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Value Assignment of Vitamin D Metabolites in Vitamin D Standardization Program (VDSP) Serum Samples

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Abstract

Assay variability has been cited as an obstacle to establishing optimal vitamin D exposure. As part of the Vitamin D Standardization Program (VDSP) effort to standardize the measurement of total 25(OH)D, value assignment of total 25(OH)D in 50 single donor serum samples was performed using two isotope-dilution LC-MS/MS methods. Both methods are recognized as reference measurement procedures (RMPs) by the Joint Committee for Traceability in Laboratory Medicine. These samples and their assigned values serve as the foundation for several aspects of the VDSP. To our knowledge, this is the first time that two RMPs have been used to assign 25(OH)D values to such a large number of serum samples.

Introduction

Measurement of circulating 25-hydroxyvitamin D [25(OH)D] is the accepted indicator of vitamin D exposure (1, 2). Several factors, including media attention, have led to considerable growth in the number of 25(OH)D tests performed each year. As a result, there has also been an increase in the number of assay platforms, both commercial and laboratory-developed, for 25(OH)D. Numerous publications have described comparisons among immunoassays for 25(OH)D or have compared immunoassay approaches to those based upon liquid chromatography coupled to mass spectrometry (LC-MS or LC-MS/MS) (3–6). Although such studies are informative from the standpoint of characterizing assay performance, they have also highlighted the lack of comparability among different assays. Assay variability has been identified as a significant obstacle to the accurate diagnosis of vitamin D deficiency (7–9).

A primary objective of the Vitamin D Standardization Program (VDSP) is the standardization of total 25(OH)D measurements over time, location, and laboratory procedure (10, 11). Total 25(OH)D is defined in the context of the VDSP as the sum of the 25(OH)D₂ and 25(OH)D₃ concentrations. Standardization of total 25(OH)D measurements is necessary for comparing data across different populations, pooling and interpreting the results of research studies, and for ensuring appropriate decision making by medical professionals (12, 13). To achieve this objective, the VDSP incorporates several components designed to link the results obtained by the end user of a routine assay to a true value as determined by a reference measurement procedure (RMP) (11). These components comprise a reference measurement system and include reference methods, reference materials, accuracy-based performance testing (PT)/external quality assessment (EQA) schemes, and an assay standardization-certification program. The VDSP has also undertaken a research program directed toward understanding and improving the laboratory measurement of total 25(OH)D.

As part of this VDSP effort, concentrations of total 25(OH)D were determined in 50 single donor serum samples selected to span a range from approximately 10 nmol/L to 150 nmol/L and which included samples with endogenous 25(OH)D₂. These samples and their assigned values will serve as the foundation for several aspects of the VDSP, including assessment of the performance of assays for 25(OH)D, evaluating the commutability of reference materials and quality assurance testing materials, and in developing study designs for standardizing data from completed national health surveys. Concentrations of total 25(OH)D were determined by two independent isotope-dilution (ID) liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods developed by the National Institute of Standards and Technology (NIST)(14) and by Ghent University (15). Both methods are recognized as RMPs by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) (16).

Each of the 50 samples was analyzed in triplicate by Ghent University and in duplicate by NIST. Standard Reference Material (SRM) 972 Vitamin D in Human Serum (17) was employed as a measurement quality assurance material by both laboratories. Each laboratory provided results for 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃. Although the biological function of the 3-epimer of 25(OH)D₃ has not yet been elucidated, it is detectable in many adult serum samples and can interfere with the determination of 25(OH)D₃ by mass spectrometry-based methods (18, 19). The protocols used for assigning values to each sample and for evaluating uncertainties associated with the assigned values are described in this manuscript along with observations about the performance of each of the RMPs.

Experimental

Materials

Single donor serum samples were collected by Solomon Park Research Laboratories (Kirkland, WA) in accordance with specifications provided by the Centers for Disease Control and Prevention (CDC) and using the procedures described in the Clinical and Laboratory Standards Institute (CLSI) C37-A protocol but without filtration (20). Serum samples were initially screened by LC-MS/MS at CDC to identify those suitable for inclusion in the study. These orientation values were provided to the two reference laboratories, NIST and Ghent University, to assist in determining appropriate calibration solution and internal standard concentrations for the RMPs.

Measurements by ID LC-MS/MS at NIST

The NIST RMPs for 25(OH)D₂ and 25(OH)D₃ have been described previously (14), and details specific to this study are included in the supplemental material. Briefly, serum samples were spiked with internal standard solution, the pH was adjusted, and the samples were subjected to liquid-liquid extraction with hexane:ethyl acetate. Each sample was analyzed on two different days, and isotopically labeled internal standards [25(OH)D₂-*d*₃, 25(OH)D₃-*d*₆, and 3-epi-25(OH)D₃-*d*₃] were utilized for the quantification of 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃, respectively. Two analysts performed the measurements at NIST, with each analyst responsible for 25 of the single donor samples. In addition, two different tandem mass spectrometry (MS/MS) instruments, an API 4000 and an API 5000 (both from AB Sciex), were employed for the measurement of the desired analytes. Limits

of quantitation (LOQs) for 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃ were 0.5 ng/g for one analyst and 1.0 ng/g for the other. Results were reported in mass fractions (ng/g) and were converted to ng/mL using the measured density of each serum sample. Density measurements were performed by Ghent University.

Measurements by ID LC-MS/MS at Ghent

The Ghent University RMPs for 25(OH)D₂ and 25(OH)D₃ have been outlined previously (15), and details specific to this study are provided in the supplemental information. Samples were spiked with internal standard, alkalized, extracted with hexane, and fractionated chromatographically. Each sample was analyzed on three different days, and isotopically labeled internal standards [25(OH)D₂-d₆ and 25(OH)D₃-d₆] were utilized for quantification of 25(OH)D₂ and 25(OH)D₃, respectively. Concentrations of 3-epi-25(OH)D₃ were estimated by assuming equivalent response factors for 25(OH)D₃ and 3-epi-25(OH)D₃. All results were converted from ng/g to ng/mL using the measured density of each serum sample. The LOQs for 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃ were 0.6 ng/mL.

Results and Discussion

Routine assays for 25(OH)D differ in the way that values for total 25(OH)D are obtained and reported. Immunoassays are generally developed to detect both 25(OH)D₃ and 25(OH)D₂, although there has been debate about the extent of cross-reactivity with 25(OH)D₂ for certain assays (21–23). Cross-reactivity with other hydroxylated metabolites can also occur and contribute to the observed total 25(OH)D concentration (24). On the other hand, cross-reactivity with 3-epimers of 25(OH)D is generally thought to be low for most immunoassays and is not likely to contribute to the value obtained for total 25(OH)D (21).

In mass spectrometry-based platforms, the individual metabolites 25(OH)D₂ and 25(OH)D₃ can be detected and quantified. Values for total 25(OH)D are then obtained by summing the concentrations of 25(OH)D₂ and 25(OH)D₃. As noted previously, the 3-epimer of 25(OH)D₃ can interfere with the determination of 25(OH)D₃ in mass spectrometry assays because it has the same molecular mass and generates similar fragmentation patterns. Failure to resolve this isomer chromatographically can result in overestimation of the concentration of 25(OH)D₃ (25). Routine mass spectrometry methods may employ chromatographic approaches that do not resolve the epimers in the interest of higher sample throughput.

Previous assessments of assay platforms for 25(OH)D have illustrated both within-method and between-method variability (13, 26, 27). Lack of method comparability and changes in assay performance over time have proven to be obstacles in establishing recommendations for optimal vitamin D exposure (8, 28, 29). The majority of assay comparisons reported to date have been limited in their ability to assess performance of 25(OH)D assays because they have not employed reference methods or reference materials as an anchor point for the comparisons. A study by Moon et al. used Standard Reference Material (SRM) 972 Vitamin D in Human Serum to evaluate the accuracy of several 25(OH)D assays, but some of the method bias observed may have been linked to the fact that certain levels of SRM 972 were prepared using non-human serum or exogenous 25(OH)D₂ (5). Nevertheless, the authors

concluded that the use of uniform decision points for identifying vitamin D deficiency was problematic without assay standardization.

As a foundation for standardization of 25(OH)D measurements, the VDSP elected to assign values for total 25(OH)D to a set of 50 single-donor human serum samples having endogenous vitamin D metabolite concentrations. These samples could be utilized in a variety of ways including an evaluation of routine assay accuracy (bias) and precision. RMPs are the preferred approach to value assignment of reference materials and other samples used in standardization efforts. Hence, the RMPs developed by NIST and by Ghent University were used to assign values for vitamin D metabolites in these VDSP samples. The serum samples were selected to span a wide range of 25(OH)D concentrations and included samples with endogenous 25(OH)D₂.

This study represents the first time, to our knowledge, that two RMPs have been used to assign 25(OH)D values to such a large number of samples. Although the methods are both based upon ID-LC-MS/MS, they differ in the specific procedures used for sample preparation and analysis. Therefore, one aspect of this study involved assessing the concordance of results from the two methods. The use of two or more independent analytical methods is a common approach to value assignment of reference materials at NIST because it reduces the likelihood of undetected measurement errors or bias (30, 31).

A few differences between the NIST and Ghent methods are worth noting prior to discussion of specific results for 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃, and total 25(OH)D. Both methods employ liquid-liquid extraction for isolation of the vitamin D metabolites. The Ghent method includes an additional fractionation step by Sephadex LH20 chromatography. The Ghent method also employs a two-dimensional chromatographic approach, with a C₄ column in the first dimension and either a C₁₈ or cyanopropyl (CN) column in the second dimension. The C₁₈ column was used for quantification of 25(OH)D₂, and the CN column provided chromatographic separation between 25(OH)D₃ and 3-epi-25(OH)D₃. In the Ghent method, the concentration of 3-epi-25(OH)D₃ in each sample was estimated by assuming equal response factors for 25(OH)D₃ and its 3-epimer. In the NIST method, a pentafluorophenyl (PFP) chromatographic stationary phase was used for the determination of 25(OH)D₂, and a CN column was used for 25(OH)D₃ and 3-epi-25(OH)D₃. Isotopically labeled internal standards were employed for all three analytes in the NIST measurements.

25(OH)D₂

Only 17 of the 50 samples had 25(OH)D₂ concentrations that were above the LOQ for both laboratories; results for those samples with 25(OH)D₂ concentrations below the LOQ are not included in Table 1. The fact that 25(OH)D₂ concentrations were below the LOQ in more than half the samples was not surprising because, in general, 25(OH)D₂ is only detected in serum if supplementation with vitamin D₂ has occurred. The concentrations ranged from approximately 1.4 nmol/L to 19 nmol/L. Good agreement between the two methods (NIST and Ghent) was consistently observed, even when concentrations of 25(OH)D₂ were < 5 nmol/L, and relative standard deviations (%RSD) for the two methods were nearly always less than 3.5%.

25(OH)D₃

In most individuals, 25(OH)D₃ is the major contributor to the observed total 25(OH)D concentration, and 25(OH)D₃ was detected at concentrations above the LOQ in all 50 samples (Table 2). Both the LC-MS/MS methods incorporated chromatographic resolution of 25(OH)D₃ from its 3-epimer to ensure that the presence of 3-epi-25(OH)D₃ in the samples did not introduce measurement bias. Concentrations of 25(OH)D₃ in the 50 samples ranged from approximately 12 nmol/L to 140 nmol/L, as shown in Table 2. Excellent measurement precision was observed for both methods across this concentration range, with %RSD values frequently below 2%.

3-Epi-25(OH)D₃

The majority of the samples (40 of 50) were found to have concentrations of 3-epi-25(OH)D₃ above the LOQ (Table 3). The observed results are in agreement with previous studies reporting that 3-epi-25(OH)D₃ is present in most adult sera (18, 19). Because the concentrations of 3-epi-25(OH)D₃ determined by the Ghent laboratory were estimated by assuming equivalent response factors for 25(OH)D₃ and 3-epi-25(OH)D₃, they were not used in the final value assignment. Nevertheless, the Ghent results provide supporting evidence for the assigned values and generally were in good agreement with the NIST data. Concentrations of 3-epi-25(OH)D₃ in the 40 samples ranged from approximately 1.4 nmol/L to 15 nmol/L.

Comparison of Method Results/Value Assignment

Figure 1 shows a Bland-Altman plot (32) comparing the NIST and Ghent results for total 25(OH)D, which as noted previously, corresponded to the sum of the 25(OH)D₂ and 25(OH)D₃ concentrations. The total 25(OH)D concentrations included values for 25(OH)D₂ that were below the LOQ. Results for each sample (blue dots) are shown with standard uncertainties (u) for both the average of method results and their difference (33). The results suggest a tendency for the NIST RMP to measure slightly lower concentrations of total 25(OH)D than the Ghent RMP, with an average difference of -0.52 ng/mL (u = 0.04 ng/mL). This corresponds to a relative average difference between methods less than 1.8% based on the median concentration (30.17 ng/mL) of total 25(OH)D in these samples. The Bland-Altman plot also suggests that the differences between methods may increase with higher total 25(OH)D concentrations, but the slope of a Deming regression line fitted to the data (34), taking into account the uncertainties in both variables, did not differ significantly from zero (results not shown). Although the method comparison does reveal a small but statistically significant difference between the averages, it is impossible to attribute any bias to one laboratory or the other, or both, without further study. Results from each laboratory for the measurement quality assurance material SRM 972 were also used to evaluate bias, and none of the biases differed significantly from zero.

Values for 25(OH)D₂ and 25(OH)D₃ were assigned to each of the 50 samples based upon the average of the averages of determinations made by NIST and Ghent. Results for 3-epi-25(OH)D₃ were based solely on the average of results from NIST. The assigned values for all 50 samples are summarized in Table 4, and the distribution of metabolites for each of the 50 samples is shown in Figure 2. An uncertainty evaluation was also performed for each

of the assigned values, including 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃, and total 25(OH)D and included uncertainty components arising from within-laboratory and between-laboratory measurement dispersion as well as from calibration of the methods (33). As can be seen in the table, most of the values have small uncertainties, and the median coefficient of variation for total 25(OH)D was 1.8%.

Conclusions

Standardization of 25(OH)D measurements is important for accurate and consistent identification of inadequate and/or deficient vitamin D levels and related health consequences in individuals and populations. Value assignment of the 50 single donor samples described here represents a fundamental component of the VDSP and supports its goal to improve clinical and public health practice worldwide. In addition, this study provided evidence of the comparability of two RMPs for vitamin D metabolites, providing further confidence in measurements based on these methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Bland-Altman plot comparing total 25(OH)D results from the NIST and Ghent methods. Each sample (blue dot) is shown with the associated standard uncertainties (u) for the method average (horizontal lines) and the method difference (vertical lines). The solid red line represents an outlier-resistant summary of the method differences, and the dashed red lines are the limits of agreement.

Figure 2. Distribution of vitamin D metabolites in the 50 VDSP single donor samples (1–50). All results in nmol/L.

Table 1

Summary of NIST and Ghent results for 25(OH)D₂ in 50 single donor samples. The mean values (nmol/L) for each laboratory are listed with relative standard deviations (% RSD) in parentheses. Samples with 25(OH)D₂ concentrations below the LOQ are not included in the table.

Sample ID	NIST	Ghent
2	3.70 (0)	3.76 (2.2)
4	7.34 (0)	7.42 (2.0)
5	1.83 (0.9)	1.85 (0)
7	5.40 (1.6)	5.45 (2.0)
12	1.44 (1.2)	1.43 (1.0)
13	2.96 (0)	2.95 (3.7)
14	5.74 (1.5)	5.83 (1.1)
18	2.10 (0)	2.14 (3.5)
26	1.90 (1.8)	1.87 (1.2)
28	1.50 (3.5)	1.44 (0.8)
36	1.74 (2.0)	1.77 (1.6)
37	1.76 (1.0)	1.75 (2.2)
41	18.74 (2.1)	19.11 (2.1)
42	3.40 (2.6)	3.28 (0.7)
45	2.32 (2.3)	2.40 (1.9)
46	2.46 (1.8)	2.41 (3.4)
47	6.34 (5.1)	6.21 (1.1)

Table 2

Summary of NIST and Ghent results for 25(OH)D₃ in 50 single donor samples. The mean values (nmol/L) for each laboratory are presented with relative standard deviations (% RSD) in parentheses.

Sample ID	NIST	Ghent
1	84.89 (0.6)	85.02 (1.2)
2	43.90 (0.6)	44.57 (0.1)
3	111.66 (0)	112.86 (1.5)
4	105.35 (0.3)	106.56 (1.0)
5	22.93 (1.3)	23.60 (1.4)
6	12.29 (0.1)	12.59 (1.7)
7	77.38 (0.1)	79.14 (2.1)
8	77.32 (0.2)	78.19 (2.7)
9	72.91 (1.6)	73.73 (1.9)
10	51.89 (2.9)	52.80 (2.3)
11	47.33 (1.7)	47.48 (1.5)
12	63.98 (0.1)	66.10 (1.0)
13	52.60 (0.2)	54.43 (1.9)
14	86.12 (0)	89.25 (1.4)
15	81.61 (0.1)	83.81 (1.9)
16	77.40 (3.5)	77.24 (1.1)
17	66.55 (0.7)	64.90 (1.4)
18	80.41 (0.3)	82.97 (0.4)
19	84.97 (0.4)	87.91 (1.1)
20	32.53 (0.8)	33.82 (3.1)
21	71.31 (0.5)	71.88 (0.3)
22	56.82 (0.5)	57.91 (0.3)
23	61.31 (4.1)	62.02 (2.3)
24	67.57 (0.6)	67.02 (0.8)
25	41.81 (0.1)	43.43 (3.1)
26	29.88 (0.3)	30.93 (2.8)
27	97.56 (0.4)	95.11 (2.9)
28	138.48 (0.2)	140.32 (2.7)
29	14.32 (0.8)	14.29 (0.9)
30	63.18 (2.4)	64.58 (0.4)
31	72.79 (2.3)	72.56 (0.5)
32	106.35 (0.5)	109.35 (1.0)
33	103.77 (0.1)	108.23 (1.2)
34	32.21 (1.8)	33.06 (1.6)
35	61.54 (0.8)	61.79 (0.8)
36	60.90 (0.2)	60.98 (1.0)

Sample ID	NIST	Ghent
37	68.03 (0.3)	68.41 (1.0)
38	58.84 (0.1)	59.55 (1.9)
39	89.14 (0.5)	90.13 (1.6)
40	39.44 (1.6)	40.45 (1.8)
41	76.14 (1.6)	78.07 (1.3)
42	138.36 (0.1)	143.39 (1.2)
43	145.69 (0.7)	150.27 (1.1)
44	36.32 (0)	37.15 (1.6)
45	111.49 (0.8)	113.29 (2.1)
46	96.89 (0.7)	97.52 (1.9)
47	93.72 (0.4)	94.50 (1.3)
48	55.66 (0.6)	56.83 (0.4)
49	78.55 (0.5)	79.44 (1.2)
50	55.01 (0)	56.89 (1.1)

Table 3

Summary of NIST and Ghent results for 3-epi-25(OH)D₃ in 50 single donor samples. The mean values (nmol/L) for each laboratory are presented with relative standard deviations (% RSD) in parentheses. Samples with 3-epi-25(OH)D₃ concentrations below the LOQ are not included in the table.

Sample ID	NIST	Ghent
1	6.09 (1.0)	6.36 (11.0)
2	1.77 (1.0)	1.78 (19.5)
3	6.21 (1.2)	6.67 (8.1)
4	10.61 (1.3)	10.32 (5.0)
7	5.21 (0.3)	5.34 (8.8)
8	2.70 (1.3)	2.92 (9.1)
12	3.92 (1.8)	4.20 (6.6)
13	2.14 (1.7)	2.60 (5.6)
14	6.35 (0.9)	5.78 (13.1)
15	4.29 (4.6)	4.13 (9.5)
16	3.48 (1.8)	3.78 (8.3)
17	3.11 (1.7)	3.34 (8.9)
18	5.23 (1.4)	4.60 (4.8)
19	4.02 (1.8)	4.03 (4.8)
20	1.67 (3.2)	1.73 (8.1)
21	4.65 (0.4)	4.82 (5.8)
22	4.84 (0.4)	5.18 (6.9)
23	3.12 (2.0)	3.20 (9.4)
24	3.98 (0)	4.11 (4.4)
25	2.41 (1.5)	2.55 (5.7)
26	1.44 (1.3)	1.61 (6.8)
27	10.32 (0.7)	10.32 (4.8)
28	15.18 (1.8)	15.91 (3.9)
30	3.45 (0.5)	3.75 (5.6)
31	5.33 (0.8)	5.84 (4.6)
32	5.03 (1.1)	5.07 (12.4)
33	9.12 (0)	9.75 (5.1)
36	2.43 (0)	2.07 (12.6)
37	3.80 (0)	3.98 (5.8)
38	4.56 (0.8)	4.43 (7.2)
39	6.03 (1.8)	6.38 (3.8)
41	5.46 (1.0)	6.16 (5.8)
42	7.80 (3.8)	7.81 (5.9)
43	11.53 (2.5)	13.11 (10.0)

Sample ID	NIST	Ghent
45	9.04 (1.9)	9.85 (2.9)
46	2.66 (3.4)	2.19 (22.7)
47	9.60 (3.1)	10.06 (2.7)
48	7.25 (4.2)	7.84 (4.1)
49	7.94 (0.3)	8.18 (10.1)
50	6.51 (2.8)	7.04 (5.1)

Table 4

Assigned values (nmol/L) for the 50 VDSP samples. Relative expanded uncertainties are given in parentheses.

Sample ID	25(OH)D ₂	25(OH)D ₃	Total 25(OH)D	3-epi-25(OH)D ₃
1		84.95 (1.74)	85.33 (1.74)	6.09 (3.12)
2	3.73 (3.44)	44.24 (1.93)	47.97 (1.90)	1.77 (4.08)
3		112.26 (1.61)	112.49 (1.61)	6.21 (3.12)
4	7.38 (2.95)	105.96 (1.61)	113.33 (1.58)	10.61 (2.83)
5	1.85 (3.99)	23.27 (2.31)	25.11 (2.26)	
6		12.43 (2.62)	12.84 (2.60)	
7	5.42 (3.21)	78.26 (1.80)	83.68 (1.76)	5.21 (3.15)
8		77.76 (1.87)	78.70 (1.86)	2.70 (3.77)
9		73.32 (1.83)	74.39 (1.82)	
10		52.34 (2.05)	53.16 (2.04)	
11		47.41 (1.98)	48.33 (1.97)	
12	1.44 (4.25)	65.04 (1.79)	66.48 (1.78)	3.92 (3.59)
13	2.96 (3.72)	53.51 (1.92)	56.47 (1.89)	2.14 (4.03)
14	5.79 (3.12)	87.68 (1.69)	93.47 (1.66)	6.35 (3.06)
15		82.71 (1.77)	83.47 (1.77)	4.29 (3.98)
16		77.32 (1.90)	78.19 (1.89)	3.48 (3.78)
17		65.73 (1.82)	67.50 (1.80)	3.11 (3.79)
18	2.12 (3.99)	81.69 (1.68)	83.81 (1.67)	5.23 (3.28)
19		86.44 (1.69)	87.03 (1.68)	4.02 (3.56)
20		33.17 (2.08)	33.79 (2.07)	1.67 (4.67)
21		71.60 (1.73)	72.67 (1.73)	4.65 (3.23)
22		57.36 (1.82)	58.64 (1.81)	4.84 (3.20)
23		61.67 (2.10)	62.32 (2.09)	3.12 (3.67)
24		67.30 (1.78)	67.72 (1.78)	3.98 (3.35)
25		42.62 (2.13)	43.14 (2.13)	2.41 (3.89)
26	1.89 (4.03)	30.40 (2.26)	32.29 (2.21)	1.44 (4.30)
27		96.33 (1.82)	97.06 (1.81)	10.32 (2.73)
28	1.46 (4.39)	139.41 (1.67)	140.88 (1.66)	15.18 (2.76)
29		14.30 (2.52)	15.32 (2.48)	
30		63.88 (1.83)	64.38 (1.83)	3.45 (3.58)
31		72.68 (1.81)	74.60 (1.79)	5.33 (3.21)
32		107.85 (1.61)	108.97 (1.60)	5.03 (3.25)
33		106.00 (1.61)	106.45 (1.61)	9.12 (2.76)
34		32.64 (2.17)	33.14 (2.16)	
35		61.67 (1.82)	62.62 (1.81)	
36	1.75 (4.11)	60.94 (1.81)	62.69 (1.80)	2.43 (3.74)

Sample ID	25(OH)D ₂	25(OH)D ₃	Total 25(OH)D	3-epi-25(OH)D ₃
37	1.76 (4.09)	68.22 (1.77)	69.97 (1.76)	3.80 (3.37)
38		59.20 (1.88)	59.77 (1.88)	4.56 (3.28)
39		89.64 (1.71)	90.32 (1.71)	6.03 (3.13)
40		39.94 (2.06)	40.53 (2.06)	
41	18.92 (2.45)	77.11 (1.76)	96.03 (1.66)	5.46 (3.14)
42	3.34 (3.57)	140.88 (1.53)	144.22 (1.52)	7.80 (3.48)
43		147.98 (1.51)	149.10 (1.50)	11.53 (2.84)
44		36.74 (2.07)	37.46 (2.06)	
45	2.36 (3.91)	112.39 (1.68)	114.76 (1.67)	9.04 (2.89)
46	2.44 (3.89)	97.21 (1.70)	99.64 (1.69)	2.66 (3.93)
47	6.27 (3.25)	94.11 (1.68)	100.38 (1.65)	9.60 (3.05)
48		56.25 (1.83)	56.60 (1.83)	7.25 (3.46)
49		79.00 (1.72)	79.66 (1.72)	7.94 (3.46)
50		55.95 (1.85)	56.62 (1.85)	6.51 (3.26)