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# The genetic determinants of circulating C3-epimers of 25-hydroxyvitamin D

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ABSTRACT

*Background:* The complexity of vitamin D metabolites especially the contribution of C3-epimers of 25-hydroxyvitamin D (C3-epimers) in human sera remains unclear. We hypothesized that genetic polymorphisms in the vitamin D-related gene pathway contribute to variation in C3-epimer levels. Therefore, we investigated candidate single nucleotide polymorphisms (SNPs) concerning C3-epimer levels.

*Methods:* The candidate SNPs, including *DHCR7/NADSYN1* (rs12785878), *CYP2R1* (rs2060793) and *GC* (rs2282679), were genotyped in 1727 members of the third project of the Electricity Generating Authority of Thailand 3/1 cohort investigation. Each SNP was tested under three genetic effects (dominant, recessive and additive models) concerning the levels of total serum 25(OH)D [the sum of $25(OH)D_{2+3}$ and 3-epi-$25(OH)D_{2+3}$], non-C3-epimers [$25(OH)D_{2+3}$] and C3-epimers [3-epi-$25(OH)D_{2+3}$], using linear regression analysis.

*Results:* Among the participants, the median (range) levels of non-C3-epimers and C3-epimers were 22.7 (6.4–49.2) ng/mL and 1.3 (0.01–14.2) ng/mL, respectively. In regression analysis, we found the genetic variation of two SNPs, the *DHCR7/NADSYN1* (rs12785878; G > T) and *GC* (rs2282679; T > G) under additive genetic models, explained the variation of C3-epimer levels about 1.5% ($p = 1.66 \times 10^{-7}$) and 1.1% ($p = 1.10 \times 10^{-5}$), respectively. Interestingly, participants carrying the minor T-allele of rs12785878 exhibited a trend to increase C3-epimer levels, while those carrying the minor G-allele of rs2282679 exhibited a trend to decrease levels of both non-C3-epimers and C3-epimers. In addition, *CYP2R1* (rs2060793; G > A) was clearly associated only with non-C3-epimer levels ($p = 2.46 \times 10^{-8}$). In multivariate analyses, sex, age and BMI were predictors for variation in C3-epimer concentration; sex and age for variation in non-C3-epimers.

*Conclusion:* To the best of our knowledge, this is the first study to demonstrate genetic models concerning the variation in C3-epimer levels. Our results emphasize that genetic determinants and the potential factors of C3-epimers differ from non-C3-epimers. This study contributes fundamental knowledge of the endogenous vitamin D pathway.

## Introduction

Vitamin D is an essential micronutrient [1] required for calcium homeostasis and bone health and is related to non-skeletal outcomes [2–5]. Vitamin D exhibits two main major forms: vitamin $D_2$ and vitamin $D_3$ [6,7]. In the body, vitamin D is obtained from food ($D_2$ and $D_3$), supplementation ($D_2$ and $D_3$), or from cutaneous synthesis ($D_3$), and is metabolized further through C25-, C1- and C24-hydroxylation pathways [8–10]. These processes produce various forms of vitamin D such as 25-hydroxyvitamin D [25(OH)D], 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], 24,25-dihydroxyvitamin D [$(24R,25(OH)_2D$] [11–14]. Importantly, vitamin D can be metabolized through an alternative pathway or C3-epimerization [15,16]. The process affects the structure of vitamin D metabolites; the spatial orientation of the hydroxyl group at carbon 3 changes from alpha to beta [17,18] and forms the C3-epimer. For example, the C3-epimeric form of $25(OH)D_3$ is 3-epi-$25(OH)D_3$. *In vitro* and *in vivo* studies have indicated that 3-epi-$25(OH)D_3$ can be further metabolized into 3-epi-$1\alpha,25(OH)_2D_3$ and 3-epi-$24(R),25(OH)_2D_3$ [15,16,18]. The biological affinity of C3-metabolites is lower than their native form [19]. This has led to growing interest in distinguishing the nature of vitamin D metabolites and C3-epimeric forms in human sera [20–24]. Currently, C3-epimerization is thought to parallel standard vitamin D metabolism and uses the same enzymes responsible for hydroxylation [25]. In addition, 3-epi-25(OH)D

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metabolites contribute to human sera and their proportion is likely relative to the total serum 25(OH)D levels [26–29]. Little is known about the impact of genetic variants on the level of the C3-epimers. We hypothesized that genetic polymorphisms in the vitamin D-related gene pathway contribute to variation in C3-epimer levels.

By providing candidate genes, genome-wide association studies (GWAS) constitute a strong approach in identifying suitable genetic variants related to total serum 25(OH)D levels. Two GWAS [30,31] reported that genetic polymorphisms in three genes were functionally related to the variation in total serum 25(OH)D levels, including 7-dehydrocholesterol reductase/nicotinamide-adenine dinucleotide synthetase 1 (*DHCR7/NADSYN1*), cytochrome P450, family 2, subfamily R, polypeptide 1 (*CYP2R1*) and group specific component (*GC*). Wang et al. [30] showed that *DHCR7/NADSYN1* (rs12785878) variants were strongly associated with total serum 25(OH)D concentrations ($p = 2.1 \times 10^{-27}$). Ahn et al. [31] reported that *CYP2R1* (rs2060793) and *GC* (rs2282679) polymorphisms were significantly associated with total serum 25(OH)D levels ($p = 1.4 \times 10^{-5}$ and $1.8 \times 10^{-49}$, respectively). Therefore, we aimed to investigate the associations between vitamin D-related polymorphisms including the *DHCR7/NADSYN1* (rs12785878), *CYP2R1* (rs2060793) and *GC* (rs2282679) and the concentration of C3-epimers.

## Materials and methods

### *Study population*

Data and specimens were obtained from 1727 participants recruited from the third project of the Electricity Generating Authority of Thailand (EGAT3/1) cohort investigation and reported in full detail [32]. Briefly, this cohort started 2009, by randomly recruiting EGAT employees aged between 24 and 54 years from the Bangkok Metropolitan Area. All subjects gave informed consent before the study. Anthropometric data and specimens were collected by medical professionals. Physical examinations and fasting blood tests were performed on the same day using standard procedures. All specimens were stored at −80 °C until analysis. This study complied with guidelines outlined in the Declaration of Helsinki. Approval was obtained from the Ramathibodi Hospital Ethics Committee.

### *Measurement of serum vitamin D levels*

Serum levels of 25(OH)$D_2$, 25(OH)$D_3$, 3-epi-25(OH)$D_2$ and 3-epi-25(OH)$D_3$ were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). An Agilent 1260 Infinity Liquid Chromatograph (Agilent Technologies, Waldbronn, Germany) was used coupled to a QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Framingham, MA, USA) using a MassChrom® 25-OH-$D_3/D_2$ serum/plasma diagnostics kit with a 3-epi-25-OH-$D_3/D_2$ upgrade (Chromsystems, Munich, Germany). An atmospheric pressure chemical ionization source, operated in positive mode, was used to ionize the target compound. Data were collected and analyzed using Analyst Software, Version 1.6.2 (Applied Biosystems, USA). All calibrators (Chromsystems 3PLUS1® Multilevel Serum Calibrator Set 3-epi-25-OH-$D_3/D_2$ and 25-OH-$D_3/D_2$) and serum control (MassCheck® 3-epi-25-OH-$D_3/D_2$ and 25-OH-$D_3/D_2$) used in this study were traceable to certified substances and standard reference materials (SRM) of the National Institute of Standards and Technology (NIST; SRM 972, Gaithersburg, MD, USA). Each batch contained a four-point calibration curve and two levels of quality control materials. Acceptable linearity of calibration curves was achieved when correlation coefficients were 0.998 or greater. In this study, total serum 25(OH)D comprised the sum of 25(OH)$D_3$, 25(OH)$D_2$, 3-epi-25(OH)$D_2$ and 3-epi-25(OH)$D_3$. Separated by C3-epimeric form, non-C3-epimers were the sum of 25(OH)$D_2$ and 25(OH)$D_3$, and C3-epimers were the sum of 3-epi-25(OH)$D_2$ and 3-epi-25(OH)$D_3$. The coefficients of variation (CV) of 25(OH)$D_3$, 25(OH)$D_2$, and 3-epi-25(OH)$D_3$ were 9.2, 19.9 and 11.8, respectively.

### *DNA extraction and genotyping*

Genomic DNA was isolated from peripheral blood leucocytes using the standard phenol-chloroform method. Isolated genomic DNA was stored at 4 °C until single nucleotide polymorphism (SNP) genotyping. *DHCR7/NADSYN1* (rs12785878), *CYP2R1* (rs2060793) and *GC* (rs2282679) were genotyped using a TaqMan assay with allele-specific probes. Amplification reactions were performed in a total volume of 10μL in optical 96-well plates. Thermal cycler conditions of Real-Time PCR were as follows: 10 min at 95 °C (hold stage), 15 s at 95 °C for DNA denaturation, and 1 min at 60 °C for annealing and extension for 40 cycles using an Applied Biosystems ViiA7™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

### *Statistical analyses*

#### *Descriptive statistics*

The Kolmogorov-Smirnov test was used for normality testing. Non-normally distributed data were reported as median and range (min–max). Categorical data including body mass index (BMI), glycemic index (fasting blood glucose) and vitamin D levels (total serum 25(OH)D), were summarized as frequencies and percentages. To access BMI and glycemic status, they were classified based on the standard BMI for adult Asian populations [33] and the American Diabetes Association fasting glucose criteria [34], respectively. In addition, according to the Endocrine Society's Clinical Guidelines, total serum 25(OH)D less than 20 ng/mL was classified as vitamin D deficiency status [35].

We performed Chi-square test to evaluate significant differences in categorical variables between groups. For non-parametric data, the Mann-Whitney *U* test was conducted to find any significant difference between two independent groups. All analyses were performed using IBM SPSS Statistics Software, Version 20.0. A *p*-value less than 0.001 was considered statistically significant.

#### *Genetic association*

A natural logarithmic (Ln) transformation was used to improve vitamin D metabolite data that did not follow normal distribution. Deviations from Hardy-Weinberg equilibrium (HWE) were assessed using the Chi-square ($\chi 2$) test. The effect of three SNPs on total serum 25(OH)D, non-C3-epimer and C3-epimer levels were analyzed under three different genetic models: dominant, recessive and additive effects by using simple linear regression analysis to predict linear relationships between genetic polymorphism and vitamin D metabolite levels. Genetic models were fitted in simple linear regression, followed by multiple linear regression analysis. The variables added in the models were sex, age and BMI. The best model was selected from the maximum adjusted $R^2$.

## Results

### *Participant characteristics*

The 1727 subjects (Table 1) had a median (range) age of 40 (25–54) years and 71.3% were male. The prevalence of overweight and obesity in men was 26.2 and 38.8%, respectively. Most women exhibited normal weight status (53.6%). The prevalence of type 2 diabetes mellitus was 2.7% only. A high prevalence of vitamin D deficiency was found in women (female, 43.1% vs. male, 14.5%). The median (range) level of total serum 25(OH)D, non-C3-epimers and C3-epimers were 24.1 (6.7–53.7) ng/mL, 22.7 (6.4–49.2) ng/mL and 1.3 (0.01–14.2) ng/mL, respectively. Serum creatinine, BMI, glycemic status, vitamin D status and total serum 25(OH)D levels differed significantly between males and females ($p < 0.001$).

**Table 1**
Characteristic profiles of the study population (N = 1727).

| Parameter | Group | | | *p*-value |
|---|---|---|---|---|
| | Total (n = 1727) | Male (n = 1231) | Female (n = 496) | |
| Age (years)[1] | 40 (25–54) | 40 (25–54) | 39 (25–54) | 0.008[3] |
| Serum creatinine (mg/dL) | 0.9 (0.8–1.0) | 1.0 (0.9–1.1) | 0.7 (0.6–0.7) | < 0.001[3] |
| BMI category (kg/m$^2$)[2] | | | | < 0.001[4] |
| Underweight | 89 (5.2) | 23 (1.9) | 66 (13.3) | |
| Normal weight | 673 (39.0) | 407 (33.1) | 266 (53.6) | |
| Overweight | 391 (22.6) | 323 (26.2) | 68 (13.7) | |
| Obese class I | 464 (26.9) | 392 (31.8) | 72 (14.5) | |
| Obese class II | 110 (6.4) | 86 (7.0) | 24 (4.8) | |
| Glycemic status[2] | | | | < 0.001[4] |
| Normal fasting plasma glucose | 1508 (87.3) | 1041 (84.6) | 467 (94.2) | |
| Pre-diabetes | 173 (10.0) | 150 (12.2) | 23 (4.6) | |
| Type 2 diabetes mellitus | 46 (2.7) | 40 (3.2) | 6 (1.2) | |
| Vitamin D status[2] | | | | < 0.001[4] |
| Deficiency (< 20 ng/mL) | 393 (22.8) | 179 (14.5) | 214 (43.1) | |
| Non-deficiency (≥ 20 ng/mL) | 1334 (77.2) | 1052 (85.5) | 282 (56.9) | |
| Total serum 25(OH)D (ng/mL)[1] | 24.1 (6.7–53.7) | 25.3 (10.6–53.7) | 20.9 (6.7–40.7) | < 0.001[3] |
| 25(OH)$D_3$ (ng/mL)[1] | 22.1 (5.8–48.5) | 23.3 (9.6.–48.5) | 19.2 (5.8–38.7) | |
| 25(OH)$D_2$ (ng/mL)[1] | 0.50 (0.00–10.6)* | 0.50 (0.0–10.6)* | 0.50 (0.0–10.0)* | |
| 3-Epi-25(OH)$D_3$ (ng/mL)[1] | 1.31 (0.01–14.20) | 1.43 (0.01–14.20) | 1.02 (0.01–5.27) | |
| 3-Epi-25(OH)$D_2$ (ng/mL)[1] | 0.00 (0.00–0.43)** | 0.00 (0.00–0.36)** | 0.00 (0.00–0.43)** | |

*Notes:* the median (range) levels of non-C3-epimers and C3-epimers were 22.7 (6.4–49.2) ng/mL and 1.3 (0.01–14.2) ng/mL, respectively.
[1] Median (range: min–max)
[2] Number and percentage
A *p*-value indicated significance between male and female by Mann-Whitney U test [3], by chi-square test [4]
* The concentration of 25(OH)$D_2$ was 0 in 17 sera subjects.
** The concentration of 3-epi-25(OH)$D_2$ was 0 in 1718 sera subjects.

*The contribution of each vitamin D metabolite on total serum 25(OH)D and their correlation*

Within the total serum 25(OH)D, 25(OH)$D_3$ showed the highest contributor to total serum 25(OH)D concentration (92.18%) (Table 2). In contrast, 25(OH)$D_2$ contribution totaled 2.06%. Concerning C3-epimer fraction, the 3-epi-25(OH)$D_3$ metabolite was quantified among all participants. This metabolite contributed 5.52% of total serum 25(OH)D levels, whereas the 3-epi-25(OH)$D_2$ metabolite was quantified in only 9 participants. Thus, the contribution of 3-epi-25(OH)$D_2$ was observed at 0%. We found a significantly positive correlation between 3-epi-25(OH)$D_3$ and total serum 25(OH)D levels ($r = 0.639$, $P < 0.001$).

**Table 2**
The non-C3-epimer and C3-epimer fractions expressed in percentage of total serum 25(OH)D levels and their correlation (N = 1727).

| Vitamin D metabolite | Percentage contribution to total serum 25(OH)D | Pearson's correlation coefficient (r) | *p*-value[2] |
|---|---|---|---|
| **Non-C3-epimers** | | | |
| 25(OH)$D_3$ | 92.18 (54.15–97.73)[1] | 0.990 | < 0.001 |
| 25(OH)$D_2$ | 2.06 (0.00*–40.79)[1] | 0.157 | < 0.001 |
| **C3-epimers** | | | |
| 3-epi-25(OH)$D_3$ | 5.52 (0.05–32.15)[1] | 0.639 | < 0.001 |
| 3-epi-25(OH)$D_2$ | 0.00 (0.00**–1.57)[1] | 0.018 | 0.045 |

* The concentration of 25(OH)$D_2$ was 0 in 17 sera subjects.
** The concentration of 3-epi-25(OH)$D_2$ was 0 in 1718 sera subjects.
[1] The percentage contribution was presented as median (min–max).
[2] All vitamin D metabolite levels used a natural logarithmic (Ln) transformation for Pearson correlation analysis.

*SNP characteristics*

SNP characteristics of the study population are shown in Table 3. The rs12785878 G > T variant (*DHCR7/NADSYN1* gene), the allele distribution was 51% GG, 41% GT and 8% TT. The prevalence of the minor allele (T) was 28.7%. Moreover, the rs2060793 G > A variant (*CYP2R1* gene), the allele distribution was 47% GG, 42% GA and 11% AA. The prevalence of the minor allele (A) was 32.5%. In addition, the rs2282679 T > G variant (*GC* gene), the allele distribution was 59% TT, 35% TG and 6% GG. The prevalence of the minor allele (G) was 23.7%. The genotype distributions of all SNPs agreed with HWE ($P_{HWE} = 0.8292$, 0.0558 and 0.2786, respectively).

*Associations between genetic polymorphisms and vitamin D metabolite levels*

In the candidate gene/SNP analysis, the effect of three SNPs on total serum 25(OH)D, non-C3-epimer and C3-epimer levels were analyzed under three different genetic models: dominant, recessive and additive effects (Table 4). Simple linear regression analysis with a natural logarithmic (Ln) transformation of each vitamin D metabolite showed that the additive genetic model provided a better fit for all SNPs.

The *DHCR7/NADSYN1* (rs12785878) polymorphism showed an effect on the variation in total serum 25(OH)D levels at *p*-value 0.001. Interestingly, after non-C3-epimers were analyzed separately, the rs12785878 polymorphism was strongly associated with C3-epimer levels ($p = 1.66 \times 10^{-7}$), but not with non-C3-epimer levels ($p = 0.002$). Participants who carried the minor allele T (GT and TT genotypes) exhibited higher levels of C3-epimers, compared with those carrying the GG genotype. The additive genetic model of the rs12785878 polymorphism (model 1) could explain the variation in C3-

**Table 3**
SNP characteristics of the study population (N = 1727).

| SNP (Gene) | Chromosome | M/m | MAF (%) | Genotype | n (%) | HWE *p*-value |
|---|---|---|---|---|---|---|
| 1. rs12785878 (*DHCR7/NADSYN1*) | 11q13.4 | G/T | 28.7 | GG<br>GT<br>TT | 880 (51)<br>703 (41)<br>144 (8) | 0.8292 |
| 2. rs2060793 (*CYP2R1*) | 11p15.2 | G/A | 32.5 | GG<br>GA<br>AA | 804 (47)<br>723 (42)<br>200(11) | 0.0558 |
| 3. rs2282679 (*GC*) | 4q12-q13 | T/G | 23.7 | TT<br>TG<br>GG | 1014 (59)<br>608 (35)<br>105 (6) | 0.2786 |

*Abbreviations*: M/m Major allele/ minor allele, MAF minor allele frequency.
Hardy-Weinberg equilibrium (HWE), HWE *p*-value was calculated from $\chi^2$ test.

epimer levels accounting for 1.5% ($R^2 = 0.015$, F = 27.63) (Table 5). In addition, the additive genetic model with the covariates of sex and age showed significance in determining C3-epimer levels accounting for 17.2% [model 2; regression equation; $y = 0.054 + 0.088$ (rs12785878) $- 0.376$(female) $+ 0.007$(age); $p = 8.13 \times 10^{-71}$]. Moreover, 17.4% of the variability in C3-epimer levels was explained by rs12785878 polymorphism, sex, age and BMI as predictors [model 3; regression equation; $y = -0.108 + 0.089$(rs12785878) $- 0.360$(female) $+ 0.007$(age) $+ 0.007$(BMI); $p = 4.29 \times 10^{-71}$].

The *CYP2R1* (rs2060793) polymorphism associated with total serum 25(OH)D levels ($P = 7.60 \times 10^{-8}$) (Table 4). After non-C3-epimers and C3-epimers were analyzed separately, the rs2060793 polymorphism was only associated with non-C3-epimer levels ($p = 2.46 \times 10^{-8}$). Increased non-C3-epimer levels were observed in individuals carrying the minor allele A (GA and AA genotypes). In Table 5, the additive genetic model for rs2060793 polymorphism (model 1) provided information to determine non-C3-epimer levels by accounting for 1.7% ($R^2 = 0.017$, F = 31.38). In addition, as shown in models 2–3, the predictors including rs2060793 polymorphism, sex, age, but not BMI could explain the variation in non-C3-epimer levels accounting for 14.4% [model 2; regression equation; $y = 2.954 + 0.049$ (rs2060793) $- 0.188$(female) $+ 0.005$(age); $p = 2.30 \times 10^{-58}$].

The *GC* (rs2282679) polymorphism associated with total serum 25(OH)D levels ($p = 1.54 \times 10^{-19}$) (Table 4). Analysis of separated C3-epimers revealed that this SNP was significantly associated with both non-C3-epimer levels ($p = 7.30 \times 10^{-20}$) and C3-epimer levels ($p = 1.10 \times 10^{-5}$). Participants who carried the minor allele G (TG and GG genotype) exhibited a trend to decrease both metabolite levels. In Table 5, the additive model for rs2282679 polymorphism (model 1) explained the variation in non-C3-epimer and C3-epimer levels accounting for 4.7% ($R^2 = 0.047$, F = 85.30) and 1.1% ($R^2 = 0.011$, F = 19.43), respectively. Interestingly, after adding covariates, 17.9% of the variability in non-C3-epimer levels was explained by rs2282679 polymorphism, sex, age, but not BMI [model 2; regression equation; $y = 3.014–0.097$(rs2282679) $- 0.190$(female) $+ 0.005$(age); $p = 4.10 \times 10^{-74}$]. On the other hand, 17.2% of the variability in C3-epimer levels was explained by rs2282679 polymorphism, sex, age and BMI [model 3, regression equation; $y = -0.033–0.086$(rs2282679) $- 0.361$(female) $+ 0.007$(age) $+ 0.007$(BMI); $p = 5.02 \times 10^{-70}$].

## Discussion

Concerning the main point of this study, the complexity of C3-epimer among humans should be elucidated. Identifying genetic polymorphisms will provide insights regarding the physiological regulation of serum C3-epimers. Here, we explored the associations between SNPs/genes related to vitamin D native pathways, *DHCR7/NADSYN1* (rs12785878), *CYP2R1* (rs2060793) and *GC* (rs2282679), and serum C3-epimer levels among Thai subjects. In 1727 participants, the percentage distribution of C3-epimers on total serum 25(OH)D concentration was 5.52%. Regression analyses revealed that genetic underlying C3-epimers differed from non-C3-epimers. The variants in *DHCR7/NADSYN1* (rs12785878) and *GC* (rs2282679) showed significant associations with C3-epimers, while the variants in *CYP2R1* (rs2060793) found significant association with only non-C3-epimers.

Regarding *DHCR7/NADSYN1* (rs12785878; G > T), slightly increased total serum 25(OH)D levels were observed among individuals carrying the minor allele T. After distinguishing C3-epimers from non-C3-epimers, the rs12785878 polymorphism was only associated with C3-epimer levels. As evidenced by reduced *p*-value thresholds (0.001 to 0.002), increased C3-epimer levels interfered with total serum 25(OH)D levels. Furthermore, because rs12785878 is located near the 7-dehydrocholesterol reductase gene [36], it is involved in vitamin $D_3$ synthesis in the skin. Two epidemiologic studies [26,29] and one study using

**Table 4**
Association of polymorphisms with serum vitamin D metabolite levels under three genetic models (N = 1727).

| SNP | M/m | Median levels (ng/mL) | | | | *p*-value* | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Additive | Dominant | Recessive |
| 1. rs12785878 (*DHCR7/NADSYN1*) | G/T | Total serum 25(OH)D | 23.80 | 24.37 | 24.54 | 0.001 | 0.001 | 0.077 |
| | | Non-C3-epimers | 22.43 | 22.94 | 22.82 | 0.002 | 0.002 | 0.155 |
| | | C3-epimers | 1.25 | 1.34 | 1.50 | **$1.66 \times 10^{-7}$** | $2.00 \times 10^{-6}$ | $3.89 \times 10^{-4}$ |
| 2. rs2060793 (*CYP2R1*) | G/A | Total serum 25(OH)D | 23.40 | 24.37 | 25.61 | **$7.60 \times 10^{-8}$** | $5.48 \times 10^{-7}$ | $3.66 \times 10^{-4}$ |
| | | Non-C3-epimers | 22.06 | 22.94 | 24.45 | **$2.46 \times 10^{-8}$** | $1.97 \times 10^{-7}$ | $2.31 \times 10^{-4}$ |
| | | C3-epimers | 1.29 | 1.33 | 1.37 | 0.093 | 0.123 | 0.252 |
| 3. rs2282679 (*GC*) | T/G | Total serum 25(OH)D | 24.92 | 23.24 | 19.78 | **$1.54 \times 10^{-19}$** | $5.80 \times 10^{-15}$ | $6.26 \times 10^{-12}$ |
| | | Non-C3-epimers | 23.57 | 21.85 | 18.48 | **$7.30 \times 10^{-20}$** | $3.16 \times 10^{-15}$ | $4.46 \times 10^{-12}$ |
| | | C3-epimers | 1.36 | 1.28 | 1.10 | **$1.10 \times 10^{-5}$** | $1.43 \times 10^{-4}$ | 0.001 |

*Abbreviations*: M/m Major allele/minor allele.
Total serum 25(OH)D levels, non-C3-epimer levels and C3-epimer levels are presented as median levels (ng/mL).
All vitamin D metabolite levels used a natural logarithmic (Ln) transformation for simple linear regression analysis.
* Simple linear regression provided *p*-values; $p < 0.001$ was considered statistically significant. Bold font indicates the additive model was a better fit for models.

**Table 5**
Linear regression models for associations between the genetic variants and vitamin D metabolite levels (N = 1727).

| SNP | M/m | Vitamin D metabolite | Model 1 | | | Model 2 | | | Model 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $R^2$ | F-value | *p*-value* | $R^2$ | F-value | *p*-value* | $R^2$ | F-value | *p*-value* | Remark |
| 1. rs12785878 (*DHCR7/NADSYN1*) | G/T | C3-epimers | 0.015 | 27.63 | $1.66 \times 10^{-7}$ | 0.172 | 120.45 | $8.13 \times 10^{-71}$ | 0.174 | 92.18 | $4.29 \times 10^{-71}$ | BMI ($p = 0.012$) |
| 2. rs2060793 (*CYP2R1*) | G/A | Total serum 25(OH)D | 0.016 | 29.16 | $7.60 \times 10^{-8}$ | 0.156 | 107.02 | $1.56 \times 10^{-63}$ | 0.155 | 80.23 | $1.71 \times 10^{-62}$ | BMI ($p = 0.832$) |
| | | Non-C3-epimers | 0.017 | 31.38 | $2.46 \times 10^{-8}$ | 0.144 | 97.63 | $2.30 \times 10^{-58}$ | 0.143 | 73.26 | $2.19 \times 10^{-57}$ | BMI ($p = 0.614$) |
| 3. rs2282679 (*GC*) | T/G | Total serum 5(OH)D | 0.046 | 83.75 | $1.54 \times 10^{-19}$ | 0.192 | 137.36 | $8.63 \times 10^{-80}$ | 0.191 | 103.01 | $1.01 \times 10^{-78}$ | BMI ($p = 0.674$) |
| | | Non-C3-epimers | 0.047 | 85.30 | $7.30 \times 10^{-20}$ | 0.179 | 126.61 | $4.10 \times 10^{-74}$ | 0.179 | 95.06 | $3.88 \times 10^{-73}$ | BMI ($p = 0.472$) |
| | | C3-epimers | 0.011 | 19.43 | $1.10 \times 10^{-5}$ | 0.170 | 118.77 | $6.49 \times 10^{-70}$ | 0.172 | 90.68 | $5.02 \times 10^{-70}$ | BMI ($p = 0.019$) |

*Abbreviations:* M/m Major allele/ minor allele, $R^2$ represented adjusted R-square in linear regression analysis.
Model 1; simple linear regression analysis with genetic polymorphism as an independent variable.
Model 2; multiple linear regression analysis with genetic polymorphism, sex and age as independent variables.
Model 3; multiple linear regression analysis with genetic polymorphism, sex, age and BMI as independent variables.
A *p*-value $< 0.001$ was considered statistically significant.

a mouse model [37] reported that 3-epi-25(OH)$D_3$ levels increased during summer season and UV exposure. Therefore, the rs12785878 polymorphism reveals a genetic explanation for the metabolic pathway of C3-epimerization created by the endogenous system.

The *CYP2R1* gene encodes biologically relevant vitamin D 25-hydroxylase among humans [36]. This enzyme is related to the conversion of vitamin D ($D_2$ and $D_3$) to 25(OH)D metabolite (the major circulating form). A number of studies have shown an association between *CYP2R1* gene polymorphisms and vitamin D status [38–43]. With our additive genetic models, the *CYP2R1* (rs2060793; G > A) polymorphism was associated with total serum 25(OH)D levels. This was consistent with related findings in Chinese populations [38,44] and of Ahn et al. [31]. However, in our study, the rs2060793 variant was clearly associated only with non-C3-epimer levels. This suggests that an enzyme responsible for vitamin D C3-epimerization may differ from vitamin D native metabolite pathways. According to related *in vitro* studies [15,16], the highest amount of 3-epi-25(OH)$D_3$ was observed in liver cells, after incubating 25(OH)$D_3$. Moreover, among adult patients with liver disease, their sera could not reveal 3-epi-25(OH)$D_3$ metabolites [20]. Taken together, the 3-epi-25-hydroxylase might be related to hepatic cells. Further studies should identify other polymorphisms in the hepatic human *CYPs* genes.

Regarding the *GC* (rs2282679; T > G) polymorphism, we observed a significant association with total serum 25(OH)D levels. This result was consistent with the findings of GWAS in European [30,31], Chinese [45] and Arab [46] populations. Although the analysis was performed using separate C3-epimers, the rs2282679 polymorphism was significantly associated with non-C3-epimer and C3-epimer levels. Notably, both metabolite levels tended to decline with each additional minor allele G (risk allele), resulting in reduced total serum 25(OH)D levels. Because the variant in the *GC* gene is involved in the vitamin D binding protein (protein transporter) (41), our results suggested that the rs2282679 polymorphism in the *GC* gene regulated total serum 25(OH)D concentrations by influencing non-C3-epimer and C3-epimer levels. On the other hand, C3-epimeric forms have shown less biological activity than their native form such as the potential for calcemic effects and vitamin D receptor affinity [19]. Therefore, the DBP-bound C3-epimer may interrupt the bioavailability of vitamin D. We recommend that further studies should focus on the clearance of these metabolites and how this affects vitamin D status.

In our adjusted additive genetic models, age was found to be a covariate. Aging is a common factor of vitamin D deficiency. However, negative associations between age and vitamin D metabolite levels were not observed. This could be explained by the characteristics of the study participants. Their median age was 40 (maximum 54); no elderly people were enrolled in the study population. Moreover, all equations revealed that being female had a major effect on varying non-C3-epimer and C3-epimer levels. This may be based on the high prevalence of vitamin D deficiency found among women. Our results were consistent with Thai studies that Thai women living in urban areas especially the Bangkok Metropolitan Area exhibited low vitamin D levels.[47]. In addition, lifestyle factors, i.e., time spent indoors, as well as sunscreen use are major risks of low vitamin D levels among women [48]. With regard to the potential predictors, BMI was classified as an independent variable for C3-epimer levels [*DHCR7/NADSYN1* (rs12785878) and *GC* (rs2282679)]. Although the predictor's *p*-value did not show statistical significance, the magnitude of R-square strongly explained variations from 17.2% to 17.4% and from 17.0% to 17.2%, respectively after adding BMI in the regression models. Furthermore, in regression model 3 of *CYP2R1* (rs2060793) and *GC* (rs2282679), BMI reduced $R^2$ and *p*-value thresholds to explain variation in non-C3-epimers. Clearly, BMI was not a potential predictor for non-C3-epimer levels. Concerning those opposite effects of genetic underlying and potential predictors on C3-epimers and its fractions from our multiple regression analyses, these indicate that further studies should focus on separating C3-epimeric forms.

Regarding all subjects in this study, the contribution of 25(OH)$D_2$ on total serum 25(OH)D was about 2%. This may be explained by the fact that vitamin $D_2$ is scarce in Thai foods. However, 25(OH)$D_3$ was abundant in all sera, implying that the participants obtained vitamin D predominantly by cutaneous synthesis, as vitamin $D_3$ (27). The 3-epi-25(OH)$D_3$ has been described across ethnic adult populations which contributed between 2.5 and 43% of total serum 25(OH)D levels (25). The correlation between 3-epi-25(OH)$D_3$ and total serum 25(OH)D or 25(OH)$D_3$ levels revealed a positive relationship using Pearson's correlation coefficient (*r*) ranging from 0.6 to 0.8 [49]. In our study, the results were consistent with related reports.

The major strengths of this study included a larger sample size, using the analytical method to quantify vitamin D metabolites, measured by LC-MS/MS. We quantified vitamin D metabolites in four forms providing exact values of non-C3-epimer and C3-epimer levels to explore genetic associations. Moreover, we used three SNPs/genes to focus on the vitamin D pathway, beginning with synthesis in the skin, the first step of vitamin D metabolites and followed by vitamin D transported in the blood circulation. Our study had limitations that should be addressed. The participants were not representative for the general Thai population. Moreover, potentially important factors including sunlight exposure, diet, sartorial habits and use of sunscreen and vitamin D supplements were not considered. In addition, we did not report subjects' ethnicities. Concerning these points, further cohort and clinical studies should be conducted to determine the relevance between sex and other ethnic groups to better understand the genetic associations related to C3-epimers of vitamin D metabolites.

## Conclusion

In summary, the *DHCR7/NADSYN1* (rs12785878) and *GC* (rs2282679) were associated with serum C3-epimer levels. This indicated a fundamental knowledge gap regarding the genetic determinants of endogenous C3-epimerization. An analysis using different C3-epimeric forms should be conducted to clarify the underlying vitamin D etiology, vitamin D related diseases, clinical relevance of vitamin D and potential factors concerning vitamin D status.

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