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Reference values for free 25-hydroxy-vitamin D based on established total 25-hydroxy-vitamin D reference values

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ABSTRACT

Measurements of total 25-hydroxyvitamin D (t25(OH)D) are currently primarily used to assess the vitamin D status. The lipophilic cell membrane can only be passed by the un-bound form of 25-hydroxyvitamin D: free 25-hydroxyvitamin D (f25(OH)D). It is thought that f25(OH)D does reflect its biological actions better than t25(OH)D. However, as of today, there are no established guidelines for the clinical use of f25(OH)D.

We analysed 5060 patients with simultaneous measurements of free and total 25(OH). Linear regression was used to study the relationship between free 25(OH)D and total 25(OH)D. We reviewed and used the established t25(OH)D reference values and determined the slope of the relationship between them to calculate reference values for f25(OH)D.

F25(OH)D and t25(OH)D showed a strong positive linear ($r = 0.8395$, $p < 0.0001$) correlation. The slope of the relationship was 0.2833 ± 0.00257. The recommended threshold level of f25(OH)D is 8.499 pg/mL, corresponding to a target concentration for t25(OH)D of at least 30 ng/mL considered as sufficient in most of the international vitamin D guidelines. The upper limit for vitamin D is less clear in the guidelines. Most experts favour an upper limit for t25(OH)D of 100 ng/mL. This is equivalent to 28.330 pg/mL f25OHD.

We established based on international guidelines for t25(OH)D reference values for f25(OH)D that are urgently needed for clinical use of f25(OH)D. However, clinical studies with f25(OH)D to confirm our suggestions are needed but will take time.

1. Introduction

Measurements of total 25-hydroxyvitamin D (t25(OH)D) is currently used to assess whether a patient is adequately supplied with vitamin D. However, t25(OH)D covers the free, biologically available vitamin D and the bound content and cannot differentiate between them [1–3]. The free form of 25(OH)D can be determined indirectly by calculating it based on measurements of albumin, vitamin D binding protein, and t25(OH)D. Recent developments enabled also a direct measurement of free 25(OH)D (f25(OH)D) using an ELISA based technology [1,2].

Vitamin D is not a vitamin, but rather a hormone. Like lipophilic steroid hormones such as testosterone or cortisol, 25(OH)D needs to be bound to a carrier protein to be transported in an aqueous solution like blood. Similar to thyroid hormone, testosterone and cortisol, 25(OH)D does not act on a cell membrane bound receptor, it acts on a nuclear receptor after passing the cell membrane, a lipid bilayer. The lipophilic cell membrane can only be passed by the un-bound free form of 25(OH)D; thus, it is thought that this unbound form of vitamin D does reflect its biological actions better than t25(OH)D. This concept is known as “free hormone hypothesis” in the endocrinological literature. Only free, unbound vitamin D is biologically active. About 85–90 % of vitamin D are bound to vitamin D-binding protein (VDBP), and only about 10 % are

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bound to albumin. Only about 1% of vitamin D is freely available. [4]. Total vitamin D measurements reflect the sum of vitamin D-binding protein and albumin bound vitamin D plus free vitamin D. Since the strength of the non-covalent vitamin D binding to albumin is much weaker as compared to the non-covalent binding of vitamin D to VDBP, the sum of free and albumin bound vitamin D is called bioavailable vitamin D, because after free vitamin D passed the lipophilic cell membrane, albumin bound vitamin D is released fast from albumin and can thus replace the free vitamin D uptaken by the cell. In contrast to free and total vitamin D concentrations which can be determined by direct ELISA`s, determinations of bioavailable vitamin D is more complex and requires the measurements of albumin, VDBP and total vitamin D followed by mathematical calculations [5]. Total vitamin D is considered a reservoir that is not immediately available to the body's cells, because the vast majority of total vitamin D cannot penetrate the cell membranes due to the hydrophobic nature of albumin and in particular VDBP.

There are only three exceptions to this general rule. In the proximal tubules of the kidney, the chief cells of the parathyroid gland and the placenta, vitamin D bound to vitamin D binding protein is uptaken by either megalin- or cubulin- mediated pathways. This is a specific and fast uptake compared to the passive, concentration dependent diffusion in all other cell types of the human body.

Measurements of total vitamin D depends on the variables (25(OH)D, albumin, and vitamin D binding protein). The concentration of vitamin D binding protein in the blood depends on factors that are not related to mineral-bone metabolism such as liver function (vitamin D binding protein is synthesised in the liver), kidney function (vitamin D binding protein is freely filtered by the glomeruli and reuptake in the proximal tubules by the megalin receptor complex; if this process is affected by diseases such as diabetic nephropathy, the human body loses huge amounts of vitamin D binding protein), and endocrine status (oestrogens for example stimulate the synthesis of vitamin D binding proteins in the liver). There is also evidence that the binding affinity of the vitamin D binding protein depends on the ethnic background. [2,4,6,7] The concentrations of f25(OH)D, on the other hands, are independent of these confounding factors. This may explain findings that free 25-hydroxy-vitamin D3 (25(OH)D3) measurements are closer linked to clinical end-points as compared to measurements of t25(OH)D3 is some recent studies [8–14].

However, as of today, there are no established guidelines for the clinical use of f25(OH)D. The guidelines for t25(OH)D that are currently used [15–29] are based on data coming from huge clinical studies in the past decades. Getting this level of evidence for f25(OH)D, however, will take a long time, maybe decades as well. We therefore translated knowledge coming from measurements of t25(OH)D measurements used for the currently available guidelines for t25(OH)D and calculated corresponding f25(OH)D values. This approach might guide clinical design making based on the more accurate measurements of f25(OH)D.

2. Methods

We performed a retrospective analysis of all patients with simultaneous measurements of total and free vitamin D from December 2018 to February 2021 in the Institute of Medical Diagnostics Berlin (IMD Berlin), Berlin, Germany. A total of 5060 patients were included in the study. All vitamin D measurements carried out during this period were included in the study. If measurements of calcium, phosphate, c-reactive protein (CRP), parathyroid hormone (PTH), fast plasma glucose (FPG), insulin, 1,25(OH)$_2$D, creatinine, total cholesterol, low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL), hemoglobin and white blood cell count were made from the sample material at the same time, these data were also included in the study.

T25(OH)D (25-hydroxyvitamin D_2 and D_3) was measured by Abbott Architect i2000 (Abbott Laboratories, Wiesbaden, Germany) using Abbott Architect 25(OH)D automated chemiluminescent microparticle immunoassay (Abbott Laboratories, Wiesbaden, Germany). This kit was standardized to standard reference materials (SRM) of National Institute of Standards and Technology (NIST) (NIST SRM 2972). Serum f25(OH)D (25(OH)D_3 and 25(OH)D_2) was measured directly using a commercial kit by Future Diagnostics Solutions B.V. (Wijchen, Netherlands), distributed by DIASource Immunoassays, Louvain-la-Neuve, Belgium) based on a two-step immunoassay procedure [30]. This assay is CE-IVD marked, i.e. it fully complies with the requirements of the European Directive regulating the quality of the products present on the marked of in vitro diagnostics.

Calcium, phosphate, CRP, PTH, FPG, insulin, 1,25(OH)$_2$D, creatinine, total cholesterol, LDL, HDL, haemoglobin and white blood cell count were measured using standard assays by the Institute of Medical Diagnostics Berlin (IMD Berlin), Berlin, Germany. The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR = FPG (mg/dL) × insulin (μIU/mL) / 405.

Continuous variables with normal distribution were presented as mean (standard deviation [SD]); non-normal variables were reported as median (interquartile range [IQR]). Analysis of the statistical difference between the patients with t25(OH)D of 30−100 ng/mL and other three patient groups (t25(OH)D 0−20, 20−30 and >100 ng/mL) was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Linear regression was used to study the linear relationship between f25(OH)D and t25(OH)D. Since there was a significant direct positive relationship between f25(OH)D and t25(OH)D concentrations, we used the established t25(OH)D reference values and the slopes of the relationship between free and t25(OH)D to calculate reference values for f25(OH)D. All analyses were performed using SPSS, version 22 and differences were considered significant if $p < 0.05$.

3. Results

5060 patients (3087 women/1970 men/3 unknown) were included into this study. The characteristics of the patients and selected biochemical and haematological parameters (such as white blood cell count, haemoglobin, glucose, calcium, phosphate, cholesterol, LDL, HDL, CRP, PTH, and creatinine) are shown in Table 1. The median (IQR) age of the subjects was 55.00 (44.00, 64.00) years. The median (IQR) serum concentration of f25(OH)D was 8.51 (6.13, 13.10) pg/mL. The test of normality indicates a non-normal distribution of free and t25(OH)

Table 1
Characteristics of patients. (n = 5060).

	Value
N	5060
Age at study entry (years)	52.90 ± 16.04
Sex (male/female/unknown, n)	1970/3087/3
WBC count (10^9/L)	5.90 ± 3.1
Hemoglobin (g/dL)	13.13 ± 1.91
Fasting Glucose (mg/dL)	99.89 ± 11.76
calcium (mmol/L)	2.39 ± 0.12
phosphate (mmol/L)	1.08 ± 0.31
Cholesterol (mg/dL)	202.71 ± 48.64
LDL (mg/dL)	121.28 ± 41.80
HDL (mg/dL)	58.18 ± 19.65
CRP (mg/dL)	1.09 ± 0.09
Creatinine (mg/dL)	0.90 ± 0.29
PTH (pg/mL)	35,01 ± 2.9
Insulin (μU/mL)	7.20 ± 0.61
HOMA-IR	2.01 ± 0.96
t25(OH)D (ng/mL)	41.43 ± 30.83
f25(OH)D (pg/mL)	11.52 ± 10.40

Data are presented as n, mean ± SD, PTH, Parathyroid hormone; CRP, C-Reactive Protein; LDL, Low density lipoprotein; HDL, High density lipoprotein; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; t25(OH)D, total 25-hydroxyvitamin D; f25(OH)D, free 25-hydroxyvitamin D.

D. The histogram rather suggests a right-skewed distribution (Fig. 1).

We first performed a linear correlation analysis between the total and free 25(OH)D serum concentration and found that there was a strong positive linear ($r = 0.8395$, $p < 0.0001$) correlation between them. The slope of the relationship was 0.2833 ± 0.002578 pg/ng (Fig. 2). Then we used the slopes of the relationship between total and f25(OH)D and the established t25(OH)D reference values to calculate corresponding reference values for f25(OH)D (Table 2). Although various published guidelines [15–29] lack consistency of the optimal t25(OH)D concentration, these guidelines either consider the optimal t25(OH)D concentration for bone health as > 20 ng/mL (50 nmol/L), or greater than 30 ng/mL (75 nmol/L) for pleiotropic effect/general health effects of vitamin D. Corresponding reference values for free vitamin D might be higher than either 5.67 pg/mL or higher than 8.50 pg/mL, depending on the guidelines and endpoints (Table 3).

Furthermore, the characteristics of all patients with total vitamin D measurements, which included 560,578 patients, seem to support that the optimal 25(OH)D concentration in serum should be greater than 30 ng/mL, as patients with t25(OH)D concentrations in the range 30−100 ng/mL have lower PTH and higher calcium levels compared to the patients with t25(OH)D concentrations of 0−20 ng/mL and 20−30 ng/mL (Supplementary Table 1).

4. Discussion

In this study, the well-established knowledge based on numerous studies analysing t25(OH)D with regard to bone and general health that were the basis of current guidelines for the treatment of vitamin D deficiency was used to calculate reference values for f25(OH)D, because establishment of guidelines for free vitamin D based on a comparable amount of clinical data as it was done for total vitamin D will last decades.

Numerous authorities and scientific organizations have developed guidelines for the optimal concentration of serum t25(OH)D. The bone centred guidelines recommend a target t25(OH)D concentration of at least 20 ng/mL (50 nmol/L), while the guidelines that focus on the pleiotropic effect of vitamin D recommend a lower threshold t25(OH)D concentration of 30 ng/mL (75 nmol/L).

There are several criteria to define optimal t25(OH)D concentration, including maximum suppression of PTH by vitamin D, adequate renal production of 1,25-dihydroxyvitamin D to ensure adequate calcium absorption in the gut, and an optimal value to prevent a defined clinical endpoint. In the analysed studies used for the establishment of the total vitamin D guidelines, most of the guidelines suggest total vitamin D concentrations of 30 ng/mL (75 nmol/L) as the necessary minimal concentration since patients with t25(OH)D concentrations greater than 30 ng/mL have overall lower PTH as well as the lowest rate of any adverse clinical events [16,17,19–22,24,27,28]. This interpretation fit very well with the observations in our own total vitamin D population of 560,578 patients, see supplementary Table 1.

As previously noted, the free hormone hypothesis postulates that only the unbound part (the free part) of the hormones that otherwise circulates in the blood and is bound to their carrier proteins can enter the cells and exert their biological effects. One of the earliest clinical examples that led to the formulation of the free hormone hypothesis is based on observations by Recant and Riggs [31] that patients with protein-loss nephropathies developed fairly low thyroid hormone levels along with increased urine losses, but without signs of hypothyroidism. Subsequent studies have established the free hormone hypothesis for sex steroid hormones and thyroid hormone [32,33]. As will be discussed subsequently, this will probably also be the case for f25(OH)D. DBP-deficient mice lose substantial amounts of the vitamin D metabolites in urine with marked reductions in their circulating levels of 25(OH)D, but do not develop signs of rickets unless they were given a low

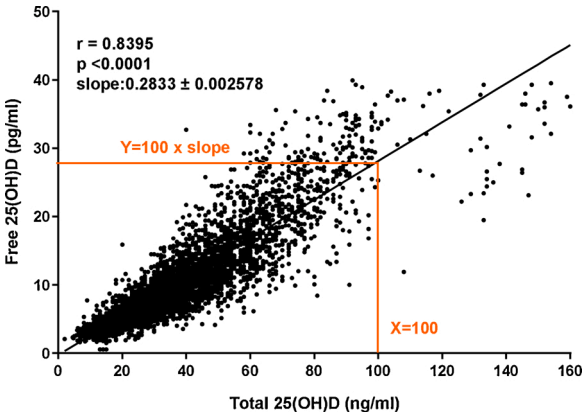

Fig. 2. Total and free 25(OH) serum concentrations are positively correlated in 5060 patients.

Table 2
F25(OH)D concentration calculated from t25(OH)D.

T25(OH)D (ng/mL)	F25(OH)D (pg/mL)
10	2.833
20	5.666
30	8.499
40	11.332
50	14.165
60	16.998
70	19.831
80	22.664
90	25.497
100	28.330
110	31.163
120	33.996
130	36.829
140	39.662
150	42.495

The slope of the relationship between total and f25(OH)D was 0.2833 pg/ng.

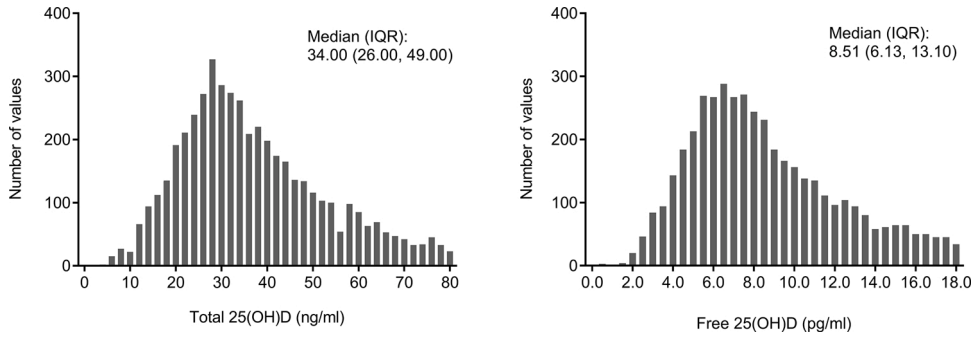

Fig. 1. Distribution of total and free vitamin D in the study population.

Table 3
Total vitamin D guidelines published since 2011.

	Organization	Countries	Target Population	Target disease	Method of guideline development	Vitamin D sufficiency (ng/mL)	Vitamin D insufficiency (ng/mL)	Vitamin D deficiency (ng/mL)	Vitamin D severe deficiency (ng/mL)	Risk of vitamin D toxicity (ng/mL)
Giustina 2019 [15]	International conference "Controversies in Vitamin D", Italy	Global	Healthy general population	Skeletal health	Expert opinion	>20	12–20	<12	–	–
Haq 2018 [16]	GULF	United Arab Emirates and the Gulf region	General population	General health	Systematic reviews of the guidelines and those vitamin D-related papers relevant to the Middle-Eastern region.	>30	20–30	<20	–	–
Okazaki 2017 [17]	Consensus of 3 organizations	Japan	General population	Bone health	Integrating Japanese clinical data and published guidelines worldwide.	>30	20–30	<20	–	–
Munns 2016 [18]	Consensus of 11 organizations	Global	Infants, children, and adolescents	General health	Systematic reviews of the nutritional rickets in children.	>20	12–20	<12	–	>100
Grant 2015 [19]	AADMD https://www.aadmd.org/		People with neuro develop mental disorders and intellectual disabilities	General health	Expert opinion	>30	–	–	–	–
Judge 2014 [20]	American Geriatrics Society https://www.americangeriatrics.org/		Elderly	Falls, fractures	Reviewing of all meta-analyses and including randomized controlled trials published before 2008.	>30	–	–	–	>60
Cosman 2014 [21]	NOF https://www.nof.org/	USA	Postmenopausal women and men age 50 and older	Osteoporosis	Developed by an expert committee of the National Osteoporosis Foundation (NOF) in collaboration with a multispecialty council of medical experts in the field of bone health convened by NOF.	>30	–	–	–	–
Maeda 2014 [22]	SBEM https://www.endocrino.org.br/	Brazil	People with osteoporosis	Prevention of secondary hyperparathyroidism, fall prevention, bone mass & density	Literature search on hypovitaminosis D using PubMed, Lilacs and SciELO.	>30				
Braegger 2013 [23]	ESPGHAN http://www.espghan.org/	Europe	Infants, children, adolescents	General health	Review of published data on vitamin D intake and prevalence of vitamin D deficiency in healthy European pediatric population.	>20	–	–	<10	–
Pludowski 2013 [24]	Vitamin D opinion leaders (EVIDAS)	Central Europe	General population	General health	Expert opinion based on both skeletal and non-skeletal effects of Vitamin D	>30	20–30	<20	–	>200
Rizzoli 2013 [25]	ESCEO http://www.esceo.org/		Elderly or postmenopausal women	Bone health	Expert opinion based on meetings of the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis	>20	<20	<10	–	–
Rizzoli 2013 [25]	ESCEO http://www.esceo.org/		Fragile elderly		Expert opinion of leading scientists of the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis	>30	<20	<10	–	–
GNS 2012 [26]	GNS https://www.dge.de/en/	Austria, Germany, Switzerland	General population	Bone health	The D-A-CH reference values for the intake of vitamin D have been	>20	–	–	–	–

(*continued on next page*)

Table 3 (*continued*)

	Organization	Countries	Target Population	Target disease	Method of guideline development	Vitamin D sufficiency (ng/mL)	Vitamin D insufficiency (ng/mL)	Vitamin D deficiency (ng/mL)	Vitamin D severe deficiency (ng/mL)	Risk of vitamin D toxicity (ng/mL)
					revised based on a critical review by the German Nutrition Society.					
Pérez-López 2012 [27]	EMAS https://www.emas-online.org/		Postmenopausal women	General health	Literature review and consensus of expert opinion.	>30	20–30	<20	<10	–
Holick 2011 [28]	Endocrine Society https://www.endocrine.org/	USA	General population	Risk of vitamin D deficiency	Expert opinion	>30	20–30	<20	–	–
Ross 2011 [29]	Institute of Medicine (IOM) http://www.iom.edu.np/	USA, Canada	General population	Bone health	Systematic reviews of studies for including both skeletal and extra-skeletal outcomes.	>20	–	–	–	–

AADMD, American Academy of Developmental Medicine and Dentistry; NOF, National Osteoporosis Foundation; SBEM, Sociedade Brasileira de Endocrinologia e Metabologia (Brazilian Society of Endocrinology and Metabology); ESPGHAN, European Society for Paediatric Gastroenterology Hepatology and Nutrition; EVIDAS, European Vitamin D Association; ESCEO, European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis; GNS, German Nutrition Society; EMAS, European Menopause Andropause Society.

vitamin D diet. Such results indicate the importance of the free fraction of 25(OH)D for biologic functions and the role of DBP as a circulating reservoir [34]. In previous clinical studies, determinations of f25(OH)D concentrations in healthy populations show highly significant correlations with t25(OH)D concentrations, but clinical conditions that alter either the concentration of DBP, the affinity of DBP for 25(OH)D metabolites or albumin may alter f25(OH)D concentrations [1]. In clinical conditions such as pregnancy, liver cirrhosis, kidney diseases with urinary protein loss and elderly women after menopause, the concentration of free vitamin is suggested to be a more accurate assessment of vitamin D status than the total levels. In this context it is important to note that estrogen concentrations have a strong impact on vitamin D binding protein concentrations, because estrogens stimulate vitamin D binding protein synthesis in the liver. Thus t25(OH)D in women varies throughout the women` life cycle substantially depending on taking estrogen containing contraceptives or not, being pregnant or having low levels of estrogens after the menopause, or being treated with estrogens or hormones that substantially increases estrogens like it is the case in women undergoing in vitro fertilization. Measurements of free vitamin D have the advantage that they are independent of the estrogen status of the women. [2,35]

Unlike the optimal reference value of t25(OH)D for general or skeletal health in different populations, which has been extensively studied and established over a long period of time, reference values of free vitamin based on clinical data do not exist so far and will not exist in the near future. Therefore, we used total vitamin D data to generate by correlation reference values for free vitamin D to guide clinical decision-making and thus contribute to improving patient prognosis.

According to the recommendation of the clinical guidelines for optimal total vitamin D concentration of 20 or 30 ng/mL, the lower threshold concentrations for f25(OH)D should be 5.67 pg/mL or 8.50 pg/mL, respectively, for vitamin D sufficiency. However, there has been controversy about what exact t25(OH)D concentrations define vitamin D deficiency and sufficiency. For the general population, the Institute of Medicine (IOM) recommended in 2010 based on bone health that the minimum 25(OH)D concentration of 20 ng/mL (50 nmol/L) covers the needs of at least 97.5 % of the population. [29] Subsequently, the German Nutrition Society [26] and the Italian International Conference "Vitamin D Controversy" [15] also indicated that 25-hydroxyvitamin D concentrations above 20 ng/mL (50 nmol/L) appear to be safe and sufficient for skeletal health in the healthy general population. However, this has been contested by many. The majority of the guidelines that included 25(OH)D concentrations to analyse relations between health and the risk of non-bone diseases pointed on higher 25(OH)D concentrations, i.e., above 30 ng/mL (75 nmol/L), [16,17,19–22,24,27,28].

Likewise the Endocrine Society in the USA made recommendations to treat and prevent vitamin D deficiency; they recommended achieving serum 25(OH)D concentrations more than 30 ng/mL (>75 nmol/L), with the preferred range of 40–60 ng/mL (100–150 nmol/L) [28]. The European guidelines recommended the use of vitamin D supplements to obtain and maintain the optimal target 25(OH)D concentration in a range of 30–50 ng/mL (75–125 nmol/L) [24]. For elderly and postmenopausal women, European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis recommended concentration of at least 20 ng/mL (50 nmol/L) [25] is controversial, and a 25(OH)D concentration >30 ng/mL (75 nmol/L) is a better fit for general or skeletal health, as proposed by the National Osteoporosis Foundation and the European Menopause Andropause Society [21,27]. For the general health of infants, children and adolescents, the pragmatic use of a serum concentration >50 nmol/L to indicate sufficiency is recommended [18,23], see also the summary of current vitamin D guidelines in Table 3.

Taken together total vitamin D concentrations of at least 30 ng/mL (75 nmol/L) are in any case safe and sufficient for bone and general health, hence we recommend this as a lower boundary for vitamin D.

For the upper concentrations of total vitamin D there are only very

few recommendations in the guidelines (Table 3). The recommendations are 60, 100, and 200 ng/mL, respectively. We are thus of the opinion that 100 ng/mL (250 nmol/L) might be a suitable compromise. This also fits to the observations in our huge total vitamin D observational analysis (supplementary Table 1), since calcium and LDL (both risk markers of cardiovascular diseases) increases significantly when total vitamin D is greater than 100 ng/mL (250 nmol/L).

The size of the core study as well the supporting total vitamin D study are clearly strength of our analysis. However, the lack of clinical data such as blood pressure or underlying diseases remains a study limitation. The justification of the upper limit for either total or free vitamin D is also based on just three guidelines and rather few patients in our own observational study. Here, more studies are clearly needed to establish robust upper limits for both free and total vitamin D.

A reference value for free vitamin D was established for the first time in a large population. The recommended threshold level of free 25(OH)D is 8.50 pg/mL, corresponding to a target concentration of total vitamin D of 30 ng/mL. With some uncertainty 28.33 pg/mL could be considered as upper limit for free vitamin D corresponding to a concentration of total vitamin D of 100 ng/mL.

Author contributions

BH conceived the idea for the study and contributed to the design of the research. CD and VvB collected the data and created the database. SZ and CC analyzed the data and wrote the paper. BH participated in the revision of the manuscript. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2021.105877.

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