ORIGINAL

Effects of consuming fish on vitamin D status and use of urinary vitamin D metabolite as a noninvasive biomarker

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Abstract : Physiological studies on the amount and duration of vitamin D intake are limited. Noninvasive biomarkers for vitamin D status also need to be explored. Thus, we conducted a dietary challenge study to assess vitamin D metabolism. This crossover study included 21 healthy Japanese individuals, with each test lasting 6 consecutive days. Participants consumed test meals during the study period for 5 days. The main dish was either fish (FD) or meat (MD), and the FD group was provided with at least 18 μg of vitamin D per day. Fasting blood samples and 24 h urine samples were collected on the first and last days. The mean serum 25(OH)D_3 level were low before the intervention and changed only slightly after intervention, with higher levels in the FD group. Serum 25(OH)D_3 demonstrated a strong positive correlation with serum 24,25$(OH)_2D_3$ but showed no correlation with urinary 25(OH)D_3. Surprisingly, it had a relatively strong positive correlation with urinary 24,25$(OH)_2D_3$. In conclusion, in healthy Japanese, adequate intake of vitamin D from fish failed to increase serum 25(OH)D_3 concentrations. On the other hand, noninvasive urinary24,25$(OH)_2D_3$ excretion may be used instead of serum 25(OH)D_3 levels to assess vitamin D status. J. Med. Invest. 72 : 308-315, August, 2025

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INTRODUCTION

Vitamin D is essential for bone and mineral metabolism (1). Thus, vitamin D deficiency may increase the risk of bone calcification or hyperparathyroidism (2). In addition, this deficiency is related to various diseases, such as immune system disorder (3), cardiovascular disease (4), diabetes (5, 6), and cancer (7, 8).

Serum 25-hydroxyvitamin D (S-25(OH)D) levels are used as biomarkers of vitamin D status. Vitamin D is considered deficient if the S-25(OH)D level is less than 20 ng/mL, and insufficient if it is 20-30 ng/mL (9). Vitamin D deficiency/insufficiency has been widely reported to be highly prevalent, with the rate reaching 70-90% (10-14).

Vitamin D has two sources : dietary intake and synthesis in the skin following exposure to sunlight in humans. In the 2019 National Health and Nutrition Survey, Japan, the average intake of vitamin D, except for those in the 70s, does not meet 8.5 μg/day, which is the adequate intake (AI) shown in the Dietary References Intakes (DRIs) for Japanese 2020 (15).

Fish is a major source of vitamin D. However, the intake of fish is decreasing as westernized diets are becoming popular (16). Fish intake is effective in maintaining health. In particular, n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are at high levels in fish, can help prevent lifestyle diseases or noncommunicable diseases (17). Therefore, consuming a sufficient amount of fish is necessary not only as a source of vitamin D but also for health promotion.

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Through recent advancements in mass spectrometry–based clinical detection of serum metabolites, we can now accurately detect serum vitamin D metabolite concentrations, including 25(OH)D_3, 25(OH)D_2, 3-epi-25(OH)D_3, and 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) (18), which is the major inactive metabolite of 25(OH)D_3 (19). The expression of 24-hydroxyrase (CYP24A1), which converts 25(OH)D_3 into 24,25$(OH)_2D_3$, is partially regulated by the vitamin D receptor (VDR), and VDR activation depends upon 1,25-dihydroxyvitamin D (1,25$(OH)_2$D), which is the active form of vitamin D (20, 21). Therefore, 24,25$(OH)_2D_3$ can be a good indicator of vitamin D status because it is dependent on both 25(OH)D_3 and CYP24A1 (22, 23). Hence, serum 24,25$(OH)_2D_3$ (S-24,25$(OH)_2D_3$) levels demonstrate a strong positive correlation between with S-25(OH)D_3 level (22-27). However, dietary interventional trials that evaluated both 25(OH)D_3 and 24,25$(OH)_2D_3$ levels under a strictly controlled diet remain unavailable. Moreover, measuring serum levels requires blood sampling, which is an invasive method. Conversely, measuring urine levels is noninvasive. Most of the vitamin D metabolites excreted into urine are 24,25$(OH)_2D_3$, which is presented in urine as glucuronidated conjugates (28). This metabolite might be helpful in assessing vitamin D status. However, liquid chromatography/mass spectrometry (LC-MS/MS), the traditional method to quantify S-25(OH)D_3 levels, might be insufficient to quantify U-24,25$(OH)_2D_3$ levels because their concentrations are predicted to be extremely low (possibly in the pg/mL range) ; thus, Ogawa *et al*. developed 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD), a new derivatization reagent that enables quantifying vitamin D metabolites including 24,25$(OH)_2D_3$ (29). Nevertheless, the usefulness of U-24,25$(OH)_2D_3$ excretion for evaluating vitamin D status remains unreported.

Therefore, this study aimed to quantify urinary vitamin D metabolites by using LC-MS/MS with electrospray ionization (ESI)-enhancing and stable isotope-coded derivatization. We first evaluated whether ingesting a sufficient amount of vitamin

D from fish, which is a vitamin D-rich food, can improve vitamin D status. Second, we assessed whether vitamin D status can be evaluated noninvasively by quantifying urinary vitamin D metabolites.

MATERIALS AND METHODS

Participants

This study recruited 21 healthy Japanese volunteers. Participants were confirmed to be currently not on medical treatment or any nutritional supplementation. Other exclusion criteria were allergies to prescribed meals, heavy alcohol consumption, currently pregnant or breastfeeding, and smoking.

This study was conducted after obtaining written informed consent from all participants. It was approved by the ethics committee of the University of Shizuoka (approval number : 3-58) and registered with the University Hospital Medical Information Network (UMIN registration number : UMIN000053385). This study also conformed to the principles of the Declaration of Helsinki.

Study Protocol

We used a crossover study design and conducted from January 2021 to July 2021 at the University of Shizuoka, Shizuoka, Japan. Each test was conducted over 6 consecutive days and separated by a washout period of at least 7 days. The study protocol is shown in Supplemental Figure 1. Heavy exercise and alcohol intake were not allowed for 3 days before each test. After consuming the prescribed meal, they were prohibited from eating and drinking other than water. They were instructed to eat and drink the same prescribed meal until 20:00 and only drink 500 mL of water until 24:00 before the test period. Fasting blood samples were collected at 7:30 on days 1 and 6. On days 1-5, participants consumed test meals at 8:00 (breakfast), 12:00 (lunch), and 18:00 (dinner), and they were instructed to drink 200 mL of water after waking up and 1,300 mL of water from 8:00 to 24:00. In the 24 h urine collection, urine on day 1 was discarded right before breakfast intake (at 8:00) and collected after breakfast intake until just before breakfast intake (at 8:00) on day 2, which was denoted as day 1. Similarly, 24 h urine collection was done on days 5 through 6, which was denoted as day 6. The participants underwent physical measurements on days 1 and 6. They responded to a food intake frequency survey using the brief-type self-administered diet history questionnaire (BDHQ) at their first visit.

Test meals

Each test meal was designed to have similar total energy (male : 2,500 kcal/day, female : 2,200 kcal/day) and protein : fat : carbohydrate ratios in terms of percentage of energy (13-20 : 20-30 : 50-65) (Table 1). Each test meal was the same except for the main dish. The main dish was fish in the fish period (FD) and meat in the meat period (MD). The FD period met the AI of vitamin D, that is 8.5 μg/day, which is based on DRIs for Japanese (15). Participants were instructed to consume each test meal within 30 min. The Japan Food Research Laboratories Foundation (Tokyo, Japan) analyzed the test meal components.

Blood and urine analysis methods and anthropometric measurements

Blood samples were centrifuged at 940 g for 10 min at 4 °C. Subsequently, they were separated into plasma and serum and stored at −80 °C until the analysis. Clinical laboratory measurements included serum 25(OH)D_3, 3-epi-25(OH)D_3, 25(OH)D_2, 24,25$(OH)_2D_3$, creatinine (S-Cre), and blood urea nitrogen (BUN). Urinary measurements were the levels of urinary 25(OH)D_3 and 24,25$(OH)_2D_3$. The blood and urine samples were analyzed by JEOL Ltd. (Tokyo, Japan). The participants underwent anthropometric measurements using the bioelectrical impedance analysis method (InBody 770 ; InBody Japan, Tokyo, Japan). Height was measured using a YL-65S stadiometer (Yagami, Nagoya, Japan).

Measurement of vitamin D metabolites

Vitamin D metabolites such as 25$(OH)D_2$, 25(OH)D_3, 24,25$(OH)_2D_3$, and 3-epi-25(OH)D_3 were measured using LC-MS/MS. Vitamin D metabolites in urine and serum were analyzed with Xevo TQ-XS (Waters, Milford, MA, USA) using an in-house method with the JeoQuant™ Kit for VD Metabolites (JEOL, Tokyo, Japan). This kit contains the caged DAPTAD (14-(4-(dimethylamino)phenyl)-9-phenyl-9,10-dihydro-9,10-[1,2] epitriazoloanthracene-13,15-dione, DAP-PA) as a derivatization reagent (30). The detailed LC-MS/MS assay is described in the supplementary methods.

Table 1. Composition of the test meals.

A. Male						
	Energy kcal/day	Protein g/day	Fat g/day	Carbohydrate g/day	NaCl g/day	Vitamin D μg/day
FD	2,496	83.0	81.8	372.9	6.8	18.7
	%Energy	13.3	29.5	57.2		
MD	2,527	80.6	81.1	381.6	8.1	3.7
	%Energy	12.8	28.9	58.3		
B. Female						
	Energy kcal/day	Protein g/day	Fat g/day	Carbohydrate g/day	NaCl g/day	Vitamin D μg/day
FD	2,154	76.7	68.6	321.9	6.3	18.5
	%Energy	14.2	28.7	57.1		
MD	2,201	74.9	69.0	331.6	7.9	2.5
	%Energy	13.6	28.2	58.2		

FD, fish diet ; MD, meat diet ; NaCl, sodium chloride.

Calculating formulas

Vitamin D metabolites ratio (VMR), urinary 25(OH)D_3 excretion per day (U-25(OH)D_3/day), and 24,25$(OH)_2D_3$ excretion per day (U-24,25$(OH)_2D_3$/day) were calculated using the following formulas (31) :

VMR (pg/ng) = S-24,25$(OH)_2D_3$ × 1,000 / S-25$(OH)_2D_3$

U-25(OH)D_3/day (ng/day) = U-25(OH)D_3 × (U-volume / 100)

U-24,25$(OH)_2D_3$/day (ng/day) = U-24,25$(OH)_2D_3$ × (U-volume / 100)

Statistical Analysis

All data are shown as mean ± standard deviation (SD) or medians (25th to 75th in-interquartile range). Shapiro–Wilk test statistic was used for data normality testing. We conducted parametric analysis for normally distributed data, and nonparametric analysis for non-normally distributed ones. Differences in serum and urinary parameters between days 1 and 6 were identified using a paired *t*-test or the Wilcoxon signed-rank test. Differences between sexes were identified using an independent *t*-test or the Mann–Whitney *U* test. The correlations between vitamin D metabolite variables were determined using Pearson's correlation coefficient or Spearman's rank correlation coefficient. *P*-values less than 0.05 were considered statistically significant. All statistical data were analyzed using SPSS for Windows, release 26 (IBM Corp., Armonk, NY).

RESULTS

Participant characteristics

Table 2 shows the participants' clinical and biological characteristics. Eighteen participants were vitamin D deficient (S-25(OH)D_3 level < 20 ng/mL) and 3 participants were vitamin D insufficient (S-25(OH)D_3 level 20–30 ng/mL). None of the participants had adequate vitamin D status. The variable parameters of serum vitamin D metabolite levels did not significantly differ between sexes. According to the data calculated from the BDHQ, the habitual vitamin D intake of 11 participants did not reach the AI and the habitual calcium intake of all participants did not reach the Recommended Dietary Allowances (Supplemental Table 1).

Changes in serum vitamin D metabolite levels

The S-25(OH)D_3 levels and S-3-epi-25(OH)D_3 levels for groups FD ($p = 0.002$, 0.002, respectively) and MD ($p = 0.002$, 0.029, respectively) were significantly higher after than before the intervention. Likewise, the S-24,25$(OH)_2D_3$ levels for the MD group after the intervention were significantly higher than those before the intervention ($p = 0.027$) (Table 3). Furthermore, changes in S-25(OH)D_3 levels were higher in the FD group than in the MD group ($p = 0.050$) (Table 4).

Table 2. Participant characteristics.

		All (n = 21)	Male (n = 10)	Female (n = 11)	*p*-value
Age	(year)	23.0 (22.0, 30.0)	23.0 (21.0, 24.5)	24.0 (23.0, 35.0)	0.118
Body weight	(kg)	57.4 (50.2, 74.4)	66.0 ± 12.5	57.0 ± 12.0	0.111
BMI	(kg/m^2)	21.0 (19.1, 24.2)	22.0 (17.2, 25.0)	20.9 (19.7, 23.1)	0.833
S-25(OH)D_3	(ng/mL)	15.1 ± 4.6	15.8 ± 6.3	14.6 ± 2.1	0.580
S-3-epi-25(OH)D_3	(ng/mL)	0.49 ± 0.15	0.54 ± 0.19	0.45 ± 0.07	0.158
S-25(OH)D_2	(ng/mL)	0.34 (0.29, 0.43)	0.33 (0.28, 0.43)	0.34 (0.29, 0.45)	0.725
S-24,25$(OH)_2D_3$	(ng/mL)	0.87 ± 0.39	0.95 (0.60, 1.34)	0.73 (0.67, 1.03)	0.573
S-Cre	(mg/dL)	0.8 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	**< 0.01**
BUN	(mg/dL)	10.5 ± 1.8	11.4 ± 1.5	9.7 ± 1.7	**0.032**

Values are presented as mean ± SD or medians (25^{th} to 75^{th} interquartile range). Differences between sexes were identified using an independent *t*-test or the Mann–Whitney *U* test. BMI, body mass index ; S-25(OH)D_3, serum 25-hydroxyvitamin D_3 level ; S-3-epi-25(OH)D_3, serum 3-epi-25-hydroxyvitamin D_3 level ; S-25(OH)D_2, serum 25-hydroxyvitamin D_2 level ; S-24,25$(OH)_2D_3$, serum 24,25-dihydroxyvitamin D_3 level ; S-Cre, serum creatinine ; BUN, blood urea nitrogen.

Table 3. Serum vitamin D metabolic parameters compared between those on days 1 and 6.

		FD			MD		
		day 1	day 6	*p*-value	day 1	day 6	*p*-value
S-25(OH)D_3	(ng/mL)	14.7 ± 4.8	16.7 ± 5.1	**0.002**	15.6 ± 4.6	16.6 ± 4.0	**0.002**
S-3-epi-25(OH)D_3	(ng/mL)	0.43 (0.40, 0.52)	0.48 (0.41, 0.63)	**0.002**	0.51 ± 0.14	0.54 ± 0.12	**0.029**
S-25(OH)D_2	(ng/mL)	0.32 (0.26, 0.41)	0.31 (0.26, 0.39)	0.140	0.36 (0.28, 0.47)	0.38 (0.30, 0.47)	0.903
S-24,25$(OH)_2D_3$	(ng/mL)	0.91 (0.56, 1.05)	0.91 (0.65, 1.07)	0.054	0.89 ± 0.40	0.95 ± 0.42	**0.027**

Values are presented as mean ± SD or medians (25^{th} to 75^{th} interquartile range). Differences between days 1 and 6 were identified using paired *t*-test or Wilcoxon signed-rank test. FD, fish diet ; MD, meat diet ; S-25(OH)D_3, serum 25-hydroxyvitamin D_3 level ; S-3-epi-25(OH)D_3, serum 3-epi-25-hydroxyvitamin D_3 level ; S-25(OH)D_2, serum 25-hydroxyvitamin D_2 level ; S-24,25$(OH)_2D_3$, serum 24,25-dihydroxyvitamin D_3 level.

Changes in urinary vitamin D metabolite excretion

The level of U-24,25(OH)$_2$D$_3$ excretion was significantly higher on day 6 than on day 1 in group FD ($p = 0.020$) (Table 5). Its change in the FD group was also significantly higher than that in the MD group ($p < 0.001$) (Table 6). Conversely, changes of U-25(OH)D$_3$ excretion showed no significant differences between the such groups.

Correlation analysis between vitamin D metabolite variables

Table 7 shows the relationship between vitamin D metabolite variables. Predictably, S-25(OH)D$_3$ and S-24,25(OH)$_2$D$_3$ showed a strong positive correlation ($r = 0.934$; $p < 0.001$). Surprisingly, S-25(OH)D$_3$ positively correlated with U-24,25(OH)$_2$D$_3$ ($r = 0.622$; $p < 0.001$).

Table 4. Changes from day 1 in serum vitamin D metabolic parameters between the FD and MD groups.

		All		
		FD	MD	*p*-value
ΔS-25(OH)D$_3$	(ng/mL)	2.1 ± 2.7	1.0 ± 1.3	0.050
ΔS-3-epi-25(OH)D$_3$	(ng/mL)	0.07 (0.00, 0.10)	0.02 (−0.02, 0.07)	0.058
ΔS-25(OH)D$_2$	(ng/mL)	−0.02 ± 0.06	0.00 ± 0.01	0.184
ΔS-24,25(OH)$_2$D$_3$	(ng/mL)	0.06 (−0.04, 0.16)	0.07 (−0.03, 0.14)	0.444

Values are presented as mean ± SD or medians (25^{th} to 75^{th} interquartile range). Differences between the FD and MD groups were compared using paired *t*-test or Wilcoxon signed-rank test. FD, fish diet ; MD, meat diet ; S-25(OH)D$_3$, serum 25-hydroxyvitamin D$_3$ level ; S-3-epi-25(OH)D$_3$, serum 3-epi-25-hydroxyvitamin D$_3$ level ; S-25(OH)D$_2$, serum 25-hydroxyvitamin D$_2$ level ; S-24,25(OH)$_2$D$_3$, serum 24,25-dihydroxyvitamin D$_3$ level.

Table 5. Urinary vitamin D metabolic parameters compared between those on days 1 and 6.

		FD			MD		
		day 1	day 6	*p*-value	day 1	day 6	*p*-value
U-25(OH)D$_3$	(ng/day)	22.3 (18.0, 31.3)	19.4 (17.1, 29.1)	0.117	22.1 (13.8, 32.0)	19.9 (16.2, 24.5)	0.678
U-24,25(OH)$_2$D$_3$	(ng/day)	75.8 ± 34.0	86.7 ± 37.9	**0.020**	68.8 (48.0, 84.0)	67.6 (39.0, 76.4)	0.339

Values are presented as mean ± SD or medians (25^{th} to 75^{th} interquartile range). Differences between day 1 and day 6 were identified using paired *t*-test or Wilcoxon signed-rank test. FD, fish diet ; MD, meat diet ; U-25(OH)D$_3$, urinary 25-hydroxyvitamin D$_3$ level ; U-24,25(OH)$_2$D$_3$, urinary 24,25-dihydroxyvitamin D$_3$ level.

Table 6. Changes from day 1 in urinary vitamin D metabolic parameters between the FD and MD groups.

		All		
		FD	MD	*p*-value
ΔU-25(OH)D$_3$	(ng/day)	−2.1 (−5.3, 1.4)	0.5 (−12.0, 6.2)	0.441
ΔU-24,25(OH)$_2$D$_3$	(ng/day)	10.9 ± 19.8	−7.6 ± 27.7	**< 0.001**

Values are presented as mean ± SD or medians (25^{th} to 75^{th} interquartile range). Differences between the FD and MD groups were identified using paired *t*-test or Wilcoxon signed-rank test. FD, fish diet ; MD, meat diet ; U-25(OH)D$_3$, urinary 25-hydroxyvitamin D$_3$ level ; U-24,25(OH)$_2$D$_3$, urinary-24,25-dihydroxyvitamin D$_3$ level.

Table 7. Correlation analysis between vitamin D metabolite variables.

		S-25(OH)D_3 (ng/mL)	S-25(OH)D_2 (ng/mL)	S-3-epi-25(OH)D_3 (ng/mL)	S-24,25$(OH)_2D_3$ (ng/mL)	VMR (pg/ng)	U-25(OH)D_3 (ng/day)	U-24,25$(OH)_2D_3$ (ng/day)
S-25(OH)D_3	(ng/mL)	-	0.059	0.931***	0.934***	0.606***	0.293	0.622***
S-25(OH)D_2	(ng/mL)		-	0.074	0.200	0.344*	−0.230	0.068
S-3-epi-25(OH)D_3	(ng/mL)			-	0.903***	0.589***	0.284	0.581***
S-24,25$(OH)_2D_3$	(ng/mL)				-	0.805***	0.219	0.642***
VMR	(pg/ng)					-	0.017	0.512**
U-25(OH)D_3	(ng/day)						-	0.602***
U-24,25$(OH)_2D_3$	(ng/day)							-

The relationship between vitamin D metabolite variables was identified using Pearson's correlation coefficient or Spearman's rank correlation coefficient. All the parameters in the table were the data of day 1 in the FD and MD groups. FD, fish diet ; MD, meat diet. S-25(OH)D_3, serum 25-hydroxyvitamin D3 level ; S-25(OH)D_2, serum 25-hydroxyvitamin D_2 level ; S-3-epi-25(OH)D_3, serum 3-epi-25-hydroxyvitamin D_3 level ; S-24,25$(OH)_2D_3$, serum 24,25-dihydroxyvitamin D_3 level ; VMR, vitamin D metabolite ratio ; U-25(OH)D_3, 25-hydroxyvitamin D_3 level ; U-24,25$(OH)_2D_3$, urinary 24,25-dihydroxyvitamin D_3 level. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

DISCUSSION

This study primarily aimed to evaluate the effect of changing the main dish to fish or meat on vitamin D status. After the intervention, the S-25(OH)D_3 levels increased significantly in both groups, with higher values in group FD than in group MD. Therefore, the S-25(OH)D_3 level increased by ingesting a meal containing fish as the main dish for 5 days. However, no participant in the FD group obtained S-25(OH)D_3 levels of 30 ng/mL or higher after the intervention ; vitamin D intake from the test meal in group FD was 18.7 μg/day for males and 18.5 μg/day for females, exceeding DRI's AI amount of 8.5 μg/day, but not sufficient to achieve vitamin D sufficiency.

In a supplementation study conducted in Norway, healthy people aged 19-48 years took 10 μg/day of vitamin D_3, which is fewer than our study, from supplements for 4 weeks ; consequently, the average S-25(OH)D level increased from 17.7 to 31.4 ng/mL (32). In another supplementation study conducted in Saudi Arabia, healthy people aged 18-60 years took 50 μg/day of vitamin D ; as a result, the average S-25(OH)D level increased from 16.5 to 20 ng/mL on day 7 and plateaued at approximately 28 ng/mL on day 90 (33). A systematic review showed that the S-25(OH)D level response to vitamin D supplementation peaks at 3-6 months (34). Accordingly, the duration of 5 days of dietary intervention was insufficient to satisfy vitamin D status even if they took an amount of vitamin D far exceeding the DRI's AI. However, the S-25(OH)D_3 level increased by approximately 2.0 ng/mL in 5 days ; therefore, vitamin D status could improve greatly if they keep having similar diet.

Moreover, this study evaluated the correlation between serum and urinary vitamin D metabolites, and there were positive correlations between excluding S-25(OH)D_2 level and U-25(OH)D_3 excretion. Vitamin D_3 is converted into 25(OH)D_3 by CYP2R1 expressed in the liver, and some of it is converted into 3-epi-25(OH)D_3 by 3-epimerase (35). CYP27B1 expressed in the kidney converts 25(OH)D_3 and 3-epi-25(OH)D_3 into 1α-25$(OH)_2D_3$ and 3-epi-1α-25$(OH)_2D_3$, or CYP24A1, which is also expressed in the kidney, converts them into 24,25$(OH)_2D_3$ and 3-epi-24,25$(OH)_2D_3$, respectively. Therefore, a positive correlation existed because 3-epi-25(OH)D_3 and 24,25$(OH)_2D_3$ increased commensurately with 25(OH)D_3.

VMR, represented by the ratio of S-24,25$(OH)_2D_3$ level and S-25(OH)D_3 level, is an indicator of CYP24A1 activity and thereby of vitamin D catabolism (27). Recently, VMR has been proposed as a biomarker of vitamin D status. Some studies indicate that VMR can predict the magnitude of the S-25(OH)D_3 level change resulting from vitamin D_3 supplementation, but they have not obtained a consistent result (24, 26, 31). In the present study, the S-25(OH)D_3 level showed a moderately strong correlation with VMR ($r = 0.606$; $p < 0.001$), but the change in S-25(OH)D_3 level did not correlate with VMR (data not shown). While a correlation between estimated glomerular filtration rate (eGFR) and S-24,25$(OH)_2D_3$ level has been reported (36), the mechanism underlying the decline in both S-24,25$(OH)_2D_3$ level and eGFR remains unclear. Additionally, CYP24A1 is highly expressed in extra-renal tissues, including small intestine and stomach (37, 38), suggesting that decreased CYP24A1 expression or activity in these tissues may be associated with kidney function decline. Therefore, while VMR may serve as an indicator of CYP24A1 activity and vitamin D catabolism, its suitability for monitoring kidney function remains uncertain and requires further investigation.

Surprisingly, the S-25(OH)D_3 and S-24,25$(OH)_2D_3$ levels showed moderately strong positive correlations with U-24,25$(OH)_2D_3$ excretion ($r = 0.622$ and 0.642, respectively ; $p < 0.001$ for both). Excessive 25(OH)D_3 was inactivated and excreted in the form of 24,25$(OH)_2D_3$. We also noted a strong positive correlation between the S-25(OH)D_3 and S-24,25$(OH)_2D_3$ levels ($r = 0.934$; $p < 0.001$), consisted with the results of many studies (22-25, 27). Therefore, relationships between not only the S-25(OH)D_3 and S-24,25$(OH)_2D_3$ levels but also between the S-24,25$(OH)_2D_3$ level and U-24,25$(OH)_2D_3$ excretion have created a relationship between the S-25(OH)D_3 level and U-24,25$(OH)_2D_3$ excretion.

LC-MS/MS is the gold standard for measuring vitamin D status, and ESI is used for ionization. However, the ionization efficiency of vitamin D_3 metabolites is not high in ESI, and the sensitivity is insufficient to measure vitamin D metabolites circulating at low concentrations. In the current study, a new derivatization reagent (DAP-PA), was used to measure urinary vitamin D metabolites, demonstrating greater accuracy and noninvasively compared with the other methods. Urine is a non-invasive biomarker, and we have reported that urine excretion could be a new biomarker of phosphorus and magnesium, which are difficult to evaluate using serum concentration (39, 40). Therefore, U-24,25$(OH)_2D_3$ excretion may be helpful in assessing vitamin D status, superseding the S-25(OH)D_3 level.

This study has some strengths. First, diet during the test

period was strictly controlled. Second, we evaluated the effect of vitamin D from the diet instead of supplementation. Third, vitamin D metabolites including S-25(OH)D_3 levels were quantified by LC-MS/MS, which is the gold standard for measuring vitamin D status. Additionally, we were able to measure U-24,25(OH)$_2D_3$ levels with greater accuracy using DAP-PA. However, this study also has some limitations. First, considering the burden of participants, the test period was set to 5 days ; it was too short to evaluate the effects of vitamin D supplementation on vitamin D status. Second, the participants have an age bias ; approximately 80% of them were in their 20s. Hence, we will target people of various ages in future studies.

In conclusion, the S-25(OH)D_3 levels increased after consuming sufficient amounts of vitamin D from fish. However, the test period was too short to improve the vitamin D status. Furthermore, the S-25(OH)D_3 level had moderate positive correlation with U-24,25(OH)$_2D_3$ excretion. Therefore, U-24,25(OH)$_2D_3$ excretion may be useful in assessing vitamin D status, superseding the S-25(OH)D_3 levels.

CONFLICT OF INTERESTS-DISCLOSURE

The authors declare that they have no competing interests.

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AUTHOR CONTRIBUTIONS

Research conception and design : YKS and HA ; experiments : XZ, YKS, MT, KA ; statistical analysis of the data : XZ and YKS ; interpretation of the data : XZ, YKS and HA ; writing of the manuscript : XZ, YKS and HA. All the authors contributed to the review of the final draft. XZ, YKS and HA contributed equally to this work.

DISCLOSURE STATEMENT

The authors declare that they have no competing interests.

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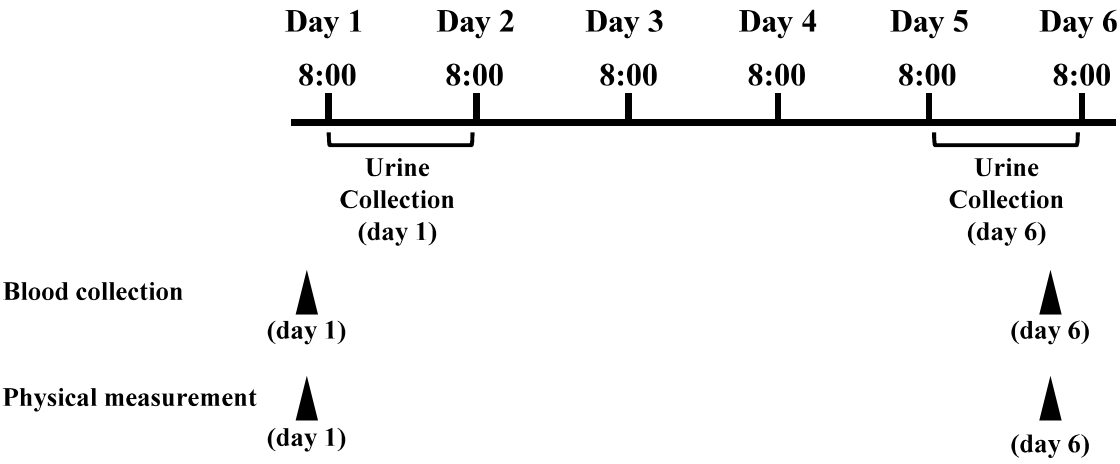

Supplemental Figure 1. Study protocol

Supplemental Table 1. The estimated habitual dietary nutrients intake assessed by BDHQ.

		All (n = 21)	Male (n = 10)	Female (n = 11)
Energy	(kcal)	1,574.6 ± 552.8	1,643.2 ± 410.1	1,512.3 ± 671.4
Protein %Energy	(%)	15.5 ± 3.3	15.3 ± 3.6	15.6 ± 3.1
Fat %Energy	(%)	28.7 ± 7.5	29.7 ± 8.8	27.8 ± 6.5
Carbohydrate %Energy	(%)	55.8 ± 10.0	55.0 ± 11.9	56.6 ± 8.6
Calcium	(mg)	366.4 ± 158.3	397.8 ± 201.7	337.9 ± 107.7
Vitamin D	(μg)	8.2 (5.7, 13.0)	11.3 ± 8.6	8.7 ± 4.0

Values are presented as mean ± SD or medians (25^{th} to 75^{th} interquartile range). BDHQ, brief-type self-administered diet histry questionnaire.