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ORIGINAL ARTICLE

Calculation of free and bioavailable vitamin D and its association with bone mineral density in Malaysian women with rheumatoid arthritis

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Abstract

Introduction: Low 25-hydroxyvitamin D [25(OH)D] levels have not been consistently associated with bone mineral density (BMD). It has been suggested that calculation of the free/bioavailable 25(OH)D may correlate better with BMD. We examined this hypothesis in a cohort of Malaysian women. *Materials and Methods:* A cross-sectional study of 77 patients with rheumatoid arthritis (RA) and 29 controls was performed. Serum 25(OH)D was measured using the Roche Cobas E170 immunoassay. Serum vitamin D binding protein (VDBP) was measured using a monoclonal enzyme-linked immunosorbent assay (ELISA). Free/bioavailable 25(OH)D were calculated using both the modified Vermuelen and Bikle formulae. *Results:* Since there were no significant differences between RA patients and controls for VDBP and 25(OH)D, the dataset was analysed as a whole. Calculated free 25(OH)D by Vermeulen was strongly correlated with Bikle ($r = 1.00$, $p < 0.001$). A significant positive correlation was noted between measured total 25(OH)D with free/bioavailable 25(OH) D ($r = 0.607$, $r = 0.637$, respectively, $p < 0.001$). Median free/bioavailable 25(OH)D values were significantly higher in Chinese compared with Malays and Indians, consistent with their median total 25(OH)D. Similar to total 25(OH)D, the free/bioavailable 25(OH)D did not correlate with BMD. *Conclusion:* In this first study of a multiethnic female Malaysian population, free/bioavailable 25(OH)D were found to reflect total 25(OH)D, and was not superior to total 25(OH)D in its correlation with BMD. Should they need to be calculated, the Bikle formula is easier to use but only calculates free 25(OH)D. The Vermuelen formula calculates both free/bioavailable 25(OH)D but is more complex to use.

Keywords: free/bioavailable 25-hydroxyvitamin D [25(OH)D], bone mineral density (BMD), vitamin D binding protein (VDBP)

INTRODUCTION

Current international guidelines recommend the measurement of total 25-hydroxyvitamin D [25(OH)D] for the assessment of vitamin D levels in the body.[1] 25(OH)D is the major circulating form of vitamin D present in the blood and has been shown to be linked to measures of bone health. However, not all studies have reported significant relationships between 25(OH)D and bone mineral density (BMD).[2,3]

Recent evidence suggests that bioavailable or free 25(OH)D may be better indicators of vitamin D status.[4] Free 25(OH)D values can be obtained by calculation[5] or measured directly using an enzyme-linked immunosorbent assay (ELISA).[6] However, some studies have reported no improvement of free or bioavailable forms over total 25(OH)D as a more accurate measure of vitamin D status.[3,4,7]

Previous studies have suggested that free and/

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or bioavailable 25(OH)D showed better correlations with BMD compared to just measuring serum 25(OH)D alone.[5,8,9] There are limited assessments in an Asian population.[9] Thus, this study aimed to determine the free and bioavailable 25(OH)D levels in a group of multiethnic Malaysian women, and to assess if they were correlated with race and BMD.

MATERIALS AND METHODS

Subjects

This study consisted of 77 patients with rheumatoid arthritis (RA), aged between 40 and 90 years that were recruited from the rheumatology clinic at Hospital Tuanku Jaafar, Seremban, and Puchong Specialist Clinic, Puchong, during the period July 2014 to March 2015, as previously described in a study by Wong *et al*. (2017).[10] Twenty-nine age-matched healthy controls were recruited from hospital or clinic staff and their relatives or friends. All subjects were females. None of the subjects were taking native vitamin D supplements.

Radiological measurements

BMD was measured by dual-energy X-ray absorptiometry (DXA) at the lumbar spine [LS] [L2–L4] and left hip (femoral neck [FN] and total hip [TH]). Both sites used a HOLOGIC Discovery W densitometer (Hologic Corporation, Bedford, MA, USA). The precision of both machines is ± 2%. The database of female Japanese population from the manufacturer was used as the reference population.

Laboratory measurements

Serum 25(OH)D was measured on the automated Cobas E170 immunoassay analyser (Roche Diagnostics Limited, Basel, Switzerland). The specified intra- and inter-assay coefficients of variation (CV) are < 6.5% and < 11.5%, respectively. The cross-reactivity of the assay to D2 is 92%. Serum VDBP was measured using ELISA technique [Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA)] that employs the quantitative sandwich enzyme immunoassay using monoclonal antibody. The intra- and inter-assay CV ranged from 5.7–6.2% and 5.1–7.4%, respectively. The recovery was 104%.

VDBP measurements were used to calculate free 25(OH)D by two methods – one was the previously developed equation by modification of the Vermuelen method for free testosterone estimation and that which was further adapted by Bikle *et al*.[5] The equations for both methods are summarised below:

Bikle Method:

$$\text{Free 25(OH)D} = \frac{\text{Total 25(OH)D}}{1 + (K_{alb} \cdot [\text{Alb}]) + (K_{DBP} \cdot [\text{VDBP}])}$$

Vermeulen Method:

$$[D] = [-b + \sqrt{b^2 - 4ac}] \div 2a$$

$$[\text{Bio D}] = [D] + [\text{DAlb}] = (K_{alb} \cdot [\text{Alb}] + 1) \cdot [D]$$

$$\text{VDBP-bound 25(OH)D} = [\text{Total 25(OH)D}] - [\text{DAlb}]$$

where

[D] = Free 25(OH)D

$a = K_{VDBP} \cdot K_{alb} \cdot [Alb] + K_{VDBP}$

$b = (K_{VDBP} \cdot [VDBP]) - (K_{VDBP} \cdot [\text{Total 25(OH)D}]) + (K_{alb} \cdot [Alb]) + 1$

$c = -[\text{Total 25(OH)D}]$

K_{VDBP} = *affinity constant between 25(OH)D and VDBP* = $7 \times 10^8\ M^{-1}$

K_{alb} = *affinity constant between 25(OH)D and albumin* = $6 \times 10^5\ M^{-1}$

[Alb] = concentration of albumin

[Bio D] = Bioavailable 25(OH)D

[DAlb] = Albumin-bound 25(OH)D = [Bio D] – [D]

Calculations of all forms of 25(OH)D were done in moles per liter (mol/L), using Vermeulen method as it provides separately for free and bioavailable 25(OH)D. Subsequently, bioavailable 25(OH)D was converted to nanomoles per litre (nmol/L) while free 25(OH)D was expressed as nmol/L and picomoles per litre (pmol/L).

Statistical analysis

Statistical analysis was done using IBM SPSS Statistics version 24 (IBM, Armonk, NY, USA). Independent samples Kruskal–Wallis test and Spearman correlation were used to analyse non-Gaussian data. A p-value of ≤ 0.05 was taken to be statistically significant.

Ethics

This study was reviewed and approved by the Ethics Committee of the International Medical University, Malaysia [BMS101/2014(07)]. In accordance with the Helsinki Declaration, before entering the study, each subject gave informed written consent.

RESULTS

This was a cross-sectional study of 106 women, consisting of 77 patients with RA and 29 age-matched controls. The baseline characteristics of these patients have been described in the previously published paper.[10] The mean age of

TABLE 1: Association between levels of total 25(OH)D and VDBP with race [adapted from ref (10)]

	Malay n = 24 (22.6%)	Chinese n = 48 (42.3%)	Indian n = 34 (32.1%)	Chi-square for the Kruskal-Wallis H *p* value
	Median (Interquartile range [IQR])			
25(OH)D (nmol/L)	29.74 (18.60)	48.54 (27.76)	32.55 (17.39)	< 0.001*
VDBP (μg/mL)	215.17 (196.17)	98.78 (175.68)	147.73 (179.40)	0.185

*Statistically significant ($p < 0.05$)

the group was 53.66 ± 6.5 years. For the RA patients, 5/77 (6.5%) were on prednisolone. There were no significant differences between the total 25(OH)D and VDBP levels between the RA patients and controls. Thus, the dataset was analysed as a whole for this study.

In this study, the median (IQR) for total 25(OH)D and VDBP levels, were 37.51 (22.12) nmol/L and 120.49 (177.08) μg/mL, respectively for the whole cohort. There was no significant correlation between VDBP and total 25(OH) D (Spearman p = 0.526). The results of total 25(OH)D and VDBP levels in the different ethnic groups are shown in Table 1.

The median serum levels of the various forms of 25(OH)D are shown in Table 2. The correlation between the various forms of 25(OH)D, BMD and osteocalcin are shown in Table 3.

Calculated free 25(OH)D by Vermeulen was strongly correlated with calculation by Bikle (r = 1.00, p < 0.001), though values from Bikle were on an average 1.7% lower (data not shown). A significant positive correlation was noted between measured total 25(OH)D, with free and bioavailable 25(OH)D (r = 0.607, r = 0.637, respectively, p < 0.001 for both). Similarly, there was a significant negative correlation between VDBP with free and bioavailable 25(OH)D (r = -0.705, r = -0.696, respectively, p < 0.001 for both) but not with total 25(OH)D (r = 0.062, p = 0.526). VDBP-bound and albumin-bound 25(OH)D were positively correlated with total 25(OH)D (r = 0.937 and r = 0.637, respectively, p < 0.001).

As shown in Table 4, there were significant differences between races in all forms of 25(OH)D, with the Chinese having the highest values compared with Malay/Indian subjects. Malays had slightly lower levels of total and VDBP-bound 25(OH)D than Indians but higher levels of albumin-bound and, hence bioavailable 25(OH)D. Age was weakly correlated with free and bioavailable vitamin D (r = 0.191 p = 0.05, r = 0.196 p = 0.44, respectively). Race was significantly associated with BMD at the TH (Fig. 1A) and FN (Fig. 1B) (Kruskal-Wallis H p = 0.023 and < 0.001, respectively), but not at the LS (Kruskal-Wallis H p = 0.363). There was no difference in the menopausal status between the three races (Pearson Chi-Square p = 0.937).

DISCUSSION

The biological action of 25(OH)D depends on the concentration of its free and/or bioavailable form as postulated by the 'free-hormone hypothesis'.[11] 85-90% of circulating 25(OH)D is bound to VDBP, another 10-15% circulates bound to

TABLE 2: Values of the different forms of serum 25(OH)D

	Median (Interquartile Range [IQR])
Total 25(OH)D (nmol/L)	37.51 (22.12)
Free 25(OH)D (nmol/L / pmol/L)	0.0177 (0.0223) / 17.7 (22.3)
Bioavailable 25(OH)D (nmol/L)	7.08 (8.49)
Albumin-bound 25(OH)D (nmol/L)	7.07 (8.47)
VDBP-bound 25(OH)D (nmol/L)	28.54 (18.21)

Measured values: Total 25(OH)D, albumin and VDBP.
Calculated values: Free, bioavailable, VDBP-bound and Albumin-bound 25(OH)D using modified Vermeulen equations.

TABLE 3: Correlation between the forms of 25(OH)D with BMD and osteocalcin

	LS BMD	FN BMD	TH BMD	Osteocalcin
		Spearman correlation *p* value		
Total 25(OH)D (nmol/L)	0.237	0.042* (r = –0.201)	0.270	0.451
VDBP (μg/mL)	0.847	0.798	0.354	0.556
Free 25(OH)D (nmol/L)	0.226	0.170	0.147	0.868
Bioavailable 25(OH)D (nmol/L)	0.181	0.147	0.140	0.849
Albumin-bound 25(OH)D (nmol/L)	0.181	0.147	0.140	0.849
VDBP-bound 25(OH)D (nmol/L)	0.307	0.073	0.426	0.388

*Statistically significant ($p < 0.05$)
LS = lumbar spine, FN = femoral neck, TH = total hip, BMD = bone mineral density

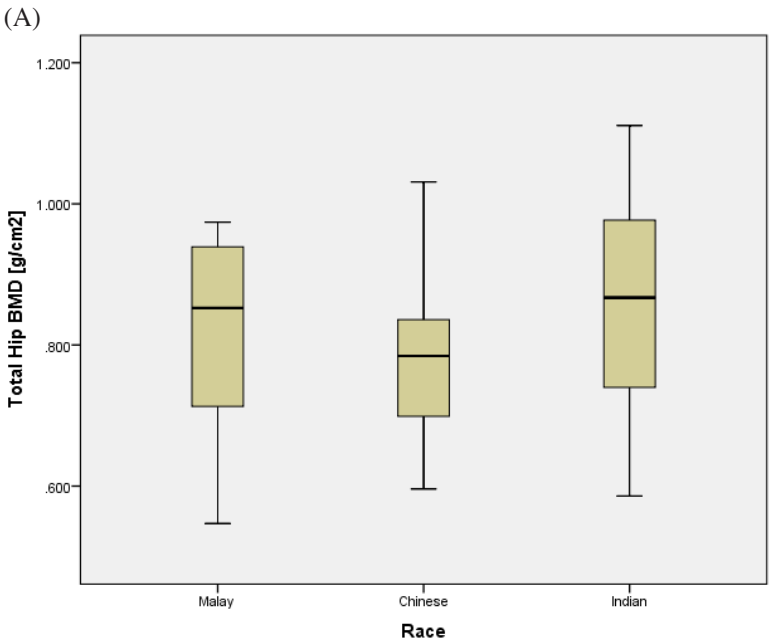
albumin, with only < 1% in its free form.[5] However, as not all studies have consistently shown a correlation between total serum 25(OH)D and BMD, we sought to determine if free 25(OH)D better reflects BMD compared to total 25(OH)D. We found that there was a negative association between total serum 25(OH)D and FN BMD, but not LS or TH BMD or osteocalcin. Studies looking at correlations between total 25(OH)D and BMD have shown differing results; some have shown a positive correlation between total 25(OH)D and BMD[12,13,14,15] while others have found no such association.[2,16,17] In a Malaysian study looking at patients with systemic lupus erythematosus, a similar negative correlation between 25(OH)D and FN BMD was found[18], similar to this study. A recent meta-analysis found that subjects who took vitamin D supplements had higher vitamin D levels with a small benefit/increase in FN BMD but not in LS or TH BMD, in keeping with this study.[19]

Despite finding the initial association of FN BMD with total serum 25(OH)D, we were unable to find any significant associations between all forms of calculated 25(OH)D with BMD at all sites. Not all the studies looking at free/

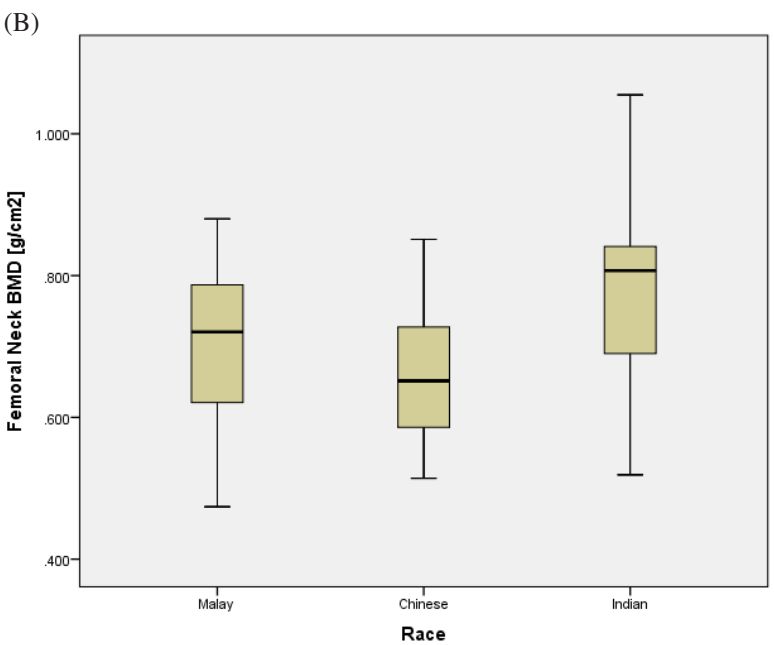
TABLE 4: Association between the forms of 25(OH)D and race

	Chinese n = 48	Malay n = 24	Indian n = 34	χ^2	*p-value
	Median (Interquartile Range [IQR])				
Total 25(OH)D (nmol/L)	48.54 (27.76)	29.74 (20.03)	32.55 (18.17)	23.336	< 0.001
Free 25(OH)D	0.028 (0.029)	0.013 (0.019)	0.013 (0.016)	19.257	< 0.001
(nmol/L) / (pmol/L)	27.8 (29.2)	12.9 (18.9)	13.3 (16.3)	19.257	< 0.001
Bioavailable 25(OH)D (nmol/L)	11.24 (13.09)	5.17 (6.79)	4.74 (5.99)	20.729	< 0.001
Albumin-bound 25(OH)D (nmol/L)	11.21 (13.06)	5.16 (6.78)	4.73 (5.98)	20.729	< 0.001
VDBP-bound 25(OH)D (nmol/L)	33.62 (22.44)	23.22 (14.95)	25.50 (15.16)	15.799	< 0.001

*Statistically significant ($p < 0.05$); Kruskal Wallis statistical test (χ^2)



$\chi^2 = 7.550$, $p = 0.023$*



$\chi^2 = 17.538$, $p < 0.001$*

FIG. 1: Boxplot showing association of race with (A) total hip BMD and (B) femoral neck BMD. *Statistically significant ($p < 0.05$). Chi-square for the Kruskal-Wallis H (χ^2).

bioavailable 25(OH)D and its correlation with BMD have been in female populations. In two studies involving both male and female subjects from USA, one found no correlation of all forms of 25(OH)D with LS, hip or radius BMD,[3] but another showed that bioavailable 25(OH) D predicted LS BMD.[5] In the latter study, hip BMD was not measured. In two studies looking at postmenopausal women,[8,9] both showed correlations between the calculated 25(OH)D values with BMD, in contrast to our study. Johnsen and colleagues showed a significant association between total body BMD and free and bioavailable 25(OH)D in a Norwegian population.[8] Li and colleagues showed that bioavailable 25(OH) D was an independent predictor on LS BMD.[9]

VDBP is a plasma protein with 3 major electrophoretic variants (Gc2, Gc1s, Gc1f) that differ by amino acid substitutions and extent of glycosylation. Initial studies used a monoclonal antibody ELISA assay which only measured one epitope, with different affinities for the different variants.[20] These alleles demonstrated distinct racial distribution patterns, with Gc1f genotype being the most abundant among Africans and Asians, whereas Gc1s and Gc2 were prevalent in Caucasians.[21] Powe and colleagues[22] used a monoclonal assay and reported that measured VDBP was lower in African Americans compared to Caucasians, which is now thought to be due to the VDBP isoform-specific measurement bias. Other studies using polyclonal immunoassays or mass spectrometric assays to measure VDBP have not shown racial differences.[23,24,25]

Although we used a monoclonal ELISA assay in this study, there was no significant difference in VDBP values between the three races; Malay, Chinese and Indians. Hence, although VDBP genotyping was not done in this study, the type of assay used most probably would not affect the accuracy of VDBP results. It is possible that the VDBP genotypes are similar within Asians, resulting in the similar levels between the three races studied here.

The accuracy of calculated free 25(OH)D depends on measurement precision of each of the individual components of the formulae. The Vermeulen formula includes total 25(OH)D, VDBP and albumin and their affinity constants (K_{VDBP} = affinity constant between 25(OH)D and VDBP = 7×10^8 M^{-1}; K_{alb} = affinity constant between 25(OH)D and albumin = 6×10^5 M^{-1}).[5] We did not use the genotype-specific affinity constants,[26] but since there was no significant difference in VDBP between races, we assumed that there is no difference in VDBP genotype, hence there was no necessity to use the genotype-specific affinity constants in the formula. In addition, we calculated free 25(OH)D using two different formulae, Bikle and Vermeulen, which were strongly correlated. We found Bikle easier to use for calculation compared to Vermeulen. However, we used Vermeulen for data analysis because the equations included both bioavailable and free 25(OH)D whereas Bikle calculates only the free form.

In this study, significant correlations were found between measured total serum 25(OH)D and VDBP with calculated free, bioavailable, albumin-bound and VDBP-bound 25(OH)D, respectively. This was to be expected, as calculations of these forms of 25(OH)D included total 25(OH)D and VDBP in the equations.[21] However, there was no significant correlation between measured serum VDBP and total 25(OH)D in contrast to other studies[5,9,27] which also used monoclonal antibody assays. Nielson and colleagues (2016) showed that total 25(OH)D was weakly correlated with VDBP, regardless of VDBP assay type ($r = 0.28$).[4] However, in those studies total 25(OH)D was analysed using liquid chromatography mass spectrometry (LCMS), whereas in our study, we used an immunoassay. Hence, our contradictory results could be due to the discrepancies that exist among commercially available 25(OH)D assays.[23,26]

Interestingly, although total 25(OH)D levels, together with the calculated free and bioavailable 25(OH)D, were found to be higher in the Chinese ethnic group, BMD at the TH and FN was actually lower in the Chinese. There was no difference in the menopausal status between the three ethnic groups that could have contributed to this. This only confirms that vitamin D is only one of the many factors that affect BMD in individuals.

This study found no significant association between all forms of 25(OH)D with osteocalcin. A study in Chinese postmenopausal women found a correlation between bioavailable 25(OH)D and osteocalcin.[9] A possible reason for this could again be linked with the VDBP genotypes and/or measurement techniques, which will require further study.

We recognise that there are some limitations in this study. Our median level of serum 25(OH) D in this study was 37.51 nmol/L in a group with an average age of 53.66 years. In another study from Malaysia, the mean level of serum 25(OH)D was 49 nmol/L in a much younger age

group with a mean age 25.2 years.[28] As vitamin D production from the skin falls with age, we feel that our study levels of 25(OH)D are in keeping with the general population and hence the results are valid. There are no data on VDBP polymorphisms in the Malaysian population, which may be a confounding factor, although VDBP levels were not significantly different between races. We are hoping to obtain funding to study VDBP polymorphisms in a larger sample of multiethnic Malaysian subjects.

CONCLUSION

In this first study of a multiethnic female Malaysian population, free and bioavailable 25(OH)D was found to reflect total 25(OH)D, and was not superior to total 25(OH)D in its correlation with BMD. The median values of calculated free and bioavailable 25(OH)D were significantly higher in Chinese compared with Malays and Indians, consistent with their median total 25(OH)D serum levels. The calculated free and bioavailable 25(OH)D levels did not correlate with BMD and thus may not be helpful in clinical practice when assessing bone health in Malaysian women. Should such a calculation be required, the Bikle formula is easier to use but only calculates free 25(OH)D. The Vermuelen formula calculates both free and bioavailable 25(OH)D but is more complex to use.

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