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Review

Challenges in vitamin D measurement and its role on bone regeneration

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Abstract: Vitamin D plays a crucial role in calcium homeostasis and is significantly involved in the maintenance of a strong mineralized skeleton, healthy teeth, and bone regeneration. Apart of this main function, the scientific evidence of vitamin D involvement in numerous physiological and pathological processes has been growing, linking deficiencies to systematic diseases, autoimmune diseases, and various infections. Over the last years, it has been made clear that modern lifestyle has been the major contributor to vitamin D deficiency globally and the extended awareness has led to a significant increase in vitamin D levels testing. There is a wide range of available testing methodologies, posing different limitations, such as cross-reactivity, low detection capacity, limited throughput, requirement of highly competent staff. Those result from not only due to assay limitations but also due to the complexity of vitamin D metabolisms and catabolism. With liquid chromatography in tandem with mass spectrometry (LC-MS/MS) still considered the gold standard to vitamin D blood level detection, novel point-of-care technologies emerge aiming to bypass the strong monitored variability between assays. The aim of this review is to discuss the crucial limitations of vitamin D measuring assays regarding accuracy and the important issues to consider when interpreting vitamin D results in the dental office.

Keywords: vitamin D, analytical performance, accuracy, standardization

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Introduction

The chemical structure of vitamin D was discovered in 1930 by Adolf Otto Reinhold Windaus and since then its biological effects have been an appealing subject in the scientific world [1]. Nowadays, vitamin D has been the focus of numerous research, particularly due to the resurgence of vitamin D deficiencies among the world population and the widespread public awareness. Modern lifestyle has been the major contributor to vitamin D deficiency globally, as most children and adults experience insufficient sun exposure and maintain an inadequate dietary intake of vitamin D. Additionally, hereditary factors resulting in compromised absorption and metabolic conversion of the molecule may also trigger a vitamin D deficiency [2]. On molecular level, the impact of fluctuating vitamin D levels has been investigated extensively. Vitamin D is a key player in calcium homeostasis and is significantly involved in the maintenance of a healthy mineralized skeleton. Initially, after the active form of vitamin D binds to the vitamin D receptor osteoblastic differentiation is promoted, whereby throughout the vitamin D receptor (VDR) the expression of osteogenic markers such as runt-related transcription factor 2 (Runx2), osterix (Osx), and alkaline phosphatase (ALP) is enhanced [3], [4]. Additionally, the formation of the vitamin D-VDR complex is known to stimulate extracellular matrix proteins which are crucial for bone formation and bone strength [4], [5]. On the other hand, the complex contributes to the regulation of bone resorption

throughout the control of osteoclast formation and activity [4], [6], [7]. Thus, studies have reported that insufficient level of vitamin D led to decreased calcium absorption and bone demineralization, ultimately resulting in decrease in bone strength and density [2], [4], [8]. Beyond the skeletal function of vitamin D, growing evidence supports its role in oral health support. There are numerous studies reporting the association of vitamin D levels with teeth mineralization, movement and even with the prevalence of caries and rickets [9]–[11]. Low levels if vitamin D have been associated with higher caries frequency in children and adults [12]–[14]. Calcitriol, which is the active from of vitamin D, is shown to stimulate antimicrobial activities of macrophages and monocytes and is known to disrupt viral envelopes and impact granulomatous inflammation [2], [15], [16]. Additionally, vitamin D is involved in dendritic cell activity and cytokine secretion, with numerous studies furtherly underlining its effects on the innate and the adaptive immune system [17], [18]. Furthermore, the anti-inflammatory action of vitamin D was also reported in periodontal health, whereby vitamin D deficiency is seen as a factor that may affect periodontal conditions [11]. Previous studies have demonstrated the action of vitamin D to inhibit oxidative stress, prevent bacterial infection development and increase collagen synthesis, which ultimately results in support of tissue regeneration during periodontitis [11], [19]. Clinically, the correlation of low vitamin D levels and systematic diseases beyond its skeletal function, such as chronic kidney disease, diabetes mellitus, cardiovascular diseases have been reported but also its association with a wide range of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis [2], [18], [20], [21]. Currently, the verification of vitamin D involvement in numerous physiological and pathological processes and the growing awareness of the risks of ongoing vitamin D deficiency, lead to the emphasis of its potential therapeutic effects and thus, resulted in increased testing of vitamin D levels.

Sources of vitamin D

Vitamin D is a generic name comprised of two forms of a steroid hormone – vitamin D2 and vitamin D3, which differ structurally only in their side chain. On one hand, vitamin D3 is produced in the skin after ultraviolet B radiation, or it can be obtained directly from the diet or supplements. On the other hand, vitamin D2 is synthetized in plants as a result of UVB radiation and can be obtained in human through a balanced diet [22]–[24]. However, the amount of vitamin D in food does not contribute highly to the general vitamin D intake, because food rich in vitamin D such as oily fish, liver, eggs and mushrooms exposed to sunlight, are not consumed on a daily basis. Additionally, most food do not contain high vitamin D levels unless fortified. Thus, vitamin D production in the epidermis remains the main source [22], [25]. The production rate depends on the intensity of UVB rays and time spent outdoors, which on the other hand is affected by latitude and season change [26]–[28]. Other factors contributing to diminished vitamin D production are skin pigmentation, age and genetic predispositions or hereditary disorders [8], [25], [29].

Vitamin D metabolism

Humans dispose of both forms of Vitamin D - vitamins D2 and D3, as part of a typical lifestyle combining sun exposure, food intake and supplements. Vitamins D2 and D3 function as prohormones and their precursors have no biological effect prior to undergoing structural changes in the body. Although, vitamin D2 and D3 undergo identical steps in hydroxylation and result in the same final metabolite, some scientific studies have reported diverse results in regard to their efficacies in raising serum 25(OH)D [30]–[32]. Disregarding the initial source of vitamin D (oral or cutaneous), the first step of the metabolic pathway is an enzymatic hydroxylation which results in 25-hydroxyvitamin D (25(OH)D). This is achieved primarily in the liver by several enzymes including CYP2R1 and CYP27A1. However, the hydroxylation can occur in other tissues as well in an autocrine/paracrine fashion. Once synthesized, 25(OH)D is secreted into the blood stream and eventually binds to vitamin D binding protein (VDBP), which acts as a transporter. Eventually the complex reaches the

kidney, where a second hydroxylation takes place by 25-hydroxyvitamin D-1α-hydroxylase (1-OHase) leading to the conversion of the biologically active metabolite calcitriol (1,25(OH)2D) [33], [34]. The activation can occur in variety of tissues and can be controlled by phosphorus and calcium in serum. Furthermore, negative feedback is carried out by the parathyroid hormone (PTH) secreted by the parathyroid glands. The circulating form of vitamin D (25(OH)D) in blood has been traditionally used as a marker for vitamin D level measurements, due to its longer plasma half-life in comparison to other vitamin D metabolites [35], [36]. There have been reports about additional vitamin D metabolites which are generated through alternative catabolic and anabolic pathways, such as C3-epimetisation, CYP24A1 hydroxylase and CYP11A1 triggered metabolism pathways [37]. However, they differ in their biologic activity. For instance, vitamin D3 can be metabolized by CYP11A1 and subsequently by CYP27B1 and/or CYP24A1 resulting in metabolites involved in cell proliferation, differentiation and display anti-inflammatory activities [37].

The interest in vitamin D levels has increased drastically due to the growing amount of published literature, linking vitamin D deficiencies to numerous diseases, such as osteoarthritis, diabetes, and autoimmune diseases [2], [25], [37]. Moreover, the discovery that 25(OH)D can be converted to its active form not only in the liver, but also in additional tissues such as brain, uterus and in vascular smooth muscle cells [38]–[41], implements the importance of the molecule in those tissues disregarding its main function in the calcium homeostasis pathway. However, the accurate measurement of vitamin D can be challenging due to various environmental, as well as genetic factors that can influence the metabolism of vitamin D and thus, the numerous factors that can affect the outcome of the analytical methods. The aim of this review is to discuss the crucial aspects of vitamin D measurement procedures and the limitations of the methods, as well as the important issues to consider when interpreting vitamin D results.

Vitamin D as a challenging metabolite

Despite the increasing awareness of the limitation of vitamin D measurement with different methods, the availability of reference materials and a vitamin D standardization program, the comparability of the laboratory results is still challenging [42]. The major testing methods are liquid chromatography in tandem with mass spectrometry (LC-MS/MS) and immunoassays such as enzyme Immunoassays (EIA), enzyme-Linked Immunosorbent Assays (ELISA), Radio-Immunoassays (RIA) and chemiluminescence Immunoassays (CLIA). LC-MS/MS are considered the gold standard in vitamin D testing as they offer a very high accuracy and sensitivity. The method is based on sample separation via liquid chromatography based on chemical properties, followed by the separation of individual vitamin D compounds via tandem mass spectrometry. Although their many advantages, LC-MS/MS assay also pose analytical challenges. For instance, a comparison of three LC-MS/MS methods carried out in different laboratories were reported to show differences [43]. Moreover, some pre-analytical and analytical aspect need to be taken under consideration. For instance, protein precipitation, analyte extraction as well as methodological considerations including chromatographic separation ionization and capabilities of the mass spectrometer may impact the measurement and need to be revised by qualified staff [44]. On the other hand, immunoassay is based on antibody affinity and solid phase separation, and they offer advantages such as high throughput, faster result generation, lower equipment and detergent costs and complete automatization. Nevertheless, commercially available immunoassay suffer from result variability due to different sample treatment and/or extraction methods, limited accuracy due to antibody specificity and cross-reactivity [42]–[44].

When circulating in the bloodstream, vitamin D is hardly ever detected in its free active form. Around 85% of the vitamin D metabolites are bound to the vitamin D binding proteins and additionally a small amount is bound to albumin. Thus, the non-bound, free vitamin D in serum can bind o vitamin D receptor [34]. Farrell et al. compared automated immunoassays from different manufacturers, a RIA and LC-MS/MS assays. It was reported that the performance of the immunoassays was variable, especially at concentrations lower that 8 ng/L. On the contrary, the two addressed LC-

MS/MS demonstrated correlating consistent measurement regardless of the differences in sample preparation and extraction [42]. The direct quantification of free vitamin D is challenging not only due to its low concentrations in serum but also because of the strong interaction with VDBP. The lipophilicity of 25(OH)D contributes to its strong affinity to VDBP, resulting in a complex with a very low dissociation constant. Moreover, 25(OH)D exerts a low affinity to albumin. Although direct methods for the measurement have been developed, in most cases the concentration is calculated from the total measurement of 25(OH)D, VDBP and albumin. However, concentration of VDBP also vary in patients, whereby in some patient's complex formation may be favored in comparison to others, furtherly enhancing the quantification issue [45]. In many analytical assays, a dissociation step has been integrated to release 25(OH)D but variations in puffer and detergent composition may also results in deviations.

As vitamin D can be obtained both from sunlight exposure and from our diet, the measurement of both form vitamin D2 and D3 is necessary to accurately assess the individual vitamin D level in blood. Therefore, the method of choice is required to recognize 25-OH vitamin D2, as well as 25-OH vitamin D3 and to provide the total sum. However, the two metabolites have different chemical structures, different half-lives, and distinct affinities to VDBP. Chromatography and mass-spectrometry-based approaches, especially the powerful LC-MS/MS methods, can separate the two molecules, based on their structure, even though the difference is minimal via detecting the two peaks of 25-OH vitamin D2 and 25-OH vitamin D3 on the chromatogram. On the contrary, distinguishing those metabolites and recognizing them equally can be challenging for both automated and non-automated assays [46], resulting in inaccurate measurements of vitamin D levels. Additionally, as numerous vitamin D metabolites are synthesized in the human body, the potential cross-reactivity in assay exerting lower sensitivity is increased. One metabolite generated from the 3-epimerization pathway has been highly discussed. Those forms are results of the isomerization of the C3-OH group of 25 OH-D und 1,25(OH)2D from their α to β orientation, leading to lower bioactivity of the metabolites [24]. Nevertheless, a study by Stepman et al. reported that the 3-epi forms are not only present in younger children and infants, as it was initially thought, but are also detectable in adults in varying concentration accounting for up to 17% of total vitamin D [47]. Thus, protein-binding assay and immunoassay may exert a level of cross-reactivity, accounting for misleading estimation of vitamin D levels. However, some LC-MS/MS methods are able to separate the epimers, thus overcoming the limitations of other assays [48]. Furthermore, only LC–MS/MS is the only technique which, when used for the measurement of VDBP, can detect isoforms [49], which can also impact accurate detection. Essentially, the assay variability and the reported deviations pose a problem when determining dose-response and clinical cut points, thus making it difficult to compare results and rely on their accuracy.

Despite the measurement type, there are pre-analytical considerations that need to be addressed. Sample storage can impact stability of vitamin D and thus, contribute to the variations in measurement. Studies done on half-life time of 25(OH)D report that stability of the molecule can be hindered regarding sample storage. At room temperature, whole blood samples are reported to be stable up to 24 hours [50]. In separated serum, it appears that long-term stability at -20C and -80C was sufficient and only minor changes were reported [51]. Nevertheless, concentrations are affected from light. A study reported that prolonged exposure to sunlight influences both 25-OH D3 and 25-OH D2 concentrations, while short-term exposure had no significant impact [52]. Thus, samples are very light sensitive and should be kept protected from UV-radiation.

Nowadays, a new generation of testing approaches has been gaining popularity as an alternative to the traditional laboratory-based models. The point-of-care tests are provided close or near the patients and allow the direct quantification of samples and the screening for various compounds. Vitamin D measurement is one of the suitable candidates for point-of-care diagnostic as it is associated with several medical conditions and can be used as an indicator. In contrast to laboratory tests, the rapid vitamin D tests bypass logistical obstacles such as safe and timely transport,

complexity of quantification, laboratory capacity and data sharing. The point-of-care tests provide results quickly, reducing chair time and improving patient experience and do not require highly trained staff (fig. 1). Nevertheless, quick results are not only convenient from a time point of view, but also achieve cost-efficiency and contribute to the simplification of the health system. Although, the accuracy of those point-of-care tests has been questioned by some, there are not any reports on deviating results so far. For instance, Albrecht et al. investigated the accuracy of one commercial rapid test and reported no significant variations between measurements and satisfying distinguishment between deficient, insufficient, and sufficient samples [53].

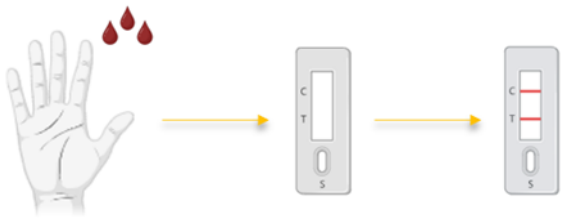

Figure 1. Overflow of point-of-care diagnostic methods. Point-of-care diagnostic methods can be performed with minimal amount of testing material, in this case blood, and require only 15 minutes of incubation to deliver vitamin D level results. The whole process can take place in the clinical office and does not require highly educated staff.

Standardization of vitamin D testing

Over the years, with the increasing number of new commercial and laboratory-based assays and the reoccurring variability of 25(OH)D assays the need for standardization process was emphasized [54], [55]. The standardization data provide the level of accuracy and reliability via the development and implementation of a reference measurement system, guidelines for assessing assay performances and external quality assessment schemes. For instance, the Vitamin D International External Quality Assessment Scheme (DEQAS) established in 1989 is used by laboratories and assay manufacturers to monitor assay performance, both for 25(OH)D and 1,25(OH)2D. Additionally, not only different assay types and manufacturers are compared but also pre-analytical factors of the samples and thus, a baseline information on the performance as well as progress within the standardizing programs is provided. DEQAS reports consistently demonstrate the great variability across various commercial assays of the mean bias from the true concentration (target value as determined by a gold standard reference method). In a DEQAS report conducted between January 2018 and January 2019 the LC-MS/MS method group was reported to produce results within the ± 25% of the target value, with only two measurements in-between + 12.5% and +25% (fig. 2).. On the contrary, assays such as Bio-Rad Bioplex 2200, Diazyme 25OHD VitD Analyzers, DIAsource Total 25OHD ELISA and IDS RIA delivered various results in discrepancy to the control. However, the assays that performed within ± 25% of the target value are the Affimedix Rapi-D 25OHD Test, Affimedix Micro-D Test, Affimedix Chemi-D Test, Abbott Architect - New (5P02), Abbott Architect - Old (3L52), Beckman Access2 Total 25OHD, bioMerieux 25OHD Vitamin D Total, DiaSorin Liaison Total, Euroimmun ELISA, HPLC, IDS EIA, LumiQuick ELISA Vitamin D. The Affimedix Rapi-D 25OHD Test, Affimedix Micro-D Test and Affimedix Chemi-D Test seemed to deliver measurements most accurately, according to the target value. Thus, it was stated that 75% or more results fell within ± 25% of the Target Value.

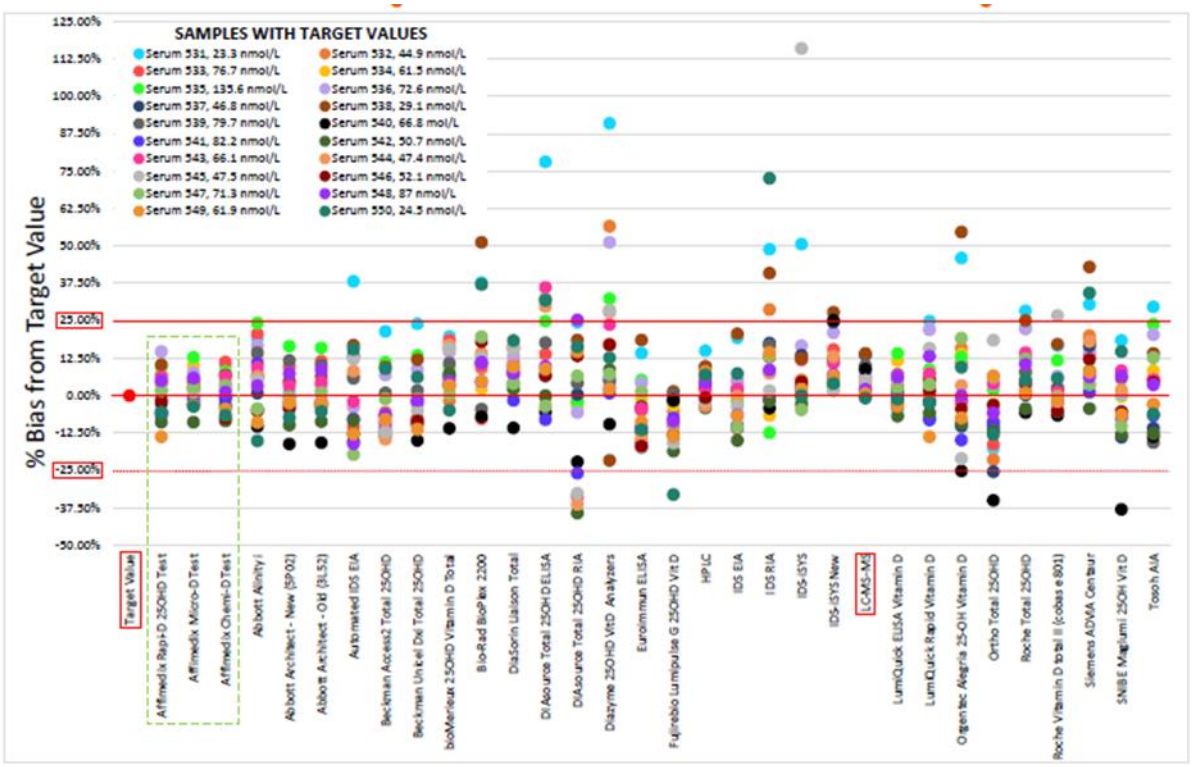

Figure 2. Summary of a DEQAS bias analysis of results conducted from January 2018 to January 2019. Figure adapted from DEQAS.

Another report from DEQAS from 2022 also demonstrates the variable performance of the assays. In the report conducted from April 2021 to January 2022 all method groups (mean % bias) were consistently within limits of the Vitamin D Standardization Program (VDSP), with exception of Siemens Centaur and Roche Total assay (positive bias) and for the Beckman Unicel and IDS iSYS methods (negative biased) (fig. 3). Additionally, it was demonstrated that the mean % CV was consistent for all groups except for Siemens Centaur, Abbott Alinity, Roche Total and Beckman Unicel methods. It was concluded that the LC-MS/MS is still the gold standard regarding bias but locks assay variability in comparison to some automated methods.

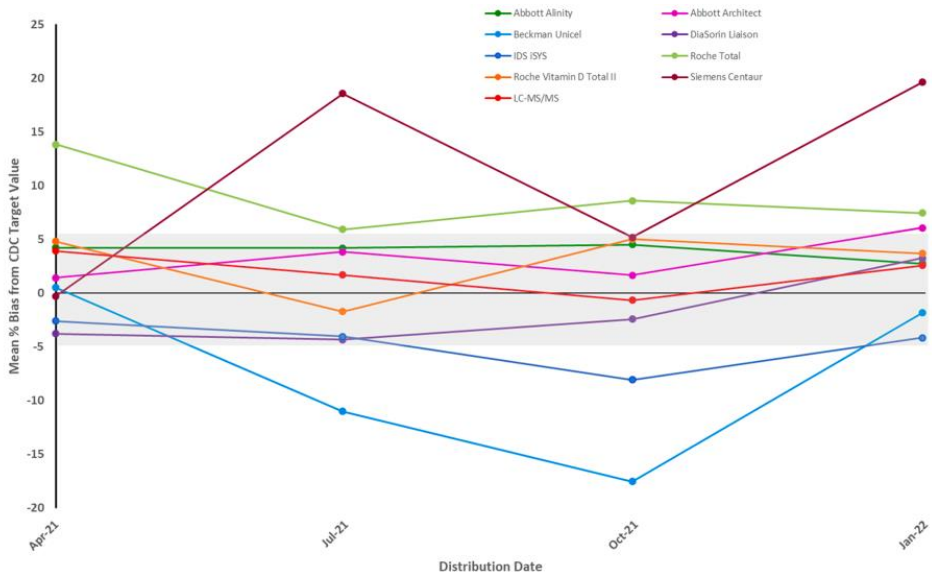

Figure 3. Mean % bias of total 25OHD results from the DEQAS report (conducted from April 2021 to January 2023). The gray shaded area displays the ±5% bias limits of acceptable performance.

Conclusion

Vitamin D is known to be a challenging molecule to detect. Over the years, the clinical demand for precise determination of vitamin D levels has been rapidly increasing in hand with the awareness of the rising vitamin D deficiency among the population. The involvement of vitamin D in dental health and bone regeneration has been the focus of researchers, not only because of its role in bone homeostasis and oral health but also due the involvement in immune response and periodontal health. There is a wide range of available testing methodologies and strong efforts have been made to integrate them into clinical settings, whereby standardizing the results accordingly. Despite this, protein binding assay and immunoassays still pose limitations, such as cross-reactivity and low antibody binding capacity. Although process has been made with standardization methods and the development of guidelines, as well as reference materials, strong variability between assays and even laboratories implementing the same methodologies are still being reported. Despite its challenges, LC-MS/MS remains the gold standard for vitamin D measurement and still demonstrates advanced performance in comparison to immunoassays and protein binding assays. However, innovative assays are being introduced to the clinical diagnostic in accordance to the standardization programs and continuously improve their accuracy and specificity.

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