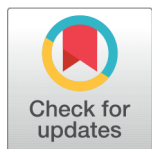


# Total and free vitamin D in type 2 diabetes mellitus patients in Baghdad city

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## ABSTRACT

**Background:** The free-form of vitamin D has been used by many researchers as an index of vitamin D status in health and disease. Several methods are there to estimate free, total, and even bioavailable vitamin D.

**Objective:** The present work was carried out to measure free vitamin D using a special formula suggested by Bikle and Schwartz in 2019, which includes the vitamin D binding protein (VDBP). The results will be used to evaluate the vitamin D status in patients with type 2 diabetes mellitus (T2DM), and its relation to the disease progression.

**Methods:** Sixty-four patients with T2DM and 73 healthy subjects, all from Baghdad city, were enrolled in the current study from March to October 2020. For each participant, fasting blood glucose, glycated hemoglobin (HbA<sub>1c</sub>), insulin resistance HOMA-IR, and body mass index (BMI) were measured in addition to the total vitamin D and VDBP. Moreover, free vitamin D was calculated by the formula of Bikle & Schwartz.

**Results:** There were highly significant correlations between total vitamin D and absolute values of free vitamin D or its percentage. The difference in total vitamin D was significant between patients and healthy controls with no significant change in VDBP, free and bio-available vitamin D, while free vitamin D% was higher in the patient's group. Correlations between vitamin D and each of BMI, fasting glucose, HbA<sub>1c</sub>, and HOMA-IR were not significant; however, there was a negative correlation with BMI and fasting glucose in the healthy control subjects only. The Receiver Operating Characteristic (ROC) curve analysis of vitamin D in the diagnosis of diabetes mellitus was poor.

**Conclusion:** Total vitamin D can represent vitamin D status, but it cannot be used as a factor for diagnosing T2DM. However, it could be of importance to change the glycemic status.

**Keywords** Bikle method, bioavailable vitamin D, free vitamin D, total vitamin D, type 2 diabetes mellitus, Vermeulen method

## OPEN ACCESS

## INTRODUCTION

Vitamin D is a fat-soluble vitamin synthesized in the skin when exposed to sunlight or from supplements or dairy products. The 1,25-dihydroxy vitamin D is the active form of vitamin D, which binds to the vitamin D receptors. However, because of its short half-life, the total vitamin D concentration has been used to evaluate vitamin D status.<sup>1-3</sup> Many pathological conditions such as reproductive dysfunction, many infections, cardiovascular disease and even cancer were attributed to low vitamin D levels or defects in its metabolism.<sup>4</sup>

It has been reported that 25-hydroxyvitamin D (25(OH)D) is converted to the 1,2-dihydroxy vitamin D in many tissues such as the brain, uterus and placenta, and vascular smooth muscle cells,<sup>5-8</sup> in an independent way of calcium homeostasis pathway.<sup>9,10</sup> Many criticisms of the different techniques of vitamin D measurement appeared in different reports.<sup>11-13</sup> The interpretations of vitamin D concentrations vary in different seasons depending on the ultraviolet light exposure. Other biological variations may associate that.<sup>14</sup> The seasonal variation may reduce the validity of vitamin D as a biomarker of vitamin D status. Several approaches have been used to account for seasonal variation in vitamin D concentrations. One approach is adjusting the exposure-outcome association for the season or month of blood draw. Another approach is to use the measured vitamin D concentrations to estimate the annual average concentration using a cosinor model<sup>14</sup> or a residuals-based approach<sup>15</sup>.

The bioavailable or free vitamin D may be more suitable to evaluate vitamin D status than total vitamin D,<sup>15</sup> similar to the steroid hormones, which are affected by the variation of hormone-binding proteins. On the other hand, when vitamin D is bound to VDBP, the entire complex can enter a cell by binding the transmembrane protein, megalin. This protein is expressed in the kidney, parathyroid, placenta, epididymis, mammary epithelium, and thyroid.<sup>16</sup> The extra-renal tissues may contain both free and bound vitamin D, which may lead to the assumption that both free and bound vitamin D may play a role in vitamin D signaling.<sup>17,18</sup>

The laboratory assays show that the free (unbound) vitamin D constitutes less than 1% of the total vitamin D, while vitamin D bound to VDBP measures about 85–90% and the rest (about 10-15%) is vitamin D bound to albumin.<sup>19</sup> Vitamin D may dissociate from albumin during tissue perfusion, making it possible to call the albumin-bound and free fractions together “bioavailable” vitamin D.<sup>20</sup>

However, measuring bioavailable vitamin D is thought to be difficult for many reasons, but the measurement of free vitamin D is possible by the immunoassay technique.<sup>20</sup> Furthermore, different reports gave different results for the correlations between total and free vitamin D.<sup>21-23</sup> The methods used for the calculation of free vitamin D play an important role in the final results. It was claimed by some scientists that direct measurement of free vitamin D or VDBP using either LC/MS-MS or a polyclonal immunoassay might give better findings. While, the relationship between free, bound vitamin D and Parathyroid hormone (PTH) is variable depending on different states of patients under study<sup>24-26</sup> Al-Oanzi et al.

(2006) showed that osteoporotic men had lower free 25(OH)D but not lower total 25(OH)D, suggesting that free 25(OH)D may be a more useful measure of biological activity.<sup>27</sup> Thus, further researches are needed to explore the functional differences between free and total vitamin D and the racial/ethnic differences observed in different studies.

The VDBP values of the present study were used to calculate free vitamin D by two methods; one was the previously developed equation by modification of the Vermeulen method<sup>28</sup> for free testosterone estimation, and the other was adapted by Bikle & Schwartz.<sup>29</sup>

The present study aimed to measure levels of free and bound vitamin D3 [25(OH)D3] by the formula mentioned above and find the relationship between them with the glycemic control in patients with T2DM.

## MATERIALS AND METHODS

### Subjects

The patient's group consisted of 64 (38 males and 26 females) patients with T2DM ranging from 26 to 75 years. They all were outpatients from the diabetic clinic in Al-Immamain Al-Kadhimain Medical City from March to October 2020. Patients with renal diseases, arthritis and cardiovascular diseases or cancers were excluded from the study. On the other hand, the control group consisted of 73 healthy individuals (43 males and 30 females) of age range from 24 to 70 years.

Body Mass Indexes (BMI) were used for subgrouping the patients and controls according to Division of Nutrition, Physical Activity, and Obesity, National Center for Chronic Disease Prevention and Health Promotion (NCCDPHP) as follows: underweight  $\leq 18.5$ , normal weight =18.5-24.9, overweight =25-29.9, and obesity  $\geq 30$  kg/m<sup>2</sup>.

Written informed consent was signed by each participant before enrolling in the current study. All questions needed for supporting the study were included in the questionnaire form.

### Biochemical markers and indexes

#### *Biochemical analysis*

Total vitamin D, VDBP, serum insulin and HbA<sub>1c</sub> were determined using automated analyzers: Cobas e411 (Roche Diagnostic, Germany) for total vitamin D, ELISA (LDN, Germany) for VDBP and serum insulin, and Nycocard reader kit (Abbott, Norway) for HbA<sub>1c</sub>.

#### *Calculations*

Free vitamin D was calculated by Bikle Method as follows:

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

Bioavailable 25(OH)D (free+albumin-bound fraction) was calculated using equations adapted from that of Vermeulen et al. (1999),<sup>28</sup>:

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

$$[Bio\ D] = [D] + [D_{Alb}] = (K_{Alb} \times [Alb] + 1) \times [D]$$

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

Where  $[D]$  = Free 25(OH)D;  $a = K_{VDBP} \bullet K_{Alb} \bullet [Alb] + K_{VDBP}$ ;  $b = (K_{VDBP} \bullet [VDBP]) - (K_{VDBP} \bullet [Total\ 25(OH)\ D]) + (K_{Alb} \bullet [Alb]) + 1$ ;  $c = -[Total\ 25(OH)D]$

BMI was calculated for all subjects involved in the study using the formula:

$$BMI = \frac{Weight\ (Kg)}{Height\ (m^2)}$$

## Statistical analysis

Data was collected, summarized, analyzed and presented using statistical package for social sciences (SPSS) version 23 and Microsoft Office Excel 2010. Qualitative (categorical) variables were expressed as numbers and percentage, whereas quantitative (numeric) variables were first evaluated for normality distribution using the Kolmogorov-Smirnov test, and then accordingly normally distributed numeric variables were expressed as mean (an index of central tendency) and standard deviation (an index of dispersion), while those numeric variables that are not normally distributed were expressed as median (an index of central tendency) and interquartile range (an index of dispersion). The following statistical tests were used: 1) A Chi-square test was used to evaluate the association between any two categorical variables, provided that less than 20% of cells have an expected count of less than 5. 2) Independent samples t-test was used to evaluate the difference in mean of numeric variables between any two groups provided that these variables were normally distributed; otherwise, Mann Whitney U test would be used instead if those variables were not normally distributed. 3) Spearman correlation was used to evaluate the correlation between any two numeric variables, and the results were expressed as correlation coefficient (r) and the level of significance (p). 4) In order to detect the cutoff value that predicts a positive finding, Receiver Operating Characteristic (ROC) curve analysis was used with its corresponding area under the curve (AUC), accuracy level, sensitivity, specificity and level of

significance ( $p$ ). The level of significance was considered at a  $p$ -value of equal or less than 0.05. The level of high significance was considered at a  $p$ -value of equal or less than 0.01.

## RESULTS

The comparison of vitamin D and vitamin D binding protein (VDBP) in patients and controls are shown in Table 1.

<b>Table 1</b> Vitamin D, vitamin D binding protein (VDBP) levels in patients and controls			
Characteristics	Control (n = 73)	Patients (n = 64)	P value
<b>Vitamin D Total (ng/ml)</b>			
Mean±SD	18.53±13.30	14.29±8.39	
Median (IQR)	14.60(15.25)	12.00(11.40)	—
Range	3.00-74.00	1.70-48.40	
<b>Log Total</b>			
Mean±SD	1.18±0.28	1.08±0.26	0.040 <sup>I,S</sup>
Range	0.48-1.87	0.23-1.68	
<b>Vitamin D VDBP (ng/ml)</b>			
Mean±SD	33.43±37.99	25.79±34.20	
Median (IQR)	11.00(52.45)	10.45(11.15)	—
Range	1.80-120.00	2.00-120.00	
<b>Log VDBP</b>			
Mean±SD	1.25±0.49	1.13±0.47	0.142 <sup>I,NS</sup>
Range	0.26-2.08	0.30-2.08	

SD, standard deviation; IQR, inter-quartile range; VDBP, vitamin D binding protein; I, independent samples  $t$ -test; S, significant at  $p \leq 0.05$ ; NS, not significant at  $p > 0.05$ .

From the statistical point of view, both total vitamin D and vitamin D binding protein (VDBP) were not normally distributed variables; therefore, they were described in terms of median and the interquartile range (IQR) as measures of central tendency and dispersion; however, both mean and standard deviation were shown in order to show the wide variation among individual measurements in each group.

Median and IQR of vitamin D levels were 12.00(11.40) and 14.60(15.25) in patients and control groups, respectively, thus it is lower in patients' group. Because it is not normally distributed, variable log transformation was carried out to normalize the distribution. Subsequently, an independent sample  $t$ -test was carried out, and the difference was significant ( $p = 0.040$ ).

The Median VDBP level was 10.45 (11.15) and 11.00 (52.45) in patients and control groups, respectively. For the same reason mentioned above, log transformation was carried out, and the difference in mean log values was not significant when patients were contrasted to the control group ( $p = 0.142$ ).

Free vitamin D, free vitamin D% and bioavailable vitamin D in patients with T2DM and control subjects are shown in Table 2.

**Table 2** Free vitamin D, free vitamin D% and bioavailable vitamin D levels in patients with T2DM and control subjects

Characteristics	Control (n= 73)	Patients (n= 64)	P value
<b>Free vitamin D (pg/ml)</b>			
Mean± SD	0.0466± 0.0357	0.0367±0.0205	—
Median (IQR)	0.0366(0.0344)	0.0315(0.0286)	0.117 <sup>M,NS</sup>
Range	0.0081-0.2276	0.0045-0.1071	
<b>Free vitamin D%</b>			
Mean ±SD	0.0002±0.0001	0.0003±0.0000	
Median (IQR)	0.0002(0.0001)	0.0003(0.0001)	0.005 <sup>M,HS</sup>
Range	0.0002-0.0003	0.0002-0.0003	
<b>Bio-available vitamin D (ng/ml)</b>			
Mean ±SD	5.6640±9.4128	3.9248±5.3121	
Median (IQR)	0.3800(7.7200)	0.7250(6.6800)	0.997 <sup>M,NS</sup>
Range	0.0000-45.3100	0.0000-19.2200	

n, number of cases; SD, standard deviation; M, median; I, independent samples *t*-test; S, significant at  $p \leq 0.05$ ; NS, not significant at  $p > 0.05$

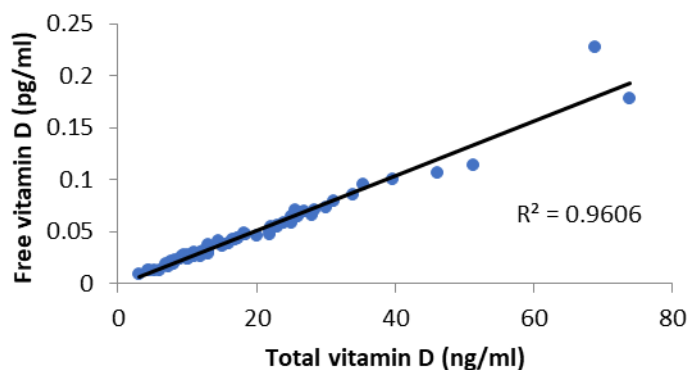
There was no significant difference in the level of free vitamin D between patients and control groups ( $p = 0.117$ ). The free vitamin level was deficient compared with total vitamin D in both control and patients' groups, 0.0366 pg/ml and 0.0315 pg/ml, and the percentage of free vitamin D level out of total was less than 0.001% in both groups. The difference in free vitamin D% between patients and control groups was highly significant ( $p = 0.005$ ). In addition, there was no significant difference in the level of bio-available vitamin D between patients and control groups ( $p = 0.997$ ).

The correlation between calculated free vitamin D and total vitamin D values is shown in Figure 1. The correlation was positive ( $r = 0.980$ ) and highly significant ( $p < 0.001$ ). In addition, the correlation line can explain approximately 96.0% of the concordance between calculated free vitamin D and total vitamin D as the  $R^2$  value was 0.960.

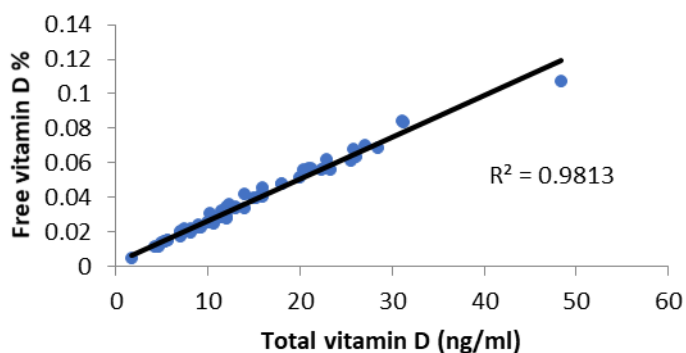
The correlation between calculated free vitamin D% and total vitamin D is shown in Figure 2. The correlation was positive ( $r = 0.991$ ) and highly significant ( $p < 0.001$ ). In addition, the correlation line can explain approximately 98.1% of the concordance between calculated free vitamin D% and total vitamin D as the R-squared value was 0.981.

The value of log total vitamin D in the discrimination between diabetes and control group was tested using the ROC curve. The results are shown in Figure 3 and Table 3. The area under the curve was 0.592, which is less than the acceptable level of 0.700; therefore, log total vitamin D is a poor predictor of diabetes mellitus. This fact is further clarified by the low specificity level of 46.6%.

The correlations of log total vitamin D with fasting plasma glucose, HbA<sub>1c</sub>, HOMA-IR and insulin level in patients with T2DM and control subjects are shown in Table 4. With respect to the control group, there was a significant negative correlation between fasting blood glucose and log total vitamin D ( $r = -0.281$ ;  $p = 0.016$ ). While in the patient's group, log total vitamin D was not correlated to fasting plasma glucose, HbA<sub>1c</sub>, HOMA-IR or



**Figure 1** Correlation between calculated free vitamin D and total vitamin D

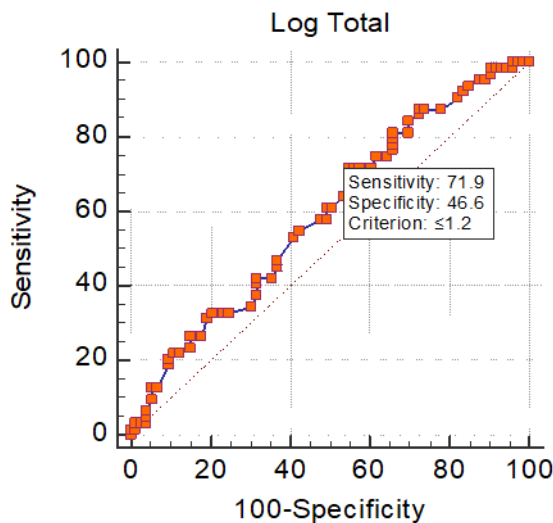


**Figure 2** Correlation between calculated free vitamin D% and total vitamin D

**Table 3** The characteristics of ROC curve shown in figure 3

Characteristic	Log Total
Cutoff	$\leq 1.2$
AUC	0.592
95% CI	0.504 to 0.675
Sensitivity (%)	71.87
Specificity (%)	45.21
<i>p</i> -value	0.060 <sup>NS</sup>
Accuracy (%)	59.2

AUC, area under curve; CI, confidence interval; NS, not significant at  $p > 0.05$



**Figure 3** Receiver Operating Characteristic (ROC) curve analysis to find the best log total vitamin D cutoff value that can predict a diagnosis of diabetes mellitus in terms of sensitivity and specificity.

insulin ( $p > 0.05$ ).

**Table 4** The correlation of log total vitamin D to fasting plasma glucose, HbA<sub>1c</sub>, HOMA-IR and insulin level in patients with T2DM and control subjects

Characteristic	Control (n = 72)		Patients (n = 64)	
	r	p	r	p
Fasting blood glucose	-0.281	0.016 <sup>S</sup>	-0.111	0.384 <sup>NS</sup>
HbA <sub>1c</sub>	-0.192	0.104 <sup>NS</sup>	-0.097	0.444 <sup>NS</sup>
HOMA-IR	-0.140	0.239 <sup>NS</sup>	-0.194	0.124 <sup>NS</sup>
Insulin	-0.097	0.418 <sup>NS</sup>	-0.181	0.152 <sup>NS</sup>

r, correlation coefficient; NS, not significant at  $p > 0.05$ ; S, significant at  $p \leq 0.05$

The correlation of log total vitamin D to BMI in both groups was negative; it was significant in the control group ( $p = 0.012$ ) and borderline in the patient's group ( $p = 0.079$ ).

## DISCUSSION

In the current study, the mean and median levels of free vitamin D was less than 1% of the total vitamin D level in both patients and the control group. This finding is in line with previous data published in many studies. For instance, it has been mentioned that in a normal non-pregnant individual, approximately 0.03% of vitamin D is free,<sup>26,29</sup> several other authors showed that the free (i.e., unbound to proteins) concentration of 25OHD or 1,25 (OH)<sub>2</sub>D was below 0.1% of their total concentration.<sup>30,31</sup>

In the present study, the calculated free vitamin D was significantly correlated to total vitamin D. In a previous study, a difference between calculated and directly measured free vitamin D was reported and was attributed to the effect of intact parathyroid hormone in the direct measurement.<sup>26</sup>

Various clinical conditions were found to affect free vitamin D, the per cent of free vitamin D, and the relationship between free and total vitamin D concentrations. Different values were seen in pregnant women, people with cirrhosis, and elderly people with multiple morbidities compared to normal or community-dwelling outpatients;<sup>29</sup> however, that study mentioned nothing about the effect of diabetes. In the present results, T2DM did not appear to affect the correlation between calculated free vitamin D and total vitamin D. Three previous studies are in line with our finding, which has shown that about 65% of the variation in free-form could be explained by total vitamin D.<sup>32-34</sup>

Several previous authors have raised the issue that free vitamin D could better reflect the vitamin D status than total vitamin D related to many clinical conditions.<sup>35-38</sup> However, the present findings showed that T2DM did not affect the correlation between free and total vitamin D. Hence, total vitamin D can represent the actual status of vitamin D in patients and healthy control subjects. This may agree with Jemielita et al. (2016).<sup>24</sup>

The present results also found that the difference in total vitamin D was significant between patients and control group with no significant variation in VDBP, free or bio-available vitamin D, but the difference in % free vitamin D was highly significant and in favour of patients; however, this is due to division of a relatively larger free portion of vitamin D in patients' group (since the total vitamin D in patients was lower than that of control and there was no significant difference in free vitamin D between both groups). The lower total vitamin D level in patients with diabetes mellitus enrolled in the current study could be considered either a consequence or a risk factor of diabetes. One of the drawbacks of a case-control study is that a causal relationship cannot be ascertained. To answer such a question, it is recommended to perform a longitudinal (cohort) study.

Over the last decade, vitamin D deficiency, or insufficiency, has emerged as a risk determinant for T2DM, and vitamin D supplementation has been hypothesized as a potential intervention to lower diabetes risk.<sup>39</sup> The hypothesis that vitamin D status may influence the risk of T2DM is biologically probable because both impaired pancreatic beta-cell function and insulin resistance have been reported with low blood vitamin D levels.<sup>40</sup> Observational studies supported an association between a low blood vitamin D level and the risk of diabetes.<sup>41</sup> In short-term mechanistic studies, vitamin D supplementation improved the disposition index, a measure of pancreatic beta-cell function, by 40%.<sup>42</sup> However, whether vitamin D supplementation lowers the risk of diabetes is unclear.<sup>43-45</sup>

In a recent multi center, randomized, placebo-controlled trial study involving persons at high risk for T2DM not selected for vitamin D insufficiency, vitamin D3 supplementation at a dose of 4000 IU per day did not result in a significantly lower risk of diabetes than placebo after a median follow-up of 2.5 years.<sup>46</sup>

When we used the ROC curve to evaluate the diagnostic potential of total vitamin D level concerning diabetes mellitus, we found that the total vitamin D cutoff value of  $\leq 1.2$

ng/ml was a poor predictor because the low area under the curve ( $< 0.7$ ) and low sensitivity, specificity and overall accuracy levels. Such a point was raised in the available previous published article when they tried to use ROC to find the cutoff value of vitamin D in relation to glycemic control in patients with diabetes mellitus.<sup>46</sup>

The significant negative correlation between total vitamin D of the present study may support the role of vitamin D in glycemic control. A study published in 2020 showed that vitamin D supplement could change the prediabetic state in non-obese persons to normal glycemia. This finding agrees with some previous studies.<sup>47-50</sup> Another recent report stressed the controversy in the literature about the effect of vitamin D on T2DM complications and the need for more studies in this respect, especially in the elderly.<sup>51</sup>

In conclusion, from the present study, we may say that the highly significant correlations between total vitamin D and the absolute value of free vitamin D or its per cent would confirm that total vitamin D can be used to represent vitamin D status and that vitamin D deficiency in T2DM could be considered as a part of the manifestation of malnutrition of this disease rather than being a risk factor. One of the supportive evidence for this hypothesis is that vitamin D deficiency has been recorded in children with type 1 diabetes, known to be an autoimmune disease.<sup>52,53</sup> Malnutrition is a well-recognized cause of vitamin D deficiency worldwide.<sup>54</sup> In general, it was believed that persons with diabetes are at significant risk for vitamin D insufficiency or deficiency. Reasons for this include diet, lack of sun exposure, obesity, renal impairment, and genetic predisposition.<sup>55</sup> Moreover, failure to get positive results from the ROC curve suggests that vitamin D status cannot be a cause of T2DM.

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## DECLARATIONS

### Authors' contributions

All authors have equally contributed to this work.

### Conflict of interest

Authors have no conflict of interest.

### Ethical approvals

This project was approved by Al-Immamain Al-Kadhimain Medical City and written informed consent was obtained from all participants before enrolling in the current study.

## Data availability

All data associated with the current paper can be requested from the corresponding author.

## Funding resources

This work didn't receive any fund.

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