

# Determining the Required Vitamin D Level for Bone Health Based on Bone Turnover Markers

Tuan Salwani Tuan Ismail<sup>1</sup>, Bayani Che Muda<sup>1</sup>, Roznie Aida Mohd Rosdi<sup>1</sup>, Azalina Zainuddin<sup>1</sup>, Salbiah Isa<sup>2</sup>, Zulkarnain Mustapaha<sup>1</sup>

<sup>1</sup>Department of Chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia

<sup>2</sup>Advanced Medical and Dental Institute, Universiti Sains Malaysia, Kelantan, Malaysia

To date, no clear threshold that has been established for defining an adequate store of vitamin D for bone health. Therefore, this study aims to determine the required level of vitamin D to maintain a healthy skeleton based on bone remodelling process among healthy adult population. This was a cross sectional study, involving a healthy adult population in Kota Bharu, Malaysia, aged 18~50 years. We measured serum 25(OH)D (vitamin D), serum parathyroid hormone (PTH), serum C-terminal telopeptide of type 1 collagen (CTX), and Procollagen 1 Intact N-Terminal (P1NP) in 120 healthy adults selected via multi stage sampling (64 males, 56 females) from 6 subdistricts in Kota Bharu. The mean level of 25(OH)D was 23.50 ( $\pm 8.74$ ) nmol/L. There was a significant difference of the vitamin D level between genders (26.81  $\pm 8.3$  nmol/L vs 19.72  $\pm 7.68$  nmol/L in males and females respectively) ( $p$  value < 0.001). More than 50% of female subjects had 25(OH)D less than 20 nmol/L, while only 20.3% of male subjects had 25(OH)D below 20 nmol/L. Based on the LOESS plot, the bone turnover markers showed a plateauing result, at the 25(OH)D level of 35 nmol/L for CTX and 20 nmol/L for P1NP. Contrastingly, PTH showed a step rise in the 25(OH)D level of 20 nmol/L. Based on the LOESS plot for CTX, P1NP and PTH versus 25(OH)D, level of vitamin D between 20 to 35 nmol/L is recommended to maintain healthy skeleton.

**Key words:** Vitamin D, Bone turnover markers, N-terminal propeptide of type 1 collagen, C-telopeptide, Parathyroid hormones

Corresponding author: Tuan Salwani Tuan Ismail  
Department of Chemical Pathology, School of  
Medical Sciences, Universiti Sains Malaysia,  
Health Campus, 16150, Kubang Kerian,  
Kelantan, Malaysia  
Tel: 609-767-6510  
E-mail: tusti@usm.my

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## INTRODUCTION

Vitamin D plays a pivotal role in the development and maintenance of a healthy skeleton. It plays a critical function in the calcium and phosphorus metabolism and helps ensure adequate levels of these minerals for bone mineralization [1]. In adults, low vitamin D can result in osteomalacia which is a defective mineralization of the collagen matrix causing a reduction of structural support and being associated with an increased risk of fracture [2]. A low prevalence of vitamin D deficiency is expected in tropical countries with abundant sun exposure. This is because the most important source of vitamin D is by

cutaneous synthesis under the action of sunlight. The sunlight converts 7-dehydrocholesterol in the skin to vitamin D<sub>3</sub>, which will be transported to the liver and hydroxylated to 25-hydroxyvitamin D [(25-OH)D]. Vitamin D<sub>3</sub> will then be conveyed to the kidney and hydroxylated to 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D]. The primary circulating form of vitamin D is 25(OH)D and it has a longer half-life (2~3 weeks) compared to 4~6 hours for 1,25(OH)<sub>2</sub>D, thus accepted as the determinant of vitamin D status [3]. Having said that, the prevalence of vitamin D deficiency in countries like Hawaii, Iran, India, and Saudi Arabia which receive sunlight throughout the year are high [4-7]. Vitamin D deficiency is now recognized as a global pan-

demic. A systematic review of vitamin D status in populations worldwide done in 2013 showed that 37.3% of the sample had vitamin D level below 50 nmol/L and 6.7% had values below 25 nmol/L [8]. In Malaysia, a study done among adults in Kuala Lumpur revealed that approximately 41% of male and 87% of females had insufficient (<50 nmol/L) level of vitamin D [9]. Another study conducted among primary school children in Kuala Lumpur revealed that 35.3% having vitamin D deficiency (<37.5 nmol/L) and 37.1% having vitamin D insufficiency (37.5~50.0 nmol/L) [10]. Despite mounting evidence of vitamin D status globally, there are still lack of studies in our local setting determining the level of vitamin D, especially among general population. Precisely defining vitamin D deficiency based on measurement of 25(OH)D concentration is still a matter of much debate. There is no optimum level or threshold that has been clearly established to define whether there is adequate store of vitamin D in the body [11]. Various vitamin D cut-off has been proposed as the indication of bone health in general population. Previous study showed a plateau in suppression of parathyroid hormone (PTH) when the 25(OH)D level reaches approximately 30 nmol/L which defines the optimum level [12]. Other study concluded that bone health in older persons are likely to improve when serum 25(OH)D is raised over at least 50~60 nmol/L [13]. There are also other studies in which the researcher considers a 25(OH)D value of equal or more than 75 nmol as an adequate or optimum level [14]. Our study aims to determine the optimum level of vitamin D required for maintenance of healthy skeleton based on bone remodelling process. Remodelling of bone involves two counteracting processes, which are bone formation and bone resorption. This balance of two processes is regulated by various substances like vitamin D, parathyroid hormone and various cytokines [15]. An imbalance of these processes leads to higher release of bone turnover markers, which are proteins originating from osteoclast and osteoblast activity or fragments released during the formation or degradation of type I collagen. In vitamin D deficiency, secondary hyperparathyroidism develops due to hypo-

calcaemia. This hyperparathyroidism causes a negative balance in the bone metabolism by stimulating bone resorption, thus releasing higher levels of bone turnover markers. These markers of bone metabolism are helpful tools to detect the dynamics of the metabolic imbalance itself [16]. Markers like osteocalcin, Procollagen 1 Intact N-Terminal (P1NP) and bone specific alkaline phosphatase are markers for bone formation while markers for bone resorption includes hydroxyproline, hydroxylysine-glycoside and C-Terminal Telopeptide of Type 1 Collagen (CTX). Many studies have shown that low serum 25(OH)D level are associated with an increased in bone turnover markers. A study done by Kuchuk et al (2009) showed that parameters of bone turnover like osteocalcin and CTX were significantly lower in the higher vitamin D group [13]. A study in German children also showed that increase in 25(OH)D was associated with a significant decrease in the bone resorption marker, CTX [17]. Across the general population, various cut-off of 25(OH)D has been proposed for bone health. Therefore our aim is to determine the desired range of vitamin D for maintenance of healthy bone in general healthy population based on bone turnover markers. It will provide a comparison with the optimum level of vitamin D established based on (bone mineral density) BMD scanning in other studies.

## SUBJECTS AND METHODS

### 1. Study design

This cross sectional study was conducted within 2 months duration, from July until August 2015. Within this period of time, the seasonal variations are minimal, whereby the rate of sunlight received are maximum. The reference population was Malay adult in Kota Bharu, aged 18~50 years. Sample size calculation done using single mean formula. One hundred and twenty subjects from Kota Bharu were randomly selected using multistage sampling. In the first stage, 6 out of 14 subdistricts in Kota Bharu were selected. Secondly, a village from each of the 6 subdistricts was randomly selected, and later, all residents in the selected villages, who aged between 18~

50 years old, and fulfil the inclusion and exclusion criteria were randomly chosen and offered to participate in the study. We randomly chose the subjects by the name list provided by the village's authorities. Subjects with chronic diseases e.g, chronic kidney disease, liver failure, diabetes mellitus, thyroid disorders and established osteoporosis were excluded. Subjects who were on any form of drug treatment with possible effect on bone metabolism (e.g., oral contraceptive, hormone replacement therapy, glucocorticoids, and vitamin D supplements) were also excluded from the study. Apart from that, subjects who took alcohol, had a history of recent fracture within past 1 year and women who were pregnant, lactating or menopause were also excluded.

The study was approved by HREC (Human Ethics Research Committee USM) No. 15030091 and informed written consent was obtained from all subjects participated in the present study.

### 2. Specimen collection

Venous blood samples were collected in the morning between 8 to 10 am after an overnight fasting period. Serum was stored at  $-20^{\circ}\text{C}$  after centrifugation at  $2,500 \times g$  for 10 min. The samples were stored until analysis for the determinations of serum 25(OH)D, CTX and P1NP. All assays were carried out according to the manufacturer's instruction.

### 3. Laboratory measurements

All analytes were measured using Elecsys Cobas e 411 by Roche Diagnostics. Serum 25(OH)D were measured by competitive protein binding assay with CVs for repeatability were between 1.7% to 7.8% and the CVs for intermediate precision were between 2.2% to 10.7%. This method is standardized against liquid chromatography-tandem mass

spectrometry (LC-MS/MS) which in turn is traceable to National Institute of Standards and Technology (NIST). The CTX and total P1NP both were measured by sandwich assay. The CVs for repeatability were between 2.1% to 3.5% and CVs for intermediate precision were 2.8% to 8.4% for CTX. The total P1NP's CVs were 1.2% to 3.0% and 1.7% to 4.1% for repeatability and intermediate precision respectively. For the PTH, the assay used was second generation assay with sandwich principle. The CVs for repeatability were 1.5% to 2.7% and the intermediate precisions were between 3.0% to 6.5%.

### 4. Statistical analysis

Data were analysed using SPSS Statistical Package. All numerical data are presented by mean and SD. Baseline data for total sample and each of gender group were calculated. The differences between the gender groups were tested by independent t-test. The locally weighted scatterplot smoothing (LOESS) plot was performed to investigate the relationships between vitamin D and the bone turnover markers and subsequently used to determine the optimum level of vitamin D.

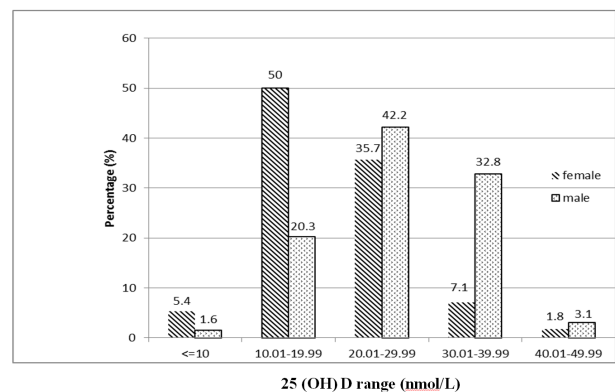
## RESULTS

One hundred and twenty study subjects were recruited which consist of 95.8% Malay, 2.5% Chinese and 1.7% Indian. The vitamin D measured among study subjects ranged between 8.59 nmol/L to 47.56 nmol/L. The mean

**Table 1.** Mean of vitamin D among study subjects (N=120)

Confidence interval	p-value	Female N=56	Male N=64	25(OH)D, (nmol/L)
4.18-9.99	<0.001	19.72±7.68	26.81±6.81	

\*Statistical significance was tested by t-test.



**Figure 1.** Distribution (%) of gender based on the range of 25(OH)D.

level of 25(OH)D in this study was 23.50 ( $\pm 8.74$ ) nmol/L. There was significant difference the level of 25(OH)D between male and female as is summarized in Table 1.

More than 50% of female subjects had vitamin D less than 20 nmol/L, while only 20.3% of male subjects had vitamin D below 20 nmol/L. Majority of female subjects have vitamin D level in the range of 11~20 nmol/L, whereas male subjects had higher range of vitamin D which was between 20~30 nmol/L. Out of 120, 4 subjects had vitamin D level below 10 nmol/L. Figure 1 showed the distribution of 25(OH)D among different gender (male and female). The proportion of vitamin D status at various cut-off classifies according to circulating 25-hydroxyvitamin

**Table 2.** Proportion of vitamin D status at various 25-(OH)D cut-off

N (%) (Total No.=120)	25(OH)D cut-off (nmol/L)
68 (56.7)	<25 (vitamin D deficiency)
52 (43.3)	25~50 (Insufficiency)
0 (0)	>50 (Hypovitaminosis)

D concentrations is summarized in Table 2.

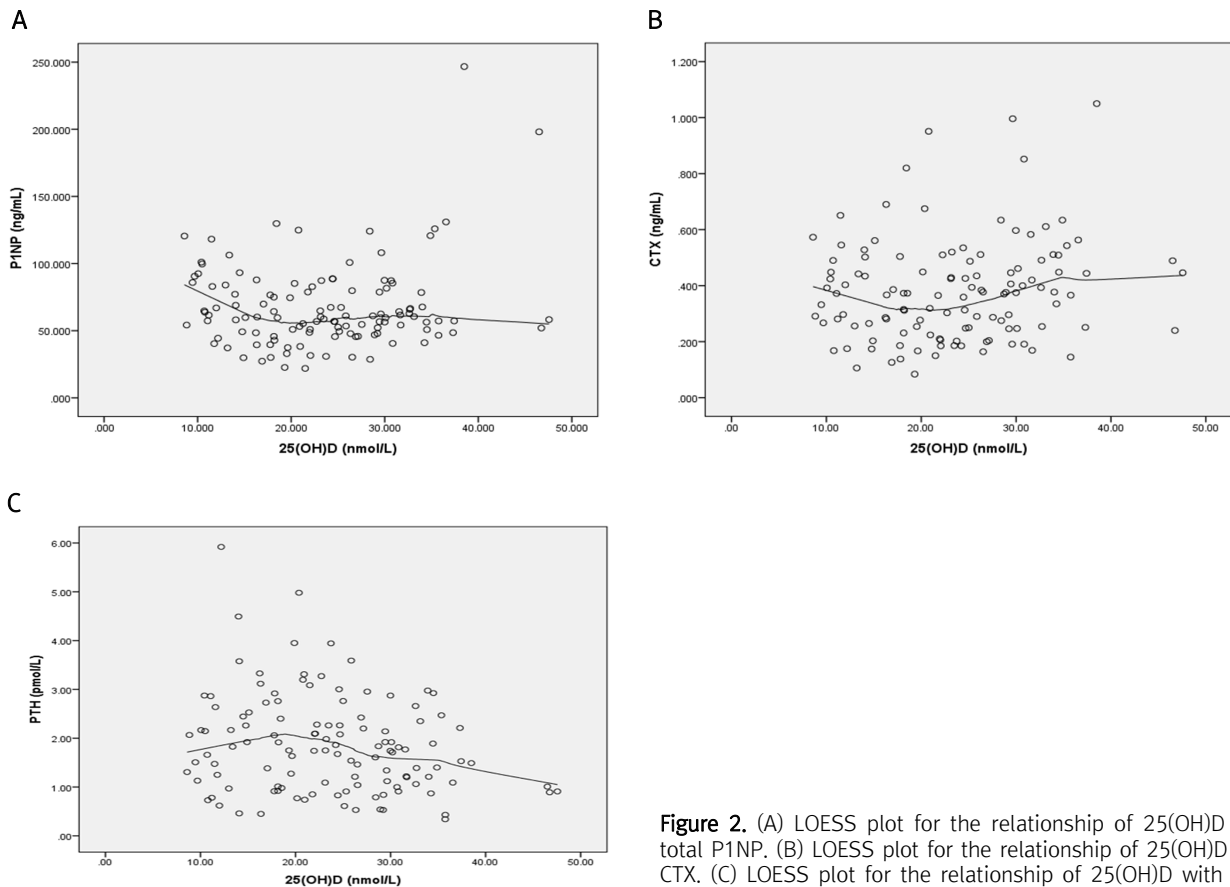
The level of bone turn over markers between male and female were measured and summarized in Table 3. The level of bone markers between male and female were significant different statistically.

The relationships between vitamin D level and bone markers are presented in Figure 2A, 2B and 2C. LOESS plots show the values of serum CTX, P1NP and PTH for each value of serum vitamin D. The level of P1NP is seen to be plateau when the vitamin D levels reach more than 20

**Table 3.** Mean levels of bone turnover markers between male and female

Confidence interval	p-value	Female N=56	Male N=64	
0.05~0.18	<0.001	0.32 (0.16)	0.43 (0.19)	CTX, ng/mL
2.74~25.37	0.015	60.25 (24.85)	74.30 (35.8)	P1NP, ng/L
-1.07~-0.38	<0.001	2.24 (0.99)	1.51 (0.88)	PTH, pmol/L

\*Statistical significance was tested by t-test.



**Figure 2.** (A) LOESS plot for the relationship of 25(OH)D with total P1NP. (B) LOESS plot for the relationship of 25(OH)D with CTX. (C) LOESS plot for the relationship of 25(OH)D with PTH.

nmol/L (Figure 2A). For serum CTX, the plot shows no significant increment in the level when the vitamin D level is more than 35 nmol/L (Figure 2B). Result for PTH shows that when the vitamin D levels reach 20 nmol/L, there is a steep decrease in the PTH level (Figure 2C).

## DISCUSSION

Vitamin D is known for decades to be important in maintaining a healthy bone in human of all ages starting from prenatal, infancy, childhood, and adulthood until the elderly. In recent years, there has been renewed interest in vitamin D because of its many other extra skeletal benefits. It has been suggested to play roles in the skin, cardiovascular system, immune system, pathogenesis of diabetes mellitus type 1, autoimmune diseases and in cancer prevention and treatment [18-21]. Inadequate vitamin D leads to bone diseases like rickets, osteomalacia and osteoporosis. These bone diseases have a huge impact on the population as a whole and especially on affected individuals and their families. It causes pain and deformities and affects functional status and also self-esteem. As it leaves a major impact to the society, it is crucial to maintain an adequate level of vitamin D for bone health. In this study, we found that the mean vitamin D level in our population was 23.50 nmol/L. The range of vitamin D levels are between 8.59 to 47.56 nmol/L. Previous study reported that mean population level of 25(OH)D values are varied considerably across the worldwide ranging from 4.9 until 136.2 nmol/L [8]. In the South East Asia region like Indonesia, the mean vitamin D level is higher with 38.7 nmol/L and 67.6 nmol/L in Thailand [22,23]. Even though both the countries are tropical countries similar like Malaysia, the result exhibits a large difference. The reason could be due to different assays being used in measuring vitamin D. The study in Thailand used liquid chromatography mass spectrometry, whereas in Indonesia, ELISA was used. Apart from that, exposure to sunlight, dietary intake of vitamin D and use of supplements may also become factors that contribute to the difference. There are conflicting evidences in the vitamin D status

between male and female subjects in the study worldwide. In our study, the mean vitamin D level for male is higher ( $26.81 \pm 8.30$ ) nmol/L compared to female ( $19.72 \pm 7.68$ ) nmol/L. The vitamin D values in women tended to be lower, especially in the Middle East regions. Previous study in Turkey and Lebanon showed lower vitamin D in the females [24,25]. The reasons are most likely due to less sun exposure, less outdoor activities, and decreased body area exposure than the male counterpart especially in the Muslim countries where women wear full clothing including hijab and veil. A meta-analysis on global vitamin D status in healthy subjects showed that female had higher 25(OH)D levels than male [26,27]. This finding could be explained by the possible usage of vitamin D supplements by the female compared to male. The definition of vitamin D deficiency remains controversial. Determination of 25(OH)D among postmenopausal Malay and Chinese women, aged 50~65 years old by Rahman et al (2004) defined the hypovitaminosis D between range of 50~100 nmol/L and vitamin D insufficiency between range of 25~50 nmol/L [28]. The liquid phase radioimmunoassay kit (Gamma B, IDS Limited, USA) was used to extract and quantify 25(OH)D in their study. In another study conducted by Moy and Bulgiba (2011) among Malay adult, mean age of 48.5 years in Kuala Lumpur, Malaysia were using the cut-off of <50 nmol/L to define vitamin D insufficiency [9]. The World Health Organization (WHO) defined vitamin D insufficiency as serum 25(OH)D below 50 nmol/L (20 ng/mL) [28]. Serum 25(OH)D >50 nmol/L is considered sufficient while serum 25(OH)D <30 nmol/L used as cut-off to start treatment based on New Therapies Subgroup (NTS) [29]. A target range of serum 25(OH)D between 50~90 nmol/L (20~36 ng/mL) was found to minimize the risk of acute coronary syndrome and all cause mortality [30]. Nurbazlin et al (2013) defined vitamin D deficient and insufficiency at lower cut-off as <30 nmol/L and 30~50 nmol/L respectively in their study, comparing vitamin D level among rural and urban population. Electrochemiluminescence immunoassay (ECLIA) method on Cobas E-411 analyzer was used to analyzed 25(OH)D in that study [31].

Difference in the study population and difference in the method used to measure 25(OH)D contribute to the variation of the used cut-off to define optimum level of vitamin D. Given the absence of uniformly accepted definitions and cut-offs, previous reviews have reported substantial variations in the prevalence of vitamin D deficiency across countries throughout the world. The estimates ranging from 2 to 90% depending on the cut-off value and study population selected. For the proportion of vitamin D deficiency, 76.7% of our subjects had 25(OH)D level less than 30 nmol/L and if the cut-off of vitamin D deficiency is taken at less than 50 nmol/L, it means that 100% of our subjects were vitamin D deficient. The required serum 25(OH)D has usually been established by assessing the threshold of serum 25(OH)D by which serum PTH maximally can be suppressed [32]. However, increases in serum PTH associated with vitamin D deficiency are usually within the normal reference range; serum PTH has a short half-life and depends on calcium intake, so different data sets could lead to different conclusions [11]. PTH also shows fluctuations related to diet, renal function and time of the day [33,34]. A threshold for optimal 25(OH)D by using bone mineral density (BMD) has been addressed recently. Numerous studies have shown significant positive correlation between vitamin D level and BMD [35,36]. In the NHANES III study, a threshold of about 80 nmol/L represented serum 25(OH)D level above which BMD increased more slowly [37]. BMD testing needs to be done in centre with special equipment using dual energy x-ray absorptiometry (DEXA) scan, performed by specialized personnel and needs the experts to interpret the result. It is not widely available as the cost is very high. Bone turnover markers on the other hand can be an alternative for estimating the threshold of vitamin D. In vitamin D deficiency, there will be higher bone turnover process due to secondary hyperparathyroidism, thus it will lead to increase in the bone turnover markers in the body. Usage of bone turnover markers is more practical as it is widely available, cheaper compared to DEXA scan and can monitor the bone turnover changes after treatment within short period (few weeks) compared to BMD by DEXA scan

that needs about 2 years for the changes to be seen. In this study, we observed that serum bone turnover markers, CTX showed plateau response when the vitamin D level reached 35 nmol/L. For serum total P1NP and PTH, the threshold of vitamin D at which these parameters started to plateau is at 20 nmol/L. The result that we had is lower compared to the previous study. Serum osteocalcin, a marker of bone formation in relation to serum vitamin D, should be higher than 40 nmol/L before it becomes plateau [12]. The difference for this threshold could be explained due to seasonal bone changes and preanalytical factors for bone turnover markers like influence of diets and diurnal rhythm. The level of 16 ng/mL (39.9 nmol/L) had been showed in another study conducted in Pakistan among postmenopausal women to maintain the PTH level of 53 pg/dL [38]. In our study, around 50% of study subjects fall between 20~35 nmol/L. In conclusion, healthy adult population in Kota Bharu have low level of vitamin D, with mean level of 23.5 nmol/L. Based on our study finding, the required level of 25(OH)D measured by electrochemiluminescence immunoassay is between 20 nmol/L to 35nmol/L for maintenance of bone health in our local adult population. There was great discrepancy of the required level of vitamin D based on bone markers and National Health and Nutrition Examination Survey (NHANES) III study which based on BMD. Therefore, a more extensive study involved bigger population and determination of optimum vitamin D level in correlation with series of bone markers and BMD scanning are required to confirm the required vitamin D level. Future study should consider several confounders related to vitamin D measurement such as age, race, duration of daily sun exposure, daily calcium intake, skin colour, body mass index and occupation of the study subjects which are not been addressed in this study.

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**Conflict of interest:** None

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