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Sempos, Christopher T
Heijboer, Annemieke C
Bikle, Daniel D
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REVIEW

Vitamin D assays and the definition of hypovitaminosis D: results from the First International Conference on Controversies in Vitamin D

Christopher T. Sempos[1] [iD], Annemieke C. Heijboer[2,3], Daniel D. Bikle[4], Jens Bollerslev[5,6], Roger Bouillon[7], Patsy M. Brannon[8], Hector F. DeLuca[9], Glenville Jones[10], Craig F. Munns[11], John P. Bilezikian[12], Andrea Giustina[13] and Neil Binkley[14]

[1]Vitamin D Standardization Program, Havre de Grace, MD, USA, [2]Endocrine Laboratory, Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands, [3]Laboratory of Endocrinology, Academic Medical Center, Amsterdam, The Netherlands, [4]San Francisco, San Francisco Department of Veterans Affairs Medical Center, Endocrine Research Unit, University of California, San Francisco, CA, USA, [5]Section of Specialized Endocrinology, Department of Endocrinology, Oslo University Hospital, Rikshospitalet, Oslo, Norway, [6]Faculty of Medicine, University of Oslo, Oslo, Norway, [7]Department of Chronic Diseases, Metabolism and Ageing, Laboratory of Clinical and Experimental Endocrinology, KULeuven, Belgium, [8]Division of Nutritional Sciences, Cornell University, Ithaca, NY, USA, [9]Department of Biochemistry, University of Wisconsin-Madison, Madison, WI, USA, [10]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada, [11]Institute of Endocrinology and Diabetes, The Children's Hospital at Westmead, Sydney, NSW, Australia, [12]Department of Medicine, Endocrinology Division, College of Physicians and Surgeons, Columbia University, New York, NY, USA, [13]Division of Endocrinology, San Raffaele University Hospital, Milan, Italy, and [14]Osteoporosis Clinical Research Program and Institute on Aging, University of Wisconsin-Madison, Madison, WI, USA

The First International Conference on Controversies in Vitamin D was held in Pisa, Italy, 14–16 June 2017. The meeting's purpose was to address controversies in vitamin D research, review the data available, to help resolve them, and suggest a research agenda to clarify areas of uncertainty. The serum 25-hydroxyvitamin D [25(OH)D] concentration [i.e. the sum of 25(OH)D$_3$ and 25(OH)D$_2$] remains the critical measurement for defining vitamin D status. Assay variation for 25(OH)D has contributed to the current chaos surrounding efforts to define hypovitaminosis D. An essential requirement to develop a consensus on vitamin D status is that measurement of 25(OH)D and, in the future, other potential vitamin D biomarkers [e.g. 1α,25(OH)$_2$D$_3$, 3-epi-25(OH)D, 24,25(OH)$_2$D$_3$, vitamin D-binding protein, free/bioavailable 25(OH)D and parathyroid hormone] be standardized/harmonized, to allow pooling of research data. Vitamin D Standardization Program tools are described and recommended for standardizing 25(OH)D measurement in research. In the future, similar methodology, based on National Institute for Standards and Technology standard reference materials, must be developed for other candidate markers of vitamin D status. Failure to standardize/harmonize vitamin D metabolite measurements is destined to promulgate continued chaos. At this time, 25(OH)D values below 12 ng ml^{-1} (30 nmol l^{-1}) should be considered to be associated with an increased risk of rickets/osteomalacia, whereas 25(OH)D concentrations between 20 ng ml^{-1} and 50 ng ml^{-1} (50–125 nmol l^{-1}) appear to be safe and sufficient in the general population for skeletal health. In an effort to bridge knowledge gaps in defining hypovitaminosis D, an international study on rickets as a multifactorial disease is proposed.

Introduction

A central controversy in vitamin D research is how to define hypovitaminosis D [1]. Many questions must be addressed to resolve this controversy. Specifically, is there a biological/biochemical marker, or set of markers, that can be used to identify patients who are at high risk for hypovitaminosis D-related diseases or conditions and, therefore, in need of intervention? If so, what is this marker, can it be measured accurately and what cut-off point(s) can be used to define low vitamin D status? Importantly, an essential laboratory requirement in the effort to develop consensus guidelines on vitamin D status is that the measurement of potential biological/biochemical markers in vitamin D research be standardized, or at least harmonized, to allow the pooling of research data.

Currently, serum total **25-hydroxyvitamin D [25(OH)D]** concentration – the sum of the $25(OH)D_3$ and $25(OH)D_2$ concentrations – is considered to be the best biomarker to define vitamin D status [2–4]. This is because 25(OH)D has a relatively long half-life of about 2–3 weeks, the hepatic conversion of the parent molecules **cholecalciferol** and ergocalciferol to 25(OH)D is unregulated, and its concentration in patients with rickets or osteomalacia – the two diseases accepted as being due, in part, to hypovitaminosis D – increases rapidly following treatment with orally administered vitamin D, with associated resolution of these diseases.

However, despite multiple meta-analyses including large randomized clinical trials, the definition of hypovitaminosis D and 'optimal' vitamin D status remains elusive [1]. In large part, this reflects the use of unstandardized 25(OH)D assays in vitamin D research, which precludes the ability to pool research data from different studies and, therefore, to allow evidence-based definitions of vitamin D status [5, 6]. Additionally, difficulties in differentiating cases of rickets and osteomalacia primarily due to hypovitaminosis D from those due to other causes (e.g. calcium deficiency) promulgate confusion [7, 8].

At the same time, given the multitude of vitamin D metabolites, it is reasonable to ask if singular measurement of circulating 25(OH)D is the best marker to define hypovitaminosis D, or if measurement of other vitamin D metabolites that possess physiological activity should be included in a so-called 'vitamin D panel' that could be used to define 'low' vitamin D status [9]. The list of such potential vitamin D metabolites at the centre of intense research includes the vitamin D_2 and D_3 forms of **$1\alpha,25(OH)_2D_3$**, and 3-epi-25(OH)D and 24,25-dihydroxyvitamin D [$24,25(OH)_2D_3$]. Vitamin D-binding protein (DBP) and free/bioavailable 25(OH)D, although not vitamin D metabolites, must also be added to the list for consideration [10]. However, as has been the case for total 25(OH)D, the lack of assay standardization/harmonization confounds research on these additional metabolites. Ultimately, vitamin D status (e.g. 'low', 'adequate' or ' optimal') should be defined based on the measurement of a vitamin D-related analyte or analytes that best predict surrogate or clear skeletal or extraskeletal outcomes for a specific target group.

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25(OH)D and the definition of vitamin D status

Consensus statement

Serum total 25(OH)D concentration remains the critical measurement for defining vitamin D status [1–4]. Serum total 25(OH)D is defined as the sum of the concentrations of $25(OH)D_3$ and $25(OH)D_2$. Measurement of vitamin D status, based on currently available data, should not include the concentration of 3-epi-25(OH)D_3 or any other vitamin D metabolite. As such, both research and clinical assays based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) measurement systems, should preferably separate 3-epi-25(OH)D_3 from 25(OH)D_3. Not excluding this epimer from the measurement of 25(OH)D_3 may lead to overestimation of serum total 25(OH)D and misclassification, bias and/or errors in clinical decision making when a fixed cut-off point is used to define 'low' vitamin D status, especially in young children [11, 12], in whom the 3-epimer is present at higher concentration.

There needs to be evidence-based consensus regarding the 25(OH)D concentration to define hypovitaminosis D. In the absence of such data, at this time, 25(OH)D values below 12 ng ml^{-1} (30 nmol l^{-1}) should be considered to be associated with an increased risk of rickets/osteomalacia, whereas 25(OH)D concentrations between 20 ng ml^{-1} and 50 ng ml^{-1} (50–125 nmol l^{-1}) appear to be safe and sufficient in the general population for skeletal health.

Laboratory standardization in vitamin D research is a necessary element in developing consensus regarding the 25(OH)D level to define hypovitaminosis D [1, 5, 10]. This standardization needs to take place in three principal areas: (i) laboratory standardization of the measurement of 25(OH)D; (ii) laboratory harmonization/standardization of the measurement of vitamin D metabolites thought to be possible measures of status [e.g. $1\alpha,25(OH)_2D_3$, 3-epi-25(OH)D_3, $24,25(OH)_2D_3$, DBP and free/bioavailable 25(OH)D_3] and other measures used to evaluate vitamin D status [e.g. **parathyroid hormone** (PTH)]; and (iii) standardization of the definitions of what constitutes vitamin D rickets and osteomalacia.

Clinical thresholds are needed for defining the 25(OH)D concentration at which intervention is essential in vitamin D deficiency (rickets/osteomalacia). These should be based on well-designed studies of the relationship of standardized 25(OH)D data with health outcomes. This includes the recommendation that follows for the development of an international rickets registry to define the relationship of 25(OH)D and other key vitamin D metabolites with clinical outcomes. Additionally, but importantly, consensus is needed to define causality both for skeletal and nonskeletal health outcomes; should they be PTH, immune function, falls etcetera? It seems likely that for the foreseeable future, vitamin D status will be related to PTH measurement. As such, it is recommended that a PTH reference measurement

procedure be developed and that PTH measurements be standardized or at least harmonized.

Standardization of serum total 25(OH)D in vitamin D research

Consensus statement

Standardization is the process whereby, within defined statistical limits, all laboratories and assays are brought into alignment with the 'true concentration' based on gold standard reference measurement procedures and certified reference materials [13, 14]. That is, standardized laboratories report the 'true' concentration – in this case, of serum total 25(OH)D – regardless of time, place and assay or measurement system.

Failure to utilize standardized 25(OH)D data is a major contributor to the confusion surrounding vitamin D status [15]. A substantial literature exists documenting 25(OH)D assay variation. Despite the existence of external quality assessment schemes [e.g. the Vitamin D External Quality Assessment Scheme (DEQAS)], it is only since the development of the US National Institute for Standards and Technology (NIST) reference measurement procedure [16] in 2010 and the introduction of the Vitamin D Standardization Program (VDSP) that it has been possible to evaluate assay variation in an unbiased way [17]. In 2013, NIST began assigning target values for DEQAS materials. Studies of DEQAS data since 2013 clearly demonstrate that there remains a great deal of sample-to-sample variation within laboratories using the same assay, and also between different assay platforms [18, 19]. Assay standardization of national nutrition survey data and epidemiological studies clearly show the dramatic differences in the prevalence of 'low'vitamin D status between unstandardized measurements and those standardized using VDSP methods. For example, it was originally thought that there had been a dramatic decline in mean 25(OH)D levels in the US population from 1990 to the period 2001–2004 [20]. However, after standardization mean 25(OH)D levels from 1990 dropped dramatically to the mean levels found during 2001–2004, documenting that during the entire period 1990–2004 US mean 25(OH)D levels were stable [21]. Additionally, in a study among Nordic countries, standardization of the Finnish Health 2011 survey led to mean 25(OH)D levels decreasing from 76 nmol l^{-1} to 68 nmol l^{-1}, and in the Danish Health 2006 survey mean 25(OH)D levels increased from 44 nmol l^{-1} to 65 nmol l^{-1} [22]. Results such as these document that standardization may lead to increasing or decreasing observed 25(OH)D levels; it is impossible to know if, or which way, an individual study's 25(OH)D data are biased if standardized data are not used. Standardization of Finnish national data and data from throughout the EU have had a profound effect on evaluating the impact of food fortification policy which would not have been possible without standardization [23]. Moreover, in cases where 25(OH)D is used to evaluate the effectiveness of drug therapies [24], the interpretation of the results is uncertain without standardized measurements. These results and others call into question the validity of meta-analyses utilizing unstandardized 25(OH)D data. As such, meta-analyses of nonstandardized

25(OH)D data are of little use; standardized 25(OH)D measurements are essential in current and future vitamin D research studies. Moreover, retrospective standardization of studies completed in the past, using validated methods developed by the VDSP, should be promoted, as the vast majority of previously published research data have reported unstandardized 25(OH)D results [25, 26]. Recently completed key research identified in the preparation of vitamin D guidelines should be recommended for retrospective standardization. Journals should require standardized 25(OH)D data as a condition for publication. Moreover, authors should document that the assay used meets the minimal performance criteria set by the VDSP – that is, precision [total coefficient of variation (CV)] ≤10% and accuracy (mean bias) ≤5%.

We recognize that LC–MS/MS is potentially the most accurate and precise method for measuring 25(OH)D in research studies, especially where multiple vitamin D metabolites are measured in addition to 25(OH)D, and across a variety of physiological states/health conditions [27, 28]. Moreover, we recognize that automated immunoassays will continue to be used in many clinical laboratories where the focus remains on serum total 25(OH)D. We encourage all assay manufacturers to standardize their assays through participation in the US Centers for Disease Control and Prevention (CDC) programme [29, 30]. At the same time, we recognize that substantial assay variation around mean bias (%) continues to exist [31, 32]. Correcting this problem requires the VDSP to re-evaluate and tighten its performance criteria to include a measure of variability around mean bias (%) and for commercial assay manufacturers to improve their assays in the development phase. A much more difficult problem for some immunoassay manufacturers to correct – and for standardization efforts – are matrix-specific interferences, especially those found in some physiological states (e.g. pregnancy), and in intensive care unit (ICU), osteoporotic and haemodialysis patients [33, 34].

Issues leading to assay variation in 25(OH)D measurements

Serum total 25(OH)D is a very difficult analyte to measure [35]. Several issues which contribute to assay variation in its measurement include antibody affinities for 25(OH)D$_2$ and 25(OH)$_3$, cross-reactivity with other vitamin D metabolites [e.g. 24,25(OH)$_2$D], DBP concentration and unknown matrix interferences.

In some immunoassays, the antibodies used to measure 25(OH)D may have low affinity for 25(OH)D$_2$. This can lead to low estimates of total 25(OH)D [36, 37]. This is primarily of importance in populations where ergocalciferol is widely used as a supplement or in the treatment of hypovitaminosis D (e.g. in the USA). Additionally, supplementation with ergocalciferol will increase 25(OH)D$_2$ while concomitantly reducing 25(OH)D$_3$ [38]. When in doubt, it is best to confirm the 25(OH)D concentration for patients prescribed ergocalciferol using a standardized LC–MS/MS assay.

Additionally, several 25(OH)D immunoassays show high cross-reactivity with 24,25(OH)$_2$D [39]. As 24,25(OH)$_2$D, can be present in serum at concentrations of up to 20% of total 25(OH)D, such cross-reactivity can potentially substantially affect the '25(OH)D' concentration [40]. By

contrast, 3-epi-25-OHD$_3$ does not appear to show cross-reactivity in immunoassays, yet does cross-react in a competitive protein-binding assay and is not always separated in high-performance liquid chromatography and LC–MS/MS methods [39, 41, 42]. This is especially of importance in young children [11, 12] but the epimer is found in individuals of all ages [43–45].

DBP concentration can also be associated with sample-specific inaccuracy of total 25(OH)D measurement. In some automated immunoassays, 25(OH)D is not well released from the binding protein, leading to discrepant results in individuals with high or low DBP concentrations, such as pregnant women or patients with liver failure [6, 34].

Finally, as stated above, matrix-specific interference with 25(OH)D assays may occur, for not always apparent reasons. For instance, in some immunoassays, sera from pregnant women, and among ICU, osteoporotic and haemodialysis

patients behave differently than those from healthy controls [6, 33, 34, 37, 46, 47].

Serum total 25(OH)D laboratory standardization methods

The VDSP was founded in 2010 to promote the standardized measurement of serum total 25(OH)D around the world [15]. Since that time, the VDSP, its partners and collaborators have developed cost-effective tools and methods to standardize 25(OH)D measurement prospectively in current and future vitamin D research [48] within defined statistical performance guidelines. Moreover, the VDSP has developed tools and methods to standardize 25-OHD measurements retrospectively from studies completed in the past, when properly banked serum samples exist [26, 49].

It is useful to think of the VDSP standardization process as being composed of four distinct phases [48] (Figure 1). The first phase – development of the reference measurement system – is essential to the remaining three phases as it includes the tools and methods to accomplish standardization (Table 1). Some elements of the reference measurement system were developed by programmes collaborating with the VDSP. Phases 2, 3 and 4 consist of a series of calibration steps, with each step connected to the previous ones to develop a chain of traceability from the routine clinical or research laboratory back up the scale to the gold standard reference measurement procedures and/or the NIST standard reference materials (SRMs) (Tables 2 and 3) [50–54]. The VDSP has developed performance criteria that reference measurement procedures and routine laboratories can both use in phases 2–4 and which must meet to be considered standardized (Table 4) [48, 55].

Reference measurement procedures are certified by the international Joint Committee for Traceability in Laboratory Medicine (JCTLM). Currently, there are only three laboratories in the world with certified 25(OH)D reference measurement procedures – NIST [16], Ghent University, in Belgium [56], and the CDC [57].

Accuracy-based performance testing/external quality assessment (PT/EQA) schemes play a role in all phases. In these schemes, the serum materials used in the programme have been value assigned by one of the three JCTLM-certified reference measurement procedures. The target value is the true concentration of serum total 25(OH)D. To our
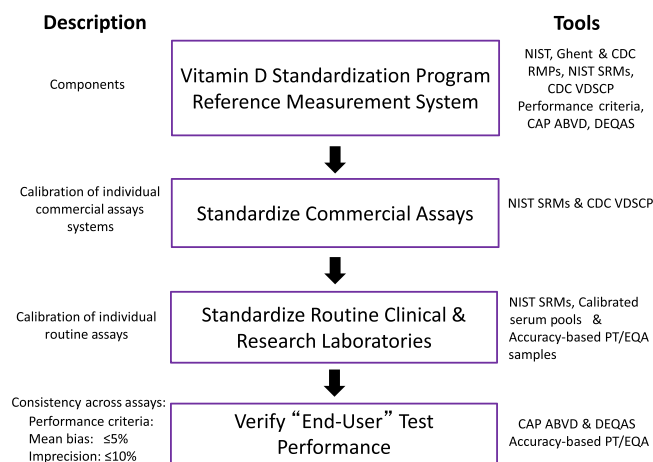
Figure 1

Vitamin D Standardization Program (VDSP): standardization phases. ABVD, accuracy-based vitamin D survey; CAP, College of American Pathologists; CDC, Centers for Disease Control and Prevention; DEQAS, Vitamin D External Quality Assessment Scheme; NIST, National Institute for Standards and Technology; PT/EQA, performance testing/external quality assessment; RMP, reference method procedure; SRM, standard reference material; VDSCP, Vitamin D Standardization-Certification Program

Table 1

Vitamin D Standardization Program (VDSP) reference measurement system components

National Institute for Standards and Technology (NIST), Ghent University and Centers for Disease Control and Prevention (CDC) reference measurement procedures
NIST standard reference materials
Performance standards for accuracy (mean bias %) and precision (total CV%)
CDC Vitamin D Standardization-Certification Program
Accuracy-based performance testing/external quality assessment schemes
College of American Pathologists' accuracy-based vitamin D survey
Vitamin D External Quality Assessment Scheme.

knowledge, there are only two accuracy-based PT/EQA schemes in the world – the College of American Pathologists (CAP) accuracy-based vitamin D (ABVD) survey and DEQAS [58, 59]. PT/EQA schemes are traditionally thought to have a role primarily in phase 4, 'verify end-user test performance', as their names imply [60]. However, as the true 25(OH)D concentration in CAP ABVD and DEQAS serum test materials is known, they can be used as low-cost substitutes for NIST SRMs 972a and 2973 in phases 2 and 3, as noted below.

Phase 2, 'calibrate commercial assay systems to reference materials', highlights their central role in 25(OH)D measurement by both routine clinical and research laboratories (Figure 1). Given the dominant role of commercially developed assay systems in serum total 25(OH)D

measurement, a principal objective of the VDSP from its beginning has been to standardize those measurement systems (Figure 2). NIST SRMs can be used in the development phase to calibrate their systems, whereas NIST SRMs 972a and 2973, along with CAP ABVD and DEQAS materials, can be used in the development phase and routinely to evaluate their accuracy and precision. Moreover, the CDC's Vitamin D Standardization-Certification Program (VDSCP) was developed by the VDSP in collaboration with the CDC to provide a rigorous programme to standardize commercial assay systems as well as large commercial or research laboratories [29, 30]. The CDC's programme is conducted over a 1-year period. CDC certification lasts for only 1 year, so that maintenance of certification requires continuous participation in the programme. The current list of the CDC-certified laboratories is given on the CDC website [29].

Phase 3 is the standardization of routine laboratories to the gold standard reference methods and/or NIST SRMs (Figure 1), and it can be described in five steps (Figure 3) [48]. The general VDSP guidelines provide scientifically rigorous procedures in each of these five steps. In addition, they provide less rigorous, but much lower cost, procedures that can be used especially by small clinical laboratories and

Table 2

Standard reference material (SRM) 2972a[a] 25-hydroxyvitamin D calibrating[b] solutions[c]

SRM 2972a consists of four separate ethanolic solutions	
Vitamin D metabolite	Concentration (nmol l^{-1})
25(OH)D$_3$ in ethanol level 1	806.2 ± 32.4
25(OH)D$_3$ in ethanol level 2	1596.5 ± 64.1
25(OH)D$_2$ in ethanol	560.4 ± 19.9
3-epi-25(OH)D$_3$ in ethanol	577.0 ± 28.5

3-epi-25(OH)D$_3$, 3-epi-25-hydroxyvitamin D$_3$; 25(OH)D$_2$, 25-hydroxyvitamin D$_2$; 25(OH)D$_3$, 25-hydroxyvitamin D$_3$
The concentration "±" the expanded uncertainty
[a]SRM 2972a is a replacement for SRM 2972, which has been redesigned
[b]Ethanolic solutions can be diluted to prepare calibration curves
[c]Please see: National Institute of Standards and Technology. Certificate of Analysis Standard Reference Material® 2972a. 25-hydroxyvitamin D calibration solutions

Table 4

Vitamin D Standardization Program assay performance limits based on biological variation[a]

Measurements	CV (%)	Bias (%)
Reference laboratories	≤5%	≤1.7%
Routine laboratories	≤10%	≤5%
CV, coefficient of variation		

[a]Stöckl et al. [55]

Table 3

Assigned values of standard reference materials (SRMs) 972a and 2973 (nmol l^{-1})[a]

SRM	Total 25(OH)D	25(OH)D$_2$	25(OH)D$_3$	3-epi-25(OH)D$_3$	24,25(OH)$_2$D$_3$
972a					
Level 1	73.1 ± 2.7	1.3 ± 0.2	71.8 ± 2.7	4.5 ± 0.2	6.38 ± 0.23
Level 2	47.2 ± 1.0	2.0 ± 0.2	45.1 ± 1.0	3.2 ± 0.2	3.39 ± 0.12
Level 3	82.9 ± 1.2	32.3 ± 0.8	49.5 ± 1.1	2.9 ± 0.4	3.88 ± 0.13
Level 4	74.9 ± 2.2	1.3 ± 0.2	73.4 ± 2.3	64.8 ± 5.4	6.32 ± 0.22
2973	100.1 ± 2.0	1.59 ± 0.05	98.4 ± 2.1	1 ± 0.2	7.51 ± 0.26

3-epi-25(OH)D$_3$, 3-epi-25-hydroxyvitamin D$_3$; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D$_2$, 25-hydroxyvitamin D$_2$; 25(OH)D$_3$, 25-hydroxyvitamin D$_3$; 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; 24,25(OH)$_2$D$_3$, 24,25-dihydroxyvitamin D$_3$
The concentration or assign value for each analyte "±" the expanded uncertainty
[a]For additional details, please see: National Institute of Standards and Technology. Certificate of Analysis Standard Reference Material® 972a. Vitamin D metabolites in frozen human serum; and Certificate of Analysis Standard Reference Material® 2973. Vitamin D metabolites in frozen human serum (high level)
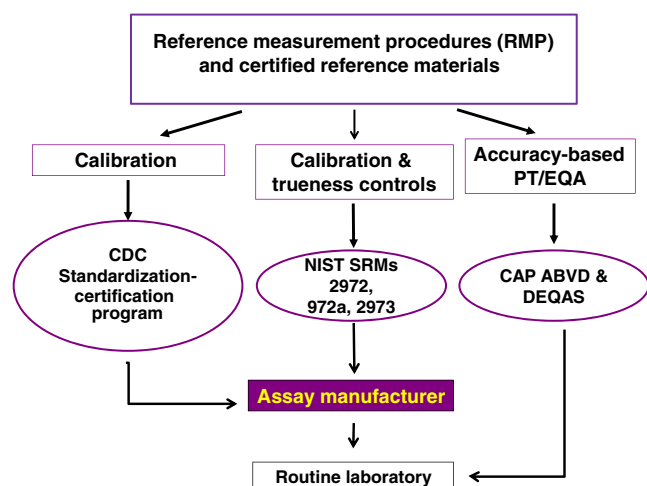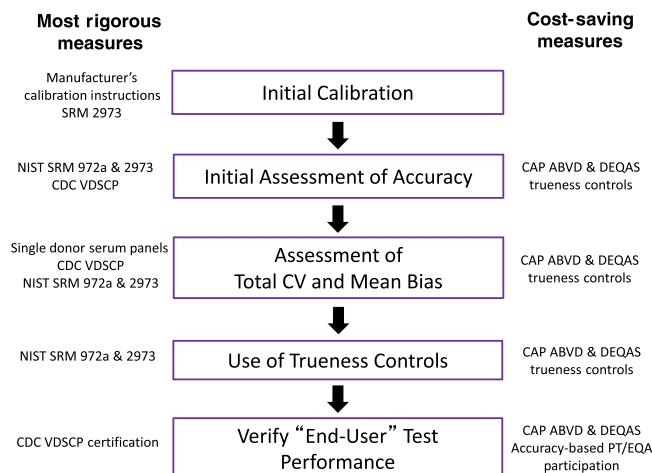
Figure 2

Vitamin D Standardization Program (VDSP): calibration traceability scheme. The VDSP calibration traceability scheme illustrates how the reference measurement system's tools are used to affect assay standardization (adapted from Myers [13]). The goal of the complex set of calibration steps is to assure an unbroken chain between the reference method procedures, or National Institute for Standards and Technology standard reference material 2972 ethanolic calibration solutions and the routine laboratory. It emphasizes the central role that commercial assay manufacturers play in the standardization process and illustrates how accuracy-based performance testing/external quality assessment (PT/EQA) are the only way to verify end-user performance in routine laboratories

research laboratories which measure serum 25(OH)D only infrequently. For example, it is recommended that in the 'initial assessment of accuracy' (step 2), samples should be measured in duplicate on each of 2 days. NIST SRMs 972a and 2973 are the preferred option, although CAP ABVD/DEQAS materials could be used as a lower-cost substitute. However, the preferred method to assess the total CV% and mean bias is to obtain 40 single donor serum samples from the CDC and measure them in duplicate on each of 2 days. CDC will provide the researcher with reference measurement procedure assigned target values for each sample in order to calculate mean bias from the true concentration. There are several lower-cost procedures, however, involving the measurement of, for example, five CAP ABVD/DEQAS samples in duplicate on each of 2 days.

The overall objective of the VDSP 'steps to standardization' for phase 3 is to determine if, after calibration, the assay meets VDSP performance criteria for precision (Total CV) ≤10% and accuracy (mean bias) ≤5%. Again, although rigorous methods, requiring the use of NIST SRMs and sets of single donor serum samples, are given, much lower-cost methods, based on using serum materials from accuracy-based PT-EQA surveys, e.g. CAP ADVD and DEQAS, are also provided. For researchers, especially those using commercial assays, if calibration is not successful and the VDSP performance criteria are not met, a strategy is described for using trueness controls – for example, NIST SRMs 972a and 2973, and CAP ABVD/DEQAS serum materials – to determine a master regression equation. That master equation can then be

Figure 3

Vitamin D Standardization Program (VDSP): steps to the standardization of an individual laboratories assay to measure serum total 25-hydroxyvitamin D. There are five steps to standardize an individual laboratory's serum total 25-hydroxyvitamin D [25(OH)D] measurement (described in greater detail in the text and in Sempos et al. [48]). Step 1, initial calibration, entails setting up the assay and calibrating it using the manufacturer's instructions. Step 2, an initial assessment of accuracy, is used to judge if the assay is performing correctly. This can be performed using NIST SRMs 972a and 2973, participating in the CDC's Vitamin D Standardization Certification Program – approaches potentially too costly for routine laboratories. A lower-cost option in steps 2–4 is to use CAP ABVD and/or DEQAS serum samples, which have RMP-assigned target values. Step 3 determines if the assay CV and mean bias meet the VDSP performance criteria – i.e. total CV ≤10% and mean bias ≤5%. In addition, a low-cost method to estimate total CV and mean bias is to measure, for example, five CAP ABVD or DEQAS serum samples in duplicate on 2 days. If the assay does not meet those criteria, laboratory chemists should contact their commercial assay representative. If it is a laboratory-developed assay, it may be necessary to start the process over. When patient/study participant samples are measured for 25(OH)D, it is recommended, in step 4, that trueness controls are used to assess the ongoing accuracy and precision. For commercial assay users, it is recommended that trueness controls be mixed in with patient/study samples. At the end of the laboratory analysis, these results should be used to develop a regression equation [i.e. RMP target values of the trueness controls (y) and routine laboratory assay results (x)]. If the laboratory does not meet VDSP performance criteria in step 3, then the regression equation may be needed to calibrate the results to RMP results [i.e. the best estimate of the true 25(OH)D concentration). All clinical and research laboratories should participate in an accuracy-based performance testing programme (e.g. CAP ABVD and/or DEQAS) [61]. ABVD, accuracy-based vitamin D survey; CAP, College of American Pathologists; CDC, Centers for Disease Control and Prevention; CV, coefficient of variation; DEQAS, Vitamin D External Quality Assessment Scheme; NIST, National Institute for Standards and Technology; PT/EQA, performance testing/external quality assessment; RMP, reference method procedure; SRM, standard reference material; VDSCP, Vitamin D Standardization-Certification Program

used to calibrate the routine assay measurements, with values assigned by a reference measurement procedure, such as NIST.

Rickets as a multifactorial disease and the rickets registry

Lipid measurement and its adverse health outcome [i.e. coronary heart disease (CHD)] can serve as a model for vitamin D status assessment and treatment. Specifically, CHD is a multifactorial disease, for which risk increases with higher serum total cholesterol concentrations [62–64]. Reducing CHD risk has required a unified approach to both treatment and public health prevention guidelines [65, 66]. Although there is no level of serum total cholesterol for which the CHD risk is zero, and an elevated level of serum cholesterol does not guarantee that an individual will develop CHD, unified clinical and public health nutrition strategies towards lowering serum total cholesterol levels and 'risk factor load' have led to reductions in CHD incidence and mortality around the world.

Similarly, rickets appears to be a multifactorial disease. In the case of vitamin D and rickets, the causal association is negative – that is, those with low vitamin D status are at a higher risk of developing rickets [7, 8]. Lack of sun exposure, absence of maternal vitamin D supplementation while breast feeding, low calcium intake and iron status, to name a few, are risk factors associated with an increased risk of rickets. As with serum total cholesterol, low vitamin D status (i.e. low 25(OH)D concentration) does not guarantee that rickets will develop, and nor does a high 25(OH)D level reduce the risk of rickets to zero.

Here, we propose the development of an international 'rickets registry' by which we can try to understand and model the vitamin D contribution to rickets, recognizing the multifactorial nature of this disease. This effort would include: (i) developing a rigorous case definition for nutritional rickets; (ii) standardized/harmonized measurement of serum 25(OH)D and other possible measures of vitamin D status [e.g. 3-epi-25(OH)D$_3$, 24,25(OH)$_2$D$_3$, DBP and bioavailable/free 25(OH)D$_3$]; and (iii) 'standardized' measurement of other known and possible rickets risk factors (e.g. sources of vitamin D exposure, calcium intake, serum alkaline phosphatase level, genetic markers and iron status). The hope is that such an effort will lead to consensus agreement on the definition of hypovitaminosis D based on standardized measurement of 25(OH)D and the use of a standardized set of criteria for defining cases of rickets.

Additional vitamin D metabolites, PTH and the definition of vitamin D status and hypovitaminosis D

Consensus statement

The following vitamin D metabolites and components of vitamin D metabolism are currently the subject of intense research to determine their role in the assessment of vitamin D status: (i) 3-epi-25(OH)D; (ii) 1α,25(OH)$_2$D; (iii) 24,25(OH)$_2$D$_3$; (iv) DBP; (v) free 25(OH)D; and (vi) PTH [10]. Currently, their importance, if any, in defining states of vitamin D status remains to be determined [1–4]. It is recommended that their measurement in vitamin D research be standardized/harmonized to prevent a recurrence of the problems due to assay variation historically, and currently

experienced with serum total 25(OH)D, that have confounded the field. Moreover, in the future, it seems likely that multiple vitamin D metabolites [e.g. cholecalciferol, 1α,25(OH)D$_3$ and 24,25(OH)$_2$D$_3$] will be measured in a single LC–MS/MS analysis (i.e. a 'vitamin D panel') [9, 67]. In such a system, care must be taken to standardize/harmonize the measurement of each analyte in the panel.

Finally, if and when new vitamin D metabolites are found to be important, standardization/harmonization will be essential, especially in vitamin D research. Such standardization/harmonization is critical as research data will be used to develop clinical and public health guidelines.

3-epi-25(OH)D

The importance of the 3 epimer of 25(OH)D, if any, remains to be determined. It was originally discovered in neonates and children, where its concentration is fairly high [11]. In adults, its concentration is generally 5–10% of the total serum 25(OH)D$_3$ concentration [43, 44], but on occasion it can be reasonably high [68]. At this time, the 3 epimer concentration should not be included in the measurement of total 25(OH)D.

Although all three reference measurement procedures for serum total 25(OH)D measure and subtract the epimer peak from 25(OH)D$_3$, there is no JCTLM-approved reference measurement procedure for the measurement of 3-epi-25(OH)D. NIST, however, does provide certified values for 3-epi-25(OH)D$_3$ in SRMs 972a and 2973, where a certified value is one for which NIST has the highest confidence in its accuracy [51–54].

24,25(OH)$_2$D$_3$

Traditionally 24,25(OH)$_2$D$_3$ was recognized only as the first step in the degradation of 25(OH)D [69]. **Cytochrome P450 (CYP) 24A1** is the primary enzyme responsible for this metabolic step from 25(OH)D to 24,25(OH)$_2$D$_3$, and serum 1α,25(OH)$_2$D$_3$ to 1,24,25(OH)$_3$D$_3$. The concentration of 24,25(OH)$_2$D$_3$ is highly correlated with that of 25(OH)D$_3$, and is reported to be, on average, between 7% and 15% of the total serum 25(OH)D$_3$ concentration [70–72]. However, 24,25(OH)$_2$D$_3$ appears to have some utility in the diagnosis and management of certain diseases. The measurement of 24,25(OH)$_2$D$_3$ and total 25(OH)D is important in patients with hypercalcaemia [72]. Moreover, the ratio of 25(OH)D$_3$ to 24,25(OH)$_2$D$_3$ is increased dramatically in patients with mutations in *CYP24A1* [73–75] but reduced in osteogenesis imperfecta [76].

The ratio of 24,25(OH)$_2$D$_3$ to 25(OH)D$_3$ appears to be predictive of vitamin D$_3$ supplementation [77, 78]. Thus, irrespective of whether the ratio of the two metabolites is expressed as 24,25(OH)$_2$D$_3$ to 25(OH)D$_3$ [70] or its inverse, 25(OH)D$_3$ to 24,25(OH)$_2$D$_3$ [72], the outcome seems to be a better overall understanding of vitamin D status *vs.* serum total 25(OH)D alone. It is claimed by some researchers that the ratio of 25(OH)D$_3$ to 24,25(OH)$_2$D$_3$ is also increased in

patients with florid vitamin D deficiency rickets, indicating that the renal vitamin D axis has switched to a 1-hydroxylation mode [40, 72, 74], although this remains somewhat controversial [79].

NIST has developed a JCTLM-approved reference measurement procedure for serum $24,25(OH)_2D_3$ [80]. $24,25(OH)_2D_3$ target values are available for SRMs 972a, and 2973 (Table 3) [50–54]. Standardization of $24,25(OH)_2D_3$ measurement in vitamin D research is needed.

DBP

DBP is the transport protein for all vitamin D metabolites [81, 82]. It has three common isoforms. It also binds fatty acids and actin monomers. Additionally, it may play a role in inflammation and serve an independent role in immune function [83–86]. It plays a key role in serum total 25(OH)D measurement, as noted previously, and in the calculation and measurement of free $25(OH)D_3$.

DBP is a highly polymorphic serum protein with three common alleles and >120 rare variants [85, 87]. These different forms have a racial and ethnic distribution. Whether different forms have different affinities for vitamin D is uncertain [88, 89].

DBP can be measured using immunoassays and LC–MS/MS [87, 90]. Some commercially available DBP immunoassays use monoclonal antibodies, which have a different affinity for the different DBP isoforms. This means that some of the DBP isoforms are better detected by these monoclonal immunoassays than others, which can lead to falsely low DBP concentrations in the serum of subjects with certain isoforms [87]. An immunoassay using polyclonal antibodies did not show these differences between the DBP isoforms, yet showed a difference with a recently developed LC–MS/MS method [87, 91]. This difference, of course, influences the calculated bioavailable or free vitamin D concentrations.

Overall, when interpreting measured vitamin D concentrations, one should be aware that: (i) the total concentration of several vitamin D metabolites may be influenced by serum DBP; (ii) the measurement of total vitamin D can be influenced by the concentration of DBP, especially in automated immunoassays, and (iii) the measurement of calculated bioavailable or free vitamin D concentration is influenced not only by the issues confounding total vitamin D measurement, but also by DBP methodology.

The recently published NIST assay for DBP measurement may eventually lead to the development of a reference measurement procedure and SRMs which can be used to promote the standardized measurement of DBP, and in turn to improvements in the calculation of Free 25(OH)D [92].

Free 25(OH)D

The free hormone hypothesis postulates that it is only the unbound fraction (the free fraction) of hormones that can enter cells and exert their biological effects [61]. 25(OH)D is bound primarily to DBP (≈85%) and to a lesser extent to serum albumin (≈15%). The unbound fraction of 25(OH)D is <1% of the total amount. Bioavailable 25(OH)D is the sum of free 25(OH)D and the 25(OH)D bound to serum albumin [93]. It is hypothesized that it is the unbound, 'free' 25(OH)D that drives many of the nonclassical actions of vitamin D [94].

Support for the importance of the free levels of vitamin D metabolites came initially from observations that the increase in $1\alpha,25(OH)_2D$ levels with administration of oral contraceptives or during the third trimester of pregnancy was not associated with changes in calcium metabolism, at least until the latter stages of pregnancy, but was accompanied by a parallel increase in DBP [95]. This role of DBP as carrier of the vitamin D metabolites was well demonstrated in DBP knockout mice. Although these mice lost substantial amounts of the vitamin D metabolites in the urine, and their circulating levels of 25(OH)D were very low, they did not develop evidence of rickets until put on a low vitamin D diet [96]. Interest in free 25(OH)D and free $1\alpha,25(OH)_2D$ levels was stimulated further by disease states in which the binding proteins are markedly altered, such as liver disease and nephrotic syndrome, as well as in normal physiological states such as pregnancy, and some reports that allelic variations in DBP may affect its affinity for the vitamin D metabolites [88].

Free 25(OH)D concentration can be calculated or measured directly [93, 97]. However, free 25(OH)D is an extremely difficult measurement to make. Currently, there is only one immunoassay for the direct measurement of free 25(OH)D. This enzyme-linked immunosorbent assay is less sensitive for $25(OH)D_2$. Assay calibration was against a symmetrical dialysis method [98]. The limit of detection for blank serum is 0.7 pg ml^{-1}; at 5.02 pg ml^{-1}, the between-run CV was 6.2% and the between-day CV was 4.5%, with a total imprecision CV of 15.7% [99]. LC–MS has been used to detect 25(OH)D in saliva, which is expected to be free of DBP and albumin and so represents free 25(OH)D [100]. In this method, 1 ml of saliva was deproteinized with acetonitrile, purified using a Strata-X cartridge, derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione, ionized by electrospray ionization and subjected to LC–MS. The limit of detection were reported as 2 pg ml^{-1}. The range of values obtained in normal controls was between 3 pg ml^{-1} and 15 pg ml^{-1}, correlating well with total serum 25(OH)D (10–30 ng ml^{-1}). The intercept was positive but the free fraction in the mid-range of the assay was approximately 0.04%, in line with the results from centrifugal ultrafiltration and the Future Diagnostics immunoassay [101]. However, the development of a JCTLM-certified reference method for the direct measurement of free 25(OH)D will be difficult as the concentration is at the limit of current LC–MS/MS technology, given the accuracy and precision required for JCTLM reference measurement procedures.

The calculated free 25(OH)D concentration, as currently measured, is influenced by DBP and albumin concentration, varies in different clinical conditions and is of unknown accuracy [93]. However, initial studies suggest that directly measured free 25(OH)D may be useful in overcoming that bias [97, 102]. The development of DBP and serum albumin reference measurement procedures will be of help in evaluating the different equations for calculating free 25(OH)D. Further research to define the utility, if any, of directly measured or calculated free 25(OH)D measurement in the assessment of vitamin D status is needed [103].

Fibroblast growth factor 23 (FGF23) as a biomarker

FGF23 is a relatively recently discovered hormone and, with the interplay among PTH, 1,25(OH)$_2$D and FGF23, it has been speculated that it may also be a biomarker of vitamin D status. However, current research on the effect of vitamin D treatment on FGF23 concentrations has not shown a clear effect [104].

The measurement of FGF23 is difficult, and assays are neither standardized nor necessarily measuring the same fragments. Some FGF23 assays measure solely the intact FGF23 (the biological active form), whereas others measure both the intact form and the C-terminal fragments of FGF23 [105]. As can be expected, the results from these assays do not correlate well with each other, especially in the physiological range [106–108].

Moreover, the quality of FGF23 assays has not always been established, and intact FGF23 assays do not always agree with each other [105, 106]. Apart from standardization differences, and apart from the fact that intact FGF23 and C-terminal FGF23 assays do not agree and give different information, there is also a problem with the analytical performance of some assays. All of this raises the question of whether these assays are measuring the same thing.

In addition to these analytical issues, pre-analysis of FGF23 must be taken into account, as the intact FGF23, in particular, is highly unstable, and protease inhibitor cocktails are needed for a reliable measurement [109]. After centrifugation, FGF23 in the plasma or serum is relatively stable [107].

1α,25(OH)$_2$ D

Serum 1α,25(OH)$_2$D is the hormone form of vitamin D. Serum 25(OH)D is hydroxylated at the one position by 1α-hydroxylase (**CYP27B1**) in the kidney and other tissues, to form 1α,25(OH)$_2$D [110, 111]. The vitamin D hormone system is essential in regulating serum calcium concentration [110, 111]. Serum 1α,25(OH)$_2$D stimulates: (i) calcium and phosphate absorption in the intestine (primarily the duodenum and jejunum); (ii) renal tubule reabsorption of calcium and phosphate in the kidney (along with PTH); and (iii) with PTH, mobilization of calcium and phosphate from bone.

As 1α,25(OH)$_2$D has a relatively short half-life and its levels are tightly controlled by PTH, FGF23, phosphate and calcium, calcitriol is not considered a useful measure of vitamin D status. 1α,25(OH)$_2$D stimulates the 24-hydroxylase enzyme (CYP24A1), which leads to its degradation [110, 111]. Mutations in *CYP24A1* are one cause of idiopathic infantile hypercalcaemia [77, 112], and increasing levels of serum phosphate/FGF23 may be correlated with the increased expression of CYP24A1 mRNA [69], although serum 24,25(OH)$_2$D$_3$ levels fall in patients with chronic kidney disease undergoing dialysis [78]. Serum concentrations of 1α,25(OH)$_2$D$_3$ can provide an insight into the aetiology of hypo- and hypercalcaemia and into the complex origin of rare cases of rickets or other metabolic bone diseases.

In prospective research studies, 1α,25(OH)$_2$D$_3$ should only be reported in association with other vitamin D metabolites [i.e. 25(OH)D], calcium, phosphate and PTH. DEQAS runs a PT/EQA programme for 1α, 25(OH)$_2$D$_3$, and all clinical laboratories that measure it should participate in it. Currently, there is no reference measurement procedure for 1α,25(OH)$_2$D$_3$, although NIST had begun work on one.

Recently, 1β,25(OH)$_2$D$_3$ was identified as a new vitamin D metabolite in human serum; however, its role in vitamin D metabolism remains to be elucidated [113].

PTH as a biomarker of hypovitaminosis D

Due to the tight physiological control of plasma calcium concentration by the calciotropic hormones regulating calcium absorption and excretion, and the interplay of PTH, 1α,25(OH)$_2$D and FGF23, it is logical to focus on one of these factors as a potential biomarker for another – here, PTH for 25(OH)D levels. As an example, intact PTH suppression by serum 25(OH)D concentration has been used to estimate the 25(OH)D level, to define hypovitaminosis D. This is an attractive idea because lower serum 25(OH)D levels are associated with higher PTH levels. However, the threshold (inflection or breaking point) at which intact PTH clearly rises when 25(OH)levels are physiologically low remains inconsistent and thereby unsolved [114]. Establishment of a threshold is hampered by differences in the standardization of PTH assays and other, preanalytical, issues, including specimen type and stability [115]. The possibility of multiple thresholds has also been suggested [116].

As with other peptide hormones, PTH is relatively unstable, and metabolized into inactive fragments both in the circulation and after venepuncture. Thus, well-defined preanalytical conditions are important for its measurement [117–119]. In clinical practice, the second-generation PTH assay, introduced in the 1980s, is widely used. The assay was developed as an 'intact PTH assay', with one antibody directed towards the C-terminal and one towards the N-terminal part of the 84-amino acid peptide. However, such assays also capture PTH fragments, mostly inactive, with some (PTH 7–84) even being inhibitory. The assays, in general, perform reasonably well in most clinical situations, with the exception of declining renal function. In renal failure, inactive PTH fragments are not readily cleared from the circulation, build up and are detected in the second-generation assay. Typically, in renal failure the elevated PTH level, as detected by the second-generation assay, will reflect both a level of true secondary hyperparathyroidism (active PTH) and the accumulation of uncleared fragments (inactive PTH). The third-generation PTH assays are theoretically more selective for measurement of the entire peptide, PTH (1–84), and not circulating fragments because the second antibody is directed towards the first four amino acids in the peptide. The so-called 'whole' PTH assays are preferred in patients with impaired renal function. However, whether the clinical specificity for third- compared with second-generation assays is improved remains to be established. While much remains to be clarified with regard to the relationship between circulating 25(OH)D and PTH, standardized 25(OH)D data will continue to be used in conjunction with 'PTH' measurements. It is apparent that reference methods and materials for PTH are needed to enhance the definition of vitamin D status.

Summary and conclusions

A central controversy in vitamin D research is how to define hypovitaminosis D. Among the possible markers, serum total 25(OH)D is currently considered to be the best marker of vitamin D status. In the absence of consensus, at this time, 25(OH)D values below 12 ng ml^{-1} (30 nmol l^{-1}) should be considered to be associated with an increased risk of rickets/osteomalacia, while 25(OH)D concentrations between 20 ng ml^{-1} and 50 ng ml^{-1} (50–125 nmol l^{-1}) appear to be safe and sufficient. To resolve this controversy, related to the definition of hypovitaminosis D, it will be necessary to: (i) standardize the measurement of serum total 25(OH)D in vitamin D research, as well as standardize/harmonize the measurement of other possible markers of vitamin D status; and (ii) develop/conduct a rickets registry which includes a precise case definition of nutritional rickets, including other risk factors for nutritional rickets and standardized measurements of 25(OH)D and vitamin D metabolites.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [120], and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18.

Competing Interests

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