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The importance of analyzing the serum C3-epimer level for evaluating vitamin D storage in some special populations

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Abstract. – OBJECTIVE: The serum 25-hydroxyvitamin D [25(OH)D] is recommended by various management agencies for evaluating the nutritional status of vitamin D (VitD). However, 25(OH)D cannot reflect the actual composition and activity of VitD *in vivo*. This study used UPLC-MS/MS to detect the levels of serum VitD metabolites in some special populations, so as to clarify its importance in accurately evaluating VitD storage *in vivo*.

SUBJECTS AND METHODS: A total of 2029 subjects were enrolled, including 1204 cases in minor health (MH), 467 in the minor disease (MD), 119 in the adult health (AH) and 239 in adult disease (AD). Serum VitD2 and VitD3 levels were measured by UPLC-MS/MS. Serum C3-epi concentrations were also measured in 144 subjects by a spot check method.

RESULTS: There were significant differences in the levels of VitD2, VitD3 and 25(OH)D among groups (all p <0.001). According to serum level of 25(OH)D, percentage of subjects with sufficient VitD in the MH, MD, AH and AD group were 65.4%, 52.7%, 29.4% and 20.9%, respectively. After converting VitD2 activity to AVitD3, subjects with sufficient VitD in MH, MD, AH and AD group accounted for 53.2%, 40.9%, 17.7% and 11.3%, respectively. C3-epi levels in the MH (z = 7.49, p <0.001), MD (z = 7.03, p <0.001) and AD group (z = 4.68, p <0.001) were higher than that in the AH group.

CONCLUSIONS: Not only the serum 25(OH)D level, but also the simultaneous detection of VitD2 and VitD3 levels will overestimate the VitD storage in some subjects. Accurate evaluation of VitD storage in these individuals also requires detection of C3-epi levels.

Key Words:
Vitamin D metabolites, UPLC-MS/MS, 3-epimer-25(OH)D3, Nutritional status.

Abbreviations
25(OH)D = 25-hydroxyvitamin D; VitD = vitamin D; MH = minor health; MD = minor disease; AH = adult health; AD = adult disease; UPLC-MS/MS = Ultra-high Performance Liquid Chromatography-tandem Mass Spectrometry.

Introduction

Vitamin D (VitD) is a kind of fat-soluble sterol derivative. It has 50 kinds of metabolites in human blood circulation but its main effective analog is 25-hydroxy vitamin D [25(OH)D]. The 25(OH)D, which mainly consists of 25(OH)D2 (VitD2) and 25(OH)D3 (VitD3), is the main storage form of VitD in the human body, accounting for more than 95% of the total amount of VitD. The 25(OH)D is considered as the optimal indicator to evaluate the nutritional status of VitD because it has long half-life (2 to 3 weeks) and its level is unaffected by blood calcium and parathyroid hormone levels[1]. VitD itself has no biological activity, which can be hydroxylated to 25(OH)D in liver and to 1, $25(OH)_2D$ in kidney, thereby exerting physiological function[2]. The main physiological function of VitD is to maintain the

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stability of blood calcium and phosphorus, and to promote osteogenesis. In addition, serum level of VitD is significantly associated with the occurrence and development of many common clinical diseases, such as diabetes[3,4], chronic urticaria[5], respiratory infections in children[6], rheumatic immune diseases[7] and tumors[8].

Although VitD2 and VitD3 are the main forms of 25(OH)D, the sources of the VitD2 and VitD3 are not the same. The former mainly comes from plants, while the latter comes mainly from animals. In setting of normal physiological conditions, the content and activity of VitD3 are significantly higher than VitD2. Therefore, both clinical and laboratory experts thought that the VitD detection method should simultaneously detect VitD2 and VitD3, thus ensuring that VitD supplements derived from plants and animals can be both detected[9]. Then, storage level of VitD can be calculated based on the content of VitD2 and VitD3, thereby accurately evaluating the nutritional status of VitD.

Recently, it has been found[10,11] that the C3-epimer (C3-epi) of VitD may play an important role in clinical research and VitD nutritional evaluation. It is well known that all major VitD metabolites can undergo epimerization at the C3 position to form C3-epi[12], which is the most common phenomenon in infants[11]. C3-epi has the same molecular weight and structural formula as VitD, but the spatial conformation is different, so it has no biological activity. The C3-epis of VitD2, VitD3 and 1,25-$(OH)_2$D3, C3-epi-VitD2, C3-epi-VitD3 and C3-epi-1,25-$(OH)_2$D3 have been reported[10-13]. It can be observed that although the exact mechanism of transformation in C3-epi is not clear. It is a frequent form of VitD metabolites *in vivo*. Because of its existence, it may affect the utilization and state evaluation of VitD in individuals. However, it is almost ignored by clinicians.

So far, immunological methods are the primary methods for the detection of serum VitD, such as radioimmunoassay, immunoluminescence and enzyme-linked immunosorbent assay[14]. This method can only detect the total 25 (OH)D level, and can not detect the VitD2 and VitD3 levels alone. With the progress of medical technology, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been regarded as the "gold standard" for detection of VitD2 and VitD3[15]. Therefore, we used an ultra-high performance LC-MS/MS (UPLC-MS/MS) system to simultaneously detect the levels of serum VitD2, VitD3 and C3-epi, so as to provide reliable evidence for the evaluation and treatment of VitD-related diseases.

Subjects and Methods

Subjects

A total of 2,029 subjects visiting Mianyang Central Hospital between June and August, 2019 were enrolled, including 1164 males and 865 females. All enrolled subjects received detection of serum VitD level. These subjects were divided into two groups. That is the minority group and the adult group. All minors with age less than 18 years were further divided into minor health (MH) group (n=1204) and minor disease (MD) group (n=467). Cases in MD group had dwarfism (134 cases), respiratory infections (215 cases), malnutrition (57 cases), and tic disorders (61 cases). Similarly, all adult subjects were also grouped into adult health (AH) group (n=119) and adult disease (AD) group (n=239). The patients in AD group consisted of cases with immune disorders (58 cases), osteoarthritis (49 cases), fractures (80 cases), and inguinal hernias (52 cases). Informed consent was obtained from the patients or their family. This study obtained ethical approval by the Ethics Committee of Mianyang Central Hospital, School of Medicine, University of Electronic Science and Technology of China (protocol code 201400048).

UPLC-MS/MS

Approximately 5 mL of venous blood was collected from each subject following fasting overnight. Serum was isolated after centrifugation at 4000 rpm for 10 min. The Jasper™ UPLC liquid chromatograph (Shimadzu, Canby, OR, USA) and the AB SCIEX Triple Quad™ 4500MD mass spectrometer (ABI, Estate, Singapore) was used to detect the VitD metabolites. Briefly, 200 µl serum sample were sequentially mixed with 10 µl internal standard solution and 1.0 ml tert-butyl methyl ether. After centrifugation at 13000 r/min for 5 min, the precipitate was collected and added with 100 µl of 65% methanol solution containing 0.1% formic acid for re-dissolution. Then, the samples were measured on UPLC-MS/MS.

Chromatographic Conditions

A C18 column (Phenomenex, CA, USA) was used for sample separation, with a column temperature of 40°C and a flow rate of 0.6 mL/min.

Mobile phase A was an aqueous solution containing 0.1% formic acid, and mobile phase B was a methanol solution containing 0.1% formic acid.

Mass Spectrometry Conditions

APCI ion source was used for mass spectrometry analysis, with a dwell time of 40 msec, and scanning mode of positive ion multiple reaction monitoring. VitD2, d6-25(OH)D2, VitD3, and d6-25(OH) D3 ion pairs (parent ion/quantitative product ion) were 395.3/269.2 m/z, 419.3/229.3 m/z, 383.3/365.3 m/z, and 389.3/363.3 m/z, repsectively. The decluster voltages for monitoring VitD2, d6-25(OH)D2, VitD3, and d6-25(OH)D3 were 110 V, 85 V, 130 V, and 130 V, respectively, with collision energies of 24 eV, 21 eV, 16 eV, and 19 eV, respectively. Data were collected using Analyst® MD software (ABI, Los Angeles, CA, USA, version number: 1.6.3) and Multiquant™ MD software (ABI, USA, version number: 3.0.2).

Total of 144 samples was randomly selected, including 87 minors (24 cases in MH group, and 63 cases in MD group) and 57 adults (32 cases in AH group, and 25 in AD group). Isotope dilution UPLC-MS/MS was used to measure serum level of 3-epimer-25(OH)D3, which is the C3-epimer (C3-epi).

Statistical Analysis

Statistical analysis was performed using SPSS 25.0 (IBM, Armonk, NY, USA) software package. The levels of VitD metabolites were not normally distributions and were thus expressed as median (interquartile range) [M($p25$, $p75$)]. The Kruskal-Wallis H rank sum test was used to conduct multiple independent sample comparisons, with Bonferroni method (i.e., the adjusted alpha level method) for inter-group comparisons. p <0.05 was considered as statistically significant.

Results

Serum VitD2, VitD3, 25(OH)D and Active VitD3(AVitD3) Levels

Serum levels of VitD2, VitD3, 25(OH)D and active VitD3 (AVitD3) of all subjects are shown in Table I. Serum VitD2 (H = 23.20, p <0.001), VitD3 (H = 194.79, p <0.001), and 25(OH)D (H = 278.30, p <0.001) levels were significantly different among groups. Serum VitD2 levels (z = 4.61, p <0.001; z = 3.20, p = 0.008; z = 3.17, p = 0.009), VitD3 levels (z = 5.45, p <0.001; z = 13.42, p <0.001; z = 3.50, p = 0.003), 25(OH)D levels (z = 8.03, z = 15.37, z = 6.00, both p <0.001) and AVitD3 levels (z = 6.67, p <0.001 Z = 14.73, p <0.001; z = 4.43, p = 0.003) in AD, AH and MD groups were significantly lower than those of MH group. Serum VitD3 levels (z = 3.80, p = 0.001), 25(OH)D levels (z = 2.82, p = 0.029) and AVitD3 levels (z = 3.59, p = 0.002) in AD group were significantly lower than those in the AH group. Serum VitD3 levels (z = 3.24, p = 0.007) and 25(OH)D levels (z = 4.34, p <0.001) and AVitD3 levels (z = 3.89, p = 0.001) in MD group were significantly higher than those in the AH group. Serum VitD3 levels (z = 9.55, p <0.001) and 25(OH) D levels (z = 9.57, p < 0.001) and AVitD3 levels (z = 10.08, p = 0.001) in AD group were significantly lower than those in the MD group.

Serum VitD2 and VitD3 Distribution According to Subgroups

Serum VitD2 and VitD3 distribution according to subgroups were shown in Table I. In the minor group, cases with tic disorder had a highest VitD2 levels, then sequentially MH group, respiratory infection group, dwarf group and malnutrition group (H=20.72; all p <0.01). Serum VitD2 levels in the malnutrition group were significantly lower than those in MH group (z = 3.78, p = 0.002). The tic disorder group had a serum VitD2 levels significantly higher than in the malnutrition group (z = 2.83, p = 0.047). In the adult group, the serum levels of VitD2 were in descending order of immunological disease group, osteoarthritis group, inguinal hernia group, and fracture group, and AH group (H = 12.58, p = 0.014). The serum VitD2 level in the immune disorders group was significantly higher than that in AH group (z = 3.44, p = 0.006).

In the minor group, the serum levels of VitD3 were in descending order from the MH group, respiratory infection group, malnutrition group, dwarf group, and tic disorder group (H = 29.17, p <0.001). Serum VitD3 levels in the dwarf group (z = 4.10, p <0.001) and tic disorder group (z = 3.52, p = 0.004) were significantly lower than those in MH group. Serum VitD3 levels in dwarf group (z = 3.14, p = 0.017) and tics disorder group (z = 2.99, p = 0.028) was significantly lower than the respiratory infection group. In the adult group, the levels of VitD3 in descending order were the AH group, the osteoarthritis group, the inguinal hernia group, the fracture group, and the immune disease group (H = 34.06, p <0.00). The serum VitD3 level in the immune disease

Table I. Serum VitD distribution according to subgroups (unit: ng/mL).

Subjects	Cases/Males	$VitD_2$	$VitD_3$	25(OH)D	$AVitD_3$	$VitD_2/VitD_3$
Minor						
MH	1204/662	1.91 (0.80, 8.09)	29.48 (22.31, 37.19)	34.29 (27.13, 42.46)	30.91 (24.02, 38.92)	0.07 (0.03, 0.26)
MD	467/297	1.54 (0.73, 4.48)[a]	26.78 (21.88, 26.78)[a,b]	30.50 (24.88, 37.79)[a,b]	27.91 (23.34, 33.93)[a,b]	0.06 (0.03, 0.15)
Dwarf	134/83	1.44 (0.70, 4.17)	25.64 (21.21, 30.43)*,⋆	28.14 (24.25, 33.76)*,⋆	26.47 (22.51, 31.36)*,⋆	0.05 (0.03, 0.15)
Respiratory inf.	215/130	1.61 (0.82, 5.71)	28.48 (23.09, 36.30)	33.21 (25.93, 40.23)	30.19 (24.08, 37.53)	0.06 (0.03, 0.19)
Malnutrition	57/32	0.99 (0.58, 2.27)*,#	25.89 (21.44, 33.07)	27.51 (22.95, 37.34)*	26.07 (22.36, 34.58)	0.04 (0.02, 0.10)*
Tic disorder	61/52	1.96 (0.93, 4.75)	24.73 (21.15, 30.42)*,⋆	30.04 (25.14, 34.74)*	26.56 (22.91, 31.94)*	0.09 (0.04, 0.19)
H, p	—	20.72, < 0.001	29.17, < 0.001	58.95, < 0.001	39.40, < 0.001	11.73, 0.020
Adults						
AH	119/92	1.12 (0.73, 2.06)[a]	25.13 (20.89, 27.95)[a]	26.88 (24.10, 30.78)[a]	25.61 (21.59, 28.68)[a]	0.05 (0.03, 0.09)[a]
AD	239/111	1.61 (0.76, 3.04)[a]	20.45 (15.58, 25.36)[a,b,c]	23.98 (18.47, 28.39)[a,b,c]	21.70 (16.86, 26.42)[a,b,c]	0.07 (0.04, 0.16)[b]
Immune disorder	58/18	2.11 (0.94, 6.79)*	19.51 (13.94, 23.17)*	24.00 (18.40, 28.06)*	21.49 (15.20, 24.13)*	0.10 (0.05, 0.39)*
Osteoarthritis	49/8	1.45 (0.76, 2.73)	22.85 (16.40, 27.48)	25.05 (20.50, 30.42)	23.02 (18.14, 28.26)	0.06 (0.03, 0.14)
Fracture	80/38	1.23 (0.68, 2.67)	20.06 (14.12, 25.22)*	23.29 (17.41, 27.58)*	20.88 (15.50, 25.80)*	0.07 (0.04, 0.14)
Inguinal hernia	52/47	1.44 (0.79, 2.37)	22.49 (16.39, 27.25)	25.27 (18.41, 30.02)	22.79 (17.80, 27.71)	0.06 (0.03, 0.12)
H, p	—	12.58, 0.014	34.06, < 0.001	26.79, < 0.001	34.11, < 0.001	20.76, < 0.001

Note: *p <0.05 compared with the minor/adult health group; ⋆p < 0.05 compared with the respiratory infection group; #p < 0.05 compared with the tic disorder group. a compared with the healthy group of minors, p <0.05; b compared with the healthy group of adults, p <0.05; c compared with the group of minor diseases, p <0.05. AH, adult health; MH, minor health; MD, minor disease; AD, adult disease; VitD, vitamin D; VitD2, 25(OH)D2; VitD3, 25(OH)D3; AVitD3, active VitD3; Respiratory inf., Respiratory infections.

group (z = 5.05, p <0.001) and fracture group was significantly lower than that in the AH group (z = 4.54, p <0.001).

Serum 25(OH)D and AVitD3 Distribution According to Subgroups

Serum 25(OH)D and AVitD3 distribution according to subgroups are shown in Table I. In the minor group, the level of 25(OH)D was in descending order of MH group, respiratory infection group, tic disorder group, dwarf group, and malnutrition group (H = 58.95, p <0.001). Serum 25(OH)D levels in the dwarf group (z = 6.37, p <0.001), tic disorder group (z = 3.27, p = 0.011) and malnutrition group (z = 3.73, p = 0.002) were significantly lower than those in the MH group. Serum 25(OH)D levels in the dwarf group were significantly lower than in the respiratory infection group (z = 4.10, p <0.001). In the adult group, the level of 25(OH)D was in descending order of AH group, the inguinal hernia group, the osteoarthritis group, the immune disease group, and the fracture group (H = 26.79, p <0.001). The serum 25(OH)D levels in the immune disease group (z = 3.17, p = 0.015) and the fracture group (z = 4.95, p <0.001) were significantly lower than those in the AH group.

The activity of VitD3 is 2 to 3 times as that of VitD2. Based on the highest ratio of 3 times, the activity of VitD2 was converted to that of VitD3. Then, AVitD3 was calculated by combing VitD3 and the converted VitD3 by VitD2(AVitD3 = VitD2/3 + VitD3). The results are shown in Table I. In the minor group, the levels of AVitD3 were in descending order of MH group, respiratory infection group, tic disorder group, dwarf group,

and malnutrition group (H = 39.40, p <0.001). Serum AVitD3 levels in the dwarf group (z = 5.03, p <0.001) and tic disorder group (z = 3.51, p = 0.004) were significantly lower than those in the MH group. Serum AVitD3 levels in the dwarf group were significantly lower than in the respiratory infection group (z = 3.62, p = 0.003). In the adult group, the levels of AVitD3 were in descending order of AH group, the osteoarthritis group, the inguinal hernia group, the immune disease group, and the fracture group (H = 34.11, p <0.001). The serum AVitD3 levels in the immune disease group (z = 4.71, p <0.001) and the fracture group were significantly lower than those in the AH group (z = 4.92, p <0.001).

Measurement of Serum C3-epi Level

Isotope dilution UPLC-MS/MS was used to measure serum C3-epi levels in 144 serum samples. By adjusting mass spectrometry conditions and changing columns, effective separation of C3-epi and VitD3 peaks was achieved (Figure 1). According to the independent sample Kruskal-Wallis test, the statistically significant differences in serum C3-epi level among the MH, MD, AH, and AD groups were observed (H = 68.96, p <0.001, Table II). Furthermore, C3-epi level in MH group (z = 7.49, p <0.001), MD group (z = 7.03, p <0.001) and AD group (z = 4.68, p <0.001) were higher than AH group. C3-epi level in AD group was lower than MH group (z = 2.70, p = 0.041). In Table II, we showed some calculation parameters related to C3-epi. However, as they were not actually measured, these data were not described in detail here.

VitD Nutritional Status Evaluation

According to the recommendation by the Institute of Medicine (IOM)[16], the nutritional status of VitD is evaluated using serum 25(OH)D (equivalent to the sum of VitD2 and VitD3 levels). Considering that children may consciously supplement VitD in summer, as showed in Figure 2, nearly 50% (41.8%-65.4%) of minor had adequate VitD. However, after converting VitD2 activity to AVitD3, only about half of the subjects in the MH group (53.2%) and respiratory infection group (51.2%) had adequate storage of VitD. More than two-thirds of the dwarf (30.6%) and tic disorder (31.2%) group had insufficient VitD. The percent of adults with sufficient 25(OH)D storage was less than 30.0%, with 29.4% in the AH group. Af-

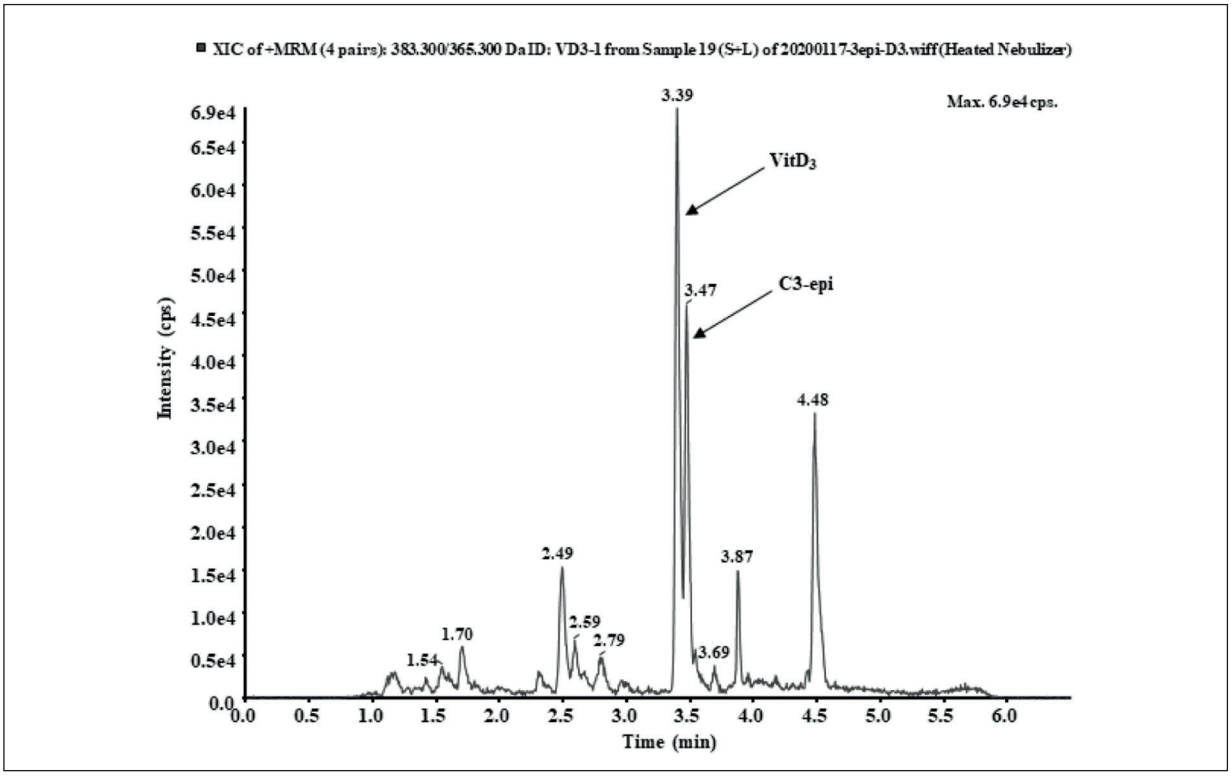

Figure 1. Representative UPLC-MS/MS spectra of serum C3-epi. The MS peak of C3-epi is closely related to $VitD_3$, and the elution time is very close. $VitD_3$ and C3-epi elute at 3.39 min and 3.47 min, respectively.

Table II. Detection range [R(Min, Max)] and estimated interval [M(p25, p75)] of Serum VitD (unit: ng/mL).

	$VitD_2$	$VitD_3$	25(OH)D	C3-epi	$C3/VitD_3$	C3/25(OH)D	$VitD_2/D_3$	Year
MD (n = 63)								
R (Min, Max)	40.64 (0.06, 40.70)	47.14 (5.70, 52.84)	61.48 (7.92, 69.40)	14.59 (0.18, 14.77)	0.71 (0.01, 0.71)	0.33 (0.01, 0.33)	3.74 (0.00, 3.74)	15.90 (0.10, 16.00)
M (p25, p75)	1.17 (0.48, 6.10)	25.20* (16.23, 30.90)	29.14* (22.17, 37.81)	1.36* (0.75, 2.76)	0.07* (0.03, 0.13)	0.06* (0.03, 0.09)	0.06 (0.02, 0.32)	4.00 (0.90, 8.00)
MH (n = 24)								
R (Min, Max)	16.52 (0.25, 16.77)	27.57 (10.07, 37.64)	23.88 (20.08, 43.96)	10.71 (0.18, 10.89)	0.32 (0.01, 0.33)	0.32 (0.01, 0.33)	1.05 (0.01, 1.06)	12.20 (0.20, 12.40)
M (p25, p75)	1.43 (0.64, 8.81)	27.8* (20.64, 32.91)	31.80* (24.83, 36.08)	2.50* (1.38, 4.04)	0.10* (0.06, 0.15)	0.09* (0.06, 0.11)	0.05 (0.02, 0.46)	0.90 (0.45, 6.98)
AD (n = 25)								
R (Min, Max)	17.56 (0.14, 17.70)	27.41 (6.39, 33.8)	23.56 (14.80, 38.36)	1.82 (0.29, 2.11)	0.04 (0.03, 0.07)	0.06 (0.01, 0.07)	2.79 (0.00, 2.80)	46.00 (42.00, 88.00)
M (p25, p75)	1.75 (1.12, 6.60)	23.60* (17.50, 27.55)	26.86* (23.29, 30.86)	1.22*,⋆ (0.75, 1.46)	0.05* (0.04, 0.06)	0.04*,⋆ (0.03, 0.05)	0.08 (0.04, 0.30)	52.00 (45.50, 63.00)
AH (n = 32)								
R (Min, Max)	1.97 (0.39, 2.36)	13.42 (8.59, 22.01)	14.56 (9.29, 23.85)	0.79 (0.02, 0.81)	0.04 (0.00, 0.04)	0.03 (0.00, 0.03)	0.17 (0.03,0.20)	34.00 (24.00, 58.00)
M (p25, p75)	0.99 (0.65, 1.27)	11.23 (10.39, 16.60)	12.37 (11.30, 17.85)	0.14 (0.04, 0.40)	0.01 (0.00, 0.03)	0.01 (0.00, 0.03)	0.08 (0.05, 0.10)	47.00 (33.00, 55.75)
H, *p*	7.22, 0.065	35.70, < 0.001	57.13, < 0.001	68.96, < 0.001	62.75, < 0.001	61.14, < 0.001	1.48, 0.686	104.42, < 0.001

M: Median, R: Range, Min: Minimum, Max: Maximum, *p*: Percentiles. * Compared with adult healthy group, $p < 0.05$; ⋆Compared with minor healthy group, $p < 0.05$. AH, adult health; MH, minor health; MD, minor disease; AD, adult disease; VitD, vitamin D; $VitD_2$, $25(OH)D_2$; $VitD_3$, $25(OH)D_3$; $AVitD_3$, active $VitD_3$; C3/C3-epi, C3-epimer.

ter converting the VitD2 activity to AVitD3, the percentage of adult subjects who had sufficient VitD storage was less than 20.0%. If the effect of C3-epi is considered, actual VitD storage would be much lower (Figure 2).

Discussion

VitD is necessary for growth, development and maintenance of human life, which can not only regulate intestinal absorption and maintain the body's balance, but also regulate calcium and phosphorus metabolism and participate in bone formation[17]. VitD deficiency can not only result in bone diseases, but it is also related to the occurrence and development of many diseases[18]. VitD deficiency has been considered as a global public health problem. The role of VitD in occurrence and development, efficacy monitoring and prognostic evaluation of various pathologies has attracted much attention[19-23]. However, most of the studies have focused on the relationship between disease and 25(OH)D levels. The correlation between VitD metabolites and disease is rarely reported.

In this study, UPLC-MS/MS was used to analyze the VitD metabolites of healthy subjects with different ages and patients with certain diseases. The proportion of VitD2 in 25(OH)D was relatively low. Although VitD2 changed in disease state, its effect on 25(OH)D level was not significant. However, the change between minors and adults seemed to be totally different. In minors, the VitD2 in the state of disease a decreasing trend, while in adults, it showed an increasing trend. VitD3 in the state of disease showed a decreasing trend in both minors and adjusts, indicating that VitD3 is the main factor affecting the storage of VitD in the state of disease. Since VitD3 is the main component of 25(OH)D, the change of VitD3 determines the change of 25(OH) D and AVitD3 levels *in vivo*. We found that I) among healthy subjects, the serum VitD2 level, serum VitD3 level, and 25(OH)D level in minors were higher than in adults. The underlying reason is speculated as follows: (1) adults may be indoors for a long time during the day due to occupational reasons, resulting in insufficient sunlight time and insufficient synthesis of VitD3[24]; (2) in summer, women deliberately avoid sunlight, resulting in insufficient synthesis of VitD3[25]; (3) children consciously supplement VitD, but adults (especially healthy adults) basically ignore VitD supplement[26]. II) Among the minor groups, the serum VitD2, VitD3 and 25(OH)D levels in the MD group were lower than those in the MH group. Similarly, in the adult group, serum VitD3 and 25(OH)D levels were lower in the AD group

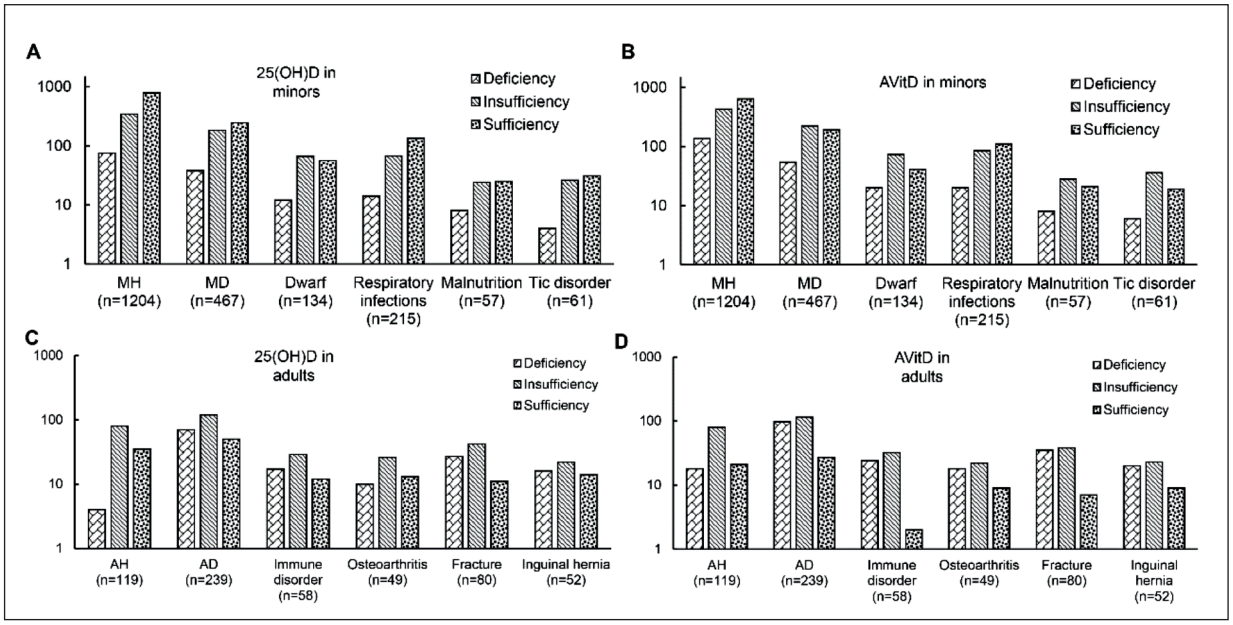

Figure 2. Nutritional status of VitD. According to the VitD evaluation criteria by IOM, the subjects' VitD nutritional status was plotted. **A,** VitD nutritional status in minors evaluated according to 25(OH)D. **B,** VitD nutritional status in minors evaluated according to AVitD3. **C,** VitD nutritional status in adults evaluated according to 25(OH)D. **D,** VitD nutritional status in adults evaluated according to $AVitD_3$. $AVitD_3 = VitD_{2/3} + VitD_3$.

than in the AH group. This indicates that the disease may lead to a decrease in VitD levels. On the other hand, lower levels of VitD may also cause disease occurrence. Although VitD has not been proven to be an antioxidant, VitD can regulate multiple cellular pathways that synthesize antioxidants, have anti-oxidation effects against reactive oxygen species and nitric oxide, and can prevent oxidative damage[27]. The median level of VitD2 in the AD group was higher than that in the AH group, but there was no statistical difference. However, the range and estimated interval of this group of patients were much higher than those of AH group. Review of the medical records revealed that VitD2 levels were extremely high in two patients with connective tissue disease. One case with Sjogren's syndrome had a VitD2 level of 17.7 ng/ml, and the other one with systemic lupus erythematosus had a VitD2 level of 13.2 ng/ml. It has been shown that serum VitD2 levels are related to the occurrence and development of Sjogren's syndrome and systemic lupus erythematosus[28]. However, whether patients with connective tissue disease have an increase in VitD2 levels in setting of decreasing of 25(OH)D[29] remains unclear. Our laboratory is currently working on this.

VitD2 and its metabolites in circulation have a lower ability to bind to VitD-binding protein than VitD3[2,30-32]. In addition, VitD2 has a non-physiological metabolic form and its half-life is shorter than VitD3. Thus, the biological efficacy of VitD2 is significantly lower than VitD3[2,30-32]. Moreover, the capacity of VitD3 to store VitD is 2 to 3 times higher than that of VitD2[2,30-32]. Therefore, in normal physiological conditions, the level of VitD3 is much higher than the level of VitD2, and the ratio of VitD2/VitD3 is extremely low. If the subject has excessively high levels of VitD2 and significantly decreased VitD3 level for some unknown reason, detecting serum 25(OH)D level only may lead to the false result of adequate VitD storage[33]. In this study, we converted VitD2 to VitD3 activity and then assessed the nutritional status of VitD *in vivo*. We found that the median level of VitD was significantly decreased in all enrolled subjects. Therefore, detecting the metabolites of serum VitD is the appropriate approach for VitD nutrition level evaluation.

VitD3 can be catalyzed by 25-hydroxyvitamin D3-3-epimerase, which reverses the position of the hydroxyl space on the third carbon atom of VitD3 from 3β to 3α, thus forming C3-epi[12]. C3-epi shares the same molecular weight and structural formula with VitD3, but it has no biological activity. Only the spatial conformation is different between them[12]. In children, especially infants under one year of age, because vitamin D metabolic pathways are not yet mature, C-3 isomerization may be one of the main metabolic pathways of 25(OH)D, leading to higher C3-epi concentrations in infants under one year of age[34], which is consistent with our results. However, with increasing age, the serum C3-epi concentration is still high, indicating that the VitD metabolic pathway matures slowly. The C3-epi concentration of the juvenile is close to the adult level. If the C3-epi concentration in the serum is high, it will lead to an overestimation of the VitD storage level in the body, and it also reflects that the subject's VitD metabolic pathway is impaired[35]. In this study, regardless of age, the median and interval of C3-epi in patient group were estimated to be higher than those in healthy subjects, indicating that certain diseases may affect the normal metabolism of VitD in the body, thereby affecting the body's VitD storage level. As for VitD3, VitD2 also has epimerization *in vivo*. However, because the content of VitD2 in the human body is relatively low, apart from research, the detection of VitD2 has little significance in evaluating VitD storage. This is the reason why VitD2 epimerization was not analyzed in this study.

Conclusions

Up to now, clinicians have only evaluated VitD storage in body based on the serum 25(OH)D level. There is no reference range for VitD2, VitD3 and their ratios (VitD2/VitD3). However, due to the different activities of VitD2 and VitD3 and the existence of C3-epi, the detection of 25(OH)D concentration may not reflect the actual VitD storage level, especially for some specific populations related to VitD metabolism (infants and patients). Therefore, accurate accession of VitD nutritional status may depend on accurate measurement of the metabolites of VitD including VitD2, VitD3 and C3-epi. This study presents a correct evaluation method for VitD status in the human body. However, research population involved are limited. Did healthy people and patients have C3-epi increase significantly? This needs further research to understand. In addition, because C3-epi-VitD2 was not measured, the nutritional status of VitD in individuals with high VitD2 level may be overestimated.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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Institutional Review Board Statement
The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Mianyang Central Hospital, School of Medicine, University of Electronic Science and Technology of China (protocol code 201400048).

Informed Consent Statement
Informed consent was obtained from the patients, or their family involved in the study.

Data Availability Statement
The data presented in this study are available on request from the corresponding author.

Authors' Contribution
Data curation: Yuchun Chen, Yiyang He, Yuanmeng Li, Bitao Wu, Yuwei Yang and Jiafu Feng; Formal analysis: Yuchun Chen, Yiyang He, Yuanmeng Li, Bitao Wu and Yuwei Yang; Investigation: Yuchun Chen; Methodology: Yuchun Chen, Yiyang He, Yuanmeng Li, Bitao Wu and Yuwei Yang; Project administration: Yuchun Chen and Jiafu Feng; Resources: Jiafu Feng; Statistical analysis: Yuchun Chen, Yiyang He, Yuanmeng Li, Bitao Wu and Yuwei Yang; Supervision, Jiafu Feng; Validation: Yuchun Chen; Writing – original draft: Yuchun Chen and Yiyang He; Writing – review & editing: Jiafu Feng.

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