


RESEARCH ARTICLE

Ethnic, geographic, and seasonal differences of vitamin D status among adults in south-west China

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Abstract

Background: There are limited data on vitamin D status of Sichuan province, and no investigation has been carried out on the correlations of 25(OH)D and BTMs between healthy Hans and Tibetans of Sichuan province. This study aimed to examine 25(OH)D levels around Sichuan province and to assess differences by ethnicity, age, gender, sunlight exposure, geographic location, and seasons.

Methods: Blood samples from 2317 healthy adults aged of 18 to 75 years and of Han and Tibetan ethnicities were collected in six regions and during four seasons. Serum 25(OH)D₂ and 25(OH)D₃ levels were measured by LC-MS/MS method. Serum total P1NP and β -CTX were measured by immunoassay.

Results: Participants aged 18-40 years showed significantly lower 25(OH)D levels than participants aged 41-75 years old ($P < .0001$). The median serum 25(OH)D level for males was significantly higher than that of females ($P < .0001$). Serum 25(OH)D levels among four seasons and different districts varied significantly ($P < .0001$). In addition, the 25(OH)D level of Tibetans was significantly lower than that of Hans, while the serum total P1NP and β -CTX levels of Tibetans were significantly higher than those of Hans ($P < .0001$).

Conclusion: Adult population was more common to have vitamin D deficiency/insufficiency among Tibetans, females, north regions and in spring and winter.

Lin Li, Kecheng Li, Jing Li and Yulei Luo contributed equally to this study.

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KEYWORDS

bone turnover marker, epidemiological study, ethnic difference, liquid chromatography-tandem mass spectrometry, vitamin D

1 | INTRODUCTION

Vitamin D is an important prohormone classically understood to play pivotal roles primarily in calcium and phosphate metabolism.¹ More recent studies have indicated vitamin D may help regulate the cell cycle which explains the link to cancer risk, increasing both interest and awareness and prompting further studies in broader clinical contexts.^{2,3} The vitamin D parent compounds are cholecalciferol (vitamin D₃, naturally occurring in humans and animals) and ergocalciferol (vitamin D₂, naturally occurring in plants). Vitamin D₃ can be obtained through dietary supplementation but is predominantly formed in the skin through the reaction of solar ultraviolet beam (UVB) in the spectrum of 290-315 nm with 7-deoxycholesterol,⁴ whereas vitamin D₂ can only be obtained from diet or dietary supplements.⁵ Both vitamin D₂ and D₃ follow the same metabolic pathways in humans having two steps activation. The first step is hydroxylation by the liver, resulting in the stable 25(OH)D forms (25(OH)D₂ and 25(OH)D₃), which circulate bound to the vitamin D binding protein (VDBP) and are subsequently converted to the biologically active metabolite 1,25-dihydroxyvitamin D (1,25(OH)₂D) under the action of renal 1- α -hydroxylase.⁶

It is widely accepted that the 25(OH)D (sum of 25(OH)D₂ and 25(OH)D₃) concentration, rather than 1,25(OH)₂D concentration, is the optimal indicator of vitamin D status owing to its 1000-fold longer half-life.⁷ Most of the total 25(OH)D in blood is represented by 25(OH)D₃, whereas the concentration of 25(OH)D₂ is normally low and only present in significant amounts in subjects taking vitamin D₂ supplements.³ US Endocrine Society recommended vitamin D deficiency as a 25(OH)D of less than 20 ng/mL and vitamin D insufficiency as a 25(OH)D of 21-29 ng/mL.⁸ Based on these cutoff values, a number of epidemiological studies have shown that vitamin D deficiency/insufficiency has become a global health problem.^{3,7,9-11} It has been estimated that 22%-97% of United States, Canadian and European population show vitamin D deficiency or insufficiency.¹²⁻¹⁴ Vitamin D deficiency is also common in Australia, the Middle East, India, Africa, and South America.^{3,15} In China, a previous report regarding 25(OH)D₂ and 25(OH)D₃ levels in 23 695 patients who requested 25(OH)D testing showed that the vitamin D deficiency/insufficiency rate was 84.1%.¹⁶ Hence, early detection and management of vitamin D deficiency have become a matter of increasing concern in clinical practice.¹⁷

In addition, findings from the 2003-2004 National Health and Nutrition Examination Survey (NHANES) suggested that ethnic differences exist in the association between 25(OH)D levels and bone mineral density (BMD), in which American whites had significantly higher 25(OH)D than American Blacks primarily because increased skin pigmentation inhibits cutaneous synthesis of cholecalciferol (pre-vitamin D₃). On the other hand, BMD significantly

decreased with the decline of serum 25(OH)D among American whites, but not blacks, indicating even though American blacks are at higher risk of vitamin D deficiency than whites, they may not have increased risk of developing osteoporosis and other skeletal diseases.¹⁸

In China, the level of 25(OH)D between male Hans and Uyghurs with osteoporosis was reported to be significantly different.¹⁹ Moreover, bone turnover markers (BTMs), specifically, N-terminal procollagen of type I collagen (P1NP, a bone formation marker) and beta C-terminal cross-linked telopeptides of type I collagen (β -CTX, a bone resorption marker) were previously reported to have significant correlations with 25(OH)D and BMD in Chinese populations.^{20,21}

Sichuan province has the second largest Tibetan population and inhabitants have variable in sunlight exposure, lifestyle, and ethnicity. Currently, there are limited data on vitamin D status of Sichuan province and no investigation has been carried out on the correlations of 25(OH)D and BTMs between healthy Hans and Tibetans of Sichuan province. In this study, a LC-MS/MS method with high selectivity for the separation of 25(OH)D₃ and 3-epi-25(OH)D₃, and for the quantification of 25(OH)D₂ and 25(OH)D₃ was firstly developed and applied to adult serum samples. A multicenter cross-sectional study was then undertaken to investigate the vitamin D status of Sichuan by age, gender, ethnicity, sunlight exposure, and season. Finally, serum 25(OH)D levels and BTMs between Hans and Tibetans were further compared to inform efforts to prevent and treat vitamin D deficiency in Sichuan province of China.

2 | MATERIAL AND METHODS

2.1 | Study population

The multi-center cross-sectional study was conducted across four seasons from May 21, 2018, to April 21, 2019, in 6 regions around Sichuan province, including Chengdu (CD), Nanchong (NC), Luzhou (LZ), Maerkang (MK), Guangyuan (GY), and Panzhihua (PH) (Figure 1). The ambient ultraviolet B (UVB) radiation levels of different areas were classified according to the corresponding dose of each participant living area from NASA Surface meteorology and Solar Energy (SSE) data. The ethnicities of CD, NC, LZ, GY, and PH population are Han, and the ethnicity of MK is Tibetan. The sample collection periods were May 21-July 21, 2018, for summer, August 23-October 23, 2018, for autumn, November 22, 2018-January 22, 2019, for winter, and February 21-April 21, 2019, for spring, making the median collection time point correspond to Vernal equinox, Summer solstice, Autumn equinox, and Winter solstice, respectively.

Samples from 2317 healthy adults aged 18 to 75 years were collected. All of the following were recorded for each subject: age,

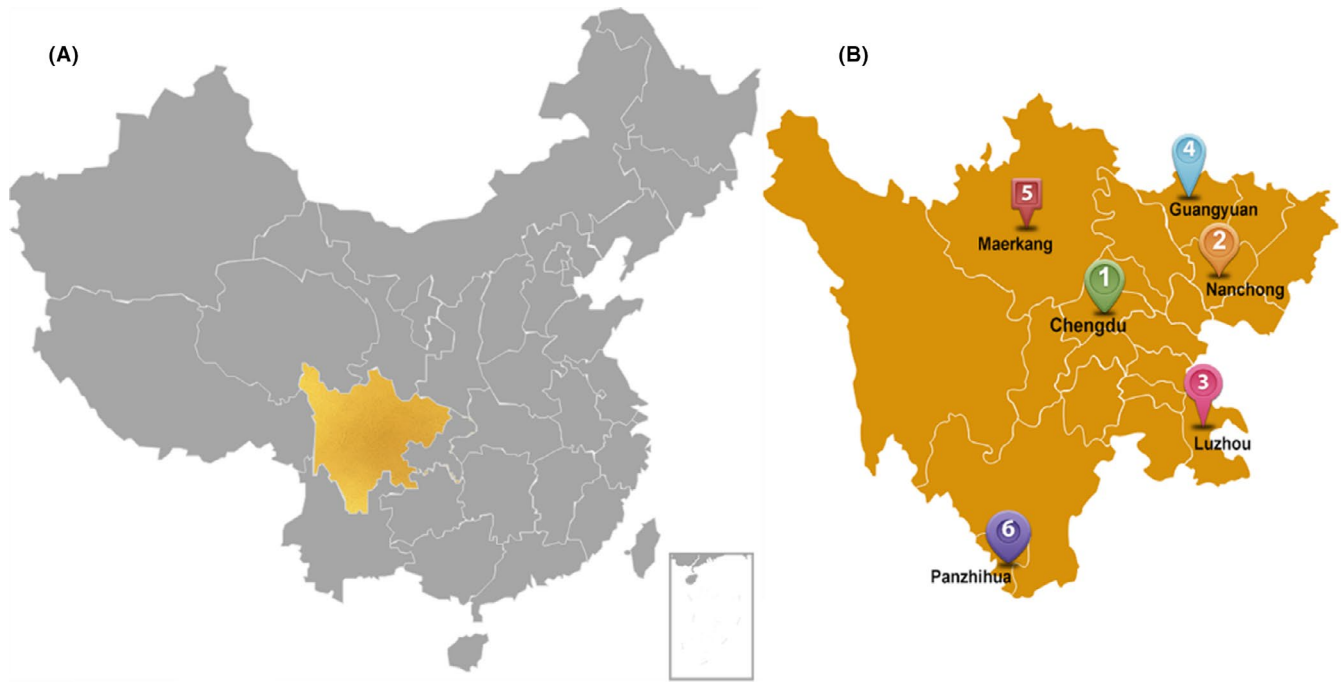


FIGURE 1 The present multicenter epidemiologic study conducted in south-west of China (A) and 6 areas around Sichuan province, including Chengdu (CD), Nanchong (NC), Luzhou (LZ), Guangyuan (GY), Maerkang (MK), and Panzihua (PH) (B)

gender, ethnicity, outdoor time, and “residence time”, that is, the period of time (prior to the study) the subject had lived in their current geographical location. Participants with age outside the 18-75 year window, levels of 25(OH) D_2 higher than 10 ng/mL and residence time less than 1 year, were excluded from data analysis. In addition, participants with previous medical history (hypertension, cardiovascular disease, diabetes, *etc*) and recent use of drugs (vitamin/calcium supplements, antiepileptic drugs, glucocorticoid, anti-tuberculosis drugs, antifungal drugs, *etc*) were also excluded from this study to eliminate the influences of disease and drugs on 25(OH) D levels. This study was reviewed and approved by the Ethics Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (2018 No. 137). All samples were de-identified before analysis.

2.2 | Laboratory analysis

Four milliliters of blood drawn in the fasting state from the antecubital vein into a 4-mL red top serum tube, followed by centrifugation at 3000 rpm at room temperature for 5 minutes. Serum samples were aliquoted into Eppendorf tubes. Samples collected from NC, LZ, MK, GY, and PH were transported to Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (Chengdu) at refrigeration temperatures after each collection period. All the samples were stored at -80°C until analysis. Serum 25(OH) D_2 and 25(OH) D_3 levels were measured by Shimadzu LC-20AD coupled with SCIEX API 3200MD LC-MS/MS system. Serum total P1NP and β -CTX were detected using the Roche Cobas e601 (Roche Diagnostics, Shanghai, China). The

intra-assay and inter-assay coefficients of variation values were 2.5 and 4.0% for P1NP, 2.7 and 5.5% for β -CTX with detection limits of 5 and 0.01 ng/mL, respectively.

2.3 | LC-MS/MS system

A Kinetex core-shell PFP column (100×2.1 mm, $2.6 \mu\text{m}$) from Phenomenex (Torrance, CA, USA) was used. The mobile phases were 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in methanol (B). The gradient elution profile was optimized for the separation of 3-epi-25(OH) D_3 from 25(OH) D_3 and was as follows: 0-7.8 minutes, 55%-80% (B); 7.8-9.0 minutes, 80% (B); 9.0-9.2 minutes, 80%-98% (B); 9.2-12.0 minutes, 98% (B); 12.0-12.2 minutes, 98%-55% (B); 12.2-14.0 minutes, 55% (B). The flow rate of mobile phase was 0.43 mL/min. The temperature of column oven was maintained at 40°C . The temperature of autosampler was maintained at 15°C . The sample injection volume was 20 μL . In these HPLC conditions, 25(OH) D_2 eluted at 10.74 minutes, 25(OH) D_3 eluted at 10.46 minutes, and 3-epi-25(OH) D_3 eluted at 10.66 minutes (Figure 2).

ESI-positive ionization mode was employed. The detailed MS parameters were as follows: curtain gas: 25 PSI; collision gas: 5 PSI; IonSpray voltage: 5500 V; source temperature 550°C ; ion source gas 1:55 PSI; ion source gas 2:55 PSI; interface heater: on. The parameters for monitoring m/z transitions of 25(OH) D_3 , 25(OH) D_2 , and 3-epi-25(OH) D_3 were shown in Table 1. The intra-assay and inter-assay coefficients of variation of the developed LC-MS/MS method were 3.99%-8.80% for 25(OH) D_2 and 3.51%-7.93% for 25(OH) D_3 , with detection limits of 2.08 and 3.00 ng/mL, respectively.

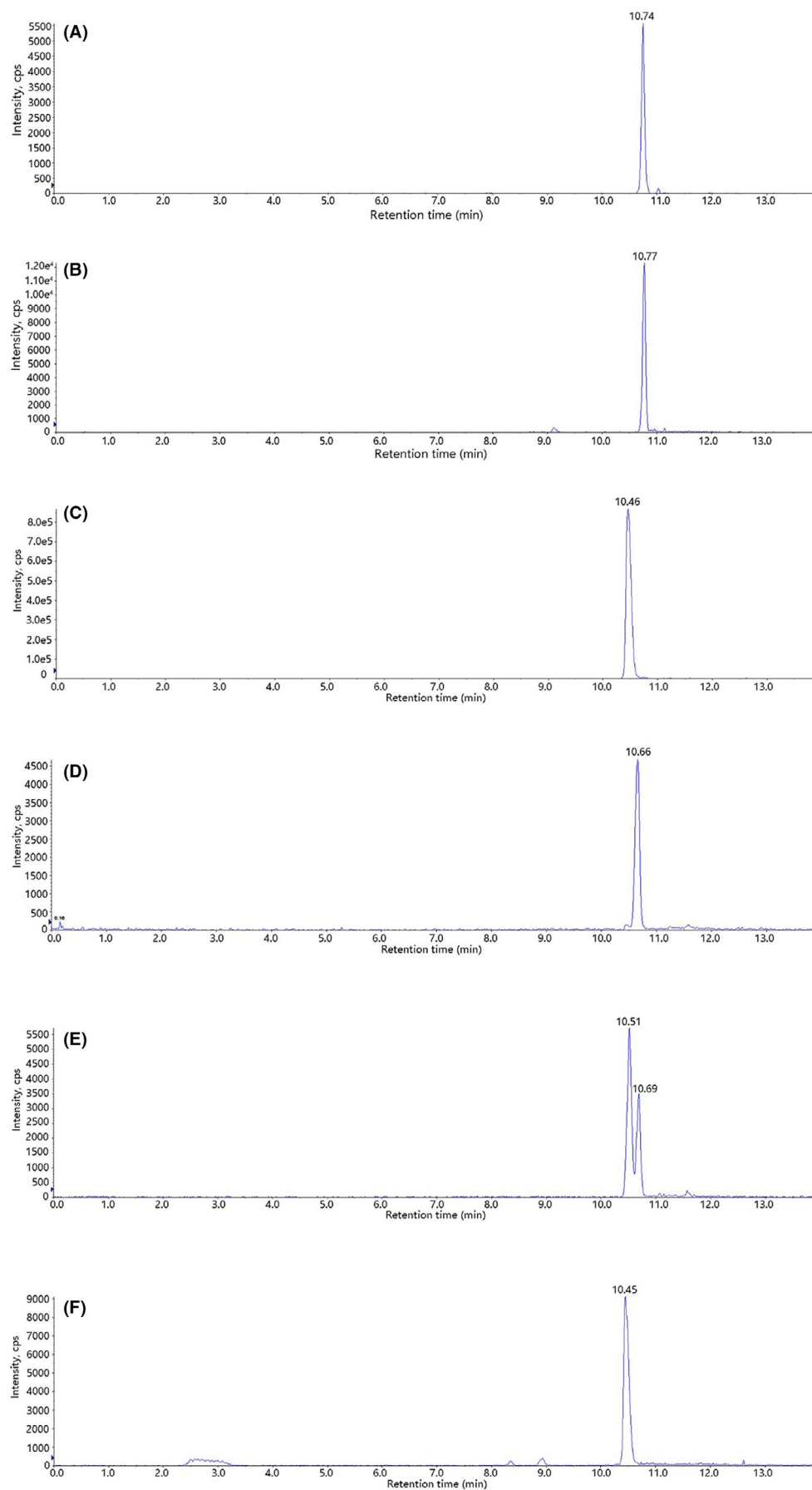


FIGURE 2 Typical MRM chromatograms of 25(OH)D₂ (A), 25(OH)D₂-d₆ (B), 25(OH)D₃ (C), 3-*epi*-25(OH)D₃ (D), mixture of 25(OH)D₃ and 3-*epi*-25(OH)D₃ (E) and 25(OH)D₃-d₃ (F) analyzed by the developed LC-MS/MS method

2.4 | Definitions of vitamin D status

Using thresholds recommended by the Endocrine Society,⁸ vitamin D deficiency was defined as 25(OH)D < 20 ng/mL and vitamin D insufficiency as 20 ≤ 25(OH)D < 30 ng/mL.

2.5 | Statistical analysis

Statistical analyses were performed using SPSS 20.0 (SPSS Inc) and GraphPad Prism[®] 7 (GraphPad Software Inc). Continuous variables were reported as median (5%-95%). Kruskal-Wallis H test for multiple independent samples and Mann-Whitney U test for two independent samples were used to evaluate the differences in 25(OH)D levels among different age groups, genders, seasons, districts, ethnicities, etc. Differences were considered statistically significant at $P < .05$.

3 | RESULTS

3.1 | Characteristics of the study participants

After exclusions, 2271 participants of 6 sample collection sites were enrolled and included in the analysis (Table 2). Of these, 301 (13.3%) were Tibetans from MK, the rest were Han Chinese. The male:female ratio was roughly 1:1 (1135/1136). The age groups of 18-30, 31-40, 41-50, 51-60, and 61-75 years accounted for 19.8%, 18.7%, 24.7%, 22.5%, and 14.3%, respectively. For CD district, serum samples were collected in 4 seasons with each season roughly 200 samples, while for the other 5 districts, serum samples were only collected in winter and summer with each season roughly 150 samples. In total, 810 (35.7%), 294 (12.9%), 292 (12.9%), 301 (13.3%), 270 (11.9%), and 304 (13.4%) were collected from CD, NC, LZ, MK, GY, and PH, respectively (Table 2).

The median 25(OH)D level for all participants was 23.41 ng/mL (range: 2.06 ng/mL-88.69 ng/mL). Of all samples, 35.5% and 38.6% were found to be vitamin D deficient and insufficient, respectively. Only 25.9% participants had sufficient vitamin D. The median male 25(OH)D was 25.42 ng/mL (95% CI 24.78-26.07 ng/mL), and the median female 25(OH)D was 21.39 ng/mL (95% CI 20.64-21.98 ng/mL). Deficiency and sufficiency rates for females were 44.8% and 18.5%, respectively. Additionally, Tibetan population showed a higher vitamin D deficiency rate and a lower sufficiency rate compared with the Han population. Further analysis is shown below.

TABLE 1 MRM parameters for monitoring 25(OH)D₂ and 25(OH)D₃

Analyte	Q1 mass (Da)	Q3 mass (Da)	Dwell time (msec)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision Cell Exit Potential (V)
25(OH)D ₂	413.3	337.3	50.0	36.0	10.0	23.0	3.0
25(OH)D ₃	401.3	365.3	50.0	38.0	6.0	22.0	5.0
25(OH)D ₂ -d ₆	419.4	159.2	50.0	35.0	5.0	36.0	2.0
25(OH)D ₃ -d ₃	404.3	386.4	50.0	50.0	5.0	17.0	3.5

3.2 | Correlation of 25(OH)D level with age and gender

Participants were divided into 5 age groups: 18-30, 31-40, 41-50, 51-60, and 61-75 years. Unexpectedly, the 18-30 y group showed significant lower serum 25(OH)D levels than the other 4 age groups ($P < .0001$, Kruskal-Wallis H test). The median 25(OH)D level of 18-30 years group was 20.44 ng/mL. The 25(OH)D levels of 41-50 years group, 51-60 years group, and 61-75 years group showed no significant difference with median values of 24.11, 24.90, and 24.09 ng/mL, respectively (Figure 3A). These results were consistent with a previous meta-analysis report showing no evidence of an age effect on vitamin D levels.²² Furthermore, the 25(OH)D levels between male and female were compared. It was determined that serum 25(OH)D level for males was statistically higher of than that of females for all 6 districts, age groups, and ethnicities ($P < .0001$, Mann-Whitney U test) (Figure 3B).

TABLE 2 Participant characteristics

Variable	Categories	No. (%)
Gender	Male	1135 (50)
	Female	1136 (50)
Age group	18-30	449 (19.8)
	31-40	424 (18.7)
	41-50	562 (24.7)
	51-60	511 (22.5)
	61-75	325 (14.3)
Ethnicity	Han	1970 (86.7)
	Tibetan	301 (13.3)
Season	Winter	935 (41.1)
	Spring	217 (9.6)
	Summer	933 (41.1)
	Autumn	186 (8.2)
Location	CD	810 (35.7)
	NC	294 (12.9)
	LZ	292 (12.9)
	MK	301 (13.3)
	GY	270 (11.9)
	PH	304 (13.4)

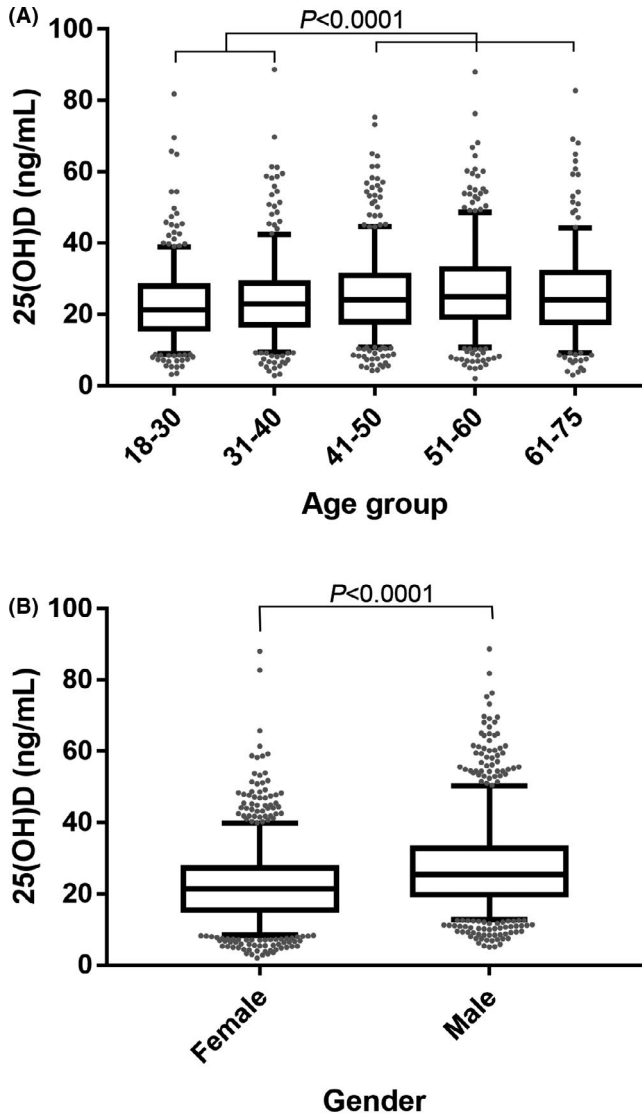


FIGURE 3 Serum 25(OH)D levels among age groups (A) and between genders (B), $P < .0001$ by independent sample Kruskal-Wallis H test for comparison of difference among different age groups; Mann-Whitney U test for comparison of difference between male and female

3.3 | Correlation of 25(OH)D level with season, districts, and ethnicity

Serum 25(OH)D levels among four seasons of CD were investigated using the 810 adult samples. The results showed that the serum 25(OH)D level of summer and autumn samples was significantly higher than those of winter and spring ($P < .0001$). The median 25(OH)D levels from summer and autumn samples were 24.29 ng/mL and 24.65 ng/mL, while winter and spring samples showed lower 25(OH)D levels of 19.42 and 20.61 ng/mL, respectively (Figure 4). Although the median 25(OH)D level of summer was slightly lower than that of autumn ($P = .85$, Mann-Whitney U test), the 25(OH)D sufficiency rate (≥ 30 ng/mL) of summer (26.42%) was higher than that of autumn (21.51%). In addition, the sufficiency rates of spring (7.83%) and winter (5.64%) were much lower than those of summer and autumn. This proved that 25(OH)D level was positively correlated with sunlight exposure time.

Furthermore, summer and winter samples from all the 6 investigated districts were used for the correlation analysis between 25(OH)D level and district. The average surface meteorology and solar energy (SSE) data from atmospheric science data center of NASA was used to represent the average sunlight of each district. The average SSEs of CD, NC, LZ, GY, MK, and PH were 4.19, 4.24, 4.02, 4.51, 5.21, 5.19 kWh/m²/day in summer, and 2.20, 1.90, 1.97, 2.52, 3.63, 4.28 kWh/m²/day in winter (Table 3). PH district which located in the south of Sichuan province has the longest sunlight exposure time among the 6 investigated districts. MK district had the second longest sunlight exposure time which nearly matches the PH district, but the participants from MK were all Tibetans. GY, NC, LZ, and CD districts are located in the north, east, and central part of Sichuan province (Figure 1) and have lower sunlight levels. The results proved that in Han population, serum 25(OH)D levels were positively correlated with sunlight exposure time of different districts (Figure 5). PH district showed the highest 25(OH)D level with median value 38.80 ng/mL for summer samples and 25.12 ng/mL for winter samples, followed by GY (30.96 ng/mL for summer samples and 16.39 ng/mL for winter samples) and NC (29.58 ng/mL for summer samples and 23.12 ng/mL for winter samples). The median

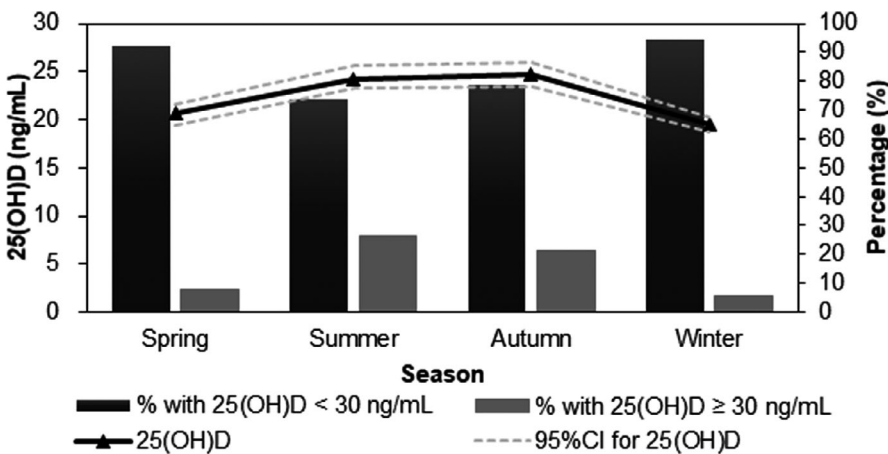


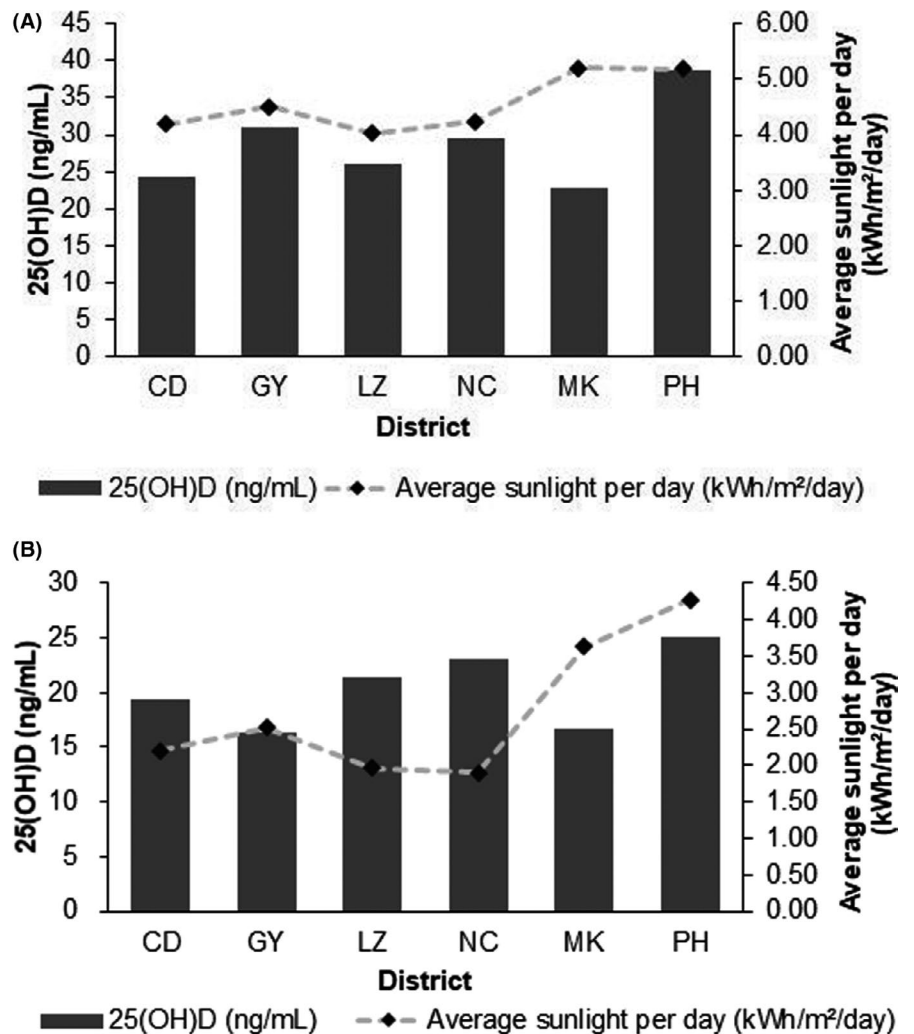
FIGURE 4 25(OH)D levels of CD district across 4 seasons. Black full curve: 25(OH)D levels; gray dash curve: 95% confidence interval for 25(OH)D; black column: percentage with 25(OH)D < 30 ng/mL; gray column: percentage with 25(OH)D > 30 ng/mL

TABLE 3 The average sunlight per day and median 25(OH)D levels in summer and winter of the 6 investigated districts

District	Summer		Winter	
	Average sunlight per day (kWh/m ² /day) ^a	Median 25(OH)D level (ng/mL)	Average sunlight per day (kWh/m ² /day) ^a	Median 25(OH)D level (ng/mL)
CD	4.19	24.29	2.20	19.42
GY	4.51	30.96	2.52	16.39
LZ	4.02	26.02	1.97	21.44
NC	4.24	29.58	1.90	23.12
MK	5.21	22.89	3.63	16.61
PH	5.19	38.80	4.28	25.12

^aAverage surface meteorology and solar energy (SSE) data from atmospheric science data center of NASA

FIGURE 5 Correlation of 25(OH)D levels and district with corresponding average sunlight data in summer and winter. Orange bars: Summer median 25(OH)D level (ng/mL) of the 6 investigated districts; yellow bars: winter median 25(OH)D level (ng/mL) of the 6 investigated districts; blue curve: summer average sunlight per day (kWh/m²/day) of the 6 investigated districts; gray curve: winter average sunlight per day (kWh/m²/day) of the 6 investigated districts. Average surface meteorology and solar energy (SSE) data from atmospheric science data center of NASA were used to represent average sunlight per day



25(OH)D levels of CD and LZ districts were lower than GY and NC, especially in summer. This may be explained by the low sunlight times of these two districts. Finally, it was interesting to note that although MK district had almost the same sunlight exposure time as PH district, the median 25(OH)D level of participants (Tibetan) from MK district was the lowest (22.89 ng/mL for summer samples and 16.61 ng/mL for winter samples, Figure 5). This indicates that 25(OH)D may differ between the Tibetan and Han ethnicities.

3.4 | Comparison of serum BTMs between Hans and Tibetans

To further clarify whether the significant difference of 25(OH)D levels between Hans and Tibetans correlate with their different BTMs, serum total P1NP and β -CTX were compared between Hans and Tibetans. After adjustment of age and gender, Tibetan population

showed significant higher serum total P1NP and β -CTX levels than Han population ($P < .0001$, Mann-Whitney U test). The median values of serum total P1NP and β -CTX for Tibetans were 58.85 ng/mL and 530.25 ng/mL, respectively. The median values of serum total P1NP and β -CTX for Hans were 44.28 ng/mL and 419.10 ng/mL, respectively (Figure 6). The median 25(OH)D values of Tibetans and Hans 14.26 ng/mL and 22.81 ng/mL, respectively. Pearson correlation analysis showed that 25(OH)D was negatively correlated with serum total P1NP and β -CTX between Tibetans and Hans.

4 | DISCUSSION

To the best of our knowledge, this is the first multicenter epidemiologic study regarding the vitamin D status in healthy adult population of Sichuan province. The results showed that vitamin D deficiency/

insufficiency was very common in the adult population of Sichuan province. Of the total samples, 35.5% and 38.6% were found to be vitamin D deficient and insufficient, respectively. Only 25.9% participants were with sufficient vitamin D. The sufficiency rate is higher than the finding of a previous report of 23,695 patients from Beijing, China, showing only 15.5% sufficiency.¹⁶ However, considering the participants used in that study were all specifically investigated for 25(OH)D testing on clinical grounds, there is likely selection bias. In addition, participants with age between 18 and 40 years old showed significantly lower 25(OH)D levels than participants with age between 41 and 75 years old. This result was at least partially concordant with a previous meta-analysis, in which no age effect on vitamin D level was found.²² Less outdoor time for younger generation was possibly one of the reasons to cause their low 25(OH)D level. At the same time, male participants showed significant higher 25(OH)D level than female participants in all categories, consistent with NHANES findings.²³

Data of different districts and seasons proved that 25(OH)D level was positively correlated with sunlight exposure level, which is also consistent with previous reported data.¹⁰ However, even under conditions of high sunlight exposure, 25(OH)D level of the Tibetan population from MK district was still the lowest among the 6 investigated districts, indicating 25(OH)D level could be affected by ethnicity. Many of studies have been performed to investigate the 25(OH)D level between different ethnicities, especially among White, Hispanic, and Black population.^{18,24} Little is known about the differences of 25(OH)D status among different Chinese ethnicities.

In order to further clarify whether the differences of 25(OH)D levels between Chinese Hans and Tibetans correlated with BTMs, serum β -CTX and total P1NP levels between Tibetans and Hans were compared. The results suggest that Tibetans have significantly higher serum β -CTX and total P1NP levels than Hans. It was previously observed that serum total P1NP and β -CTX concentrations had a negative linear correlation with BMD at the lumbar spines and the femoral neck.²⁵ However, no study has been carried out on the correlations of serum total P1NP and β -CTX concentrations and the rates of osteoporosis, fracture risk, and any other extra-skeletal diseases between Chinese Tibetans and Hans. Future study is required to elucidate this issue.

Taken together, it is necessary to promote vitamin D supplementation and healthy lifestyles (*ie*, appropriate outdoor activity and sun exposure) in order to safely improve vitamin D level in Sichuan population, especially for Tibetan, female and in the seasons of spring and winter.

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CONFLICT OF INTERESTS

The funding organizations played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication. The authors declare no competing interests.

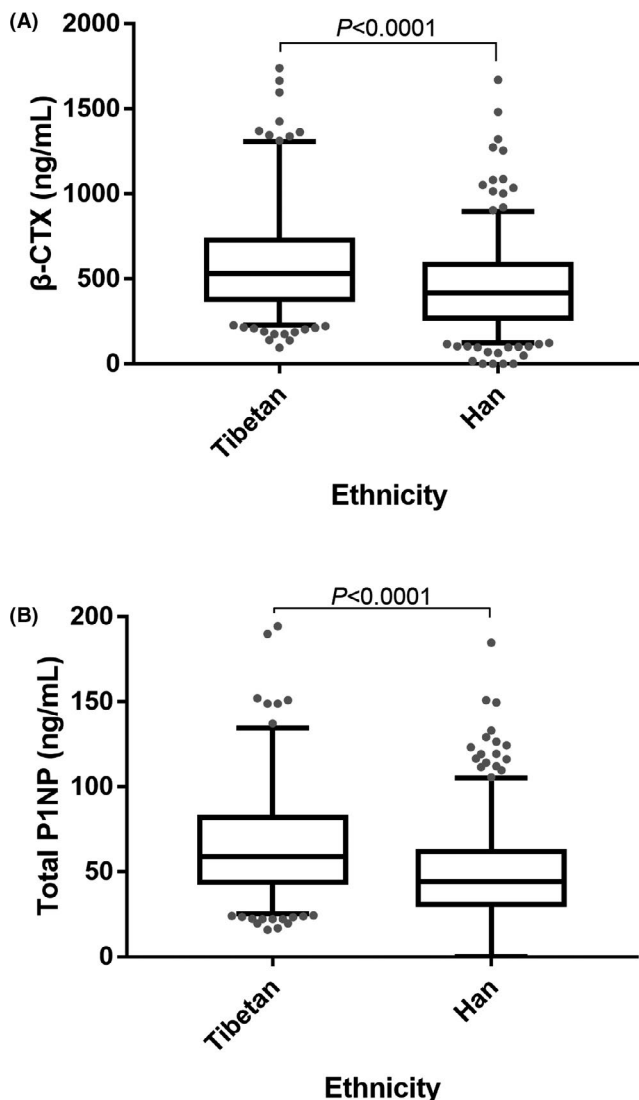


FIGURE 6 Comparison of serum β -CTX (A) and total P1NP (B) between Tibetans and Hans. $P < .0001$ for both β -CTX and total P1NP between Tibetan and Han population by Mann-Whitney U test

ETHICAL APPROVAL

This study was approved by the Ethics Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (2018No.137).

EMPLOYMENT OR LEADERSHIP

None declared.

HONORARIUM

None declared.

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