Review

Vitamin D Assay and Supplementation: Still Debatable Issues

MARIO PLEBANI * , Martina Zaninotto , Sandro Giannini , Stefania Sella , Maria Fusaro , Giovanni Luigi Tripepi , Maurizio Gallieni , Markus Herrmann , Mario Cozzolino

Posted Date: 18 July 2024

doi: 10.20944/preprints202407.1461.v1

Keywords: Vitamin D; PTH; FGF23; Supplementation; Standardization; Decision limits

Opinion

VITAMIN D Assay and Supplementation: Still Debatable Issues

MARIO PLEBANI [1,2,*], Martina Zaninotto [2], Sandro Giannini [3], Stefania Sella [3], Maria Fusaro [4], Giovanni Luigi Tripepi [5], Maurizio Gallieni [6] and Markus Herrmann [7]and Mario Cozzolino [8]

[1] University of Padova
[2] QI.LAB.MED, Spin-off of the University of Padova, Italy
[3] Clinica Medica 1, Department of Medicine, -DIMED, University of Padova, Italy
[4] National Research Council (CNR), Institute of Clinical Physiology (IFC), Pisa, Italy.
[5] National Research Council (CNR), Institute of Clinical Physiology (IFC), Reggio Calabria, Italy.
[6] Department of Biomedical and Clinical Sciences 'Luigi Sacco', University of Milano, Milano, Italy.
[7] Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Austria
[8] Renal Division, Department of Health Sciences, University of Milan
* Correspondence: mario.plebani@unipd.it

Abstract: Over the last decades, in addition to the improvement of pathophysiological knowledge regarding the role and mechanisms of action of Vitamin D, there has been a progressive advancement in analytical technologies for its determination, as well as in methodological standardization. A significant number of scientific works, meta-analyses, and guidelines have been published on the importance of Vitamin D and the need for supplementation in deficient individuals. However, it appears necessary to clarify the fundamental elements related to the determination of Vitamin D (both at the strictly analytical and post-analytical levels) and the scientific evidence related to the efficacy/safety of supplementation. Additionally, given the important interrelations between Vitamin D, parathyroid hormone (PTH), and fibroblast growth factor-23 (FGF23), the analytical issues and clinical utility of these biomarkers will be discussed.

Keywords: Vitamin D; PTH; FGF23; supplementation; standardization; decision limits

Introduction

Vitamin D deficiency remains a highly prevalent condition in developed countries that impairs bone mineralization and skeletal muscle function [1,2]. In addition to its fundamental role in the regulation of calcium and phosphorus homeostasis, numerous studies over the last few decades have demonstrated that Vitamin D has pleiotropic functions affecting virtually all organs and tissues. For example, Vitamin D modulates cell growth and differentiation, immunity regulation, glucose homeostasis, cognitive functions, and the activity of many hormones, impacting the association between vitamin deficiency and various pathological conditions, including cardiovascular diseases [3–5]. In these decades, alongside the improvement of pathophysiological knowledge, there has been a progressive advancement in analytical technologies for its determination, as well as in assay standardization. A significant number of scientific works, meta-analyses, and guidelines have been published on the importance of the Vitamin D and the need for supplementation in deficient individuals. However, there is a clear perception of a lack of clarity on fundamental elements related to the determination of Vitamin D (both at the strictly analytical and post-analytical levels, especially related to the expression of concentrations with different measurements units), as well as on the scientific evidence related to the efficacy/safety of supplementation. This paper aims to clarify the fundamental aspects mentioned above so they are brought to the attention of both the clinical world and laboratory medicine professionals, as well as other stakeholders such as patient representatives, and, of course, administrators and policy makers. Additionally, the document aims to provide

 doi:10.20944/preprints202407.1461.v1

evidence on the use of integrative and complementary tests to the measurement of the traditional vitamer 25 (OH)D.

Vitamin D Determination

There is broad consensus and evidence that the study of Vitamin D status should be carried out through the determination of the 25-hydroxylated form (25-OH Vitamin D) for four main reasons: a) its half-life is sufficiently long to allow the determination of the "stable" portion in the blood and thus a reliable indicator of vitamin status; b) its concentration in the blood is 1000 times greater than that of the di-hydroxylated form [1α,25(OH)2], allowing for the availability of measurement methods with adequate analytical sensitivity; c) its concentration is the sum of endogenous production and the intake of the vitamin from the diet, thus enabling a reliable estimate of the "overall" vitamin status; d) total serum 25(O)D is also the sum of 25(OH)D_3 and 25(OH)D_2 [6–8]. Therefore, this vitamer is the widely accepted biomarker of Vitamin D status and its determination is carried out for two main reasons: a) to determine the nutritional status of the vitamin; b) to monitor the effectiveness of supplementation.

Methods for Analytical Determination of Vitamin D

The determination of the vitamer 25(OH)D presents numerous analytical challenges due to its strong binding to the Vitamin D-Binding Protein (VDBP), the need to determine the equimolar amount of 25(OH)D_2 and 25(OH)D_3, the coexistence of numerous substances with similar chemical compositions that can cause cross-reactions, and the matrix effects such as interference from heterophilic antibodies or changes in protein composition [9]. Analytical techniques for determining Vitamin D can be divided into two major groups: a) methods with complete removal of proteins and lipids before the analytical phase using organic solvents, including (liquid chromatography-mass spectrometry (LC-MS/MS), high performance liquid chromatography (HPLC), and radioimmunoassays (RIA); b) automated immunoassays that do not use organic solvents but alternative strategies to release the vitamin from the binding proteins [9–12]. The first method for determining 25(OH)D was published in 1971 and was a competitive method using rachitic rat serum as a source of the binding protein [13]. In the late 1970s, several HPLC methods were developed, and in 1984, the first radioimmunoassay based on the use of a specific antibody [14] was introduced. Subsequently, to overcome issues related to handling radioisotopes, enzyme immunoassays (EIA, ELISA) and chemiluminescent immunoassays (CLIA) were developed [15]. These methods have become widely used in clinical laboratories due to progressive automation. However, these methods long suffered from poor standardization, preventing comparability of results obtained with different methods and from different laboratories. To address these issues, in 2010, the National Institutes of Health (NIH) initiated the Vitamin D Standardization Program (VDSP) in collaboration with the National Institute of Standards and Technology (NIST), the Centers for Disease Control and Prevention (CDC), Ghent University (Belgium), the American Association for Clinical Chemistry (AACC), the IFCC, and nutritional surveillance programs from various countries, including Australia, Canada, Germany, Ireland, Mexico, South Korea, the United Kingdom, and the USA [16]. Thanks to this initiative, three reference measurement procedures (RMP) based on ID-LC-MS/MS and recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) are now available. Additionally, the National Institute of Standardization (NIST) developed a reference material (SRM) 972 and 972a, representing the second essential element for metrological traceability and standardization of measurement methods. Although the procedures of the reference methods are too complex and time-consuming for routine clinical practice, they provide reference (target) values that can be used to standardize or re-standardize methods used in clinical practice and make the results comparable. The issue of standardizing methods and the impact of this standardization on decision levels, which identify deficiency and desirable levels, is crucial given the significant discrepancies between the results of clinical studies conducted in recent years without methodological standardization. The lack of standardization has been shown to significantly alter the recommended levels for defining deficiency and insufficiency. For example, Binkley et al.,

 doi:10.20944/preprints202407.1461.v1

commenting on two large clinical studies—the Third National Health and Nutrition Examination Survey (NHANES III, 1988-1994) and the German Health Interview and Examination Survey for Children and Adolescents (KIGGS, 2003-2006)—documented significant differences after reanalyzing the samples with a standardized method. After standardization, the percentage of vitamin D values below 30, 50, and 70 nmol/L in the KIGGS study increased from 28% to 47%, 13% to 87%, and 64% to 85%, respectively, while in the NHANES III study, the percentage of values below 30, 50, and 75 nmol/L increased from 4% to 6%, 22% to 31%, and 55% to 71%, respectively [17]. Other authors have reported similar results [18]. These significant differences lead to the conclusion that the levels recommended to date for identifying deficiency, insufficiency, and toxicity of vitamin D are compromised by the lack of method standardization. Consequently, new clinical trials with valid experimental designs and standardized methods need to be planned to accurately define the decision limits for deficiency, insufficiency, and possible toxicity of vitamin D. It also seems appropriate to revisit the literature data from recent years in light of these new findings, i.e., the variations related to methodological standardization. Regarding the methods to be used in the clinical laboratory, the choice between an automated immunoassay and an LC-MS/MS method depends on various factors such as the number of requests, the availability of qualified personnel in mass spectrometry techniques, and the specific instrumentation. Generally, data from external quality assessment programs demonstrate a continuous improvement in analytical performance: LC-MS/MS methods generally appear superior, with lower bias towards reference methods, but they exhibit greater variability reflecting existing differences in instrumentation, chromatographic separation, and calibration [19]. Recent data show that only 20% of laboratories participating in EQA programs use LC-MS/MS methods, highlighting that automated immunoassays are still the most widely used [10]. Very recently, Herrmann et al. proposed an innovative approach for diagnosing functional vitamin D deficiency based on the combined determination of 25(OH)D and its main catabolite (24, 25-dihydroxy-vitamin D) to calculate the so-called vitamin D metabolite ratio (VMR). According to the authors, the VMR allows for better identification of individuals with vitamin deficiency, as it is associated with significantly higher levels of PTH, accelerated bone metabolism, and mortality [20]. These data, undoubtedly of interest, require further confirmation in clinical studies using appropriate experimental protocols. Conversely, the proposal to determine the free fraction of 25(OH)D, given that about 85-90% of the circulating fraction is bound to the specific protein (VDBP) and 10-15% to albumin, has been shown to have limited clinical utility for various reasons and could be reserved only for individuals with clinical conditions that significantly alter the concentration or affinity of the vitamin D-binding protein, such as cirrhosis, pregnancy, or acute inflammatory diseases [21–24].

Vitamin D Measurement and Supplementation

A series of scientific studies published in recent years [25,26] and campaigns such as "Choosing Wisely" (https://www.choosingwisely.org) which, started in 2012, highlighted serious concern on the issue of appropriateness in laboratory medicine, as Vitamin D was placed at the top of the list of the so-called “inappropriate laboratory tests”. In some Countries, including Italy, scientific organizations and regulatory bodies developed guidelines and recommendations to regulate both test request and the prescription of vitamin D supplementation. In particular, the AIFA (Agenzia Italiana del Farmaco) Note 96, initially introduced in 2019 and updated in February 2023 [27], was released to regulate reimbursement of vitamin D supplementation. Other guidelines, such as those from SIOMMMS (Italian Society for Osteoporosis, Mineral Metabolism, and Skeletal Diseases) released in 2022 [28], and the more recently published Endocrine Society Clinical Practice guideline [29] agree to not recommend screening for 25-(OH)D levels in the general population due to a lack of favorable cost/benefit evidence. These recommendations, however, have been subject to comments and criticisms from various organizations and scientific societies, highlighting both analytical and clinical issues [30]. Specifically, from a purely analytical point of view, the criticisms are essentially:

a) the evidence that the standardization of methods used by clinical laboratories is modest, especially before the identification of a reference method and studies demonstrating the need to recalibrate commercial methods and avoid the bias compared to the reference method, resulting in

 doi:10.20944/preprints202407.1461.v1

significant differences in results that could reflect inadequacy concerning recommended decision levels. This means that currently adopted decision limits are affected by a significant analytical bias and that further studies are needed to establish evidence-based decision limits by adopting reference measurement procedures (RPMs) and/or methods standardized against these RMPs.

b) the non-uniformity of measurement units in published papers and guidelines which translate into the identification of two different measurement units, with the consequent possible sources of error and confusion.

c) some recommendations establish a single threshold value (50 nmol/L or 20 ng/mL) tied to reimbursement, while clinical laboratories used different levels closer to those recommended by the Endocrine Society, for example, 75 nmol/L [28].

Based on currently available evidence, decision limits that should be adopted and reported by clinical laboratories are shown in Table 1.

Table 1. Decision limits for 25(OH)D assay.

1.	**deficiency: <30 nmol/L (**12 ng/mL)
2.	**insufficiency: 30-50 nmol/L (**12- 20 ng/mL)
3.	**adequacy: 50-125 nmol/L** (12-50 ng/mL)
4.	**optimal levels in patients with osteoporosis or clinical conditions at risk of Vit D deficiency: 75-125 nmol/L (**30-50 ng/mL)
5.	**excess: 250 nmol/L (**100 ng/mL)

Although the issues of Vitamin D assay/decision levels and supplementation are clearly distinct, unfortunately they have been mixed up. Therefore, focusing on the latter, it should underline that some regulatory documents which discourage the Vitamin D supplementation are mainly based on the results of two large randomized clinical trials, the American VITAL study [25] and the European DO-HEALTH study (Bischoff-Ferrari HA et al.) [26]. In both studies, Vitamin D supplementation did not show to prevent fracture events, and for this reason, the prescribability for reimbursement purposes was reduced from 50 to 30 nmol/L (or from 20 to 12 ng/mL) of the maximum circulating 25(OH)D level, with or without specific symptoms and in the absence of other associated risk conditions. However, both cited studies present significant limitations. In the first study, LeBoff and colleagues tested the hypothesis that Vitamin D3 supplementation might reduce fracture risk compared to placebo. Participants in this study were not enrolled on the basis of Vitamin D deficiency (average Vitamin D level 30 ng/mL), low bone mass, or osteoporosis. The primary endpoints were total, non-vertebral, and hip fractures reported by participants and validated by an independent scientific committee. Supplemental Vitamin D3, compared to placebo, did not show a significant effect on total fractures ($p = 0.70$), non-vertebral fractures ($p = 0.50$), or hip fractures ($p = 0.96$). In a subgroup of 16,757 participants out of 25,871 (about 65%), baseline 25(OH)D concentrations were also available. The average (SD) 25(OH)D concentrations were 76.6 ± 25 nmol/L (30.7±10 ng/mL) and 87% of patients had 25(OH)D levels >50 nmol/L (>20 ng/mL) (25). This means that about 9 out of 10 patients enrolled in this ancillary VITAL study had a 25(OH)D concentration >20ng/mL, a cut-off above which, based on epidemiological considerations, no benefit from supplementation on fracture incidence rates was expected [25]. The same bias regarding Vitamin D levels is also found in the other study considered by AIFA [26]. Tripepi and coll., after an exhaustive analysis of the studies considered by AIFA to draft the determination, highlight how crucial it is to investigate the effect of Vitamin D supplementation on clinical outcomes within a range of 25(OH)D values where a beneficial effect of the same supplementation is at least presumable based on large-scale observational studies. In other words, etiological research, aimed at analyzing cause-effect relationships through interventional studies, must maintain consistency between its observational and experimental components [31].

Regarding excess values, some guidelines are based on only two studies [25,26], that state that 25-(OH) D values >112 nmol (45 ng/mL) are associated with a progressive increase in the risk of adverse events, including mortality. More recent studies document a progressive reduction in

 doi:10.20944/preprints202407.1461.v1

mortality as 25-(OH) blood levels increase up to about 50 nmol/L, followed by a "steady state" up to values of 125 nmol/L, without identifying threshold values associated with mortality risk from various causes [32]. The most recent prospective study by Takacs and coll. did not find any increase in the risk of falls, adverse events, hypercalcemia, and bone metabolism alterations up to values of 150 nmol/L [33].The reported supposed risk of prostate and pancreatic cancer for values >100 nmol/L, noted by AIFA Note 96, has not been confirmed in more recent studies that, instead, show a reduction in the risk of metastatic cancer incidence and cancer mortality for the same blood levels (<100 nmol/L) [34,35]. Therefore, some guidelines consider Vitamin D supplementation mandatory in all the categories of individuals/patients shown in Table 2, regardless of the Vitamin D blood levels; however, they recommend measuring it when "essential for the clinical management of the patient, for example, for differential diagnosis or after starting supplementation to ascertain the achievement of optimal levels after 3-6 months.".

Table 2. Subjects at risk of Vitamin D deficiency according to the clinical evaluation (from ref 50, modified).

Older people

Housebound people:

- disabled people;
- institutionalized people;

People working long time indoors:

- office workers;
- factory workers;

People with dark skin

People with chronic/debilitating diseases:

- diabetes;
- chronic kdney disease
- gastrointestinal malabsorptive syndromes;
- parathyroid disorders;
- liver diseases;

Obese people, particularly those with very high levels of Body Mass Index (BMI)

People after bariatric surgery

People taking drugs increasing Vitamin D catabolism:

- Carbamazepine;
- Desamethasone;
- Rifampicin;
- Sironolattone;

Childrens of mother with Vitamin D deficiency

When is the Request and Determination of 25-(OH)D Indicated?

The second issue to be discussed is when is the Request and Determination of 25-(OH)D Indicated? Firstly, some key principles and requirements must be clearly identified to make the determination of Vitamin D appropriate, which are not clearly outlined in some guidelines:

a) given the variability of vitamin levels linked to seasonality, the assessment of vitamin status should be recommended during the period between the end of winter and the beginning of summer.

b) the results obtained by clinical laboratories, whether performed with immunoassays or LC-MS methods, show a bias compared to the reference method. The measurement uncertainty that can be reasonably proposed is to consider a variability equal to ±10% compared to the reported value. This aspect is particularly important when the value is close to the decision level, especially the level identifying "deficiency," i.e., a value <30 nmol/L (12 ng/mL).

 doi:10.20944/preprints202407.1461.v1

c) finally, it is recommended to harmonize the measurement units, identifying nmol/L as the metrologically correct and most internationally adopted unit

Ultimately, the determination of Vitamin D appears appropriate and recommended:

- In all cases of clinical suspicion of osteomalacia (confirmation of the diagnostic hypothesis and/or differential diagnosis) and should be repeated at intervals of 3-6 months.
- In all cases of suspected hyperparathyroidism, both primary and secondary, and in patients diagnosed with this condition.
- In patients with chronic renal insufficiency for the evaluation and monitoring of chronic kidney diseases with mineral and bone metabolism disorders (CKD-MBD).
- In pediatric subjects with risk factors for deficiency, with growth delays, and in the case of long periods of hospitalization/institutionalization.

It should be noted that a recently published review, which summarizes the analytical issues and clinical implications of Vitamin D determination in light of the ongoing problems, concludes by suggesting the importance of further studies on supplementation that use standardized methods and enroll only patients with Vitamin D deficiency, reinforcing previously reported concerns [36]. In addition, given the important interrelations between vitamin D and other measurands (e.g. calcium, phosphate, parathyroid hormone, fibroblast growth factor 23, $1,25(OH)_2D$, in the regulation of bone metabolism, an integrated interpretation of all laboratory biomarkers is now recommended.

1,25. Dihydroxyvitamin D ($1,25(OH)_2D$)

While there is broad consensus on using the determination of the biological form 25-hydroxylated (25-OH Vitamin D), it is known that the biologically active form of the vitamin is the 1,25-dihydroxylated form [$1,25(OH)_2D$]. This evidence, in turn, raises the question of whether and when the determination of the latter form should be combined with that of 25-OH Vitamin D. Determination of $1,25(OH)_2D$ is significantly more complicated than that of 25(OH)D because its concentration in serum/plasma is much lower. Moreover, there are neither reference materials nor a reference measurement procedure available. The first method for determining $1,25(OH)_2D$, developed in 1974, was a radioreceptor assay [25], while in subsequent years other determination techniques such as HPLC, EIA, Gas Chromatography-Mass Spectrometry, and finally LC-MS/MS were developed [37,38]. The development of automated immunoassays [4,39]) has significantly changed the situation, making the determination of this vitamer available in many clinical laboratories. The most recent data from EQA programs demonstrate that 75% of participants use automated methods, 15% manual immunoassays, and only 9% LC-MS/MS techniques [10]. Some automated immunoassays, particularly the one proposed by DiaSorin and applied to the Liaison XL instrument, significantly correlate with LC-MS/MS methods and offer clear advantages in routine diagnostic management, such as high productivity and savings in time and human resources. Therefore, their development can expand and improve the clinical use of $1,25(OH)_2D$ determination [40,41]. Unlike 25(OH)D, decision levels have not been identified, and reference intervals vary depending on the method used. In adults, the range of values obtained with a radioimmunological method varies between 43 and 168 pmol/L, while with an automated immunometric method (IDS iSYS), the range is between 63 and 228 pmol/L [39], and with the DiaSorin Liaison XL method, the range varies between 77–471 pmol/L in pediatric subjects aged 0 to 1 year, 113–363 pmol/L between 1 and 3 years, and 108–246 pmol/L in children over 3 years of age [42,43]. In addition, higher serum levels of $1,25(OH)_2D$ have been observed in individuals of African descent, which have been associated with higher PTH levels. To date, the clinical utility of determining $1,25(OH)_2D$ is poorly recognized. Its serum concentration has little correlation with vitamin status and is instead strongly regulated by PTH. Thus, its determination has been considered useful in the evaluation of patients with hypercalcemia of unknown origin, sarcoidosis, pseudo-vitamin D deficiency, rickets, tumor-induced osteomalacia, hyperparathyroidism, and CYP24A1 deficiency situations. Additionally, its determination can be useful in the differential diagnosis of FGF23-dependent phosphopenic rickets [44]. Decreased values are found in chronic kidney disease (CKD) and hyperparathyroidism, while serum levels increase in sarcoidosis and tuberculosis [44]. Certain drugs reduce $1,25(OH)_2D$ levels,

 doi:10.20944/preprints202407.1461.v1

including some antifungals used to treat hypercalcemia associated with tuberculosis. During pregnancy, circulating levels increase due to the induction of 1α-hydroxylase activity, which is also expressed at the placental level. Certain genetic mutations influence 1,25(OH)$_2$D levels and cause rare bone metabolic diseases, including hereditary vitamin D-resistant rickets (VDR), type A vitamin D-dependent rickets (CYP7B1), type B (CYP2R1), and idiopathic infantile hypercalcemia (CYP24A1). Some mutations of the metalloprotease PHEX also cause X-linked hypophosphatemia, characterized by normal-low concentrations of 1,25(OH)$_2$D (45). However, recent experimental data [46,47] provide useful elements for a possible revision of the current recommendations on its use and the combined use of both biological forms, in addition to the clinical situations and conditions in which 1,25(OH)$_2$D determination is currently suggested.

New Experimental Evidence

It is known that the hydroxylation of both biological forms (Vitamin D2 and Vitamin D3) occurs in the liver due to the enzyme D-25-hydroxylase (CYP2R1). The further hydroxylation into the biologically active form, 1,25(OH)$_2$D, was previously thought to occur only in the kidneys via the action of 25-hydroxyvitamin D-1-alpha-hydroxylase (CYP27B1). It is important to note that this second hydroxylation depends on circulating parathyroid hormone (PTH) levels, confirming the complex regulation mechanisms of the formation of the biological forms of vitamin D. More recently, however, evidence has emerged showing the formation of 1,25-dihydroxyvitamin D not only in the kidneys but also in many other types of cells, including endothelial cells, cardiomyocytes, vascular smooth muscle cells, astrocytes, and microglia. These forms do not enter circulation but still regulate metabolism through paracrine and/or autocrine actions. In particular, a recent review reports evidence from numerous studies showing that the "second" hydroxylation by the enzyme 25-hydroxyvitamin D-1-alpha hydroxylase (CYP27B1) occurs not only in renal tubules: the expression of the CYP27B1 gene has been found in endothelial cells, vascular smooth muscle cells, cardiomyocytes, fibroblasts, astrocytes, epithelial cells, and microglia [46]. Specifically, it has been observed that 1,25-dihydroxyvitamin D3 can protect various types of cells from different types of stress, such as hydrogen peroxide, radiation, and high glucose levels. Additionally, it has an anti-fibrotic effect on cardiomyocytes and fibroblasts. Based on this recently collected data, clinical studies will be needed to better understand if these new pathophysiological insights could lead to revised and expanded uses in clinical practice, beyond experimental settings. Furthermore, data have been reported on the significant correlation between vitamin status and fatty liver diseases and the effect of supplementation with 1,25(OH)$_2$D, which significantly reduced triglyceride content, lipid peroxidation, and cellular damage [47].

Current Recommendations on the Request and Determination of 1,25(OH)$_2$D

The most recent and accredited recommendations on the determination of 1,25(OH)$_2$D are those published by an IFCC working group in 2021 [48]. The authors highlight, in the preamble, the analytical issues, particularly the poor standardization of methods, which have certainly hampered its use in clinical practice. To date, its determination is suggested in the following conditions:

a) Hypercalcemia;

b) Osteomalacia and calcipenic rickets;

c) Differential diagnosis of FGF23-mediated or non-mediated phosphopenic rickets;

d) Genetic disorders involving CYP27B1, the VDR receptor, and extra-renal production of 1,25(OH)2D;

e) X-linked hypophosphatemia;

f) Rare diseases such as McCune–Albright syndrome, epidermal nevus syndrome, neurofibromatosis, Jansen metaphyseal chondrodysplasia, and hypophosphatemic rickets with hyperparathyroidism.

The authors also suggest not recommending the determination of 1,25(OH)$_2$D in the monitoring of patients with Chronic Kidney Disease (CKD), while further studies are deemed necessary to ensure the development of a reference method, standardization of currently available methods, and the

 doi:10.20944/preprints202407.1461.v1

identification of appropriate reference values in adults and pediatric subjects. In a more recent study, Herrmann [49] identified the most relevant clinical situations in which the determination of the dihydroxylated form is indicated, namely:

a) Hypercalcemia of unexplained nature;
b) Sarcoidosis and other granulomatous diseases;
c) Tumor-induced osteomalacia;
d) Primary hyperparathyroidism.

Moreover, its determination is important in conditions induced by genetic mutations that lead to altered vitamin D metabolism and cause rare bone metabolic diseases such as hereditary vitamin D-resistant rickets, type 1 vitamin D-dependent rickets, idiopathic infantile hypercalcemia, and X-linked hypophosphatemia. Giustina et al. have recently confirmed the appropriateness of determining 1,25(OH_2)D in the aforementioned clinical situations [50]. Therefore, given the availability of validated and automated methods, the determination of 1,25($OH)_2$D is suggested in association with 25-OH Vitamin D in numerous clinical situations, summarized as shown in Table 3.

Table 3. Clinical conditions in which the combined measurement of both 25-OHD and 1,25(OH)2D should be recommended.

a) Forms of hypercalcemia without immediate clinical explanation.
b) Primary hyperparathyroidism.
c) Forms of hypovitaminosis not responding to therapy.
d) Sarcoidosis and granulomatous diseases.
e) Phosphopenic and calciopenic rickets.
f) Clinical situations that may suggest rare and genetic diseases related to Vitamin D metabolism.

Parathyroid Hormone (PTH) and Fibroblast Growth Factor 23 (FGF23)

The understanding of the pathophysiology regulating calcium-phosphate metabolism has significantly advanced, providing crucial clinical implications and leading to more refined diagnostic testing for better classification, prognostic indications, targeted therapies, and patient monitoring. Notably, it has long been established that PTH is essential for converting vitamin D into its biologically active (dihydroxylated) form. More recent evidence highlights the importance of fibroblast growth factor 23 (FGF23) in maintaining calcium and phosphate homeostasis in concert with PTH and the active form of vitamin D, 1,25($OH)_2$D. Elevated levels of circulating phosphates and vitamin D stimulate FGF23 production in bones, which in turn acts on the kidneys to bind FGF receptors and the co-receptor Klotho, promoting phosphate clearance (increased phosphaturia) and reducing circulating levels of 1,25($OH)_2$D. Agoro and White [51] have comprehensively summarized these new findings. Thus, understanding the interconnected mechanisms regulating calcium-phosphate homeostasis and bone metabolism underpins the rationale for evaluating not just individual parameters but their collective interactions. These disorders are common in the general population, influenced by genetic, metabolic, and environmental factors.

Combined Determination of PTH and Vitamin D

Given the pathophysiological background, combined determination of PTH and Vitamin D is particularly rational for diagnosing hypercalcemia and hypocalcemia. In primary hyperparathyroidism, circulating PTH levels are higher than expected based on calcium levels alone, whereas in other forms of hypercalcemia, PTH values are low. In secondary hyperparathyroidism, PTH levels are elevated due to hypocalcemia and/or vitamin D deficiency. Smit et al.'s review [52] convincingly underscores the necessity of this "combined" request for PTH and Vitamin D.

 doi:10.20944/preprints202407.1461.v1

Particularly in diagnosing and monitoring chronic kidney disease mineral and bone disorders (CKD-MBD), the KDIGO 2017 [53,54] guidelines recommend this combination across various stages:

a) CKD Stage G3a: Baseline measurements of PTH, Calcium (Ca), Phosphorus (P), and alkaline phosphatase (ALP); Ca and P every 6-12 months; PTH to assess disease progression.
b) CKD Stage G4: PTH every 6-12 months; Ca and P every 3-6 months.
c) CKD Stages G3a-G5D: Add 25-OH Vitamin D and repeat measurements based on treatment and disease progression, including collagen synthesis (Propeptide C-terminal of type I collagen, PINP) and degradation markers (C-terminal telopeptide of type I collagen, CTx) in specific cases.

A critical aspect highlighting the necessity of combined PTH and Vitamin D measurement is the influence of circulating vitamin D levels on PTH reference intervals. Literature shows that PTH reference intervals excluding subjects with vitamin D deficiency are approximately 20% lower than those including subjects with hypovitaminosis D. This observation is well-supported and integrated into recent PTH review [52]. Additionally, PTH levels are higher in African Americans and those with darker skin compared to whites, correlate with body mass index (BMI), and increase with age due to reduced glomerular filtration volume in individuals over 60.

Third-Generation PTH

Current literature and guidelines suggest abandoning first-generation methods and continuing the use of second-generation methods primarily due to the earlier limited commercial availability of third-generation methods. Analytical advantages of third-generation methods (especially Liaison 1-84 PTH) include reduced variability between matrices (serum and EDTA plasma) and lower interference and cross-reactivity with fragments 7-84. Clinical benefits have been demonstrated in hemodialysis patients, with third-generation methods providing a more accurate correlation with therapeutic response (55). Third-generation methods uniquely determine the biologically active N-terminal form of PTH. In chronic kidney disease, third-generation methods provide PTH values approximately 50% lower than second-generation methods, with significant intra-individual differences, as they are not influenced by N-terminal residues [56,57]. In clinical practice, many academic laboratories have long adopted third-generation methods for routine PTH determination, demonstrating significant clinical benefits [58–60]. The most significant evidence for using third-generation methods is during parathyroidectomy, where PTH values drop more rapidly post-surgery, enabling quicker and more effective surgical evaluations [61,62].

Fibroblast Growth Factor 23 (FGF23)

Discovered in 2003, mutations or excessive production of FGF23 cause rare diseases like vitamin D-resistant hypophosphatemic rickets and tumor-induced osteomalacia [63]. FGF23 is a hormone orchestrating calcium and phosphate homeostasis alongside PTH and 1,25-dihydroxyvitamin D, requiring the transmembrane protein Klotho. FGF23 regulates phosphate homeostasis through the "FGF23/Klotho" system [64]. Methods to determine FGF23, measuring intact (iFGF23) and C-terminal (cFGF23) fragments, have increased its clinical relevance, particularly in chronic kidney disease, cardiovascular diseases, and erythropoiesis regulation [65–67]. Determining both FGF23 forms and the iFGF23/cFGF23 ratio is proposed as a "liquid biopsy" for FGF23 dynamics [67]. Given the therapeutic potential of the FGF23/Klotho axis, clinical determination of circulating levels is increasingly important [68,69].

Conclusions

Despite a long history, the saga of vitamin D measurement and supplementation requires further concern. First, the lack of analytical standardization strongly affects data on decision levels, as they were obtained in clinical trials which used non-standardized assays. The current availability of reference procedure measurements (RPM) and reference materials allows to identify evidence-based decision levels to be applied in clinical practice and laboratory reports. Therefore, harmonization of currently recommended decisional levels and of measurement units is mandatory to avoid further

 doi:10.20944/preprints202407.1461.v1

confusion and to allow the right interpretation of laboratory results. Second, the adoption of current recommendations on the appropriate request of vitamin D measurement is needed. Third, even if still controversial, the adherence to current recommendations on the categories of individuals/patients who benefit from Vitamin D supplementation is strongly suggested. Fourth, the integrated evaluation of laboratory tests (e.g. 25(OH)D, calcium, phosphorus, PTH, and in selected cases 1,25(OH)$_2$D and FGF-23) is recommended or an appropriate interpretation of bone metabolism disorders.

References

1. Giuliani S, Barbieri V, Di Pierro AM, Rossi F, Widmann T, Lucchiari M et al. LC-MS/MS based 25(OH)D status in a large Southern European outpatient cohort: gender- and age-specific differences. Eur J Nutr 2019; 58:2511-2520.
2. Stoica AB, Mărginean C. The Impact of Vitamin D Deficiency on Infants' Health. Nutrients 2023;15(20):4379doi: 10.3390/nu15204379.
3. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, molecular mechanism of action, and pleiotropic effects. Physiol Rev 2016; 96:365-408,
4. Pilkey NG, Novosel O, Roy A, Wilson TE, Sharma J, Khan S et al. Does Native Vitamin D Supplementation Have Pleiotropic Effects in Patients with End-Stage Kidney Disease? A Systematic Review of Randomized Trials. Nutrients 2023; 15: 3072. https://doi.org/10.3390/nu15133072.
5. Cosentino N, Campodonico J, Milazzo V. Vitamin D and cardiovascular disease: current evidence and future perspectives. Nutrients 2021; 13:3603. https://doi.org/10.3390/nu13103603.
6. Holick MF. The use and interpretation of assays for vitamin D and its metabolites. J Nutr 1990;120 Suppl 11:1464-9.
7. Lips P. Relative value of 25(OH)D and 1,25(OH)2D measurements. J Bone Miner Res 2007; 22:1668-71.
8. Amrein K, Scherkl M, Hoffmann M, Neuwersch-Sommeregger S, Köstenberger M, Tmava Berisha A et al. Vitamin D deficiency 2.0: an update on the current status worldwide. Eur J Clin Nutr 2020;74: 1498-1513.
9. Alonso N, Zelzer S, Eibinger G, Herrmann M. Vitamin D Metabolites: Analytical Challenges and Clinical Relevance. Calcif Tissue Int 2023; 112:158-177.
10. Wallace AM, Gibson S, de la Hunty A, Lamberg-Allardt C, Ashwell M. Measurement of 25- hydroxyvitamin D in the clinical laboratory: current procedures, performance characteristics and limitations. Steroids 2010;75: 477-88.
11. Altieri B, Cavalier E, Bhattoa HP, Pérez-López FR, López-Baena MT, Pérez-Roncero GR et al. Vitamin D testing: advantages and limits of the current assays. Eur J Clin Nutr 2020;74: 231-247.
12. Bikle DD. Vitamin D Assays. Front Horm Res 2018;50: 14-30.
13. Belsey R, DeLuca HF, Potts Jr, JT Competitive binding assay for vitamin D and 25-OH vitamin D. J Clin Endocrinol Metab 1971; 33: 554-557.
14. Bouillon R, Van Heck E, Jans I, Tan BK, Van Baelen H, De Moor P. Two direct (nonchromatographic) assays for 25-hydroxyvitamin D. Clin Chem 1984;30:1731–6.
15. Le Goff C, Cavalier E, Souberbielle JC, González-Antuña A, Delvin E. Measurement of circulating 25-hydroxyvitamin D: A historical review. Pract Lab Med 2015; 12:1-14.
16. Centers for Disease Control and Prevention (CDC). Vitamin D Stardardization-Certification Program (VDSCP). Accessed April 24, 2024. https://www.cdc.gov/labstandards/csp/vdscp_procedures.htlm
17. Binkley N, Dawson-Hughes B, Durazo-Arvizu R, Thamm M, Tian L, Merkel JM et al. Vitamin D measurement standardization: The way out of the chaos. J Steroid Biochem Mol Biol. 2017; 173:117-121
18. Durazo-Arvizu RA, Tian L, Brooks SPJ, Sarafin K, Cashman KD, Kiely M et al. The Vitamin D Standardization Program (VDSP) Manual for Retrospective Laboratory Standardization of Serum 25-Hydroxyvitamin D Data. J AOAC Int2017;100: 1234-1243.
19. Briggs LE, Whitewood JK, Williams EL. Analytical variation concerning total 25-hydroxyvitamin D measurement, where are we now? A DEQAS review of current assay performance J Steroid Biochem Mol Biol 2023;231:106328. https://doi.org/10.1016/j.jsbmb.2023.106328.
20. Herrmann M, Zelzer S, Cavalier E, Kleber M, Drexler-Helmberg C, Schlenke P et al. Functional Assessment of Vitamin D Status by a Novel Metabolic Approach: The Low Vitamin D Profile Concept. Clin Chem 2023; 69:1307-1316.
21. Schwartz JB, Gallagher JC, Jorde R, Berg V, Walsh J , Eastell R et al. Determination of free 25(OH)D concentrations and their relationships to total 25(OH)D in multiple clinical populations. J Clin Endocrinol Metab 2018; 103:3278-3288.
22. Nielson CM, Jones KS, Bouillon R. Osteoporotic Fractures in Men (MrOS) Research Group. Role of assay type in determining free 25-hydroxyvitamin D levels in diverse populations. N Engl J Med 2016;374: 1695-1696.

23. Nielson CM, Jones KS, Chun RF, Jacobs JM, Wang Y, Hewison Met al. Free 25-hydroxyvitamin D: impact of vitamin D binding protein assays on racial-genotypic associations. J Clin Endocrinol Metab 2016; 101:2226-2234.
24. El Sabeh M, Ghanem P, Al-Shaar L, Rahme M, Baddoura R, Halaby G et al. Total, bioavailable, and free 25(OH)D relationship with indices of bone health in elderly: a randomized controlled trial. J Clin Endocrinol Metab 2021; 106: e990-e1001.
25. LeBoff MS, Chou SH, Ratlif KA, Cook NR, Khurana B, Kim E, Cawthon PM et al Supplemental vitamin D and incident fractures in midlife and older adults. N Engl J Med 2022; 387:299–309.
26. Bischoff-Ferrari HA, Vellas B, Rizzoli R, Kressig RW, da Silva JAP, Blauth M,et al.; DO-HEALTH Research Group. Effect of Vitamin D Supplementation, Omega-3 Fatty Acid Supplementation, or a Strength-Training Exercise Program on Clinical Outcomes in Older Adults: The DO-HEALTH Randomized Clinical Trial. JAMA2020; 324:1855-1868.
27. Agenzia Italiana del Farmaco, "Nota 96 per la prescrizione di farmaci a base di vitamina D,". Allegato 1, 2023, [Online]. Available: https://www.aifa.gov.it/Nota-96.
28. SIOMMMS "Sintesi delle nuove raccomandazioni 2022 della Società Italiana dell'Osteoporosi, del Metabolismo Minerale e Malattie dello Scheletro (SIOMMMS) per la gestione della carenza di vitamina D,"2022. https://www.siommms.it/wp-content/uploads/2023/03/Sintesi-nuove-raccomandazioni-SIOMMMS-2022-carenza-vitamina-D.pdf
29. Demay MB, Pittas AG, Bikle DD, Diab DL, Kiely ME, Lazaretti-Castro M, et al. Vitamin D for the Prevention of Disease: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab. 2024 Jun 3:dgae290. https://doi.org/10.1210/clinem/dgae290. Epub ahead of print
30. Dittadi R, Corbetta S, Banfi G, Bertoldo F, Migliaccio S, Gonnelli S et al. Documento congiunto di SIBIOC, della Società Italiana dell'Osteoporosi, del Metabolismo Minerale e delle Malattie dello Scheletro (SIOMMMS), della Sezione Italiana della European Ligand Assay Society (ELAS), e della Associazione Medici Endocrinologi (AME) relativo alla nota AIFA 96 sulla prescrivibilità dei farmaci per la carenza di Vitamina D, e raccomandazioni per la refertazione. Biochim Clin 2020; 44:400-406.
31. Tripepi G, Fusaro M, Arcidiacono G, Sella S, Giannini S. Evaluating benefit from vitamin D supplementation: defining the area for treatment. Osteoporos Int 2023; 34:1531-1533.
32. Cummings SR, Rosen C. VITAL findings — a decisive verdict on vitamin D supplementation. New Engl J Med2022; 387:368–370.
33. Takács I, Tóth BE, Szekeres L, SzabóB, Bakos B, Lakatos P. Randomized clinical trial to comparing efficacy of daily, weekly and monthly administration of vitamin D (3). Endocrine. 2017;55: 60-65.
34. Lv Q-B, Gao X, Liu X, Shao ZX, Xu QH,Tang L et al. The serum 25-hydroxyvitamin D levels and hip fracture risk: a meta-analysis of prospective cohort studies. Oncotarget 2017; 8:39849–39858.
35. Stroomberg HV, Vojdeman FJ, Madsen CM, Helgstrand JT, Schwarz P, Heegaard AM, et al. Vitamin D levels and the risk of prostate cancer and prostate cancer mortality. Acta Oncol 2021;60: 316-322.
36. Cavalier E, Makris K, Heijboer AC, Herrmann M, Souberbielle JC. Vitamin D: analytical advances, clinical impact, and ongoing debates on health perspectives. Clin Chem 2024 May 7:hvae056. https://doi.org/10.1093/clinchem/hvae056. Online ahead of print
37. Tsugawa N, Okano T. Bone and bone related biochemical examinations. Hormone and hormone related substances. Vitamin D (25D, 1,25D); measurements and clinical significances. Clin Calcium 2006;16: –42.
38. Hollis BW, Horst RL The assessment of circulating 25(OH)D and 1,25(OH)2D: where we are and where we are going. J Steroid Biochem Mol Biol 2007; 103:473-6.
39. Hussein H, Ibrahim F, Boudou P. Evaluation of a new automated assay for the measurement of circulating 1,25-dihydroxyvitamin D levels in daily practice. Clin Biochem 2015; 48:1160– 1162.
40. Valcour A, Zierold C, Podgorski AL, Olson GT, Wall JV, DeLuca HF et al. A novel, fully automated, chemiluminescent assay for the detection of 1,25-dihydroxyvitamin D in biological samples. J Steroid Biochem Mol Biol 2016; 164:120–126.
41. Spanaus K, von Eckardstein A. Evaluation of two fully automated immunoassay-based tests for the measurement of 1α,25-dihydroxyvitamin D in human serum and comparison with LC-MS/MS. Clin Chem Lab Med 2017;55:1305-1314.
42. Higgins V, Truong D, White-Al Habeeb NMA, Fung AWS, Hoffman B, Adeli K. Pediatric reference intervals for 1,25-dihydroxyvitamin D using the DiaSorin LIAISON XL assay in the healthy CALIPER cohort. Clin Chem Lab Med 2018; 56:964–972.
43. Souberbielle JC, Cavalier E, Delanaye P, Massart C, Brailly-Tabard S, Cormier C et al. Serum calcitriol concentrations measured with a new direct automated assay in a large population of adult healthy subjects and in various clinical situations. Clin Chim Acta 2015; 451:149-53.
44. Adams JS, Hewison M. Extrarenal expression of the 25-hydroxyvitamin D-1-hydroxylase. Arch Biochem Biophys 2012; 523:95-102.

45. Goltzman D. Functions of vitamin D in bone. Histochem Cell Biol 2018; 149:305–312.
46. Janubová M, Žitňanová I. The effects of vitamin D on different types of cells. Steroids 2024;202: 109350. https://doi.org/10.1016/j.steroids.2023.109350.
47. Chang E. Vitamin D Mitigates Hepatic Fat Accumulation and Inflammation and Increases SIRT1/AMPK Expression in AML-12 Hepatocytes. Molecules 2024;29: doi 10390 /molecules 2906140.
48. Makris K, Bhattoa HP, Cavalier E, Phinney K, Sempos CT, Ulmer CZ, et Recommendations on the measurement and the clinical use of vitamin D metabolites and vitamin D binding protein - A position paper from the IFCC Committee on bone metabolism. Clin Chim Acta 2021; 517:171-197.
49. Herrmann M. Assessing vitamin D metabolism - four decades of experience. Clin Chem Lab Med 2023;61: 880-894.
50. Giustina A, Bilezikian JP, Adler RA, Banfi G, Bikle DD, Binkley NC et al. Consensus Statement on Vitamin D Status Assessment and Supplementation: Whys, Whens, and Hows. Endocr Rev 2024;. https://doi.org/10.1210/endrev/bnae009.
51. Agoro R, White KE. Regulation of FGF23 production and phosphate metabolism by bone-kidney interactions. Nat Rev Nephrol 2023; 19:185-193.
52. Smit MA, van Kinschot CMJ, van der Linden J, van Noord C, Kos S. Clinical Guidelines and PTH Measurement: Does Assay Generation Matter? Endocr Rev 2019; 40:1468-1480.
53. Ketteler M, Block GA, Evenepoel P, Fukagawa M, Herzog CA, McCann L et al. Executive summary of the 2017 KDIGO Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD) Guideline Update: what's changed and why it matters. Kidney Int. 2017; 9:26-36
54. Ketteler M, Block GA, Evenepoel P, Fukagawa M, Herzog CA, McCann L, et al. Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder: Synopsis of the kidney disease: Improving Global Outcomes 2017 Clinical Practice Guideline Update. Ann Intern Med 2018;168(6):422-430.
55. Valcour A, Zierold C, Blocki FA, Hawkins DM, Martin KJ, Rao SD et al. Trueness, precision and stability of the LIAISON 1-84 parathyroid hormone (PTH) third-generation assay: comparison to existing intact PTH assays. Clin Chem Lab Med 2018;56: 1476-1482.
56. Einbinder Y, Benchetrit S, Golan E, Zitman-Gal T. Comparison of intact PTH and bio-intact PTH assays among non-dialysis dependent chronic kidney disease patients. Ann Lab Med 2017; 37:381–387.
57. Gao P, Scheibel S, D'Amour P, John MR, Rao SD, Schmidt-Gayk H,et al. Development of a novel immunoradiometric assay exclusively for biologically active whole parathyroid hormone 1- 84: implications for improvement of accurate assessment of parathyroid function. J Bone Miner Res 2001; 16:605–614.
58. Sella S, Bonfante L, Fusaro M, Neri F, Plebani M, Zaninotto M et al. Efficacy of weekly administration of cholecalciferol on parathyroid hormone in stable kidney-transplanted patients with CKD stage 1-3. Clin Chem Lab Med 2020; 59:343-351.
59. Censi S, Iacobone M, Simmini S, Manso J, Franceschet G, Plebani M et al. PTH: Redefining Reference Ranges in a Healthy Population-The Role of Interfering Factors and the Type of Laboratory Assay. Int J Endocrinol 2020;. https://doi.org/10.1155/2020/1053719
60. Chiang C. The used of bone turnover markers in chronic kidney disease-mineral and bone disorders. Nephrol 2017; Suppl 2; 11-13
61. Cavalier E, Schleck ML, Souberbielle JC. Spurious intraoperative PTH results observed with 2nd, but not with 3rd generation PTH assays. Clin Chim Acta 2018; 477:72–73.
62. Yamashita H, Gao P, Cantor T, Noguchi S, Uchino S, Watanabe S, et al. Comparison of parathyroid hormone levels from the intact and whole parathyroid hormone assays after parathyroidectomy for primary and secondary hyperparathyroidism. Surgery 2004; 135:149–156.
63. Jonsson KB, Zahradnik R, Larsson T, White KE, Sugimoto T, Imanishi Y et al. Fibroblast growth factor 23 in oncogenic osteomalacia and X-linked hypophosphatemia. N Engl J Med 2003; 348:1656-63.
64. Donate-Correa J, Muros-de-Fuentes M, Mora-Fernández C, Navarro-González JF. FGF23/Klotho axis: phosphorus, mineral metabolism and beyond. Cytokine Growth Factor Rev 2012;23:37-46
65. Moe SM, Chertow GM, Parfrey PS, Kubo Y, Block GA, Correa-Rotter R et al.; Evaluation of Cinacalcet HCl Therapy to Lower Cardiovascular Events (EVOLVE) Trial Investigators*. Cinacalcet, Fibroblast Growth Factor-23, and Cardiovascular Disease in Hemodialysis: The Evaluation of Cinacalcet HCl Therapy to Lower Cardiovascular Events (EVOLVE) Trial. Circulation. 2015; 132:27-39.
66. Edmonston D, Grabner A, Wolf M. FGF23 and klotho at the intersection of kidney and cardiovascular disease. Nat Rev Cardiol 2024; 21:11-24.
67. Edmonston D, Wolf M. FGF23 at the crossroads of phosphate, iron economy and erythropoiesis. Nat Rev Nephrol 2020; 16:7-19.

 doi:10.20944/preprints202407.1461.v1

68. Smith ER, McMahon LP, Holt SG. Fibroblast growth factor 23. Ann Clin Biochem 2014;51: 203-27.
69. Ferraro S, Biganzoli G, Calcaterra V, Zuccotti G, Biganzoli EM, Plebani M. Fibroblast growth factor 23: translating analytical improvement into clinical effectiveness for tertiary prevention in chronic kidney disease. Clin Chem Lab Med 2022; 60:1694-1705.