Published in final edited form as:

Clin Endocrinol (Oxf). 2017 May ; 86(5): 674–679. doi:10.1111/cen.13319.

The vitamin D metabolite ratio (VMR) as a predictor of functional biomarkers of bone health

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Summary

Context—The vitamin D metabolite ratio (VMR) (serum 24,25 (OH)₂D₃/25(OH)D₃) has been proposed as a biomarker of vitamin D sufficiency to replace serum 25(OH)D.

Objective—To examine the relationships of 24,25(OH)₂D₃ and VMR to functional biomarkers of bone health following vitamin D supplementation.

Setting—An ambulatory research centre.

Design—Serum from a previous research study of dose response of PTH, calcium absorption and bone turnover to vitamin D supplementation was analysed for vitamin D metabolites (25 (OH)D, 24,25(OH)₂D₃).

Outcome—The relationship of serum 24,25(OH)₂D₃ and VMR to calcium absorption, PTH and bone turnover markers was examined.

Results—Although there were strong correlations of serum 25 (OH)D with 24,25(OH)₂D₃ and free 25(OH)D, its correlation with VMR was lower. After vitamin D supplementation, the change in 25(OH)D, 24,25(OH)₂D₃ and VMR was associated with the change in calcium absorption, PTH and CTX. The correlation of the change in PTH with the change in metabolites was the lowest for VMR. Moreover, estimated dose response for standardized values of vitamin D metabolites showed a beta-coefficient for VMR that was significantly less in magnitude compared to other metabolites.

Conclusion—Serum 24,25(OH)₂D₃ is closely associated with the dose response of serum 25(OH)D to vitamin D supplementation. However, the VMR does not appear to be equivalent to either of these metabolites in its response to increasing vitamin D intake or its association with PTH. It is unlikely that VMR will replace 25(OH)D as a biomarker for vitamin D sufficiency.

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Conflict of interest

The authors have nothing to disclose.

Introduction

In 2011, the Institute of Medicine published recommendations for vitamin D intake.¹ These recommendations were based on skeletal health because there was insufficient evidence to establish the presence of extraskeletal benefits of vitamin D.^{1,2} As vitamin D intake derives from dermal synthesis as well as nutritional intake, serum 25(OH)D has been used to assess vitamin D status. Recommended nutritional intake was back-calculated from the dose response of serum 25(OH)D to vitamin D intake.

It has become increasingly clear that 25(OH)D may not be an appropriate biomarker of vitamin D status in the African American population. African Americans have lower serum 25(OH)D throughout life, in part because of their deeply pigmented skin.¹ Despite these lower levels, they have superior bone health. They have higher bone mineral density starting in childhood and have fewer fractures in old age.²⁻⁹ Further, there appears to actually be an increase in fractures at higher levels of 25(OH)D in African Americans.¹⁰ In two randomized controlled trials, vitamin D supplements did not prevent postmenopausal bone loss.^{11,12} Clearly, bone health cannot be used as an indicator for vitamin D sufficiency in African Americans.

It would be highly desirable for future research, nutritional recommendations for African Americans and clinical care to have a multiracial biomarker of vitamin D status.¹³⁻¹⁸ Free 25(OH)D may be a better candidate biomarker for vitamin D status than total 25(OH)D because it is not influenced by genetic polymorphisms or medical conditions that affect VDBP.

In addition to free 25(OH)D, a new candidate biomarker has emerged that also appears to result in equivalent values between races.¹⁹⁻²² The catabolism of 25(OH)D and 1,25(OH)₂D occurs through the hydroxylase enzyme CYP24A1 so that the first step in vitamin D catabolism is reflected in the serum level of 24,25(OH)₂D₃.⁴ Knockout mice for this enzyme develop hypercalcaemia resulting in death in half the animals.²³⁻²⁵ These animals respond to vitamin D metabolite administration with elevated 1,25(OH)₂D levels as they cannot catabolize 25(OH)D or 1,25(OH)₂D. The equivalent to this condition was found in infants with idiopathic infantile hypercalcaemia and may also be found in some adults with hypercalcaemia. The CYP24A1 enzyme is regulated in part by vitamin D receptor activity so levels of 24,25(OH)₂D₃ reflect not only substrate 25(OH)D but vitamin D regulated enzyme expression. These findings led to the concept that control of this enzyme and consequently of serum levels of 24,25-(OH)₂D₃ might indicate physiologic sufficiency of vitamin D.

The development of methods for the measurement of 24,25(OH)₂D₃ concurrently with 25(OH)D by LC-MS/MS led to testing the concept of serum 24,25(OH)₂D₃ as an indicator of vitamin D sufficiency.^{19-22,26} It was noted that when this value was normalized for substrate levels by its expression as a ratio to 25(OH)D (VMR or vitamin D metabolite ratio), there was racial equivalence of levels. Moreover, the response to vitamin D supplementation appears to be closely correlated between these two metabolites. Thus,

VMR is of interest as a candidate biomarker for vitamin D sufficiency that is equivalent between races.

To become established as a biomarker for vitamin D status, it should be shown not only that levels are equivalent between races but that the biomarker predicts functional outcomes of vitamin D. We previously performed a study of varied doses of vitamin D₃ (placebo, 800, 2000 and 4000 IUs) on calcium absorption, PTH, collagen type I N-terminal pro-peptide [PINP] and C-terminal telopeptide of type I collagen [CTX].^{27,28} Calcium absorption was measured at baseline and at 10 weeks using stable dual isotopes of calcium. Using stored serum samples from this study, we were able to measure vitamin D metabolites and calculate the VMR. We wished to answer the following question: is VMR or 24,25(OH)₂D₃ associated with functional biomarkers of bone health (calcium absorption, PTH and bone turnover) in the basal state and in response to vitamin D supplementation?

Materials and methods

In the previous study, we had 76 participants enrolled that had baseline and final results available. Serum was available from 60 participants at baseline and 68 participants at follow-up. However, only a total of 58 participants had complete baseline and follow-up data, and these were included in this analysis. Calcium absorption values and laboratory analyses from the previous publication were used for this analysis as well.^{27,28}

Serum calcium was measured with *O*-Cresolphthalein complex by using automated equipment (Dimension-RXL). Serum parathyroid hormone (PTH) was measured with the Immulite 2000 Analyzer for the quantitative measurement of intact PTH (Diagnostic Products Corporation, Los Angeles, CA, USA). Serum CTX was measured with a Serum Crosslaps ELISA kit made by Nordic Bioscience Diagnostics. Samples were sent to Future Diagnostics (Nieuweweg 279, 6603 BN Wijchen, The Netherlands) for free 25(OH)D testing. The free 25(OH) Vitamin D ELISA is based on a two-step immunoassay procedure performed in a microtitre plate.

Serum samples were analysed for vitamin D metabolites by the Department of Laboratory Medicine at the University of Washington (Seattle, WA, USA) using calibrators and controls (400 µl) that were spiked with deuterated internal standards and immunoaffinity purified using anti-1,25(OH)₂D beads from ALPCO. After incubation, the beads were washed and bound analytes were eluted with organic solvent. The eluent was dried down, and the residue reconstituted with the derivatizing agent PTAD in acetonitrile. After incubation at room temperature, the reaction was quenched with water. A portion of the mixture was analysed on a Waters Xevo TQ tandem mass spectrometer equipped with an Acquity UPLC. Analytes enriched during immunoaffinity purification and analysed by liquid chromatography–tandem mass spectrometry include 25(OH)D₂, 25(OH) D₃, 24,25(OH)₂D₃, 1,25(OH)₂D₂ and 1,25(OH)D₃ with deuterated internal standards for each analyte included. Standards were prepared in stripped human serum [PMID: 22968104, 21768219]. Concentrations of 25(OH)D₂, 25(OH)D₃ and 24,25 (OH)₂D₃ were standardized to NIST SRM 972a [PMID: 27091017, 22141317].

Statistical analysis

Means and standard deviations were generated for all continuous variables, and frequencies (%) were generated for all categorical variables to describe the data set at hand. Continuous marker concentrations were compared across dose group via analysis of variance. The overall F -test or Fisher's exact test was used to evaluate whether group means or frequencies were significantly different with a two-sided P -value less than 0.05 considered statistically significant. Spearman's correlation coefficients (ρ) were generated to examine associations between all markers of vitamin D status and bone health at a particular time point as well as between 10-week changes in markers. The strength of the linear association between 25(OH)D₃ and several vitamin D metabolites was assessed and more formally compared using Clarke's test for non-nested models. The relationship between several standardized 10-week markers levels and dose adjusting for baseline marker levels were estimated via separate linear regression models. Dose was treated as a continuous variable with values: 0 (placebo), 04 (800 IU), 10, (2000 IU) or 20 (4000 IU). The regression coefficients for dose from each standardized regression model may be compared with respect to magnitude. Estimates, 95% confidence intervals (CIs) and P -values corresponding to the standardized beta-coefficients for dose were also generated. Model R^2 values corresponding to the proportion of variability in 10-week marker levels accounted for by the model both with before and after adjusting for dose levels were computed to further summarize the strength of the relationship. SAS version 93 (SAS Institute Inc., Cary, NC, USA.) was used for all analyses.

Results

The baseline biomarker levels are given in Table 1. These are listed by supplementation group. It can be noticed that all groups were equivalent except for VMR.

Table 2 delineates the correlations between the various vitamin D metabolites and the bone health indicators. These correlations are given as baseline values and 10-week values which are the values after vitamin D supplementation. In addition, the correlation between the changes from baseline to the final values is given. It may be seen that at baseline, there is a strong correlation between serum 25(OH)D and serum 24,25(OH)₂D₃ ($\rho = 0.81$, $P < 0.0001$) (Figure 1) and with free 25(OH)D ($\rho = 0.66$; $P < 0.0001$). The correlation with VMR, however, although significantly different than zero, is also observed to be significantly lower ($\rho = 0.32$; $P < 0.05$) than for the other two vitamin D metabolites (95% upper confidence bound = 0.55; Clarke's test P -value < 0.001). At baseline, serum 25-(OH)D was inversely related to serum PTH with a moderately strong correlation ($\rho = -0.40$, $P < 0.005$). There is a similar correlation between serum 24,25-(OH)₂D₃ and PTH ($\rho = -0.42$, $P < 0.05$). However, expressed as the VMR the correlation is lower ($\rho = -0.27$, $P < 0.05$). Free 25-(OH)D was not significantly related to PTH. The bone turnover markers and calcium absorption efficiency were not related to any of the vitamin D metabolites at baseline.

Although relationships may be difficult to detect in a small cross-sectional sample, the response to vitamin D supplementation would be expected to be more sensitive. At 10 weeks (post-supplement), significant correlations are noted between total 25 (OH)D and

24,25(OH)₂D₃ with parathyroid hormone ($\rho = -0.44$; $P < 0.005$ and $\rho = 0.41$, $P < 0.005$) with a lesser correlation with free 25(OH) D ($\rho = -0.30$, $P = 0.05$). VMR was not significantly related to PTH. At this final visit, significant correlations were noted of CTX with 25(OH)D with a lesser correlation with free 25(OH) D. None of the other metabolites showed a relationship with CTX. The 10-week value was not correlated with PINP for any of the variables nor was calcium absorption sufficiency.

In the last column of Table 3, correlations between the changes (final minus baseline) are given for each of the variables. Importantly, each of the variables showed a significant correlation with the change in calcium absorption, including 24,25(OH)₂D₃ and VMR. Changes in 24,25(OH)₂D₃ and total 25(OH)D were related to the change in PTH; the correlation was strongest with total 25(OH)D. Correlations were similar between the change in CTX and the change in 24,25(OH)₂D₃, VMR and total 25(OH)D. There were no significant correlations noted with the change in PINP.

Table 3 shows the dose–response estimates and 95% CIs derived from adjusted regression models for standardized 10week 25(OH)D, VMR, free 25(OH)D and 24,25(OH)₂D₃ concentrations. A strong and consistent dose response was observed for 25(OH)D, free 25(OH)D and 24,25(OH)₂D₃ such that a 2000 IU increase in dose is associated with a 045–050 standard deviation increase in 10-week marker levels. Additionally, model R^2 values are substantially increased when accounting for dose level in the model (an additional 47%–56% of the variance is accounted for when adjusting for dose compared to simply adjusting for baseline marker levels). A significantly weaker dose response (a 0.25 standard deviation increase in 10-week marker levels per 2000 IU increase in dose) and partial R^2 (incremental $R^2 = 0.14$) is seen for VMR.

Discussion

The availability of mass spectrometry methods that permit measurement of 25(OH)D, 1,25(OH)₂D and 24,25(OH)₂D₃ on a single sample led to exploration of 24,25(OH)₂D₃ as a biomarker of vitamin D status.²⁶ Indeed, 24,25(OH)₂D₃ is of particular interest because it not only reflects intake, as shown in this study, but also reflects the first step in vitamin D catabolism. Thus, it presumably reflects the physiologic response to sufficient vitamin D as well. Indeed, we found that change in serum 24,25(OH)₂D₃ is associated with change in calcium absorption, bone turnover and PTH. However, the 24,25(OH)₂D₃ response to increasing intakes of vitamin D showed no advantage over total or free 25(OH)D. Although change in VMR was also associated with change in calcium absorption and CTX, it was not associated with change in PTH and at baseline had a weaker correlation with PTH than 25(OH)D or 24,25(OH)₂D₃. Finally, when examining the dose response of vitamin D metabolites, the standardized beta-coefficient for VMR was 0.25 compared to 0.46 for 25(OH)D₃, 050 for free 25(OH)D and 045 for 24,25 (OH)₂D₃. Thus, the use of VMR as an indicator of vitamin D status is not supported by our study

The VMR equalizes biracial values for vitamin D status. This may be due to 24,25(OH)₂D₃ proportionality to 25(OH)D in African Americans. We found in a sample matched for age and BMI that free 25(OH)D measured by a direct ELISA is equivalent between races.¹³

Recently, this has been challenged by studies using quantitative mass spectrometry as well as direct assays for free 25(OH)D.^{14–18} This is an issue that is important to resolve because opposite approaches to vitamin D supplementation in African Americans would be concluded. Of course, if the newest studies are correct, there would be little rationale for VMR in the first place.

The current study demonstrates that the increase in 24,25 (OH)₂D is similar to the increase in 25(OH)D in response to vitamin D supplementation. In the range of 25(OH)D₃ of our study, there was a proportional increase in 25(OH)D with increasing doses of vitamin D. Our population did not have sufficient numbers with vitamin D deficiency (very low D (<125 nmol/l) to determine a cut-off point where 24,25 (OH)₂D₃ changes significantly.

There are similarities and differences in our findings compared to prior publications concerning VMR. We confirmed the strong relationship of VMR with 25(OH)D found by Wagner *et al.*²² who noted a R^2 of 0.82 compared to our observed R^2 of 0.64. Further, we also found a consistent dose response across dose levels. The correlation between baseline PTH and VMR was similar to another study ($R = -0.26$; $P = 0.001$) in whites compared to $r = -0.26$, $P < 0.001$ in our study. However, we did not find that baseline VMR was predictive of changes in 25(OH)D₃ after supplementation. Wagner *et al.*²² found a moderate correlation between baseline VMR and 6-week changes in 25(OH)D₃ ($r = 0.38$, $P = 0.004$). As we did not observe any significant associations of baseline VMR and final changes in 25(OH)D, PTH, CTX or calcium absorption in both 2000 IU and 4000 IU groups, VMR was not useful in our study as a predictor of response to vitamin D supplementation.

We recognize several weaknesses in our study. The population size was small and did not include many individuals with very low 25(OH)D (vitamin D deficiency). Our population also included healthy postmenopausal women up to age 70 years so these findings may not be generalizable to the population as a whole. The use of the concept of the VMR is based on the desire to equalize intraracial values for vitamin D status.

In conclusion, we observed a close association between serum 24,25(OH)₂D₃ and the serum 25(OH)D response over a range of vitamin D supplementation. However, the VMR does not appear to be equivalent to either serum 25(OH)D or 24,25(OH)₂D₃ in its dose response to vitamin D administration or its association with PTH. The concept of the use of this ratio is to relate levels of 24,25(OH)₂D to the concentration of its substrate (25(OH) D). It does not appear that there is any utility of using a ratio as opposed to considering the two metabolites individually.

Acknowledgements

This work was supported by National Institute for Health Grant RO1-AG032440-01A2.

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Fig. 1. Association between 24,25(OH)₂D₃ and 25(OH)₂D₃ (nmol/L) at baseline visit with estimated regression. In the overall dataset ($n = 58$), 24,25(OH)₂D₃ and 25(OH)₂D₃ were highly correlated (Spearman's $\rho = 0.81$; P -value < 0.0001).

Table 1.Baseline patient characteristics and biomarker levels by vitamin D dose^{*}

	Control (<i>n</i> = 14)	800 IU (<i>n</i> = 13)	2000 IU (<i>n</i> = 15)	4000 IU (<i>n</i> = 16)	<i>P</i> -value [†]
Age	59.2 ± 4.3	58.4 ± 4.7	58.9 ± 6.2	59.8 ± 4.6	0.91
BMI	25.4 ± 3.4	25.8 ± 3.5	28.2 ± 4.9	25.9 ± 4.1	0.24
White	78.6%	76.9%	66.7%	56.3%	
Nonwhite [‡]	21.4%	23.1%	33.3%	43.7%	0.52
25(OH)D ₃ (nmol/l)	54.34 ± 14.97	59.83 ± 23.15	58.03 ± 20.63	60.91 ± 15.73	0.80
24,25(OH) ₂ D ₃ (nmol/l)	3.61 ± 1.64	4.98 ± 2.07	3.85 ± 1.81	4.91 ± 1.67	0.09
VMR	6.43 ± 1.59	3.85 ± .81	6.50 ± 1.76	8.10 ± 1.97	0.01
1,25(OH) ₂ D ₃ (nmol/l)	117.01 ± 36.44	107.90 ± 36.41	122.36 ± 36.37	127.14 ± 55.56	0.66
Free D (pmol/l)	10.65 ± 4.35	12.44 ± 3.91	11.14 ± 3.01	13.20 ± 3.79	0.28

^{*} All values are mean ± SD unless otherwise specified; VMR = 24,25(OH)₂D₃/25(OH)D₃ × 100.[†] Corresponds to overall *F*-test for differences in group means (continuous) or Fisher's exact test of association (categorical).[‡] Black, non-Hispanic (*n* = 7); Hispanic (*n* = 6); Asian (*n* = 3); and unknown (*n* = 2).

Table 2.

Correlations* between vitamin D metabolites and bone health indicators

	10 weeks				Baseline				(10 weeks – baseline)			
	PTH	CTX	PINP	CA absorption	PTH	CTX	PINP	CA absorption	PTH	CTX	PINP	CA absorption
25(OH)D ₃ (ng/ml)	-40 [†] (-60, -14)	0.05 (-23, .32)	0.08 (-20, .34)	0.06 (-22, .33)	-44 [†] (-63, -20)	0.40 [†] (.16, .60)	0.33 [‡] (.08, .55)	0.08 (-.18, .34)	-44 [†] (-64, -19)	0.32 [‡] (.05, .55)	0.13 (-.15, .39)	0.37 [‡] (-10, .58)
24,25(OH) ₂ D ₃ (ng/ml)	-42 [†] (-62, -16)	-07 (-34, 20)	-02 (-29, .26)	-05 (-31, .23)	-41 [†] (-61, -17)	0.21 (-.05, .45)	0.22 (-.04, .46)	0.04 (-.23, .46)	-36 [‡] (-58, -09)	0.28 [‡] (.01, .52)	0.23 (-.05, .52)	0.36 [‡] (.09, .58)
VMR [24,25(OH) ₂ D ₃ /25(OH)D ₃ × 100]	-27 [‡] (-51, .00)	-15 (-41, .13)	-09 (-35, .19)	-09 (-35, .19)	-19 (-43, -08)	-08 (-33, .19)	0.01 (-.25, .27)	0.02 (-.24, .28)	-27 (-50, .01)	0.28 [‡] (.00, .51)	0.21 (-.07, .46)	0.35 [‡] (.09, .57)
1,25(OH) ₂ D ₃ (ng/ml)	0.01 (-.27, .28)	0.12 (-.15, .28)	0.23 (-.04, .48)	0.13 (-.15, .39)	0.08 (-.19, .33)	0.09 (-.17, .35)	0.17 (-.1, .41)	0.35 [‡] (.10, .56)	-0.05 (-.32, .23)	0.21 (-.06, .46)	0.11 (-.17, .37)	-0.09 (-.36, .19)
Free vitamin D (pg/ml)	-10 (-37, .17)	0.01 (-.26, .28)	-01 (-.28, .26)	0.21 (-.07, .45)	-30 [‡] (-52, -.04)	0.32 [‡] (.06, .53)	0.19 (-.08, .43)	0.12 (-.14, .37)	-22 - . (46, .06)	0.17 (-.11, .43)	0.20 (-.08, .45)	0.28 [‡] (.00, .52)

* Spearman's rho presented with 95% confidence intervals in parentheses.

[†] P-value <0.005.[‡] P-value <0.05.

Table 3.

Model-based estimates^{*} of dose response for 25(OH)D₃, VMR, free 25(OH)D and 24,25(OH)₂D₃

10-week outcome	Estimate [†]	P-value	Partial R ^{2‡}
25(OH)D ₃	0.46 (0.38, 0.55)	<0.001	0.51
VMR	0.25 (0.18, 0.31)	<0.0001	0.14
free 25(OH)D	0.50 (0.40, 0.59)	<0.001	0.56
24,25(OH) ₂ D ₃	0.45 (0.37, 0.52)	<0.001	0.47

^{*} From a regression model for standardized 10-week concentration adjusting for baseline marker levels and randomized dose as a continuous variable.

[†] Based on a linear regression model adjusting for baseline marker levels and dose

[‡] Increase in model R² value attributable to dose after accounting for baseline marker levels.