(OH)D_2, which is routinely captured by immunoassays and LC-MS/MS methods. Only in patients who supplement vitamin D_2, relevant amounts of other vitamin D_2 metabolites can be encountered. Throughout the remaining section of this review, vitamin D_2 and D_3 metabolites will be mentioned separately only when necessary. In order to gain biological activity, vitamin D requires hydroxylation in position 1 and 25. The hepatic cytochromes P450 CYP2R1 (microsomal) and CYP27A1 (mitochondrial) catalyse the first hydroxylation in position 25. Although the resulting 25(OH)D represents the most abundant vitamin D metabolite in human blood, it is still inactive. CYP27B1 is responsible for the second hydroxylation step that produces the active metabolite 1,25$(OH)_2$D. While the kidneys are the primary source of circulating 1,25$(OH)_2$D, many extra-renal tissues can also express CYP27B1 leading to local synthesis of 1,25$(OH)_2$D with primarily autocrine and paracrine function. Under most circumstances, the extra-renal production of 1,25$(OH)_2$D does not relevantly contribute to the circulating concentration of this metabolite [16]. However, in granulomatous disease, such as sarcoidosis, the excessive activity of CYP27B1 in macrophages of granulomas can result in raised plasma concentrations [17].

Catabolism of vitamin D

Excessive amounts of 25(OH)D and 1,25$(OH)_2$D require efficient elimination as they can cause hypercalcaemia with neuromuscular symptoms and renal calcifications. The principal pathway of vitamin D degradation starts with another hydroxylation in position C24 resulting in the formation of 24,25-dihydroxy-vitamin D [24,25$(OH)_2$D] and 1,24,25-trihydroxy-vitamin D [1,24,25$(OH)_3$D] [18, 19]. These catabolites are further processed and are ultimately converted into inactive calcitroic acid [20–22]. In addition to 24-hydroxylation, there is an alternative pathway of vitamin D catabolism that starts with hydroxylation in position C23 and ends with the synthesis of calcitriol lactone, which has been reported to possess biological activity. Both catabolic pathways are mediated by the enzyme CYP24A1, which has both 24-hydroxylase and 23-hydroxylase activity [23]. Although 1,25$(OH)_2$D is the preferred substrate for 24-hydroxylase, 25(OH)D is also metabolized by this enzyme. Interestingly, calcitriol lactone has been reported to have unique actions that are different from those of 1,25$(OH)_2$D [24]. Shima et al. reported that this metabolite may stimulate bone formation, which is supported by a reduction of serum calcium, increased collagen production, and an inhibition of bone resorption. Also, the intermediates 1,24,25$(OH)_3$D and 24,25$(OH)_2$D appear to have some biological activity [22]. The latter has been reported to be required for optimal endochondral ossification in the growth plate [25]. However, existing evidence that supports a relevant role of these catabolites in humans is largely lacking and the primary role of CYP24A1 remains the elimination of excess amounts of 1,25$(OH)_2$D and 25(OH)D.

C3-epimerisation of vitamin D

Another biochemical aspect of vitamin D catabolism is 3'epimerisation where the C3 hydroxy group of the A ring changes from α to β orientation. This enzymatic process is supposed to be catalysed by the enzyme 3-epimerase, which does not belong to the cytochrome P450 family. While the gene encoding this enzyme is still unidentified, it seems to require NADPH as cofactor. All naturally occurring metabolites of vitamin D can be epimerized. The resulting epimers can be further metabolized by the same enzymes involved in the metabolism of 25(OH)D_3 so that individual C3 epimer metabolites can be converted to the respective downstream

metabolites. Although 3-epi-25(OH)D_2 has been detected in blood, epimerization of vitamin D_2 metabolites has not been studied in detail. It is also worth mentioning that the affinity of 3-epi-25(OH)D for VDBP and the vitamin D receptor (VDR) is only 36–46% and 2–3%, respectively, of 25(OH)D [19]. Own analyses have shown quantifiable 3-epi-25(OH)D concentrations in 40% of an unselected cohort of adult patients with concentrations between 5 and 14 nmol/L and corresponding 25(OH)D concentrations between 60 and 136 nmol/L [26]. The circulating concentration of 3-epi-25(OH)D appears to be relatively stable ranging between 4 and 10% of the non-epimerized 25(OH)D concentration [19]. However, in infants younger than 2 years of age higher proportions have been reported [27]. During the first year of life, a median 3-epi-25(OH)D concentration of 6.9 nmol/L has been reported by Singh et al., which accounts for 10–20% of the 25(OH)D concentration in most cases. In contrast, other studies that measured 3-epi-25(OH)D concentrations in maternal and embryonic umbilical cord blood reported average quantities of less than 10% [28, 29]. Of note, in individual cases the proportion of 3-epi-25(OH)D can be much higher reaching up to 60% (reviewed in [30]). In addition to the discussion on 3-epi-25(OH)D concentrations in various clinical conditions, there is an ongoing debate whether or not C3 epimer metabolites are of any clinical relevance. *In vitro* studies suggest that they have reduced biological activity. Amongst the different C3 epimer metabolites, 3-epi-1α,25$(OH)_2D_3$ seems to be most active with similar PTH suppressing properties than 1α,25$(OH)_2D_3$, but markedly lower anti-proliferative capacity [19]. Also, 3-epi-1α,25$(OH)_2D_3$ contributes less to calcium homeostasis, the activation of osteocalcin and the CYP24 gene. The extremely low plasma concentration of 3-epi-1α,25$(OH)_2D_3$, which ranges between 0.12 and 1.06 pmol/L, further questions its biological relevance. Finally, most existing knowledge on the biological activity of C3-epimer metabolites has been produced by *in-vitro* studies, which impedes a direct translation to humans.

Measurement of vitamin D metabolites

General analytical aspects

In clinical practice, 25(OH)D is the most widely measured metabolite of vitamin D as it is believed to reflect vitamin D stores that are available for metabolism. From an analytical point of view, concentrations in the nmol/L range, a half-life of approximately 2 weeks [31] and a rather small biological variation make 25(OH)D the preferred analyte. In contrast, the most active metabolite 1,25$(OH)_2D$ circulates in the pmol/L range and has a plasma half-life of just 4–6 h [32], which renders quantitation much more demanding. Moreover, this metabolite is produced on demand through CYP27B1, which makes it less useful for the assessment of vitamin D status. In recent years, the measurement of 24,25$(OH)_2D$, the principal catabolite of 25(OH)D, has gained substantial interest in clinical and experimental studies. With the technological advent of LC-MS/MS, several other metabolites, such as 3-epi-25(OH)D_3, 3-epi-25(OH)D_2, 25,26$(OH)_2D$ and others, can be measured in serum and plasma [9, 33, 34], but their clinical relevance is not yet understood. Therefore, these metabolites will not be reviewed here. Over the last five decades Clinical Chemistry and Laboratory Medicine has contributed substantially to the enormous progress in our analytical and diagnostic capabilities of assessing patient's vitamin D status by publishing hundreds of articles in this field.

Historical aspects of vitamin D testing

Vitamin D testing in serum/plasma has a history of more than five decades. In 1966, Lund and DeLuca reported the existence of a polar vitamin D metabolite that was later identified as 25(OH)D [35, 36]. The presence of this metabolite in human serum was demonstrated one year later by DeLuca et al. after the administration of tritiated vitamin D_3 to patients with familial vitamin D-resistant rickets [37]. In the early 1970s, serum 25(OH)D was measured radioactively [38]. For this purpose, radioactively labelled vitamin D was administered by injection. Later, serum from these patients was extracted with organic solvents and the lipid fraction was isolated by column chromatography. Then, the lipid fraction was separated by thin-layer chromatography. Finally, radioactivity in the 25(OH)D band was quantitated using a gamma counter. While this method was useful for physiologic studies, it did not allow measurement of the native 25(OH)D concentration in patient's serum. At around the same time, first competitive protein-binding assays were developed for the quantitation of 25(OH)D in human serum [39–41]. These assays employed VDBP from different species and used various radioactive tracers for detection. Early 25(OH)D assays were quite cumbersome and required a large sample volume. For example, the radio-ligand assay developed by Bayard et al. required 3 mL of plasma and used tritium labelled ^{3}H-25(OH)D_3 for recovery [41]. After extraction and purification with thin-layer chromatography, diluted plasma from an osteomalacia man was added as VDBP source. Following another incubation step, free and bound fractions were separated and analysed. Measuring plasma samples from normal individuals revealed a 25(OH)

D_3 concentration of 37.5 ± 10.5 nmol/L (mean ± SD), whereas in vitamin D deficient patients concentrations ranged between 5 and 17.5 nmol/L. Aiming to simplify the measurement of serum 25(OH)D, several non-chromatographic methods were developed in the 1970s [42–44]. However, these assays gave much higher values than chromatographic methods and they showed unacceptable linearity and accuracy [45]. In 1974, Preece et al. developed a competitive protein binding assay for the separate quantitation of 25(OH)D_3 and 25(OH)D_2 [46]. With this assay a mean total 25(OH)D (sum of 25[OH]D_3 and 25[OH]D_2) concentration of 29.3 (range of 9.5–82) nmol/L was found in healthy British subjects.

The next milestone in vitamin D testing was the development of high-performance liquid chromatography (HPLC) based methods with ultraviolet light detection [47–49]. These methods offered significant advantages over traditional competitive protein-binding assays, as they were faster, more specific, offered better precision, and did not require radioactive tracers. In the following decades, numerous HPLC methods were developed with the aim to further improve analytical quality [50–52]. With the advent of immunometric methods, radioimmunoassays (RIA) were developed for the measurement of 25(OH)D [53–55]. However, early method comparison studies revealed a problematic analytical performance of RIA assays, and HPLC was recommended as preferable method for clinical laboratories [56, 57]. At the end of the last century, an assay comparison study by Lips et al. showed that competitive binding protein assays, RIA and HPLC agreed poorly. Even different competitive binding protein assays varied substantially in their accuracy.

Measurement of 25(OH)D by LC-MS/MS

Considering the apparent difficulties of measuring 25(OH)D in human serum, efforts continued to develop analytical methods with better sensitivity, accuracy, and specificity. In the late 1970s and early 1980s, first mass spectrometric methods were presented [58–60]. Prior to quantitation in the mass spectrometer, serum samples have to be extracted and purified with strong organic solvents. In view of the high affinity of all vitamin D metabolites to VDBP and other carriers, this step is critical for analytical accuracy. The purified samples are then separated chromatographically and finally introduced in the mass spectrometer. HPLC is the most widely used technique for sample separation, but gas chromatographic (GC) methods have also been published [61–63]. Due to the extremely high complexity of GC-MS methods, they have not gained wider use. Compared to GC-MS, LC-MS/MS methods require substantially less time for sample preparation and allow shorter analytical run times. Today, LC-MS/MS is considered the gold standard for the measurement of 25(OH)D and other related metabolites [10]. Due to its high sensitivity and specificity, this technology allows accurate quantitation of individual vitamin D metabolites despite numerous other, chemically related vitamin D metabolites that coexist in human serum at very different concentrations ranging from a few pmol/L to hundreds of nmol/L. In addition, mass spectrometric methods are relatively immune to common matrix effects, such as heterophilic antibodies, hemolysis, icterus and lipemia. The simultaneous determination of multiple vitamin D metabolites is another key advantage of this technique. Already in 1989, Coldwell et al. described a method that allowed the simultaneous measurement of 25(OH)D_2, 25(OH)D_3, 24,25(OH)$_2D_2$ and 25,26(OH)$_2D_2$ [63]. Recently, Jenkinson et al. developed a method for the parallel measurement of 13 vitamin D metabolites [9], but a rather high complexity impedes a wider use of this method. Despite the advantages listed before, LC-MS/MS methods can vary in their analytical performance [64]. Recognizing the substantial analytical variability between different methods and laboratories in measuring 25(OH)D_3, in 2004, Vogeser et al. developed a first candidate reference method for this metabolite [65]. A few years later, the Office of Dietary Supplements from the U.S. National Institutes of Health in collaboration with the National Institute of Standardization (NIST) developed the serum-based standard reference material SRM 972 (4 levels) with certified concentrations for 25(OH)D_3, 25(OH)D_2 and 3-epi-25(OH)D_3 [66]. A later version of this material (SRM 972a) also contained certified concentrations for 24,25(OH)$_2D_3$. In 2014, NIST released SRM 2972, which consists of two separate solutions of 25(OH)D_3 and 25(OH)D_2 in ethanol, which are intended for use in calibration. Until today, three additional candidate reference methods have been developed and validated [67–69]. All these methods allow the accurate determination of 25(OH)D_3 and 25(OH)D_2 without interferences from the respective C3-epimers, which has been identified as a major confounder in LC-MS/MS methods for 25(OH)D [64]. 3-epi-25(OH)D_3 co-elutes and has identical mass as 25(OH)D_3, and they can only be separated by high resolution chromatography. To date, the consensus is that 3-epi-25(OH)D_3 should be measured in pediatric cohorts but there is no agreement yet about adult populations. Considering all of the above, laboratories that use LC-MS/MS should carefully validate their method and participate in an External Quality Assessment (EQA) program that addresses this issue. Despite the superior analytical performance of LC-MS/MS, high instrument cost, methodological complexity, limited throughput, and the lack of competent staff still detain many laboratories from adopting this technology in clinical practice.

Measurement of 25(OH)D by automated immunoassays

The introduction of fully automated 25(OH)D immunoassays in the early 2,000 years was another milestone in the history of vitamin D testing [70, 71]. With these tests, laboratories were able to cope with the rapidly growing number of requests. However, these assays were characterized by a highly variable analytical performance. For example, the electrochemiluminescence immunoassay from Roche Diagnostics for the E170 analyser showed good overall agreement with LC-MS/MS, but large variations were observed in individual patient samples [71]. In an own assay comparison study with LC-MS/MS, this test showed systematic bias of −25% and a poor concordance correlation coefficient of only 0.66 [72]. Likewise, the first generation of automated 25(OH)D assays from other manufacturers also exhibited a highly variable performance with systemic bias between −25% and +25%, and constant bias of up to 15 nmol/L [72]. Later studies identified 25(OH)D_2 as the main source of error in automated 25(OH)D immunoassays [26], whereas the impact of C3-epi-25(OH)D is limited [72]. Also matrix effects, such as heterophilic antibodies, or specific patient conditions, such as pregnancy, renal failure or acute illness, can cause analytical bias [10]. Over the last 15 years, many more fully automated 25(OH)D assays have entered the market and existing assays have been updated repeatedly by the manufacturers. The development of reference measurement procedures and reference materials has clearly contributed to a better agreement of existing methods, but considerable variability still persists. A very recent inter-laboratory comparison has shown that an assay bias of more than 20% is still relatively common, especially in the presence of specific confounders, such as C3-epi-25(OH)D, 25(OH)D_2 or 24,25(OH)$_2$D [64]. Also, the results from EQA programs show that a bias of ±20% and more is not rare. A main hurdle for automated immunoassays is that they cannot use strong organic solvents to release 25(OH)D from its carriers. Therefore, they have to employ alternative strategies that have an inferior dissociation efficacy. These strategies are optimized for the expected matrix composition. However, in situations where the matrix is altered, such as in pregnant women, patients with chronic kidney disease or individuals with a polymorphic variant of VDBP, these approaches may be less efficient and may thus introduce analytical bias. In contrast, the organic solvents used in LC-MS/MS are strong enough to precipitate all proteins and detach all vitamin D metabolites from their carriers.

The Vitamin D Standardization and Certification Program (VDSCP) from the Centre of Disease Control is another initiative aiming to align the results of different 25(OH)D methods to the reference system [73]. Currently, 39 certified assays are listed at the CDC homepage [74]. However, the VDSCP certification process is flawed by the fact that it requires a mean bias of ≤5% obtained on a standard set of samples, regardless of the scatter that these samples produce. Wise et al. have proposed the percentage of samples with a bias ≤10% as a better criterion for accuracy, but so far, the VDSP certification requirements have not been adapted.

Measurement of 1,25(OH)$_2$D

Although 25(OH)D is by far the most frequently measured vitamin D metabolite in clinical practice, 1,25(OH)$_2$D is a useful marker in some situations and is thus offered by many medical laboratories. 1,25(OH)$_2$D has a short half-life of approximately 6 h and circulates at concentrations in the pmol/L range [75]. Therefore, highly sensitive analytical methods are indispensable. In addition, the serum concentration of 1,25(OH)$_2$D has a rather high biological variability, which is due to its short half-life and a production that is tightly regulated on the basis of the specific demand. Unlike 25(OH)D, the measurement of 1,25(OH)$_2$D is not yet standardized. In 1974 Brumbaugh PF et al. developed the first radio-receptor binding assay, where 1,25(OH)$_2$D in the sample displaced the tritiated ligand from a cytosol-chromatin receptor preparation isolated from chick small intestine [76, 77]. This assay yielded a 1,25(OH)$_2$D concentration of 144 pmol/L in plasma from renal patients. Over the following decades, competitive protein binding assays [78], RIAs [79], enzyme immune assays (EIAs) [80], HPLC [48, 50, 81], GC-MS [82] and LC-MS/MS [83] methods were developed. The principles and capabilities of the different methods have been reviewed by Tsugawa [84] and Hollis [85]. Until the recent introduction of fully automated immunoassays [80, 86], clinical laboratories measured 1,25(OH)$_2$D mostly with commercial RIAs. Nowadays, automated immunoassays are widely used and represent more than 75% of the participants in the DEQAS program. In contrast, LC–MS/MS is used by approx. 10% of the participating laboratories. Despite a broad adoption by clinical laboratories, comparability between automated 1,25(OH)$_2$D immunoassays is not ideal [87]. LC-MS/MS methods do also show significant variability [64], which is at least partly due to different calibration procedures [88]. Furthermore, particular strategies are needed to deal with the very low serum concentration of 1,25(OH)$_2$D. LC-MS/MS methods typically use a dual column system, where the first column serves for analyte enrichment and the second one for separation. In order to enrich 1,25(OH)$_2$D and to reduce interferences from isobaric compounds, such as 1β-25-dihydroxy-vitamin D, some methods employ an

immuno-purification step in their preanalytical sample preparation procedure [86, 87, 89]. Derivatization with compounds, such as PTAD (4-phenyl-1,2,4-triazoline-3,5-dione), is an alternative strategy to increase analytical sensitivity [90]. In addition to calibration and derivatization, the ionization mode is another factor that influences the analytical performance of LC-MS/MS methods [91]. Electrospray ionization (ESI) is typically used for derivatization methods, whereas atmospheric pressure chemical ionisation (APCI) works well without prior derivatization. However, in the absence of proper standardization, it is impossible to decide if one approach is preferable over another.

Measurement of 24,25(OH)$_2$D

Although the measurement of 24,25(OH)$_2$D is not yet recommended for clinical purposes, this metabolite is of increases interest. When used in conjunction with 25(OH)D, 24,25(OH)$_2$D can help to identify patients with reduced 24-hydroxylase activity and functional vitamin D deficiency. The two results can be used for the calculation of the vitamin D metabolite ratio (VMR), a functional indicator of vitamin D metabolism [10]. Although 24,25(OH)$_2$D is exclusively measured by LC-MS/MS, results can vary widely between laboratories [92]. The recent introduction of a standard reference material [93, 94] and a reference method [95] are important prerequisites for a better alignment of 24,25(OH)$_2$D results from different laboratories. Also, 24,25(OH)$_2$D has been included in EQA programs, such as DEQAS [96]. The efficacy of these measures is shown by a recent comparison study where two independent LC-MS/MS methods from different laboratories were found to agree closely [97]. So far, alternative methods for the measurement 24,25(OH)$_2$D, such as immunoassays, have not been developed. Reference intervals for 24,25(OH)$_2$D were determined in healthy young army recruits (1.1–13.5 nmol/L; [98]) and middle-aged adults (0.4–8.9 nmol/L, [99]).

Vitamin D testing in clinical practice – present and future

25(OH)D

Vitamin D status should be assessed in individuals at increased risk of deficiency, such as patients with previous fragility fractures, chronic kidney disease, malabsorption, and abnormalities of calcium and phosphate metabolism [100]. While there is solid evidence that the serum 25(OH)D concentration is inversely associated with total and hip fracture risk [101, 102], several large meta-analysis demonstrated that supplementation of vitamin D alone or in combination with calcium does not significantly reduce fracture risk [103–105]. Current guidelines unanimously recommend evaluating vitamin D status by measuring the serum 25(OH)D concentration [106–109]. In addition to the identification of individuals with vitamin D deficiency, 25(OH)D is the biomarker of choice when hypervitaminosis D or intoxication is suspected. The differential diagnosis of rickets, osteomalacia, and the monitoring of vitamin D supplementation also requires the determination of serum 25(OH)D. While numerous studies support associations between the serum 25(OH)D concentration and a broad range of non-osseous diseases, such as cardiovascular disease [110, 111], malignancies [112, 113], dementia [114, 115], and autoimmune disease [116, 117], potential functional relationships are still matter of ongoing debate and thus 25(OH)D measurement is not yet recommended for the assessment of such patients [10]. In particular, it is unclear whether vitamin D deficiency promotes cardiovascular disease and cancer or if it is the result of poor health and disease specific alterations of vitamin D metabolism in such patients. In line with studies investigating fracture risk, randomized intervention studies did not find beneficial effects of vitamin D supplementation on the incidence of invasive cancer or cardiovascular disease [118, 119]. Since the outbreak of COVID-19 pandemic, 25(OH)D deficiency has also been discussed as a risk factor for SARS-CoV2 infection, severe disease course and adverse outcome. However, most existing studies are of poor quality and a recent meta-analysis did not find significant relationships between serum 25(OH)D levels and various outcomes including mortality, intensive care unit admission and ventilation requirement [120]. Moreover, another meta-analysis of nine smaller intervention studies showed that vitamin D supplementation reduced intensive care unit admissions, but not mortality [121]. Therefore, existing evidence does not support vitamin D testing or supplementation for the management of SARS-CoV2-infected patients.

In contrast to the majority of laboratory test results, the serum 25(OH)D concentration is interpreted on the basis of fixed cut-offs rather than a reference range. Most guidelines distinguish between sufficiency, insufficiency, and deficiency [67, 73, 122]. Some guidelines also provide cut-offs for severe deficiency [123, 124] and toxicity [91, 125, 126]. Although the cut-offs and risk categories vary between different guidelines, 25(OH)D concentrations <50 nmol/L are usually considered deficient [102, 127]. Endocrinologists often prefer a more conservative cut-off of 75 nmol/L. Levels

of 25(OH)D below 30 nmol/L are associated with an increased risk of rickets and osteomalacia, while concentrations between 50 and 125 nmol/L are sufficient to maintain bone health [101]. Toxicity should be considered when 25(OH)D exceeds 150–500 nmol/L [127]. It is important to recognize that the commonly used 25(OH)D cut-offs apply to Caucasians and Asians. People with dark skin have 30–40% lower 25(OH)D serum concentrations than Caucasians, but comparable or higher bone mineral density (BMD) and lower fracture risk [128].

The use of reference intervals for serum 25(OH)D is hampered by its pronounced seasonal variation of 20–30% with the highest concentrations in summer and autumn [129]. Furthermore, own studies in large central European cohorts have shown that lower reference limits would fall between 12 and 29 nmol/L and upper reference limits between 136 and 159 nmol/L. However, there is substantial evidence that within such reference intervals higher 25(OH)D concentrations are associated with better bone health and calcium metabolism [130–132]. For example, PTH continuously decreases with increasing concentrations of 25(OH)D [133], and in contrast to previous concepts there is no threshold above which this relationship plateaus. While these observations are based on statistical analyses of large cohorts, it is also clear that the serum PTH concentration varies substantially between individuals with the same 25(OH)D concentration [128, 134, 135]. The inter-individual variability of PTH is particularly pronounced with serum 25(OH)D concentrations between 20 and 50 nmol/L [134], which raises the question if all individuals in this range, are actually vitamin D-deficient and will benefit from supplementation. This point is further strengthened by data from Priemel et al. showing that only a small fraction of individuals with 25(OH)D concentrations in this range have impaired bone calcification [136]. The concept of using 25(OH)D as the sole marker for vitamin D deficiency is further flawed by the fact that the concentration of this analyte is influenced by many factors including the concentration of its carrier VDBP [137], age [126], body fat content [138], pregnancy [139] and therapy with antiepileptic drugs [140]. For example, the expression of CYP2R1, which encodes 25-hydroxylase, decreases with age, and thus contributes to lower serum 25(OH)D concentrations in this age group [141]. Also, in obese individuals the serum 25(OH)D concentration [142, 143] and the 25-hydroxylase activity are reduced [144]. Furthermore, lipid soluble 25(OH)D may be sequestered in adipose tissue. Finally, pregnant women have higher VDBP concentrations and express CYP2R1, CYP27B1 and CYP24A1 in the placenta [145].

$1,25(OH)_2D$

$1,25(OH)_2D$ is a key regulator of blood calcium levels through intestinal absorption, renal reabsorption, and release from bone stores [146]. Furthermore, $1,25(OH)_2D$ modulates osteoblast [147] and osteoclast activity [117] in bone. In addition to its role in bone metabolism, $1,25(OH)_2D$ is also a regulator of cell proliferation, differentiation, and apoptosis [148]. The synthesis of $1,25(OH)_2D$ is a tightly regulated enzymatic process that is driven by calcium and phosphate homeostasis. In order to ensure an adequate cellular supply with calcium, the $1,25(OH)_2D$ concentration will be kept constant for as long as a minimum amount of 25(OH)D is available. Consequently, the serum concentration of $1,25(OH)_2D$ has little relationship to the bodies vitamin D stores [149, 150]. In fact, it starts do drop below a 25(OH)D of 20 nmol/L, which makes it a very insensitive marker for vitamin D deficiency [151]. Also, supplementation of vitamin D increases serum 25(OH)D, but not $1,25(OH)_2D$ [152, 153]. In patients with renal insufficiency, $1,25(OH)_2D$ is usually low, but quantitation is only indicated in the presence of severe, progressive hyperparathyroidism [154].

While measurement of $1,25(OH)_2D$ is not recommended for the assessment of patient's vitamin D status, it is a helpful marker for the investigation of patients with unexplained hypercalcaemia, sarcoidosis, granulomatous disorders, pseudo vitamin D deficiency, rickets, tumour-induced osteomalacia, and hyperparathyroidism [155]. Furthermore, abnormal $1,25(OH)_2D$ levels, may reflect mutations of genes involved in $1,25(OH)_2D$ metabolism [156], that cause rare hereditary metabolic bone disease, like hereditary vitamin D-resistant rickets (VDR), vitamin D-dependent rickets type 1A (CYP27B1), type 1B (CYP2R1) or idiopathic infantile hypercalcemia (CYP24A1). Mutations of the cell surface zinc-metallopeptidase *PHEX* gene (phosphate-regulating gene with homologies to endopeptidase on the X chromosome) cause X-linked hypophosphatemia (XLH), which is characterized by low-normal 1,25(OH)2D concentrations [157]. Moreover, measurement of this vitamin D metabolite can be helpful to differentiate between FGF23-dependent and – independent phosphopenic rickets [154].

From an analytic point of view, circulating concentrations in the lower pmol/L range and a half-life of only 4–6 h hamper an accurate quantitation that provide meaningful results. In contrast to 25(OH)D, where results are interpreted on the basis of clinical cut-offs, method specific reference intervals are recommended for $1,25(OH)_2D$. For a separate quantitation of $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$ most methods are not sensitive enough as the circulating level of the latter

has been reported to be <17 pmol/L [158]. In addition, immunoassays may be interfered by chemically related vitamin D metabolites that cross-react. Blood levels of $1,25(OH)_2D$ in children are higher than in adults with highest levels between 0 and 1 year [159].

In clinical practice, several influencing factors require consideration when requesting and interpreting $1,25(OH)_2D$. Antimycotic drugs, such as ketoconazole reduce the circulating $1,25(OH)_2D$ concentration [160, 161], whereas pregnancy causes an increase [162]. Higher $1,25(OH)_2D$ concentrations during pregnancy are the result of an increased renal production [162] and placental expression of 1α-hydroxylase. Serum $1,25(OH)_2D$ levels are also higher in African individuals of all age-groups [163, 164], which may be due to increased PTH levels in this population [165].

$24,25(OH)_2D$

As the primary catabolite of 25(OH)D, $24,25(OH)_2D$ has recently moved into the focus of researchers, as it may provide additional metabolic information beyond the simple assessment of vitamin D stores by 25(OH)D. In the presence of adequate vitamin D stores, CYP24A1 converts excess amounts of 25(OH)D into $24,25(OH)_2D$. Under physiological conditions the serum concentration of $24,25(OH)_2D$ ranges between 6 and 9% (unpublished data) from that of 25(OH)D. Due to the tight correlation with 25(OH)D, the measurement of $24,25(OH)_2D$ by itself does not contribute relevant information that goes beyond 25(OH)D [166, 167]. However, there is mounting evidence that the simultaneous quantitation and interpretation of both vitamin D metabolites helps to identify patients with genetic enzyme defects, such as CYP24A1 deficiency [97], and to better assess vitamin D status in particular population groups, such as blacks [167] or children [168]. CYP24A1 deficiency, also known as idiopathic infantile hypercalcemia (IIH), typically presents with a 25(OH)D concentration in the desirable range, but a very low $24,25(OH)_2D$/25(OH)D ratio (VMR) of less than 1% [97]. The lacking enzymatic activity results in an excessive production of $1,25(OH)_2D$, hypercalcaemia and suppressed PTH. Several CYP24A1 loss-of function mutations have been described in IIH. Supplementing IIH patients with vitamin D may trigger serious adverse effects [169]. Determination of the VMR also aids the differential diagnosis of hypercalcaemia, which can be caused by a broad spectrum of diseases including hyperparathyroidism, malignancies, vitamin D intoxication, granulomatous disease, milk alkaline syndrome and genetic defects, such as CYP24A1 deficiency or Williams-Beuren syndrome with a mutated SLC34A1 gene [10]. In addition, the VMR can help to better target expensive genetic testing in patients with a suspected gene defect.

The VMR is a very useful tool for the assessment of vitamin D status in blacks, which have approximately 40% lower 25(OH)D concentrations than Caucasians despite comparable bone health and fracture risk [10, 167]. Genetic polymorphisms in the VDBP gene reduce the affinity for 25(OH)D and other vitamin D metabolites without affecting the availability of free or bioavailable vitamin D. Recent data from Cavalier et al. suggests that VMR assessment in infants, children and adolescents may improve the diagnosis of vitamin D deficiency as it provides functional insights in the patient's vitamin D metabolism [168].

The lower the 25(OH)D concentration the more individuals have been found with undetectable $24,25(OH)_2D$. In contrast, virtually all individuals with a 25(OH)D concentration above 50 nmol/L had detectable $24,25(OH)_2D$. While these results support the current 25(OH)D cut-off of 50 nmol/L for vitamin D deficiency, they also demonstrate that a substantial number of individuals below this cut-off are still adequately supplied with 25(OH)D so that they can afford to catabolize considerable amounts of this inactive vitamin D storage form. Together with another study by Berg et al. [167], these findings show that individuals with the same $24,25(OH)_2D$ concentration can have a 25(OH)D concentration that varies by factor 3–4 [167]. In can be speculated that these individuals are not equally vitamin D sufficient. In summary, the studies discussed before, suggest that the simultaneous analysis of $24,25(OH)_2D$ and 25(OH)D by LC-MS/MS may allow a better assessment of a patients vitamin D status than the measurement of 25(OH)D alone where the result is interpreted using a fixed cut-off. While there is strong support for this approach in patients with genetic enzyme defects and blacks, first evidence also supports the potential of such an approach in otherwise healthy Caucasians. Ginsberg et al. have found the VMR, but not 25(OH)D, to be associated with hip fracture risk in older adults [170]. In addition, higher $24,25(OH)_2D$ concentrations were associated with a BMD. In chronic kidney disease, the $24,25(OH)_2D$ concentration decrease with decreasing renal function and is more strongly correlated with PTH than 25(OH)D and $1,25(OH)_2D$ [171]. In the Seattle Kidney Study, a $24,25(OH)_2D$ concentration below the cohort median of 6 nmol/L was associated with an increased unadjusted risk of mortality.

In addition to potential diagnostic benefits, it has also been hypothesized that the VMR may assist to better target vitamin D supplementation [172]. However, evidence that supports this concept is lacking. In all existing vitamin D supplementation studies, baseline values of $24,25(OH)_2D$ or the VMR were not superior to 25(OH)D in predicting the

increase of 25(OH)D upon supplementation [150, 173, 174]. Also, the effects on BMD are predicted equally well by 25(OH)D and 24,25$(OH)_2$D [175]. However, existing studies are flawed by the fact that the supplemented vitamin D doses were rather high, which limits the margin for a differentiated response. Also, baseline 24,25$(OH)_2$D concentrations were relatively high so that a significant proportion of individuals was actually not vitamin D deficient. So far, no study compared the response of vitamin D supplementation in individuals with and without measurable concentrations of 24,25(OH)2D, but comparable serum 25(OH)D concentrations. Current guidelines do not yet recommend the measurement of 24,25(OH)2D in clinical practice. However, existing studies suggest that this practice should be changed, at least in specific patient groups.

VDBP, free and bioavailable 25(OH)D

Only 0.1% of all 25(OH)D in plasma circulates unbound and thus can freely enter the cytosol where the VDR is located. Similar to other steroid hormones, it is believed that this small fraction is responsible for the majority of vitamin D related effects [10]. Most of the circulating vitamin D metabolites (approximately 85%) are bound to VDBP, a highly polymorphic protein with over 100 isoforms that is closely related to albumin and alpha-fetoprotein [176]. It is primarily synthesized by the liver [177] and expression is regulated by estrogens, which explains higher levels in pregnancy [176]. The remaining 15% of 25(OH)D and other vitamin D metabolites in plasma are bound to albumin, which has a much lower affinity for these compounds so that they can easily dissociate and become metabolically active. Together with free 25(OH)D, the albumin-bound 25(OH)D fraction is referred to as bioavailable 25(OH)D [178]. Free [179] and bioavailable [180] 25(OH)D are calculated from total 25(OH)D, albumin and VDBP. Support for the free hormone theory comes from observations that lacking VDBP causes very low serum 25(OH)D and 1,25$(OH)_2$D concentrations, but not necessarily secondary hyperparathyroidism [177, 181, 182]. In contrast, several studies failed to show superior associations between free 25(OH)D and bone related outcomes when compared to total 25(OH)D [180, 183, 184]. For example, liver cirrhosis patients, who have an increased osteoporosis risk, are characterized by a markedly higher free 25(OH)D fraction despite lower VDBP concentrations [185]. Moreover, pregnant women have higher VDBP concentrations, but lower free 25(OH)D levels than non-pregnant controls [186]. Therefore, the free hormone theory is still a matter of ongoing debate [89]. In addition to lacking evidence from clinical studies, a wider use of free and bioavailable 25(OH)D is also hampered by the fact that they require an accurate quantitation of 25(OH)D and VDBP by LC-MS/MS. Especially the measurement of VDBP by LC-MS/MS is only available at some tertiary teaching centres. Due to these limitations and some other unresolved analytical issues, routine use of free 25(OH)D and bioavailable 25(OH)D is not recommended. Therefore, additional research is needed to address open analytical issues and to demonstrate the clinical utility of these markers.

Conclusions

Over the past five decades, intensive research activities have led to substantial advances of our chemical, (patho)physiological and analytical knowledge on vitamin D. An assessment of the vitamin D status is recommended for patients with established metabolic bone disease or individuals at increased risk of developing such conditions (e.g., nursing home residents), and the monitoring of vitamin D supplementation. For non-bone-related diseases, existing evidence does not justify a routine evaluation of vitamin D metabolism. Measurement of 25(OH)D in serum or plasma remains the test of choice for the assessment of patients vitamin D status. The results should be interpreted on the basis of fixed cut-offs that are based on clinical risk. However, the limitations of this approach become more and more evident. Recent research indicates a dynamic evaluation of patients vitamin D status on the basis of a simultaneous measurement of 25(OH)D and 24,25$(OH)_2$D, and calculation of the VMR, may overcome many of these limitations and may thus provide additional information that goes beyond a simple assessment of vitamin D stores as represented by 25(OH)D. The determination of VDBP, free and bioavailable 25(OH)D is compromised by unresolved analytical and clinical issues, which hamper a wider use. Despite substantial progress over the past five decades, it can be expected that intensive research in the area of vitamin D will continue for another 50 years, and that this knowledge will help to better tailor vitamin D analytics and supplementation to patients need.

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