Significance of Serum 24,25-Dihydroxyvitamin D in the Assessment of Vitamin D Status: A Double-Edged Sword?

Kevin D. Cashman, Aoife Hayes, Karen Galvin, Joyce Merkel, Glenville Jones, Martin Kaufmann, Andrew N. Hoofnagle, Graham D. Carter, Ramon A. Durazo-Arvizu, and Christopher T. Sempos

BACKGROUND: 24,25-Dihydroxyvitamin D [24,25(OH)₂D₃] in serum may be both a nuisance and nutritionally valuable.

METHODS: We investigated the impact of 24,25(OH)₂D₃ on the performance of commercially available immunoassays for serum total 25-hydroxyvitamin D [25(OH)D] using (a) serum from a nationally representative sample of adults, (b) serum from a spiking experiment, and (c) data from the UK Vitamin D External Quality Assurance Scheme (DEQAS). We also investigated the utility of the serum ratio of 24,25(OH)₂D₃ to 25(OH)D as an index of inactivation and of response to vitamin D supplementation using randomized controlled trial (RCT) data. Measurement of 24,25(OH)D in sera by a LC-MS/MS method allowed for an investigation of its impact on immunoassay-derived serum 25(OH)D values as well as its clinical utility. We report data from a nationally representative sample of adults, a recent vitamin D RCT in older adults, and DEQAS.

RESULTS: 24,25(OH)₂D₃ contributed to the positive bias observed in some immunoassays relative to LC-MS/MS-derived estimates for total 25(OH)D. A spiking experiment showed that the degree of cross-reactivity with 24,25(OH)₂D₃ was high and may underpin this positive bias. Adjustment for 24,25(OH)₂D₃ concentration brought estimates closer to true values. Data from the vitamin D RCT showed that the ratio of 24,25(OH)₂D₃ to 25(OH)D was associated with serum 25(OH)D₃ and with response of serum 25(OH)D to vitamin D supplementation.

CONCLUSIONS: Our findings highlight that the effect of 24,25(OH)₂D₃ in serum is a double-edged sword—an interferent for some immunoassays, yet potentially informative of nutritional status.

© 2015 American Association for Clinical Chemistry

Within the vitamin D metabolism pathway, the 24-hydroxylase (CYP24A1) enzyme converts serum 25-hydroxyvitamin D [25(OH)D] to 24R,25-dihydroxyvitamin D [24,25(OH)₂D₃]. It is possible that 24,25(OH)₂D₃ in the circulation is at the same time a nuisance and a clinically relevant value, highlighting the importance of its measurement in serum. In vitro experiments with purified CYP24A1 suggest that this 24-hydroxylation is just the first step in a 5-step, vitamin D–inducible pathway to water-soluble truncated degradation products (1). Not unsurprisingly, assessment of serum 24,25(OH)₂D₃, particularly when expressed as a molar ratio to 25(OH)D, has found favor as an index of vitamin D deficiency and catabolism in healthy individuals (2–5), as well as in those with rare genetic mutations in the CYP24A1 gene (5, 6). The ratio of 24,25(OH)₂D₃ to 25(OH)D may also be an indicator of response to vitamin D supplementation (3) and may help to explain some of the well-documented interindividual differences in response of serum 25(OH)D to the same administered dose of vitamin D. Older data from animal experiments also suggest that 24,25(OH)₂D₃ [or the 24-hydroxylated product of 1,25(OH)₂D₃, 1,24,25(OH)₃D₃] may stimulate intestinal calcium absorption and bone calcium mobilization (6, 7). More recently, there is evidence for a role in fracture healing (8, 9). Thus, the recent advancement of liquid chromatographic methods for measurement of serum 25(OH)₂D₃ (3), in some cases in par-
allel with serum 25(OH)D (5), is of note and should help address some of the knowledge gaps in relation to these putative roles of 24,25(OH)₂D in human biology.

Binkley and Wiebe (10) recently suggested that important challenges continue to vex the measurement of circulating 25(OH)D, despite this being recognized as the best clinical indicator of vitamin D status (11). Although chromatography-based approaches are currently considered the research gold standard (12), immunoassay procedures are in widespread clinical use (10). The presence of 24,25(OH)₂D₃, as well as other metabolites, in serum may contribute to the positive bias of some immunoassay-based methods relative to that of chromatography-based measurements (10, 13, 14). Because 24,25(OH)₂D may range from 2% to 20% of total serum total 25(OH)D (15), Binkley and Wiebe (10) illustrated its theoretical effect on 25(OH)D measurement and the inflation of the true 25(OH)D value. However, although these estimates are telling, they do not account for the fact that some commercial immunoassays cross-react >100% with 24,25(OH)₂D (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/ vol61/issue4). The UK Vitamin D External Quality Assurance Scheme (DEQAS) showed in 1 of their quarterly cycles in 2012 that different immunoassays overestimated serum total 25(OH)D by 144%–750% relative to a mean chromatographic estimate, when a single serum sample was spiked with 24S,25(OH)₂D (personal communication from author G.D. Carter, October 2014). Of note, however, the 24R isomer is the physiologically relevant one, since the 24S isomer is not found in humans. Not unsurprisingly, gaining a better understanding of the contribution of 24,25(OH)₂D to 25(OH)D measurement is an area that the Vitamin D Standardization and Certification Program (VDSP) has prioritized for further research (14).

The objective of this work was to investigate the impact of 24R,25(OH)₂D₃ at a physiologically relevant concentration, on the performance of a commercially available immunoassay for serum total 25(OH)D compared with that from a certified LC-MS/MS method, and to use this data to explore whether the 24,25(OH)₂D₃ concentration in serum of healthy adults from a nationally representative sample explains the positive bias in the immunoassay-based estimates of serum 25(OH)D concentrations (16). Unpublished data from 6 recent cycles of DEQAS were used to test the bias of the most commonly used immunoassays in the scheme relative to the target serum 25(OH)D values as measured by NIST with and without adjustment for measured 24,25(OH)₂D₃ concentration. Finally, the utility of serum 24,25(OH)₂D₃; 25(OH)D₃ as an index of inactivation and also of response to supplementation was further explored by use of data from a recent vitamin D RCT in older adults (4).

Participants and Methods

THE NATIONAL ADULT NUTRITION SURVEY SAMPLE
A detailed description of the participant sampling and recruitment procedures and methods of data collection used in the Irish National Adult Nutrition Survey (NANS) (n = 1500) has been reported elsewhere (17, 18). In the present work, data on serum 24, 25(OH)₂D₃ concentration was available for a subset of NANS participants (n = 134) in whom the impact of low and high calcium intake and low and high serum total 25(OH)D concentrations on serum 24,25(OH)₂D₃, as an index of vitamin D catabolism, was investigated. Thus, the data did not cover the full distribution of participants but rather those with the lowest and highest calcium intake/total 25(OH)D within the population, which is of particular use in the present study. Serum total 25(OH)D concentration data as measured by enzyme immunoassay (EIA) (Octeia® 25-Hydroxy Vitamin D, Immuno Diagnostic Systems), and reported previously (18), was also available for the current work.

THE VITAMIN D RANDOMIZED CONTROLLED TRIAL IN OLDER ADULTS
The 15-week winter-based, randomized, placebo-controlled, double-blind vitamin D₃ intervention (20 μg/day) study (which we will refer to in short as VitD-Ca RCT) has been described in detail elsewhere (4). Study participants were free-living women and men (ratio approximately 2.5:1, age ≥ 50 years, n = 125), stratified according to calcium intake (moderate-low, <700 mg/day, or high, >1000 mg/day). The study was registered on ClinicalTrials.gov (identifier: NCT01990872). Serum total 25(OH)D and 24,25(OH)₂D₃ increases and decreases, respectively, in the vitamin D₃ and placebo groups were of magnitudes similar to those with calcium intakes <700 and >1000 mg/day (4). In the present study, since there was no interaction with dietary calcium (P = 0.2), we examined the effect of vitamin D₃ supplementation or placebo on serum 25(OH)D₃ and 24, 25(OH)₂D₃ concentrations and their ratio over winter as potential indices of low vitamin D status.

ANALYSIS OF SERUM 25(OH)D₂ AND 24,25(OH)₂D₃
The concentrations of total 25(OH)D [i.e., 25(OH)D₂ plus 25(OH)D₃] as well as 24,25(OH)₂D₃ in serum samples were measured by the Vitamin D Research Group at University College Cork with a LC-MS/MS method, as described in detail elsewhere (4, 16), and as monitored on an ongoing basis by participation in DEQAS (Charing Cross Hospital, London, UK). In addition, the Vitamin D Research Group is a participant in the VDSP (13) and is certified by the CDC’s Vitamin D Standardization Certification Program (19).
SERUM SPIKING STUDY WITH 24,25(OH)2D3
We selected 4 baseline serum samples from the VitD-Ca RCT for measurement of serum total 25(OH)D by immunoassay (Octeia) before and after spiking the samples with the R isomer 24,25(OH)2D3 (Isosciences). We used the measured naturally present concentration of 24,25(OH)2D3 in serum to calculate the amount of exogenous 24,25(OH)2D3 spike to be added to each sample to have a final total 24,25(OH)2D3 concentration in the range of 6.3–7.5 nmol/L (close to the 75th percentile in NANS and VitD-Ca RCT). A minimal volume (<0.1134910.9262 L) of the 24,25(OH)2D3 spiking solution (120 nmol/L of methanol) was added to a 1.5-mL conical polypropylene microtube. The methanol was removed by drying under nitrogen, and serum (180 µL) was added, mixed, and allowed to equilibrate at room temperature overnight. We analyzed spiked and unspiked serum from each of the 4 individuals for total 25(OH)D by immunoassay and LC-MS/MS. The final concentration of total 24,25(OH)2D3 was also confirmed in spiked samples by LC-MS/MS.

IMPACT OF 24,25(OH)2D3 IN SERA FROM DEQAS SAMPLES ON PERCENT BIAS FROM TARGET VALUES AS MEASURED BY THE NIST REFERENCE MEASUREMENT PROCEDURE
In the present analysis, we used data from 6 recent cycles of DEQAS quarterly cycles (October 2012 to April 2014) on the mean returned serum total 25(OH)D for each of the most commonly used analytical platforms (e.g., HPLC, LC-MS/MS, DiaSorin Liaison, IDS Octeia, IDS iSYS, Roche Total, Abbott, and Siemens) for each of the 30 sera as well as the NIST-assigned target values and 24,25(OH)2D3 concentrations (as measured by a recently reported LC-MS/MS method (5)). The percentage bias of the mean returned values from the NIST target value was calculated for each grouping of analytical platforms with and without accounting for the measured concentration of 24,25(OH)2D3 in sera.

DATA INTERPRETATION AND STATISTICAL ANALYSIS
We conducted data and statistical analysis with SPSS version 20.0 for Windows, Stata 12 (StataCorp), and CBStat5 (Kristian Linnet). Descriptive statistics (frequencies, means, medians, and percentiles) were generated for serum 25(OH)D3 and 24,25(OH)2D3 data. Pearson correlation coefficients were used to explore correlations, and linear regression analysis was used to explore associations between serum 25(OH)D3 and 24,25(OH)2D3. We used Bland–Altman plots to test for differences in 25(OH)D concentrations with and without adjustment for 24,25(OH)2D3, and we used paired t tests to compare within-treatment-group changes in serum 25(OH)D3, 24,25(OH)2D3, and 24,25(OH)2D3: 25(OH)D3 from baseline to end point. We used unpaired t tests to compare baseline, end point, and change in these parameters between the vitamin D and placebo groups. A P value of <0.05 was taken as being statistically significant.

RESULTS

DISTRIBUTIONS OF SERUM 25(OH)D3 AND 24,25(OH)2D3 IN BASELINE VIID-Ca RCT AND NANS SUBSET
Data on the distribution of serum 25(OH)D3 and 24,25(OH)2D3 in the 2 populations are shown in Table 1. On average, serum 24,25(OH)2D3 represented 9.0 and 10.1% of serum 25(OH)D3 in the baseline VitD-Ca RCT and NANS subset populations, respectively.

Linear regression analyses of serum 24,25(OH)3D3 vs 25(OH)D3 in both samples showed strong correlations (Pearson coefficients) of 0.86 and 0.80 (P < 0.0001) for the VitD-Ca RCT and NANS subsets, respectively, and both exhibited linear relationships (R2 = 0.75 and 0.64, respectively; P < 0.0001). Likewise, in the 30 DEQAS sera used in the present work, there was an R2 of 0.96 (P < 0.0001).

SERUM 24,25(OH)2D3 AS POTENTIAL INTERFERENT IN MEASUREMENT OF 25(OH)D

Impact of 24,25(OH)2D3 in serum on the assessment of serum total 25(OH)D by immunoassay. The influence of spiking serum with 24,25(OH)2D3 on the assessment of

---

**Table 1. Distribution of serum 25(OH)D₃ and 24,25(OH)₂D₃ in 2 populations.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>25th Percentile</th>
<th>Median</th>
<th>75th Percentile</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 25(OH)D₃, nmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VitD Ca RCT</td>
<td>125</td>
<td>51.3</td>
<td>19.8</td>
<td>20.8</td>
<td>35.4</td>
<td>49.5</td>
<td>65.6</td>
<td>115.8</td>
</tr>
<tr>
<td>NANS subset</td>
<td>134</td>
<td>58.1</td>
<td>29.4</td>
<td>17.2</td>
<td>31.1</td>
<td>66.3</td>
<td>75.1</td>
<td>167.6</td>
</tr>
<tr>
<td>Serum 24,25(OH)₂D₃, nmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VitD Ca RCT</td>
<td>125</td>
<td>5.1</td>
<td>3.5</td>
<td>0.4</td>
<td>2.5</td>
<td>4.5</td>
<td>6.8</td>
<td>20.1</td>
</tr>
<tr>
<td>NANS subset</td>
<td>134</td>
<td>6.0</td>
<td>4.1</td>
<td>0.4</td>
<td>2.7</td>
<td>5.0</td>
<td>8.8</td>
<td>19.2</td>
</tr>
</tbody>
</table>
serum total 25(OH)D by immunoassay is shown in Table 2. The serum total 25(OH)D concentration of all 4 participants’ unspiked sera as measured by immunoassay was higher than the equivalent measured by LC-MS/MS (by 2.7–12.6 nmol/L), and accordingly, the mean serum total 25(OH)D concentration was significantly higher ($P < 0.045$) for the immuno-based vs LC-MS/MS-based measurements (Table 2).

The increase in serum total 25(OH)D in spiked samples, as measured by immunoassay, ranged from 14 to 38 nmol/L and far exceeded the concentration of spiked or total (spike plus natural content) serum 24,25(OH)2D3 present (Table 2). Spiking a separate aliquot of each of the same 4 sera with 6.6 nmol/L 3-epimer 25(OH)D3, in the same manner as 24,25(OH)2D3, led to no change in immunoassay-measured total 25(OH)D [mean (SD) 38.6 (9.2) and 39.2 (7.7) nmol/L for unspiked and spiked sera, respectively]. By accounting for the nanomole-per-liter increase in total serum 25(OH)D in each sample upon spiking with 24,25(OH)2D3, and using this increase together with information on the amount of native 24,25(OH)2D3 present in the unspiked sample, we were able to derive a mean factor [nmol/L serum total 25(OH)D increment per mg 24,25(OH)2D3] by which the antibody in the immunoassay overresponded to the 24,25(OH)2D3 content in serum. We used this mean factor (2.79) in conjunction with the measured naturally occurring 24,25(OH)2D3 concentration in the 4 sera to adjust the immunoassay-measured total serum 25(OH)D and compare the mean to that of the LC-MS/MS estimates. We also adjusted the immunoassay-measured total serum 25(OH)D concentrations for measured 24,25(OH)2D3 concentration without applying the antibody overreaction factor. Both adjustments for 24,25(OH)2D3 concentration brought the mean of the 4 individual sera’s 25(OH)D concentration as measured by immunoassay (which was significantly higher than that by LC-MS/MS; $P < 0.05$) closer to the LC-MS/MS estimate ($P > 0.1$ for both) (Table 2).

We also used the measured 24,25(OH)2D3 concentration in sera from the NANS subset (with and without the mean factor of 2.79) to adjust the immunoassay-measured total serum 25(OH)D concentration and compare the mean to that from LC-MS/MS estimates. Fig. 1A shows the significant positive bias (11.1%; $P < 0.001$) of the immunoassay-measured serum total 25(OH)D in the NANS subset samples compared with the equivalent measured by the LC-MS/MS procedure. Adjusting the immunoassay-measured estimates of total serum 25(OH)D for the measured 24,25(OH)2D3 concentration in each sample lowered this mean bias (to 1.6%), which was not significant ($P > 0.1$). Also, the discrepancy at the level of individual samples was improved (Fig. 1B), such that, for example, the percentage of samples with >20% absolute bias from LC-MS/MS values decreased from 35.4% to 14.6% for immunoassay unadjusted and adjusted for measured 24,25(OH)2D3 concentration, respectively. When the mean antibody overreaction factor was applied, the original mean positive bias became an overall negative mean bias of about the same absolute magnitude (−15.2%; $P < 0.001$), and the discrepancy in the individual samples was even greater than unadjusted immunoassay values (Fig. 1C). For example, the percentage of samples with >20% absolute bias from LC-MS/MS values increased

<table>
<thead>
<tr>
<th>Sample</th>
<th>Serum total 25(OH)D, nmol/L</th>
<th>LC-MS/MS (unspiked)</th>
<th>EIA</th>
<th>Spiked</th>
<th>Unspiked</th>
<th>Spiked a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.9</td>
<td>43.9</td>
<td>65.8</td>
<td>3.6</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>36.9</td>
<td>49.5</td>
<td>78.2</td>
<td>1.2</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>26.1</td>
<td>28.9</td>
<td>60.5</td>
<td>0.8</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>24.1</td>
<td>32.0</td>
<td>52.9</td>
<td>1.0</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>All, mean (SD)</td>
<td>31.5 (7.5)</td>
<td>38.6 (9.7)b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1c</td>
<td>36.9 (9.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>34.1 (8.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Representing naturally present 24,25(OH)2D3 plus that added to each serum.

b $P < 0.05$ vs mean for LC-MS/MS estimate, unpaired t-test.

c Model 1 is immunoassay-measured total 25(OH)D values minus the measured 24,25(OH)2D3 content. Model 2 is immunoassay-measured total 25(OH)D values minus the measured 24,25(OH)2D3 content multiplied by the average antibody overreactivity factor.
Fig. 1. Bland–Altman plots of the percentage differences between serum total 25(OH)D in the NANS subset as assessed by immunoassay vs LC-MS/MS (A), by immunoassay [but adjusted for serum 24,25(OH)₂D content] versus LC-MS/MS (B), and by immunoassay [but adjusted for serum 24,25(OH)₂D content and then multiplied by antibody overreactivity factor] versus LC-MS/MS (C) (n = 134). Difference of mean was calculated as [(overall mean − reference value)/reference value] × 100.
from 35.4% to 52.8% for immunoassay unadjusted and adjusted for measured 24,25(OH)2D3 concentration, respectively.

Impact of 24,25(OH)2D3 in sera from DEQAS samples in percent bias from target values as measured by the NIST reference measurement procedure. The percent mean bias for 2 of 6 of the selected immunoassays (Abbott and Diasorin Liaison Total) were negative to begin with and become more negative after adjustment for the measured 24,25(OH)2D3 concentration of each sample (Table 3). For the other 3 immunoassays, the positive bias of IDS EIA and Siemens ADVIA Centaur was improved to a more favorable quantitatively negative bias; for the Roche Total, the positive bias was reduced to a negative bias but of a greater magnitude (Table 3).

SERUM 24,25(OH)2D3 AS INDEX OF LOW VITAMIN D STATUS AND RESPONSE TO SUPPLEMENTATION

Response of serum 24,25(OH)2D3 and ratio of 24,25(OH)2D3:25(OH)D3 to altered serum 25(OH)D status. The response of serum 24,25(OH)2D3 and 24,25(OH)2D3:25(OH)D3 to changes in serum 25(OH)D3 arising from supplementation during winter with 20 μg/day vitamin D3 or placebo is shown in online Supplemental Table 2. There were no significant differences in serum metabolites or their ratio (P > 0.4 for all) between groups at baseline. The placebo group had a significantly (P < 0.0001) lower mean serum 25(OH)D3, 24,25(OH)2D3, and 24,25(OH)2D3:25(OH)D3 at end point compared with the vitamin D3–supplemented group. The within-treatment-group changes from baseline to end point are also shown in online Supplemental Table 2. Both serum metabolites or their ratio significantly decreased (P < 0.0001, in all cases) from baseline to end point in the placebo group and increased (P ≤ 0.05, in all cases) in the vitamin D3–supplemented group. There was no significant interaction with sex (P > 0.5).

Linear regression analysis showed that change in serum 25(OH)D3 (i.e., week 15 from week 0) was significantly associated with 24,25(OH)2D3:25(OH)D3 at baseline in the vitamin D3–supplemented group [β = −0.350; B (SE) −161 (67); P = 0.021], even when sex, age, body mass index, serum calcium, and serum parathyroid hormone (PTH) were accounted for (see online Supplemental Fig. 1). There was no significant association in the placebo group (P = 0.53) (data not shown).

Ratio of 24,25(OH)2D3:25(OH)D3 as an index of vitamin D deficiency and insufficiency. The association between serum 24,25(OH)2D3:25(OH)D3 and 25(OH)D3 is shown in Fig. 2A (R2 = 0.405; P < 0.0001, n = 222) and indicates that a ratio of approximately 0.05 in a population corresponds to vitamin D deficiency [serum 25(OH)D <25 nmol/L (20, 21)] and a ratio of approximately 0.09 to vitamin D sufficiency as defined by the Institute of Medicine as serum 25(OH)D

### Table 3. Percentage bias of mean returned serum total 25(OH)D values for 8 different vitamin D analytical platforms compared with NIST-assigned target values for sera in 6 cycles of DEQAS (n = 30), unadjusted and adjusted for 24,25(OH)2D3 concentration.

<table>
<thead>
<tr>
<th>Assay (%)</th>
<th>Proportion in DEQASa</th>
<th>Mean bias vs NIST-assigned total 25(OH)D, %</th>
<th>Unadjusted</th>
<th>Adjusted for 24,25(OH)2D3b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immuno-based</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roche Total 25OHD</td>
<td>13.7</td>
<td>1.8</td>
<td>−5.9c</td>
<td></td>
</tr>
<tr>
<td>IDS EIA</td>
<td>5.7</td>
<td>4.4</td>
<td>−3.4c</td>
<td></td>
</tr>
<tr>
<td>Siemens Advia Centaur</td>
<td>5.8</td>
<td>7.0</td>
<td>−0.8c</td>
<td></td>
</tr>
<tr>
<td>IDS-iSYS</td>
<td>12.5</td>
<td>13.1</td>
<td>5.4c</td>
<td></td>
</tr>
<tr>
<td>Abbott Architect</td>
<td>7.5</td>
<td>−3.9</td>
<td>−11.7c</td>
<td></td>
</tr>
<tr>
<td>Diasorin Liaison Total</td>
<td>28.3</td>
<td>−13.2</td>
<td>−21.0c</td>
<td></td>
</tr>
<tr>
<td>Chromatography basedd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>3.1</td>
<td>8.3</td>
<td>NA*</td>
<td></td>
</tr>
<tr>
<td>LC-MS</td>
<td>15.0</td>
<td>4.1</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

a Percentage of total methods (n = 998) represented by immunoassay within DEQAS (April 2014).

b 24,25(OH)2D3 concentration as measured; no antibody overreactivity factor.

c P < 0.0001, percentage bias significantly different from that of NIST-assigned total 25(OH)D without adjustment (n = 30); paired t test.

d Chromatographic methods included purely for illustrative purposes, as they should not be prone to the same artificial elevation in total 25(OH)D by 24,25(OH)2D3 as some immunoassays.

* NA, not applicable.
Fig. 2. The relationship between serum 25(OH)D₃ and 24,25(OH)₂D₃, 25(OH)D₃ by use of data from baseline and end point in the VitD-Ca RCT sample (n = 222). $R^2 = 0.328; P < 0.0001$, power best-fit line.
>50 nmol/L (22), whereas a ratio above this suggests sufficiency.

The inverse association between serum 25(OH)D$_3$: 24,25(OH)$_2$D$_3$ and 25(OH)D$_3$ ($R^2 = 0.328$) was much stronger than that between serum PTH and 25(OH)D$_3$ ($R^2 = 0.087$) (Fig. 2, B and C, respectively).

Discussion

The findings of the present study, which included data from 6 recent cycles of DEQAS and the returns for its 6 commonly used immunoassays, each representing >5% of all results returned in the DEQAS scheme (April 2014), clearly showed that adjustment for concentration of measured 24,25(OH)$_2$D$_3$ in serum diminished the significant positive bias in measurement of serum total 25(OH)D by some immuno-based assays compared with LC-MS/MS. For other immunoassays, which had relatively small biases to begin with, it led to artificially larger negative biases. This simple analysis did not attempt to adjust for the fact that some of the antibodies cross-react with 24,25(OH)$_2$D$_3$ by >100%.

We wished to explore further the nature of the impact of serum 24,25(OH)$_2$D$_3$ on total serum 25(OH)D as measured by 1 of these commonly used commercial immuno-based assays, as it might explain, at least in part, the significant positive bias and underestimation of the prevalence of the population with serum 25(OH)D concentrations <30, <40, and <50 nmol/L, which we have reported previously when serum total 25(OH)D was measured in our nationally representative sample of adults by immunoassay compared with LC-MS/MS (16). Adjustment of the immunoassay-derived serum total 25(OH)D value for the measured 24,25(OH)$_2$D$_3$ concentration in serum in the present work showed that it brought the values closer to that measured by LC-MS/MS, but the mean was still 17% higher. This might be expected because for some immunoassays, the antibody cross-reacts >100% with 24,25(OH)$_2$D$_3$, and simple adjustment for measured content will be an underestimation of its contribution to apparent total 25(OH)D.

DEQAS showed in 1 of their quarterly cycles in 2012 that different immunoassays overestimated serum total 25(OH)D by 144%–750% relative to a mean chromatographic estimate, when a single serum sample was spiked with 24,25(OH)$_2$D$_3$ at a single high concentration (57.9 nmol/L) and as the nonphysiologically relevant 24S isomer. Thus, to get better insight into the impact of the potential cross-reactivity with 24,25(OH)$_2$D$_3$ as the R isomer and at a more physiological concentration, we performed a relatively small, proof-of-principle spiking experiment. The data showed that for the 4 sera [25(OH)D range 24.1–38.9 nmol/L] spiked with 24R, 25(OH)$_2$D$_3$ to achieve 6.3–7.5 nmol/L, the antibody in the immunoassay significantly overreacted to serum 24,25(OH)$_2$D$_3$ relative to 25(OH)D. The mean increase in serum total 25(OH)D was such that the percentage cross-reactivity was on the order of >300%, but lower than the approximately 600% found in the 2012 DEQAS exercise with the 24S isomer. Although the result was in only 1 commercial assay, which is certainly a limitation of the current study, results of a very recent DEQAS investigation (unpublished data provided by G.D. Carter) has revealed very high cross-reactivity of 24R,25(OH)$_2$D$_3$ in some, but not all, nonextraction and extraction immunoassays. This high degree of cross-reactivity is a possible artifact associated with the spiking process. The anomalous behavior of exogenous 25(OH)D has also been reported (23), although this metabolite was underrecovered in spiked samples. In addition, the independent spiking of the 4 same sera with 6.7 nmol/L 3-epimer of 25(OH)D$_3$ (which has been reported to have extremely low cross-reactivity) led to no increase in the immunoassay-measured total 25(OH)D in the present study, possibly suggesting that spiking artifacts are not the sole factor involved, something that will need to be tested and confirmed in additional work.

Whatever the underlying reason for this potentially anomalous behavior of exogenous 24R,25(OH)$_2$D$_3$ in the present study and that of the recent DEQAS cycle, it may explain why adjusting for the 24,25(OH)$_2$D$_3$ concentration and its overreactivity within our NANS dataset led to an exaggeration of the effect of 24,25(OH)$_2$D$_3$ and resulted in an overall negative mean bias in 25(OH)D. However, the same crude correction factor applied to our 4 sera brought the estimates of serum total 25(OH)D from the immunoassay closer to that of the LC-MS/MS than did just adjustment for measured serum 24,25(OH)$_2$D$_3$ concentration.

In relation to the purposeful measurement of serum 24,25(OH)$_2$D$_3$ as an additional index of vitamin D status, the vitamin D RCT findings of the present work support those of Kaufmann et al. (5) and Wagner et al. (3) and show that measurement of serum 24,25(OH)$_2$D$_3$, and expression of its molar ratio to 25(OH)D$_3$, are indices of vitamin D deficiency and likely inactivation. Supplementation with vitamin D$_3$ significantly increased serum 24,25(OH)$_2$D$_3$ concentration and 24,25(OH)$_2$D$_3$:25(OH)D$_3$, as well as serum 25(OH)D$_3$, suggesting induction of the catabolic pathway via increased CYP24A1 activity.

As 24,25(OH)$_2$D$_3$:25(OH)D$_3$ increased in the present work, the response of serum 25(OH)D$_3$ to 15 weeks of vitamin D$_3$ supplementation decreased, which is similar to results reported by Wagner et al. (3) in their 8-week vitamin D$_3$ supplementation study. In that study, however, because of unavailability of baseline data, the ratio was from week 2 of the intervention and the change in serum 25(OH)D$_3$ was from week 2 to week 6 of intervention (3). These findings are likely related to the
well-reported greater increase in serum 25(OH)D in individuals with lower baseline status (24). In contrast to Wagner et al. (3), who reported that the ratio was higher in vitamin D–supplemented women than men (young adults), at week 6 of their RCT, but not at week 2, there was no sex difference at baseline or end point (week 15) in our trial. Our data point to no difference in the rate of vitamin D catabolism in older adult men or women. In agreement with the findings of Wagner et al. (3), the moderate inverse correlation with 24,25(OH)2D3:25(OH)D3 was similar to that commonly reported with more conventional correlates of vitamin D response and status, such as body mass index and PTH. Expressing the more conventional correlates of vitamin D response and status—has been high-lighted by the present findings. We support the view of Wagner et al. (3) that the ratio of 25(OH)D3 to 25(OH)D measurement potentially informative in terms of status—has been high-lighted by the present findings. We support the view of the VDSP that further priority research is needed to gain a better understanding of the contribution of 24,25(OH)2D3 to 25(OH)D measurement (14), but also believe this additional research should explore the additional benefit, if any, of 24,25(OH)2D3 and its molar ratios with 25(OH)D in terms of informing vitamin D status.

In conclusion, the effect of 24,25(OH)2D3 in serum—interferent for some immunoassays and yet potentially informative in terms of status—has been highlighted by the present findings. We support the view of the VDSP that further priority research is needed to gain a better understanding of the contribution of 24,25(OH)2D3 to 25(OH)D measurement (14), but also believe this additional research should explore the additional benefit, if any, of 24,25(OH)2D3 and its molar ratios with 25(OH)D in terms of informing vitamin D status.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

References

19. Rahmani YE, Botello JC, Vesper HW. CDC Vitamin D Standardization Certification Program. Endocr Rev