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Determination of 24,25-dihydroxyvitamin D_3 in Vitamin D External Quality Assessment Scheme samples using a reference measurement procedure

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ABSTRACT

Ninety archived human serum samples from the Vitamin D External Quality Assessment Scheme (DEQAS) were analyzed using a reference measurement procedure (RMP) based on isotope dilution liquid chromatography – tandem mass spectrometry (ID LC-MS/MS) for the determination of 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$]. These 24,25$(OH)_2D_3$ results, in conjunction with concentration values assigned using RMPs for 25-hydroxyvitamin D_2 [25$(OH)D_2$] and 25-hydroxyvitamin D_3 [25$(OH)D_3$], provide a valuable resource for assessing the accuracy of measurements for 24,25$(OH)_2D_3$ and for investigating the relationship between 24,25$(OH)_2D_3$ and 25$(OH)D_3$. Results for 24,25$(OH)_2D_3$ using the RMP were compared to DEQAS consensus values demonstrating that the consensus values were not sufficient to assess the accuracy of measurements among different laboratories and methods. A multivariable regression analysis approach using historical DEQAS consensus values for various total 25(OH)D assays was used to assess the contribution of 24,25$(OH)_2D_3$ concentration on the assay response. The response of several ligand binding assays for total 25(OH)D was shown to be impacted by the presence of 24,25$(OH)_2D_3$.

1. Introduction

The Vitamin D External Quality Assessment Scheme (DEQAS) was established in 1989 [1] with a goal of ensuring the quality of analytical measurements for the determination of total serum 25-hydroxyvitamin D [25(OH)D], which is the primary marker for vitamin D status and is defined as the sum of 25-hydroxyvitamin D_2 [25$(OH)D_2$] and 25-hydroxyvitamin D_3 [25$(OH)D_3$]. DEQAS distributes four sets of five human serum samples annually to over 1000 participating laboratories worldwide [1]. Initially, individual participant results in DEQAS were compared to laboratory consensus results using an All-Laboratory Trimmed Mean (ALTM) [1]. However, since 2013, DEQAS has been an accuracy-based program with participant results compared to target values established using reference measurement procedures (RMPs) initially performed at the U.S. National Institute of Standards and Technology (NIST) [2,3], and since 2018 at the U.S. Centers for Disease Control and Prevention (CDC) [4]. The College of American Pathologists (CAP) has also provided an accuracy-based vitamin D (ABVD) proficiency testing program since 2011 [5] with target values for total 25(OH)D currently assigned using an RMP [4]. In 2010, NIST issued the first of several Standard Reference Materials® (SRMs®) for the determination of vitamin D metabolites in human serum [6–11], and currently there are five SRMs with certified values assigned for 25(OH)D_2 and 25$(OH)D_3$.

The DEQAS, CAP ABVD, and the availability of SRMs with values assigned for vitamin D metabolites have significantly supported accurate and comparable measurements of total 25(OH)D within the vitamin D testing and research community [12–16]. However, there is considerable interest in other vitamin D metabolites beyond 25$(OH)D_2$ and 25$(OH)D_3$ including dihydroxyvitamin D metabolites such as 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] and 1,25-dihydroxyvitamin D [1,25$(OH)_2D_3$]. 24,25$(OH)_2D_3$ is the major product of 25(OH)D catabolism and the major dihydroxyvitamin D species in serum typically at levels of 10–15 % of the 25$(OH)D_3$ [17–19]. 1,25$(OH)_2D_3$ is the main bioactive form of vitamin D with circulating levels three orders of magnitude lower than 25$(OH)D_3$, which is a significant challenge for both

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immunoassay and LC-MS/MS methods. Recent reviews provide an overview of the various vitamin D metabolites, their importance in investigating vitamin D metabolism and deficiency, and the analytical challenges in measuring the various metabolites [14,15,20–24].

In 2011, Mizwicki et al. [25] depicted vitamin D metabolism in a diagram with over 40 various metabolites. Slominski et al. [26,27] demonstrated novel pathways of vitamin D_2 and vitamin D_3 metabolism that result in the production of numerous metabolites including dihydroxyvitamin D_2 and D_3 metabolites. In 2019, Tuckey et al. [28] proposed an alternative pathway for vitamin D metabolism that focuses on enzymatic hydroxylation of vitamin D_3 to two main monohydroxy species, 20S(OH)D_3 and 22(OH)D_3, with further hydroxylation to dihydroxy metabolites including 1α,20S$(OH)_2D_3$, 20,22$(OH)_2D_3$, 20S,23S$(OH)_2D_3$, and 20S,24R$(OH)_2D_3$. The biological significance of many of these vitamin D metabolites is unknown, and analytical methods to measure them in clinical studies are only now starting to be developed. Recently, Jones and Kaufmann [24,29] proposed a simplified version of vitamin D metabolism adding three metabolites (24,25$(OH)_2D_3$, 25(OH)D_3-26,23-lactone, and 1,24,25$(OH)_3D_3$) to the usual 25(OH)D and 1,25$(OH)_2D_3$ that could potentially be used to diagnose calcium and phosphate-related diseases.

The recent review by Jones and Kaufmann [24] emphasizes the potential diagnostic utility of vitamin D metabolite profiling that has emerged due to the emergence of LC-MS/MS technology providing the capability to simultaneously monitor multiple metabolites. Jenkinson et al. [30] reported an LC-MS/MS method for simultaneous determination of 13 vitamin D_3 and D_2 mono and dihydroxy metabolites in human serum using derivatization with PTAD (4-phenyl-1,2,4-triazole-3,5-dione) to achieve added sensitivity. In addition to monohydroxy metabolites 25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3, and 20(OH)D_3, three dihydroxy vitamin D metabolites 24,25$(OH)_2D_3$, 1α,25$(OH)_2D_3$, and 1α,20S$(OH)_2D_3$ were routinely quantified in human serum samples. Additional metabolites were monitored in the method but were not routinely observed including 3-epi-25(OH)D_2, 22(OH)D_3 1α,25$(OH)_2D_2$, 20S,24$(OH)_2D_3$, 20,22$(OH)_2D_3$, and 1,24,25$(OH)_3D_3$.

While there is considerable interest and capability to monitor numerous dihydroxyvitamin D isoforms, a major current focus is accurate measurement of 24,25$(OH)_2D_3$ using isotope dilution liquid chromatography – tandem mass spectrometry (ID LC-MS/MS). During the past 15 years, ID LC-MS/MS methods for the determination of multiple vitamin D metabolites including 24,25$(OH)_2D_3$ have been reported such as Wagner et al. [31], Ding et al. [32], Laha et al. [33], Dowling et al. [34], Kaufmann et al. [17], Fabregat-Cabello et al. [35], Zelzer et al. [36], and Jenkinson et al. [30,37,38]. The simultaneous measurement of both 25(OH)D_3 and 24,25$(OH)_2D_3$ has gained increased interest since Wagner et al. [31] proposed the use of the ratio of 24,25$(OH)_2D_3$ to 25(OH)D_3 as a predictor of serum 25(OH)D_3 response to vitamin D_3 supplementation. Berg et al. [39] demonstrated the use of the vitamin D metabolite ratio (VMR) to better assess vitamin status differences among black and white population groups whereas Cavalier et al. [40] applied the VMR to assess vitamin D deficiency in infants, children and adolescents. Zelzer et al. [36] used VMR to identify patients with genetic enzyme defects (e.g., CYP24A1deficiency). Other researchers have also employed the VMR including Ginsberg et al. [41,42] for investigations of bone fracture risk, Aloia et al. [43] for vitamin D deficiency in African Americans, and Ketha et al. [44,45] for identifying CYP24A1 mutations and investigating vitamin D_3 catabolism for lactating women.

In 2015, as part of the Vitamin D Standardization Program (VDSP) [46,47], NIST developed an ID LC-MS/MS method for the determination of 24,25$(OH)_2D_3$ [48], which was later recognized as an RMP by the Joint Committee for Traceability in Laboratory Medicine (JCTLM). Using this RMP, certified values for the concentration of 24,25$(OH)_2D_3$ were assigned to SRM 972a Vitamin D metabolites in Frozen Human Serum [7] and SRM 2973 Vitamin D Metabolites in Frozen Human Serum (High level) [8]. In 2015, DEQAS introduced a pilot scheme for the determination of 24,25$(OH)_2D_3$ in the regular serum sample distributions for the determination of 25(OH)D with a limited number of participants providing results for 24,25$(OH)_2D_3$ and a consensus mean based on the participant results. Since 2018, information values for 24,25$(OH)_2D_3$ in the DEQAS samples have been provided based on results provided by the CDC routine LC-MS/MS method, which is not designated as an RMP.

There have been few studies to assess the comparability of various custom ID LC-MS/MS methods for the determination of 24,25$(OH)_2D_3$. In 2017, Wise et al. [19] reported the results of an interlaboratory comparison among five laboratories using ID LC-MS/MS methods compared with the NIST RMP for 24,25$(OH)_2D_3$ for the determination of 24,25$(OH)_2D_3$ in two SRMs and 30 archived DEQAS samples (nos. 421–450). Recently, Zelzer et al. [36,49] compared results using two in-house custom ID LC-MS/MS methods for the determination of 24,25$(OH)_2D_3$ in two different sets of 20 DEQAS samples (nos. 551–570 [36] and 571–590 [49]). Zelzer et al. [36,49] compared their results to DEQAS consensus concentrations for 24,25$(OH)_2D_3$; unfortunately, they did not analyze any SRMs with certified values assigned for 24,25$(OH)_2D_3$ for comparison with their results. Alshabrawy et al. [50] developed an LC-MS/MS method for simultaneous determination of 25$(OH)D_3$, 3-epi-25$(OH)D_3$, and 24,25$(OH)_2D_3$ using DAPTAD (4-(4'-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione) derivatization, and they used five DEQAS samples (nos. 546–550) as part of the method validation.

In an effort to improve the comparability of measurements of 24,25$(OH)_2D_3$ in human serum, the NIST RMP for 24,25$(OH)_2D_3$ was used to provide accuracy-based target concentrations for 90 archived DEQAS samples (nos. 421–510). The results for the content of 24,25$(OH)_2D_3$ in the first 30 DEQAS samples were reported in the interlaboratory comparison study reported by Wise et al. [19]. In this paper, we report the results for the determination of 24,25$(OH)_2D_3$ in an additional 60 DEQAS samples (nos. 451–510). The availability of RMP-determined concentrations for 24,25$(OH)_2D_3$ in these archived DEQAS samples provides a valuable resource, in addition to the SRMs, for use in assessing the accuracy of measurements for 24,25$(OH)_2D_3$ in human serum. In this paper, the results for 24,25$(OH)_2D_3$ in the DEQAS samples determined using RMPs were used to: (1) compare with 24,25$(OH)_2D_3$ results from participants in DEQAS, (2) demonstrate the relationship of 25(OH)D_3 and 24,25$(OH)_2D_3$ using accuracy-based RMPs, and (3) assess the contribution of 24,25$(OH)_2D_3$ in DEQAS consensus mean values for each of the various assays reported in DEQAS exercises (i.e., ligand binding assays, LC, and LC-MS/MS).

2. Materials and methods

2.1. DEQAS samples

A total of 90 archived DEQAS samples (i.e., samples that were used in past DEQAS exercises) were analyzed at NIST using the RMP for 24,25$(OH)_2D_3$ [48]. The first 30 DEQAS samples were from the October 2012 through January 2014 DEQAS exercises (nos. 421–450) and were analyzed in 2015 with results reported in 2017 [19]. Sixty additional DEQAS samples from the April 2014 to January 2017 DEQAS exercises (nos. 451–510) were analyzed in 2019 and are reported here. DEQAS sample 465 was spiked with 24,25$(OH)_2D_3$ and is therefore not representative of natural levels of 24,25$(OH)_2D_3$ in human patient/donor samples, and this sample was removed for some of the calculations and plots. The DEQAS samples were also analyzed for the determination of 25(OH)D_2 and 25(OH)D_3 using RMPs [3] as described previously [2] and for the determination of 3-epi-25(OH)D_3 using an ID LC-MS/MS method similar to the RMPs for 25(OH)D_2 and 25(OH)D_3.

2.2. Determination of 24,25$(OH)_2D_3$

All DEQAS samples were analyzed using the NIST RMP for the determination of 24,25$(OH)_2D_3$ as described in Tai et al. [48]. As part of

Table 1
Summary of Results for NIST Determination of $25(OH)D_2$, $25(OH)D_3$, Total 25(OH)D, 3-epi-$25(OH)D_3$, and $24,25(OH)_2D_3$ in DEQAS Samples Nos. 421–510.

DEQAS no.	$25(OH)D_2$ (nmol/L)			$25(OH)D_3$ (nmol/L)			Total 25(OH)D (nmol/L)			3-epi-$25(OH)D_3$ (nmol/L)			$24,25(OH)_2D_3$ (nmol/L)		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
421	0.95	0.07	7.5	57.28	0.37	0.6	58.23	0.37	0.6	2.32	0.055	2.4	4.56	0.01	0.1
422	1.68	0.03	2.1	36.53	0.34	0.9	38.21	0.34	0.9	1.69	0.061	3.6	1.97	0.01	0.6
423	0.99	0.05	4.6	84.49	0.95	1.1	85.48	0.95	1.1	5.68	0.073	1.3	6.84	0.02	0.2
424	0.99	0.04	3.7	46.13	0.40	0.9	47.12	0.41	0.9	2.58	0.084	3.3	3.29	0.02	0.6
425	0.95	0.05	5.0	46.11	0.08	0.2	47.06	0.09	0.2	2.53	0.048	1.9	3.24	0.02	0.7
426	1.05	0.03	3.0	33.45	0.41	1.2	34.50	0.41	1.2	0.41	0.013	3.1	1.46	0.01	0.7
427	2.06	0.05	2.5	75.09	0.36	0.5	77.15	0.36	0.5	4.52	0.076	1.7	6.17	0.06	1.0
428	2.48	0.07	2.7	52.14	0.91	1.7	54.62	0.91	1.7	2.74	0.013	0.5	3.53	0.03	0.7
429	0.56	0.02	4.2	58.91	2.27	3.9	59.47	2.27	3.8	3.02	0.119	3.9	4.63	0.02	0.4
430	22.43	0.27	1.2	17.62	0.16	0.9	40.06	0.31	0.8	0.80	0.038	4.8	0.60	0.01	0.8
431	1.27	0.04	2.9	22.49	0.23	1.0	23.76	0.23	1.0	1.22	0.043	3.6	1.03	0.02	1.6
432	2.73	0.12	4.3	48.54	0.22	0.5	51.27	0.25	0.5	2.86	0.320	11.2	3.34	0.02	0.5
433	1.19	0.04	3.5	90.27	0.70	0.8	91.46	0.70	0.8	11.66	0.459	3.9	7.56	0.10	1.3
434	4.44	0.04	0.8	74.00	0.49	0.7	78.44	0.49	0.6	4.57	0.214	4.7	5.21	0.03	0.6
435	0.54	0.02	4.4	45.99	0.22	0.5	46.53	0.22	0.5	2.38	0.061	2.5	3.12	0.01	0.5
436	1.26	0.05	4.2	76.61	0.99	1.3	77.87	0.99	1.3	4.00	0.075	1.9	6.55	0.09	1.4
437	1.40	0.01	1.0	33.24	0.32	1.0	34.63	0.32	0.9	1.45	0.128	8.8	1.99	0.01	0.4
438	1.82	0.03	1.7	54.70	0.51	0.9	56.52	0.51	0.9	2.70	0.088	3.3	3.89	0.05	1.4
439	1.19	0.05	4.5	39.68	0.34	0.8	40.87	0.34	0.8	2.27	0.042	1.8	2.83	0.02	0.8
440	1.32	0.01	1.1	47.05	0.40	0.8	48.38	0.40	0.8	2.27	0.067	2.9	3.62	0.01	0.3
441	1.66	0.06	3.9	88.80	0.71	0.8	90.45	0.71	0.8	7.07	0.347	4.9	7.94	0.07	0.8
442	1.66	0.03	1.9	73.61	1.53	2.1	75.27	1.53	2.0	4.61	0.205	4.4	6.19	0.05	0.8
443	1.89	0.12	6.1	29.48	1.12	3.8	31.37	1.12	3.6	1.27	0.070	5.5	1.56	0.03	2.0
444	1.65	0.03	1.9	41.70	0.96	2.3	43.35	0.96	2.2	1.91	0.062	3.3	3.17	0.03	0.9
445	14.45	0.29	2.0	39.26	0.75	1.9	53.71	0.80	1.5	1.43	0.143	10.0	2.38	0.01	0.1
446	0.87	0.08	9.7	91.62	0.51	0.6	92.49	0.52	0.6	5.67	0.70	12.4	9.00	0.04	0.5
447	1.11	0.07	6.5	46.94	0.18	0.4	48.05	0.19	0.4	2.17	0.39	17.9	3.70	0.05	1.4
448	1.85	0.05	2.9	30.65	0.18	0.6	32.51	0.18	0.6	1.27	0.12	9.1	1.73	0.01	0.7
449	1.50	0.06	3.9	70.66	1.39	2.0	72.16	1.39	1.9	4.13	0.28	6.7	5.66	0.05	0.9
450	0.99	0.09	8.9	63.36	0.26	0.4	64.36	0.27	0.4	2.43	0.37	15.1	5.30	0.04	0.7
451	1.90	0.03	1.8	27.22	1.90	7.0	29.12	1.90	6.5	1.34	0.09	6.8	1.52	0.03	1.7
452	1.09	0.02	1.9	98.83	2.42	2.5	99.92	2.42	2.4	7.82	0.10	1.2	10.32	0.19	1.8
453	2.34	0.23	9.6	63.96	3.11	4.9	66.30	3.12	4.7	3.17	0.09	2.7	3.97	0.07	1.7
454	2.30	0.03	1.5	45.10	0.74	1.6	47.40	0.74	1.6	2.46	0.06	2.6	3.49	0.04	1.0
455	1.45	0.03	2.1	50.80	0.94	1.8	52.25	0.94	1.8	9.12	0.81	8.8	3.67	0.01	0.3
456	1.52	0.04	2.8	72.45	1.23	1.7	73.97	1.23	1.7	3.92	0.07	1.9	7.46	0.10	1.4
457	2.00	0.01	0.6	38.97	0.43	1.1	40.97	0.43	1.0	1.37	0.13	9.4	2.14	0.04	1.8
458	1.30	0.01	1.1	69.79	0.68	1.0	71.09	0.68	1.0	5.79	0.12	2.0	5.91	0.07	1.2
459	0.92	0.01	1.3	92.39	1.29	1.4	93.31	1.29	1.4	4.75	0.17	3.5	9.35	0.11	1.1
460	1.79	0.04	2.1	50.87	0.79	1.5	52.66	0.79	1.5	7.90	0.17	2.2	3.45	0.07	2.2
461	2.14	0.05	2.2	55.07	0.75	1.4	57.21	0.75	1.3	2.68	0.09	3.2	3.81	0.02	0.6
462	1.17	0.04	3.6	79.90	1.87	2.3	81.08	1.87	2.3	4.48	0.03	0.7	6.20	0.08	1.3
463	0.66	0.03	4.7	85.54	0.37	0.4	86.20	0.37	0.4	5.57	0.16	2.8	7.64	0.05	0.6
464	1.98	0.03	1.8	57.57	1.21	2.1	59.55	1.21	2.0	3.08	0.09	2.8	3.44	0.06	1.8
465	1.98	0.05	2.3	57.77	0.83	1.4	59.75	0.83	1.4	3.11	0.12	4.0	17.89	0.20	1.1
466	1.04	0.03	3.0	63.31	1.41	2.2	64.36	1.41	2.2	3.07	0.06	2.0	4.36	0.08	1.9
467	1.30	0.07	5.5	43.06	1.01	2.4	44.36	1.02	2.3	2.26	0.04	1.7	2.49	0.04	1.7
468	1.20	0.02	2.1	67.01	0.88	1.3	68.21	0.88	1.3	4.42	0.08	1.7	4.16	0.08	1.9
469	1.37	0.04	3.1	67.83	0.62	0.9	69.20	0.63	0.9	3.60	0.11	3.1	4.95	0.08	1.6
470	1.95	0.05	2.3	116.3	1.08	0.9	118.3	1.08	0.9	9.88	0.20	2.0	11.93	0.20	1.7
471	11.08	0.20	1.8	53.30	0.73	1.4	64.38	0.75	1.2	3.37	0.28	8.4	4.04	0.07	1.6
472	1.69	0.12	7.0	37.90	0.78	2.0	39.60	0.78	2.0	1.67	0.08	4.7	1.88	0.03	1.8
473	2.66	0.11	4.1	62.48	0.86	1.4	65.14	0.87	1.3	4.12	0.04	0.9	6.00	0.11	1.9
474	1.29	0.05	4.2	77.24	0.21	0.3	78.53	0.21	0.3	5.49	0.18	3.3	6.70	0.09	1.3
475	1.21	0.05	3.9	73.91	0.53	0.7	75.12	0.53	0.7	5.56	0.33	6.0	5.83	0.04	0.7
476	0.75	0.02	3.3	100.2	0.62	0.6	101.0	0.62	0.6	5.29	0.13	2.4	9.06	0.20	2.2
477	1.84	0.01	0.8	43.41	0.33	0.8	45.25	0.33	0.7	1.81	0.06	3.1	2.41	0.05	2.0
478	1.21	0.04	3.1	73.34	0.25	0.3	74.54	0.25	0.3	2.53	0.13	5.1	5.97	0.11	1.8
479	1.43	0.03	2.2	30.86	0.10	0.3	32.29	0.11	0.3	0.99	0.08	7.7	1.15	0.03	2.3
480	57.11	0.21	0.4	47.00	0.49	1.0	104.1	0.53	0.5	2.97	0.07	2.4	4.25	0.07	1.7
481	2.11	0.04	1.8	58.58	0.84	1.4	60.68	0.84	1.4	2.58	0.07	2.8	4.30	0.04	0.8
482	0.81	0.02	2.9	34.75	0.57	1.6	35.56	0.57	1.6	1.76	0.02	1.4	1.85	0.01	0.7
483	0.80	0.02	3.0	115.2	0.74	0.6	116.0	0.74	0.6	5.21	0.13	2.6	10.47	0.18	1.7
484	0.74	0.00	0.0	77.32	0.84	1.1	78.06	0.84	1.1	3.58	0.10	2.7	6.88	0.05	0.7
485	1.13	0.01	1.1	47.00	0.66	1.4	48.13	0.66	1.4	1.81	0.05	2.9	2.18	0.04	1.7
486	0.47	0.02	4.3	28.22	0.41	1.5	28.69	0.42	1.4	2.26	0.01	0.6	1.82	0.03	1.4
487	0.77	0.07	9.6	92.02	1.71	1.9	92.79	1.71	1.8	6.99	0.32	4.5	9.68	0.14	1.4
488	1.21	0.07	5.5	65.51	1.90	2.9	66.72	1.90	2.9	4.79	0.35	7.3	4.95	0.03	0.6
489	1.04	0.05	4.9	45.56	1.24	2.7	46.60	1.24	2.7	3.33	0.23	6.9	2.93	0.02	0.6
490	0.41	0.01	3.5	116.3	1.66	1.4	116.7	1.66	1.4	8.99	0.35	3.9	12.15	0.11	0.9
491	1.50	0.08	5.5	31.84	0.52	1.6	33.34	0.53	1.6	2.08	0.07	3.4	1.51	0.04	2.4
492	1.10	0.07	6.7	50.43	0.69	1.4	51.53	0.70	1.4	3.11	0.12	3.7	3.56	0.07	1.9
493	1.29	0.07	5.5	90.39	1.53	1.7	91.68	1.53	1.7	7.36	0.37	5.0	8.40	0.15	1.8

(*continued on next page*)

Table 1 (*continued*)

DEQAS no.	25(OH)D_2 (nmol/L)			25(OH)D_3 (nmol/L)			Total 25(OH)D (nmol/L)			3-epi-25(OH)D_3 (nmol/L)			24,25$(OH)_2D_3$ (nmol/L)		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
494	1.12	0.05	4.2	70.69	0.81	1.1	71.82	0.81	1.1	3.72	0.22	5.8	4.71	0.07	1.5
495	34.76	0.49	1.4	63.02	0.97	1.5	97.78	1.08	1.1	4.74	0.04	0.8	4.62	0.06	1.3
496	2.38	0.05	2.3	73.43	0.74	1.0	75.81	0.74	1.0	4.89	0.13	2.6	5.37	0.07	1.3
497	1.43	0.03	2.4	48.50	0.10	0.2	49.93	0.11	0.2	3.58	0.21	5.7	3.72	0.08	2.0
498	0.65	0.01	1.9	28.24	0.42	1.5	28.89	0.42	1.5	1.22	0.02	1.7	1.40	0.03	1.9
499	1.42	0.01	0.9	49.70	1.13	2.3	51.11	1.13	2.2	3.44	0.04	1.0	3.81	0.07	1.8
500	1.03	0.04	4.2	129.0	1.60	1.2	130.1	1.60	1.2	11.69	0.36	3.1	11.21	0.12	1.1
501	2.85	0.06	2.2	93.27	0.35	0.4	96.12	0.36	0.4	6.48	0.18	2.8	8.56	0.09	1.0
502	1.24	0.02	1.8	38.69	0.31	0.8	39.93	0.31	0.8	1.42	0.05	3.2	2.61	0.04	1.5
503	1.40	0.05	3.7	77.65	0.54	0.7	79.06	0.54	0.7	5.50	0.18	3.2	7.06	0.09	1.3
504	2.06	0.11	5.3	54.05	0.36	0.7	56.11	0.38	0.7	2.93	0.39	13.3	4.20	0.08	1.8
505	0.74	0.05	6.8	20.95	0.20	0.9	21.69	0.20	0.9	0.88	0.07	8.2	0.81	0.01	1.6
506	1.34	0.04	3.1	54.47	0.89	1.6	55.81	0.89	1.6	2.40	0.09	3.8	3.70	0.04	1.0
507	1.59	0.03	1.6	73.55	0.76	1.0	75.14	0.76	1.0	4.60	0.15	3.3	5.59	0.11	1.9
508	1.93	0.10	5.3	30.10	0.23	0.8	32.03	0.25	0.8	1.67	0.09	5.2	1.34	0.03	2.0
509	1.14	0.06	5.4	71.21	1.07	1.5	72.35	1.08	1.5	3.66	0.10	2.7	5.26	0.07	1.3
510	0.50	0.06	11.2	132.6	4.23	3.2	133.1	4.23	3.2	12.53	0.16	1.3	12.43	0.08	0.6

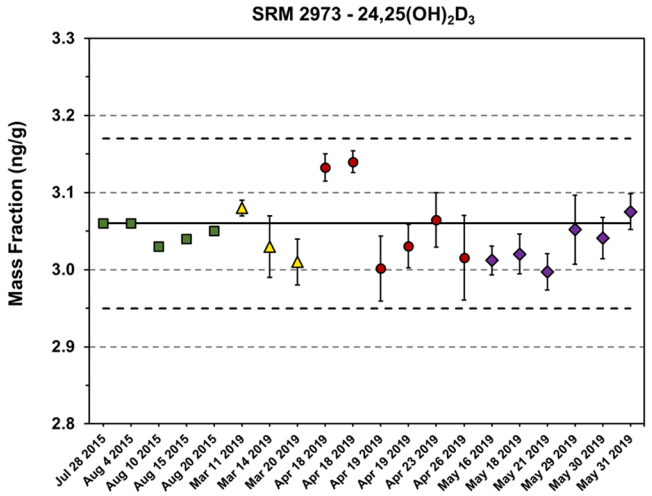

Fig. 1. Results for the determination of 24,25$(OH)_2D_3$ in SRM 2973 used as a control sample during the analyses of 90 DEQAS samples. The various colors and shapes for the measurements represent different measurement campaigns, i.e., green squares (measurements of 24,25$(OH)_2D_3$ in SRM 972, SRM 2973, and DEQAS samples 421–450) [8,19]), yellow triangles (measurements in commutability study [52]), red circles (analysis of DEQAS samples 451–480 (this study), and purple diamonds (analysis of DEQAS samples 481–510 (this study). Error bars represent ± standard deviation of the measurements with n = 4; only one replicate for 2015 measurements.

the development and validation of the RMP, potential interference from other dihydroxy vitamin D metabolites was assessed including 24S,25$(OH)_2D_3$, 3-epi-24R,25$(OH)_2D_3$, 23,25$(OH)_2D_3$, 1,25$(OH)_2D_3$, and 25,26$(OH)_2D_3$ [48]. The NIST ID LC-MS/MS method is currently the only method for the determination of 24,25$(OH)_2D_3$ that is recognized by the JCTLM as an RMP. Details of the analysis of DEQAS samples 421 through 450 were reported previously [19]. The details of the analysis of DEQAS nos. 451 through 510 are provided in the Supplementary information. Briefly, the ID LC-MS/MS method uses gradient elution chromatography on a C_{18} column with MS/MS detection in the positive ion mode with multiple reaction monitoring (MRM) with transitions monitored at *m/z* 417 → *m/z* 381and *m/z* 423 →*m/z* 387 for 24,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$-d_6, respectively. For the sample preparation, duplicate preparations of 2 g samples of DEQAS serum samples (i.e., the combined contents of four vials each containing approximately 0.5 mL) were spiked with 24,25$(OH)_2D_3$-d_6 and then extracted with hexane-ethyl acetate mixture (twice), the upper layer removed, dried, and reconstituted in 100 μL of methanol for the LC-MS/MS analysis with a 10 μL injection.

2.3. Multivariable data analysis

Results from DEQAS exercises using samples 421 through 510 were compiled from DEQAS summary reports for each study [51]. The mean results for each assay were used as the assay response in the multivariable analysis. The number of assay results for each sample analyzed were also compiled, and the mean number of assay results for the 90 samples is reported. Note that the DEQAS data represents assays used from October 2012 through January 2017 and the interlaboratory study to which the results are compared [52,53] was conducted from September to November 2016. The multivariable regression analysis was performed using Analyze-It., a statistical analysis add-in for Microsoft Excel (Analyze-It Software, Leeds, UK).

3. Results and discussion

The results for the determination of 25$(OH)D_2$, 25$(OH)D_3$, total 25(OH)D, 3-epi-25$(OH)D_3$, and 24,25$(OH)_2D_3$ in the 90 DEQAS samples are summarized in Table 1. The results for the first 30 samples (nos. 421 through 450) were published previously [19]; however, they are included in this summary for completeness. A summary of the measurements for 24,25$(OH)_2D_3$ in the DEQAS samples as determined in ng/g and converted to nmol/L is provided in Supplementary material as Table S1. For the determination of 24,25$(OH)_2D_3$, SRM 2973 was used as a control sample, and the results of analyses of SRM 2973, shown in Fig. 1, indicate that the analyses were in control during the time period of these analyses. The concentrations of all four measured vitamin D metabolites are illustrated in Fig. 2 with the 90 samples arranged according to increasing concentration of total 25(OH)D, which ranges from 24 nmol/L to 133 nmol/L. The concentrations of 24,25$(OH)_2D_3$ range from 0.60 nmol/L to 12.43 nmol/L with one sample (no. 465) spiked with 24,25$(OH)_2D_3$ to a concentration of 17.89 nmol/L. Half of the samples have concentrations of 24,25$(OH)_2D_3$ less than 5 nmol/L, and there are 17 samples with concentrations greater than 7 nmol/L.

3.1. Comparison of NIST RMP results with DEQAS results

Since the April 2015 DEQAS exercise (sample no. 471), results for 24,25$(OH)_2D_3$ in the regular samples distributed for the determination of 25(OH)D have been compiled and a consensus mean of the participant results reported. Of the 90 DEQAS samples analyzed using the NIST RMP, 40 of these samples (nos. 471–510) have DEQAS consensus means for 24,25$(OH)_2D_3$ based on results from 4 to 10 exercise participants as summarized in Table S2 in the Supplementary information. Table S2

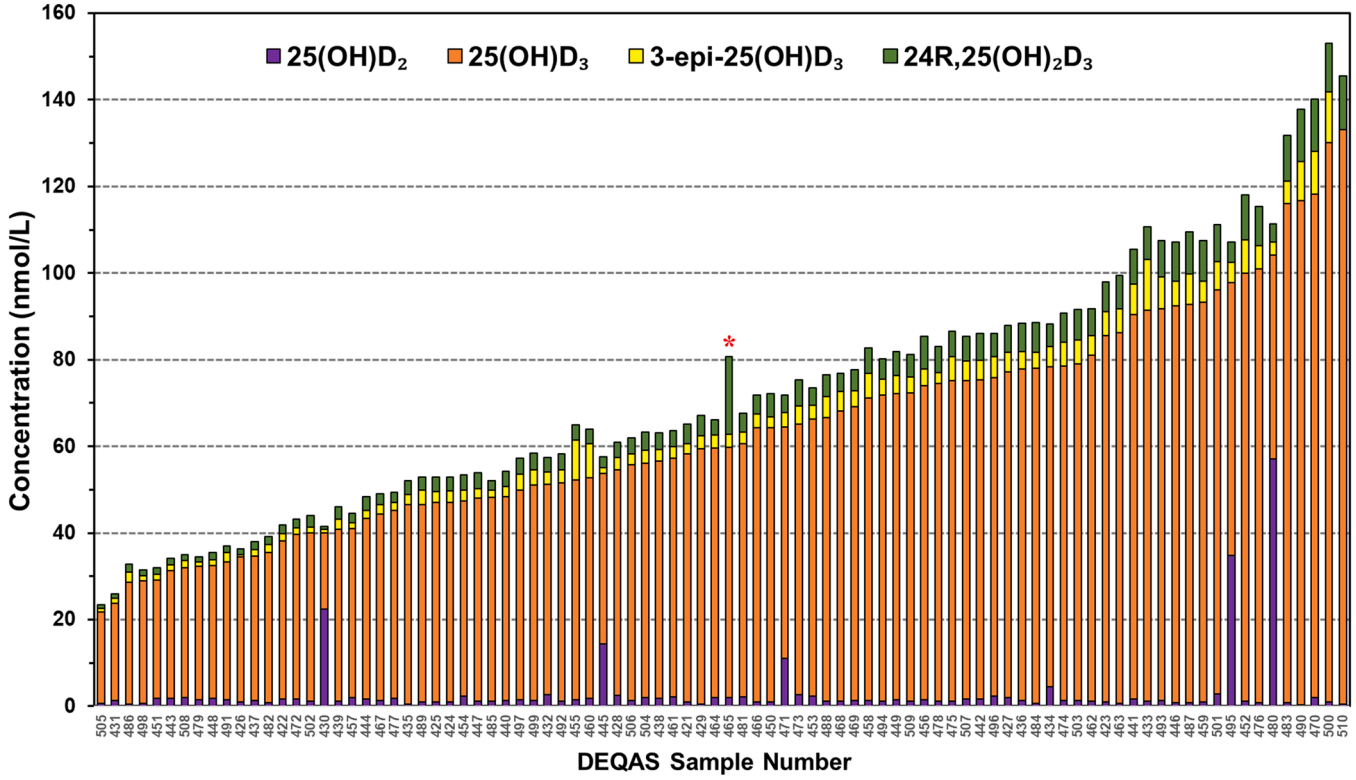

Fig. 2. Distribution of 90 DEQAS samples from lowest to highest molar concentration (nmol/L) of serum total 25(OH)D. Orange bar represents $25(OH)D_3$ molar concentration, purple bar represents $25(OH)D_2$ molar concentration, yellow bar represents the 3-epi-$25(OH)D_3$ molar concentration, and green bar represents the $24R,25(OH)_2D_3$ molar concentration, all in nmol/L. The figure is based on results provided in Table 1. Note that the total serum 25(OH)D includes only $25(OH)D_2$ and $25(OH)D_3$ (the orange plus purple envelope) and does not include the 3-epi-$25(OH)D_3$ or $24,25(OH)_2D_3$. DEQAS sample no. 465 (marked with a red asterisk) was spiked with $24,25(OH)_2D_3$ and is therefore not representative of a normal patient sample.

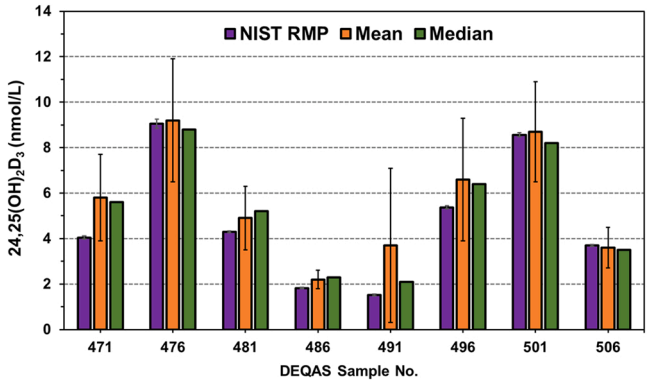

Fig. 3. Comparison of the NIST RMP results compared with the consensus mean and median values from the participant results for selected DEQAS samples (see Table S4). Error bars for NIST RMP represent ± standard deviation of duplicate measurements and error bars for the DEQAS consensus mean represent ± standard deviation of the mean results from multiple laboratories with n = 4 (no. of laboratories) for study DEQAS 471, n = 6 for DEQAS 476, n = 7 for DEQAS 481, n = 5 for DEQAS 486, n = 8 for DEQAS 491 and 496, n = 10 for DEQAS 501, and n = 9 for DEQAS 506.

also contains the NIST RMP values, the DEQAS median value, and the percent bias of the DEQAS consensus mean and median values from the NIST RMP results. The biases for the DEQAS consensus mean values range from − 11 % to 91 % with a mean bias of 26 % for the 40 samples. Individual laboratory results for $24,25(OH)_2D_3$ are provided in Table S3, and the percent bias for individual laboratory results compared with the NIST RMP is provided in Table S4 in the Supplementary information. Fig. 3 is a bar graph comparing the NIST RMP value with the DEQAS consensus mean and median value for eight selected samples (the first sample of each exercise was selected). The mean percent bias for the nine individual laboratory results for the 40 samples ranged from − 40.5 % to 45.6 %. Several laboratories (e.g., Labs 52 and 189) were consistently biased lower than the NIST RMP values while other laboratories were consistently biased higher (e.g., Labs 528, 1455, and 1920). The results of least squares linear regression of the individual laboratory results and the consensus mean and median versus the NIST RMP are summarized in Table S5 including slope, R^2, and width of the 95 % prediction interval (PI). Three laboratories had R^2 values of > 0.92 and the narrowest width of the PI (Labs 52, 1455, and 2123).

Comparison of the DEQAS consensus values with the NIST RMP values demonstrates that the consensus values do not provide an adequate assessment of the accuracy of $24,25(OH)_2D_3$ measurements. Since 2018 when the CDC started to provide target values for $25(OH)D_2$ and $25(OH)D_3$, they have also provided information values for 24,25 $(OH)_2D_3$ based on measurements using their routine ID LC-MS/MS method. The CDC routine method is not an RMP; however, a study to compare the NIST RMP for $24,25(OH)_2D_3$ with the CDC routine method and methods from several other laboratories providing measurements of $24,25(OH)_2D_3$ is currently in progress.

3.2. Relationship of $25(OH)D_3$ and $24,25(OH)_2D_3$ in the DEQAS samples

A number of papers have reported the relationship of $24,25(OH)_2D_3$ and $25(OH)D_3$ concentrations in human serum based on LC-MS/MS measurements [17,31,38,54–60] as discussed previously [19]. The well-known correlation between concentrations of $24,25(OH)_2D_3$ and $25(OH)D_3$ is illustrated in Fig. 3 for the DEQAS samples with a slope of 0.110 ± 0.005 (95 % confidence interval) and a correlation coefficient

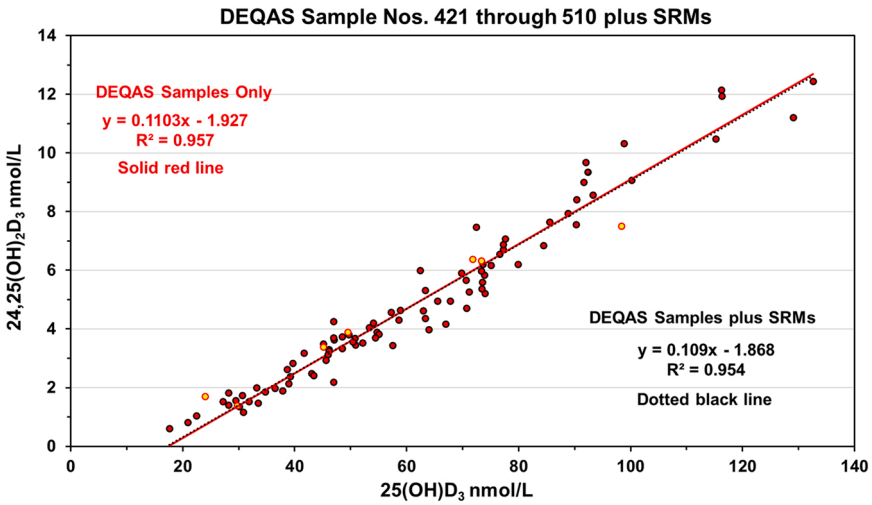

Fig. 4. Regression analysis of concentration of $24,25(OH)_2D_3$ (red circles, red solid line) versus the concentration of $25(OH)D_3$ as determined in 89 DEQAS samples (excluding no. 465) (red circles) and 7 SRM serum pools (yellow circles) using RMPs. The red solid line is the regression line for the DEQAS samples only. The black dotted line is the regression line for the DEQAS samples plus the SRMs. Plots are based on results provided in Tables 1 and S2.

of $R^2 = 0.957$. For a 50 single-donor sample set used in a commutability study [61] and analyzed using the same RMPs, a similar linear regression of $24,25(OH)_2D_3$ and $25(OH)D_3$ concentrations resulted in a slope of 0.098 ± 0.015 (95 % confidence interval) and $R^2 = 0.793$ [52]. The 50 single-donor samples and the DEQAS samples are shown together in Fig. S1 (Supplemental information). The correlation for the regression line for the single-donor samples is lower than the DEQAS samples probably because the DEQAS samples are sample pools from several donors rather than from single donors. Similar plots of the relationship of $24,25(OH)_2D_3$ and $25(OH)D_3$ are reported by Wagner et al. [31], Kaufmann et al. [17], ($R^2 = 0.80$, slope 0.13, x-intercept 25.6 nmol/L) for 672 patient samples, and Kaufmann et al. [60] ($R^2 = 0.83$, slope = 0.068, x-intercept 13.2 nmol/L) for 156 patients. The x-intercept of 17.5 ± 3.0 nmol/L from our study is between the x-intercepts of 25.6 nmol/L and 13.2 nmol/L reported by Kaufmann et al. [17,60] for similar plots from larger data sets where the authors attribute the x-intercept value to the biological threshold to vitamin D deficiency where there is no production of $24,25(OH)_2D_3$. Fig. 3 also includes data points for the seven SRM serum pools, i.e., SRM 972a (four levels), SRM 2969 Vitamin D Metabolites in Frozen Serum (Total 25-Hydroxyvitamin D Low Level), SRM 2970 Vitamin D Metabolites in Frozen Serum (25-Hydroxyvitamin D_2 High Level), and SRM 2973, which cover a range of $24,25(OH)_2D_3$ concentrations from 1.4 nmol/L to 7.5 nmol/L. SRM 2969 and SRM 2970 are recently released SRMs with low levels of $25(OH)D_3$ [10], and therefore low levels of $24,25(OH)_2D_3$. The linear regression correlation in Fig. 3 for the DEQAS samples and SRMs has a slope of 0.109 ± 0.005 (95 % confidence interval) and $R^2 = 0.955$.

As described in the Introduction, the ratio of molar concentrations of $24,25(OH)_2D_3$ to $25(OH)D_3$ offers the potential for better assessment of vitamin D status. The relative concentrations of $24,25(OH)_2D_3$ to 25 $(OH)D_3$ presented as percent and the ratio of the molar concentrations of $25(OH)D_3$ to $24,25(OH)_2D_3$ (i.e., VMR) are provided in Table S5 in Supplementary information. Similar information for the seven SRM serum pools is provided in Table S6. For the DEQAS samples, the mean relative molar concentration of $24,25(OH)_2D_3$ to $25(OH)D_3$ was 7.3 % (ranging from 3.4 % to 10.5 %, median of 7.3 %). The molar ratio 25 $(OH)D_3/24,25(OH)_2D_3$ versus molar concentration of $25(OH)D_3$ is shown in Fig. 4 for the DEQAS and SRM samples. A similar plot combining the data for the DEQAS and the 50 single donor samples is provided in Fig. S2. Similar plots of the ratio $25(OH)D_3/24,25(OH)_2D_3$

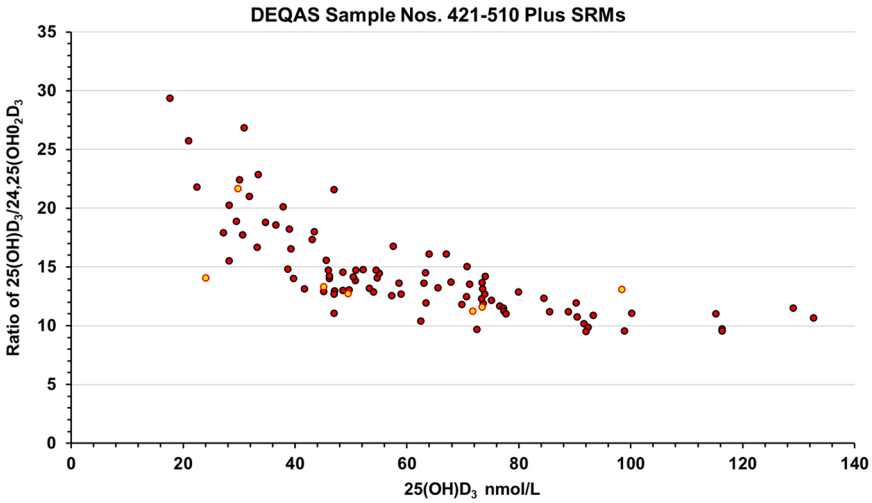

Fig. 5. Plot of the ratio $25(OH)D_3/24,25(OH)_2D_3$ versus molar concentration of $25(OH)D_3$ (nmol/L) in 89 DEQAS samples and 7 SRMs based on NIST RMP measurements. Red circles are DEQAS samples (nos. 421–510, excluding no. 465); yellow circles are SRM samples. All results are from analyses reported in Tables 1 and S2.

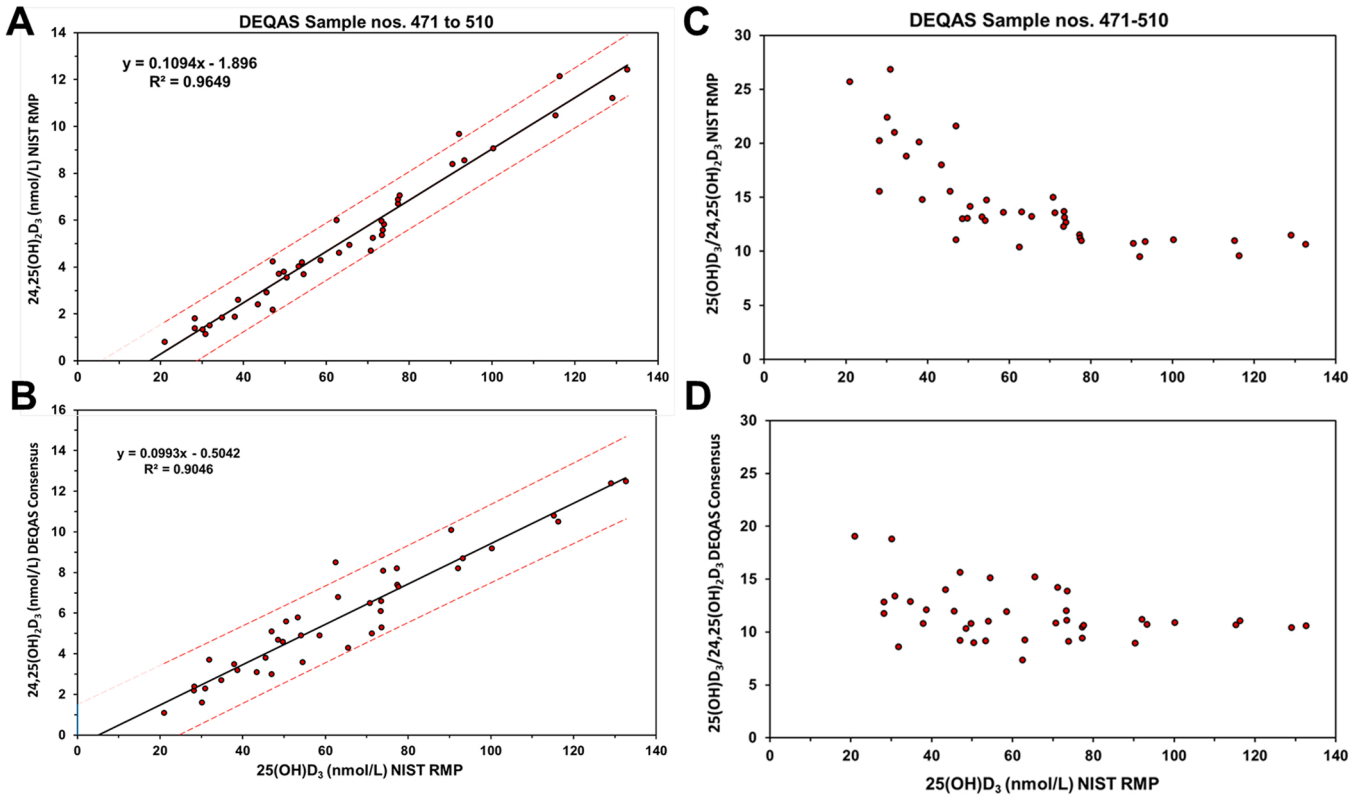

Fig. 6. Plots of the regression analysis of concentration of $24,25(OH)_2D_3$ (nmol/L) versus the concentration of $25(OH)D_3$ (nmol/L) and the ratio $25(OH)D_3/24,25(OH)_2D_3$ versus concentration of $25(OH)D_3$ (nmol/L) for 40 DEQAS samples (nos. 471–510) for which DEQAS consensus means are available. (A) Regression line using NIST RMP values $24,25(OH)_2D_3$ and (B) Regression line using DEQAS consensus means for $24,25(OH)_2D_3$. (C) Plot of ratio $25(OH)D_3/24,25(OH)_2D_3$ versus concentration of $25(OH)D_3$ (nmol/L) using NIST RMP values for $24,25(OH)_2D_3$ and (B) Plot of ratio $25(OH)D_3/24,25(OH)_2D_3$ versus concentration of $25(OH)D_3$ (nmol/L) using DEQAS consensus means for $24,25(OH)_2D_3$.

versus concentration of $25(OH)D_3$ have been reported by Kaufmann et al. [17,60] and by Cashman et al. [57] as a useful parameter to predict vitamin D deficiency.

The usefulness of plots of concentrations of $24,25(OH)_2D_3$ versus $25(OH)D_3$ as in Fig. 4 and the ratio $25(OH)D_3/24,25(OH)_2D_3$ versus $25(OH)D_3$ as in Fig. 5 requires accurate measurements of both $25(OH)D_3$ and $24,25(OH)_2D_3$, and the importance of the accuracy of the $24,25(OH)_2D_3$ measurements is enhanced due to the low concentrations as the divisor in the ratio. Zelzer et al. [49] have emphasized the importance of precise and accurate measurements to calculate and utilize the VMR. To illustrate the need for accurate measurements, the regression analysis of the 40 DEQAS samples for which DEQAS consensus values and NIST RMP values for $24,25(OH)_2D_3$ are available is shown in Fig. 6. As shown in Fig. 5B, when the DEQAS consensus values for $24,25(OH)_2D_3$ are used in the correlation, there is more scatter, a lower correlation coefficient, and a significantly different x-axis intercept (17.3 nmol/L versus 5.1 nmol/L $25(OH)D_3$), which could influence the interpretation of the results. A comparison is shown in Fig. 6 of the use of DEQAS consensus values for $24,25(OH)_2D_3$ or NIST RMP-determined values for plots of the ratio $25(OH)D_3/24,25(OH)_2D_3$ versus $25(OH)D_3$. As demonstrated in Fig. 6D, the use of the consensus values for $24,25(OH)_2D_3$ in the plot provides a somewhat different relationship when compared with the RMP values in Fig. 6C which could limit the intended interpretation.

3.3. Impact of $24,25(OH)_2D_3$ on total 25(OH)D assay performance

With target values for $25(OH)D_2$, $25(OH)D_3$, 3-epi-$25(OH)D_3$, and $24,25(OH)_2D_3$ determined in the 90 DEQAS samples using RMPs, we evaluated the potential contributions of each of these metabolites to total 25(OH)D assay response using the DEQAS consensus assay mean results from various assays used in the exercises. A similar multivariable evaluation for 25(OH)D assays was reported for both ligand binding [53] and LC-MS/MS assays [52] using results from a commutability study using 50 single-donor patient samples. Multivariable regression analysis was performed for the DEQAS assay mean result for serum total 25(OH)D using the NIST measured values for each metabolite as independent variables for the following equation:

$$y\ [25(OH)D\ Test\ Assay] = Constant + a[25(OH)D_2\ NIST] + b[25(OH)D_3\ NIST] + c[3\text{-epi-}25(OH)D_3\ NIST] + d[24,25(OH)_2D_3\ NIST].$$

The multivariable regression analysis provides scaling parameters (a, b, c, and d) that when multiplied by the NIST metabolite concentrations provide the test assay result. Since the test assay result for serum total 25(OH)D should be the sum of only $25(OH)D_2$ and $25(OH)D_3$, in the ideal case, the scaling parameters a and b should be close to 1.0, and the parameters c and d (for 3-epi-$25(OH)D_3$ and $24R,25(OH)_2D_3$, respectively) should be very small. The results of the multivariable regression analysis are summarized in Table 2 for the various assays used in DEQAS exercises with the values of the scaling parameters color coded to distinguish the level of individual metabolite contributions.

Similar multivariable analysis results for 13 different ligand binding assays and 14 LC-MS/MS assays were reported by Wise et al. [52,53]. Using the DEQAS assay results, we evaluated a similar number of different assays including two additional assays (LC and Tosoh). The multivariable regression results confirmed results reported previously [52,53] with the same assays demonstrating an underestimation (Abbott, bioMérieux, DiaSorin, IDS-EIA, and IDS-iSYS) or overestimation (Siemens) of the response for $25(OH)D_2$. The Tosoh and Fujirebio Inc. assays demonstrated a near unity response for both $25(OH)D_2$ and $25(OH)D_3$ using the DEQAS results. The combined DEQAS

Table 2
Multivariable linear regression analysis for 25(OH)D assays for 90 DEQAS samples (Nos. 421–510).

Assay[a]	n[b]	Average No. of results[c]	R^2	25(OH)D_2	SE	25(OH)D_3	SE	3-epi-25(OH)D_3	SE	24,25$(OH)_2D_3$	SE
Abbott Architect (Old)	90	66	0.973	0.472	0.074	0.512	0.056	0.25	0.40	5.67	0.35
Abbott Architect (New)	25	44	0.993	0.442*	0.105	0.679	0.138	0.31	0.90	4.38*	1.14
Beckman Unicell	65	25	0.950	1.09	0.10	0.889	0.078	1.71	0.59	0.17	0.46
Beckman Access 2	65	3	0.939	0.942	0.107	0.805	0.082	2.17*	0.62	0.049	0.49
bioMérieux	35	3	0.986	0.661	0.076	0.608*	0.158	0.09	0.71	5.69*	1.40
DiaSorin	90	278	0.974	0.682	0.060	0.581	0.046	0.18	0.32	3.36	0.28
DIAsource	85	2	0.609	0.480	0.326	0.926*	0.248	-1.83	1.76	2.70	1.54
Diazyme	85	2	0.756	1.12	0.20	0.920	0.152	-0.26	1.09	0.36	0.94
Fujirebio Inc.	60	2	0.978	0.915	0.064	0.956	0.049	0.035	0.37	0.39	0.29
IDS-EIA	90	46	0.973	0.702	0.059	0.527	0.045	0.22	0.32	3.52	0.28
IDS-iSYS	90	117	0.940	0.613	0.099	0.515	0.075	0.75	0.53	4.07	0.47
Roche	90	146	0.950	0.965	0.106	1.037	0.081	0.42	0.57	1.49**	0.50
Siemens	90	63	0.950	1.13	0.08	0.530	0.58	1.31**	0.41	2.04	0.36
SNIBE	75	2	0.563	0.277	0.326	0.396	0.249	1.90	1.89	3.20	1.49
Tosoh	90	5	0.859	0.942	0.174	0.909	0.133	1.34	0.94	1.18	0.82
LC	90	28	0.986	0.832	0.048	1.051	0.036	0.41	0.26	0.0012	0.22
LC-MS/MS	90	152	0.993	1.11	0.035	1.029	0.026	0.38	0.19	0.19	0.17

[a]Assay names/designations are based on DEQAS nomenclature from DEQAS reports.
[b]n = the number of DEQAS samples for which assay results were available.
[c]Average number of assay results for each sample.

Estimated as expected (0.90–1.10).

Underestimated (< 0.90).

Overestimated (> 1.10).

Significant contribution to the estimate ($p < 0.0001$).

No significant contribution to the estimate ($p > 0.0001$).

*Indicates possible contribution to the estimate ($0.0001 > p < 0.001$).
**Indicates possible contribution to the estimate ($p < 0.005$).

results for LC-MS/MS assays demonstrated a slight overestimation of 25(OH)D_2 response similar to the results found in the interlaboratory study [52], but no significant impact for 3-epi-25(OH)D_3 was observed as was the case for several of the individual LC-MS/MS assays in the interlaboratory study [52]. The LC assay results indicated an underestimation for the response of 25(OH)D_2. Of particular significance was the confirmation using the DEQAS results of a significant contribution from 24,25$(OH)_2D_3$ to the assay response for the Abbott, DiaSorin, IDS-EIA, and IDS-iSYS assays. The DEQAS results also indicated that there was a significant contribution from 24,25$(OH)_2D_3$ for the Siemens assay; however, the interlaboratory study [53] did not indicate a contribution from 24,25$(OH)_2D_3$ for this assay.

4. Conclusions

The 90 archived DEQAS samples with concentration values assigned for 25(OH)D_2, 25(OH)D_3, and 24,25$(OH)_2D_3$ using RMPs, as well as 3-epi-25(OH)D_3 using a similar ID-LC-MS/MS approach, represent a valuable resource for researchers for assessing the accuracy of their methods for measurement of these vitamin D metabolites and for standardizing them to RMPs [62]. Selected DEQAS samples from these past exercises are available for purchase from DEQAS [51]. The comparison of the DEQAS 24,25$(OH)_2D_3$ consensus values with the NIST RMP values demonstrates that the consensus values do not provide an adequate assessment of the accuracy of 24,25$(OH)_2D_3$ measurements among participant laboratories. In addition, few laboratories provided results with low bias compared to the NIST RMP values. These 90 DEQAS samples represent the largest number of human serum samples with values assigned for 24,25$(OH)_2D_3$, 25(OH)D_2, and 25(OH)D_3 using RMPs, and thereby provide an assessment of the potential relationship of these two metabolites using accuracy-based results. Additional efforts are needed to improve the comparability of 24,25$(OH)_2D_3$ measurements including the use of SRMs and better assessment of the accuracy of routine ID LC-MS/MS methods. The results of the multivariable analysis using DEQAS assay specific results confirmed that the 24,25$(OH)_2D_3$ concentration does contribute to the response for some 25(OH)D ligand binding assays.

CRediT authorship contribution statement

Stephen A. Wise: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Grace Hahm:** Investigation. **Carolyn Q. Burdette:** Investigation. **Susan S.-C. Tai:** Investigation. **Johanna E. Camara:** Project administration, Resources, Investigation, Writing – review & editing. **Christopher T. Sempos:** Conceptualization. **Emma L. Williams:** Conceptualization, Resources.

Human subject ethics

The National Institute of Standards and Technology Research Protections Office reviewed the protocol for this project and determined it is "not human subjects research" as defined in 15 CFR 27, the Common Rule for the Protection of Human Subjects.

Disclaimer

Certain commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such

identification does not imply recommendation or endorsement by the National Institute of Standards and Technology or the National Institutes of Health, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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Declarations of Competing Interest

The authors declare that they have no competing financial interests.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106318.

References

[1] G.D. Carter, J. Berry, R. Durazo-Arvizu, E. Gunter, G. Jones, J. Jones, H.L.J. Makin, P. Pattni, C.T. Sempos, P. Twomey, E.L. Williams, S.A. Wise, Hydroxyvitamin D assays: an historical perspective from DEQAS, J. Steroid Biochem. Mol. Biol. 177 (2018) 30–35.

[2] C.Q. Burdette, J.E. Camara, F. Nalin, J. Pritchett, L.C. Sander, G.D. Carter, J. Jones, J.M. Betz, C.T. Sempos, S.A. Wise, Establishing an accuracy basis for the vitamin D external quality assessment scheme (DEQAS), J. AOAC Int. 100 (2017) 1277–1287.

[3] S.S.C. Tai, M. Bedner, K.W. Phinney, Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry, Anal. Chem. 82 (2010) 1942–1948.

[4] E.M. Mineva, R.L. Schleicher, M. Chaudhary-Webb, K.L. Maw, J.C. Botelho, H. W. Vesper, C.M. Pfeiffer, A candidate reference measurement procedure for quantifying serum concentrations of 25-hydroxyvitamin D-3 and 25-hydroxyvitamin D-2 using isotope-dilution liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 407 (2015) 5615–5624.

[5] P. Erdman, D.E. Palmer-Toy, G. Horowitz, A. Hoofnagle, Accuracy-based vitamin D survey six years of quality improvement guided by proficiency testing, Arch. Pathol. Lab. Med. 143 (2019) 1531–1538.

[6] K.W. Phinney, M. Bedner, S.S.C. Tai, V.V. Vamathevan, L.C. Sander, K.E. Sharpless, S.A. Wise, J.H. Yen, R.L. Schleicher, M. Chaudhary-Webb, C.M. Pfeiffer, J.M. Betz, P.M. Coates, M.F. Picciano, Development and certification of a standard reference material for vitamin D metabolites in human serum, Anal. Chem. 84 (2012) 956–962.

[7] K.W. Phinney, S.S.C. Tai, M. Bedner, J.E. Camara, R.R.C. Chia, L.C. Sander, K. E. Sharpless, S.A. Wise, J.H. Yen, R.L. Schleicher, M. Chaudhary-Webb, K.L. Maw, Y. Rahmani, J.M. Betz, J. Merkel, C.T. Sempos, P.M. Coates, R.A. Durazo-Arvizu, K. Sarafin, S.P.J. Brooks, Development of an improved standard reference material for vitamin D metabolites in human serum, Anal. Chem. 89 (2017) 4907–4913.

[8] S.S.C. Tai, M.A. Nelson, M. Bedner, B.E. Lang, K.W. Phinney, L.C. Sander, J.H. Yen, J.M. Betz, C.T. Sempos, S.A. Wise, Development of standard reference material (SRM) 2973 vitamin D metabolites in frozen human serum (high level), J. AOAC Int. 100 (2017) 1294–1303.

[9] A.S.P. Boggs, L.E. Kilpatrick, C.Q. Burdette, D.S. Tevis, Z.A. Fultz, M.A. Nelson, J. M. Jarrett, J.V. Kemp, R.J. Singh, S.K.G. Grebe, S.A. Wise, B.L. Kassim, S.E. Long, Development of a pregnancy-specific reference material for thyroid biomarkers, vitamin D, and nutritional trace elements in serum, Clin. Chem. Lab. Med. 59 (2021) 671–679.

[10] G. Hahm, M. Nelson, J. Camara, B. Toman, Certification of Standard Reference Materials 2969 and 2970: Vitamin D Metabolites in Frozen Human Serum (Total 25-Hydroxyvitamin D Low Level) and (25-Hydroxyvitamin D2 High Level), National Institute of Standards and Technology (NIST), Gaithersburg, MD, 2021.

[11] K.W. Phinney, G. Ballihaut, M. Bedner, B.S. Benford, J.E. Camara, S.J. Christopher, W.C. Davis, N.G. Dodder, G. Eppe, B.E. Lang, S.E. Long, M.S. Lowenthal, E. A. McGaw, K.E. Murphy, B.C. Nelson, J.L. Prendergast, J.L. Reiner, C.A. Rimmer, L. C. Sander, M.M. Schantz, K.E. Sharpless, L.T. Sniegoski, S.S.C. Tai, J.B. Thomas, T. W. Vetter, M.J. Welch, S.A. Wise, L.J. Wood, W.F. Guthrie, C.R. Hagwood, S. D. Leigh, J.H. Yen, N.F. Zhang, M. Chaudhary-Webb, H.P. Chen, Z. Fazili, D. J. LaVoie, L.F. McCoy, S.S. Momin, N. Paladugula, E.C. Pendergrast, C.M. Pfeiffer, C.D. Powers, D. Rabinowitz, M.E. Rybak, R.L. Schleicher, B.M.H. Toombs, M. Xu, M. Zhang, A.L. Castle, Development of a standard reference material for metabolomics research, Anal. Chem. 85 (2013) 11732–11738.

[12] N. Binkley, B. Dawson-Hughes, R. Durazo-Arvizu, M. Thamm, L. Tian, J.M. Merkel, J.C. Jones, G.D. Carter, C.T. Sempos, Vitamin D measurement standardization: the way out of the chaos, J. Steroid Biochem. Mol. Biol. 173 (2017) 117–121.

[13] C.T. Sempos, N. Binkley, 25-Hydroxyvitamin D assay standardization and vitamin D guidelines paralysis, Public Health Nutr. 23 (2020) 1153–1164.

[14] A. Alexandridou, P. Schorr, C.S. Stokes, D.A. Volmer, Analysis of vitamin D metabolic markers by mass spectrometry: recent progress regarding the "gold standard" method and integration into clinical practice, Mass Spectrom. Rev. (2021) 1–41.

[15] B. Altieri, E. Cavalier, H.P. Bhattoa, F.R. Perez-Lopez, M.T. Lopez-Baena, G. R. Perez-Roncero, P. Chedraui, C. Annweiler, S. Della Casa, S. Zelzer, M. Herrmann, A. Faggiano, A. Colao, M.F. Holick, Vitamin D testing: advantages and limits of the current assays, Eur. J. Clin. Nutr. 74 (2020) 231–247.

[16] N. Binkley, G.D. Carter, Toward clarity in clinical vitamin D status assessment: 25 (OH)D assay standardization, Endocrinol. Metabol. Clin. N. Am. 46 (2017) 885–899.

[17] M. Kaufmann, J.C. Gallagher, M. Peacock, K.P. Schlingmann, M. Konrad, H. F. DeLuca, R. Sigueiro, B. Lopez, A. Mourino, M. Maestro, R. St-Arnaud, J. S. Finkelstein, D.P. Cooper, G. Jones, Clinical utility of simultaneous quantitation of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D by LC-MS/MS involving derivatization with DMEQ-TAD, J. Clin. Endocrinol. Metab. 99 (2014) 2567–2574.

[18] J.C.Y. Tang, H. Nicholls, I. Piec, C.J. Washbourne, J.J. Dutton, S. Jackson, J. Greeves, W.D. Fraser, Reference intervals for serum 24,25-dihydroxyvitamin D and the ratio with 25-hydroxyvitamin D established using a newly developed LC-MS/MS method, J. Nutr. Biochem. 46 (2017) 21–29.

[19] S.A. Wise, S.S.C. Tai, M.A. Nelson, C.Q. Burdette, J.E. Camara, A.N. Hoofnagle, T. J. Laha, G.D. Carter, J. Jones, E.L. Williams, Z.J. Barclay, G. Jones, M. Kaufmann, N. Binkley, A. Kapoor, T. Ziegler, K.D. Cashman, K.G. Dowling, C.T. Sempos, Interlaboratory comparison for the determination of 24,25-dihydroxyvitamin D-3 in human serum using liquid chromatography with tandem mass spectrometry, J. AOAC Int. 100 (2017) 1308–1317.

[20] N. Alonso, S. Zelzer, G. Eibinger, M. Herrmann, Vitamin D metabolites: analytical challenges and clinical relevance, Calcif. Tissue Int. 112 (2023) 158–177.

[21] M. Herrmann, Assessing vitamin D metabolism – four decades of experience, Clin. Chem. Lab. Med. 61 (2023) 880–894.

[22] M. Herrmann, Towards a personalized assessment of vitamin D status, Clin. Chem. Lab. Med. 58 (2020) 149–151.

[23] M. Herrmann, C.J.L. Farrell, I. Pusceddu, N. Fabregat-Cabello, E. Cavalier, Assessment of vitamin D status – a changing landscape, Clin. Chem. Lab. Med. 55 (2017) 3–26.

[24] G. Jones, M. Kaufmann, Diagnostic aspects of vitamin D: clinical utility of vitamin D metabolite profiling, Jbmr 5 (2021).

[25] M.T. Mizwicki, A.W. Norman, Vitamin D sterol/VDR conformational dynamics and nongenomic actions, in: D. Feldman, J.W. Pike, J.D. Adams (Eds.), Vitamin D, Academic Press, Waltham, MA, 2011, pp. 271–297.

[26] A.T. Slominski, T.K. Kim, H.Z. Shehabi, I. Semak, E.K.Y. Tang, M.N. Nguyen, H.A. E. Benson, E. Korik, Z. Janjetovic, J.J. Chen, C.R. Yates, A. Postlethwaite, W. Li, R. C. Tuckey, In vivo evidence for a novel pathway of vitamin D-3 metabolism initiated by P450scc and modified by CYP27B1, Faseb J. 26 (2012) 3901–3915.

[27] A.T. Slominski, T.K. Kim, H.Z. Shehabi, E.K.Y. Tang, H.A.E. Benson, I. Semak, Z. T. Lin, C.R. Yates, J. Wang, W. Li, R.C. Tuckey, In vivo production of novel vitamin D_2 hydroxy-derivatives by human placentas, epidermal keratinocytes, Caco-2 colon cells and the adrenal gland, Mol. Cell. Endocrinol. 383 (2014) 181–192.

[28] R.C. Tuckey, C.Y.S. Cheng, A.T. Slominski, The serum vitamin D metabolome: what we know and what is still to discover, J. Steroid Biochem. Mol. Biol. 186 (2019) 4–21.

[29] M. Kaufmann, K.P. Schlingmann, L. Berezin, A. Molin, J. Sheftel, M. Vig, J. C. Gallagher, A. Nagata, S.S. Masoud, R. Sakamoto, K. Nagasawa, M. Uesugi, M. L. Kottler, M. Konrad, G. Jones, Differential diagnosis of vitamin D-related hypercalcemia using serum vitamin D metabolite profiling, J. Bone Miner. Res. 36 (2021) 1340–1350.

[30] C. Jenkinson, R. Desai, A.T. Slominski, R.C. Tuckey, M. Hewison, D.J. Handelsman, Simultaneous measurement of 13 circulating vitamin D3 and D2 mono and dihydroxy metabolites using liquid chromatography mass spectrometry, Clin. Chem. Lab. Med. 59 (2021) 1642–1652.

[31] D. Wagner, H.E. Hanwell, K. Schnabl, M. Yazdanpanah, S. Kimball, L. Fu, G. Sidhom, D. Rousseau, D.E.C. Cole, R. Vieth, The ratio of serum 24,25-dihydroxyvitamin D_3 to 25-hydroxyvitamin D_3 is predictive of 25-hydroxyvitamin D_3 response to vitamin D_3 supplementation, J. Steroid Biochem. Mol. Biol. 126 (2011) 72–77.

[32] S.J. Ding, I. Schoenmakers, K. Jones, A. Koulman, A. Prentice, D.A. Volmer, Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS, Anal. Bioanal. Chem. 398 (2010) 779–789.

[33] T.J. Laha, F.G. Strathmann, Z.C. Wang, I.H. de Boer, K.E. Thummel, A. N. Hoofnagle, Characterizing antibody cross-reactivity for immunoaffinity purification of analytes prior to multiplexed liquid chromatography-tandem mass spectrometry, Clin. Chem. 58 (2012) 1711–1716.

[34] K.G. Dowling, G. Hull, J. Sundvall, C. Lamberg-Allardt, K.D. Cashman, Improved accuracy of an tandem liquid chromatography-mass spectrometry method measuring 24R,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D metabolites in serum using unspiked controls and its application to determining cross-reactivity of a chemiluminescent microparticle immunoassay, J. Chromatogr. A 1497 (2017) 102–109.

[35] N. Fabregat-Cabello, J. Farre-Segura, L. Huyghebaert, S. Peeters, C. Le Goff, J. C. Souberbielle, E. Cavalier, A fast and simple method for simultaneous measurements of 25(OH)D, 24, 25(OH)(2)D and the Vitamin D Metabolite Ratio (VMR) in serum samples by LC-MS/MS, Clin. Chim. Acta 473 (2017) 116–123.

[36] S. Zelzer, A. Meinitzer, D. Enko, S. Simstich, C. Le Goff, E. Cavalier, M. Herrmann, W. Goessler, Simultaneous determination of 24,25- and 25,26-dihydroxyvitamin

D_3 in serum samples with liquid-chromatography mass spectrometry – a useful tool for the assessment of vitamin D metabolism, J. Chromatogr. B-Anal. Technol. Biomed. Life Sci. 1158 (2020).
[37] C. Jenkinson, R. Desai, M.D. McLeod, J.W. Mueller, M. Hewison, D.J. Handelsman, Circulating conjugated and unconjugated vitamin D metabolite measurements by liquid chromatography mass spectrometry, J. Clin. Endocrinol. Metab. 107 (2022) 435–449.
[38] C. Jenkinson, A.E. Taylor, Z.K. Hassan-Smith, J.S. Adams, P.M. Stewart, M. Hewison, B.G. Keevil, High throughput LC-MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum, J. Chromatogr. B-Anal. Technol. Biomed. Life Sci. 1014 (2016) 56–63.
[39] A.H. Berg, C.E. Powe, M.K. Evans, J. Wenger, G. Ortiz, A.B. Zonderman, P. Suntharalingam, K. Lucchesi, N.R. Powe, S.A. Karumanchi, R.I. Thadhani, 24,25-Dihydroxyvitamin D_3 and vitamin D status of community-dwelling Black and White Americans, Clin. Chem. 61 (2015) 877–884.
[40] E. Cavalier, L. Huyghebaert, O. Rousselle, A.C. Bekaert, S. Kovacs, L. Vranken, S. Peeters, C. Le Goff, A. Ladang, Simultaneous measurement of 25(OH)-vitamin D and 24,25(OH)2-vitamin D to define cut-offs for CYP24A1 mutation and vitamin D deficiency in a population of 1200 young subjects, Clin. Chem. Lab. Med. 58 (2020) 197–201.
[41] C. Ginsberg, A.N. Hoofnagle, R. Katz, J. Hughes-Austin, L.M. Miller, J.O. Becker, S. B. Kritchevsky, M.G. Shlipak, M.J. Sarnak, J.H. Ix, The vitamin D metabolite ratio is associated with changes in bone density and fracture risk in older adults, J. Bone Miner. Res. 36 (2021) 2343–2350.
[42] C. Ginsberg, R. Katz, I.H. de Boer, B.R. Kestenbaum, M. Chonchol, M.G. Shlipak, M. J. Sarnak, A.N. Hoofnagle, D.E. Rifkin, P.S. Garimella, J.H. Ix, The 24,25 to 25-hydroxyvitamin D ratio and fracture risk in older adults: the cardiovascular health study, Bone 107 (2018) 124–130.
[43] J. Aloia, M. Mikhail, R. Dhaliwal, A. Shieh, G. Usera, A. Stolberg, L. Ragolia, S. Islam, Free 25(OH)D and the vitamin D paradox in African Americans, J. Clin. Endocrinol. Metab. 100 (2015) 3356–3363.
[44] H. Ketha, T.D. Thacher, S.S. Oberhelman, P.R. Fischer, R.J. Singh, R. Kumar, Comparison of the effect of daily versus bolus dose maternal vitamin D_3 supplementation on the 24,25-dihydroxyvitamin D_3 to 25-hydroxyvitamin D_3 ratio, Bone 110 (2018) 321–325.
[45] H. Ketha, R. Kumar, R.J. Singh, LC-MS/MS for identifying patients with CYP24A1 mutations, Clin. Chem. 62 (2016) 236–242.
[46] C.T. Sempos, H.W. Vesper, K.W. Phinney, L.M. Thienpont, P.M. Coates, VDSP, Vitamin D status as an international issue: national surveys and the problem of standardization, Scand. J. Clin. Lab. Invest. 72 (2012) 32–40.
[47] S.A. Wise, S.S.C. Tai, C.Q. Burdette, J.E. Camara, M. Bedner, K.A. Lippa, M. A. Nelson, F. Nalin, K.W. Phinney, L.C. Sander, J.M. Betz, C.T. Sempos, P. M. Coates, Role of the National Institute of Standards and Technology (NIST) in support of the vitamin D initiative of the National Institutes of Health, Office of Dietary Supplements, J. AOAC Int. 100 (2017) 1260–1276.
[48] S.S.C. Tai, M.A. Nelson, Candidate reference measurement procedure for the determination of (24R),25-dihydroxyvitamin D_3 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry, Anal. Chem. 87 (2015) 7964–7970.
[49] S. Zelzer, C. Le Goff, S. Peeters, C. Calaprice, A. Meinitzer, D. Enko, W. Goessler, M. Herrmann, E. Cavalier, Comparison of two LC-MS/MS methods for the quantification of 24,25-dihydroxyvitamin D_3 in patients and external quality assurance samples, Clin. Chem. Lab. Med. 60 (2022) 74–81.
[50] A.K. Alshabrawy, A. Bergamin, D.K. Sharma, S.M. Hickey, D.A. Brooks, P. O'Loughlin, M.D. Wiese, P.H. Anderson, LC-MS/MS analysis of vitamin D_3 metabolites in human serum using a salting-out based liquid-liquid extraction and DAPTAD derivatization, J. Chromatogr. B-Anal. Technol. Biomed. Life Sci. 1173 (2021).
[51] DEQAS, Vitamin D External Quality Assessment Scheme (DEQAS), ⟨www.deqas.org⟩.
[52] S.A. Wise, J.E. Camara, C.T. Sempos, C.Q. Burdette, G. Hahm, F. Nalin, A.J. Kuszak, J. Merkel, R. Durazo-Arvizu, A.N. Hoofnagle, E.L. Williams, F. Ivison, R. Fischer, J. M.W. Van den Ouweland, C.S. Ho, E.W.K. Law, J.-N. Simard, R. Gonthier, B. Holmquist, S. Meadows, L. Cox, K. Robyak, M.H. Creer, R. Fitzgerald, M. W. Clarke, N. Breen, P. Lukas, E. Cavalier, Interlaboratory comparison of 25-hydroxyvitamin D assays: vitamin D Standardization Program (VDSP) intercomparison study 2 – Part 1 liquid chromatography – tandem mass spectrometry (LC-MS/MS) assays – impact of 3-epi-25-hydroxyvitamin D_3 on assay performance, Anal. Bioanal. Chem. 414 (2022) 333–349.
[53] S.A. Wise, J.E. Camara, C.T. Sempos, C.Q. Burdette, G. Hahm, F. Nalin, A.J. Kuszak, J. Merkel, R. Durazo-Arvizu, E.L. Williams, C. Popp, C. Beckert, C. Schultess, G. Van Slooten, C. Tourneur, C. Pease, R. Kaul, A. Villarreal, M.C. Batista, H. Pham, A. Bennett, E. Jansen, D.A. Khan, M. Kilbane, J. Freeman, N. Parker, J. Yuan, S. Mushtaq, C. Simpson, P. Lukas, E. Cavalier, Interlaboratory comparison of 25-hydroxyvitamin D assays: Vitamin D Standardization Program (VDSP) intercomparison study 2 – Part 2 ligand binding assays – impact of 25-hydroxy-vitamin D2 and 24R,25-dihydroxyvitamin D_3 on assay performance, Anal. Bioanal. Chem. 414 (2022) 351–366.
[54] M. Kaufmann, J.C. Gallagher, M. Peacock, G. Jones, Ratio of 25-OH-D3:24,25-(OH) 2D3 is a novel and sensitive measure of predicting vitamin D deficiency, J. Bone Miner. Res. 28 (2013).
[55] S. Baecher, A. Leinenbach, J.A. Wright, S. Pongratz, U. Kobold, R. Thiele, Simultaneous quantification of four vitamin D metabolites in human serum using high performance liquid chromatography tandem mass spectrometry for vitamin D profiling, Clin. Biochem. 45 (2012) 1491–1496.
[56] C.R. Bosworth, G. Levin, C. Robinson-Cohen, A.N. Hoofnagle, J. Ruzinski, B. Young, S.M. Schwartz, J. Himmelfarb, B. Kestenbaum, I.H. de Boer, The serum 24,25-dihydroxyvitamin D concentration, a marker of vitamin D catabolism, is reduced in chronic kidney disease, Kidney Int. 82 (2012) 693–700.
[57] K.D. Cashman, A. Hayes, K. Galvin, J. Merkel, G. Jones, M. Kaufmann, A. N. Hoofnagle, G.D. Carter, R.A. Durazo-Arvizu, C.T. Sempos, Significance of serum 24,25-dihydroxyvitamin D in the assessment of vitamin D status: a double-edged sword? Clin. Chem. 61 (2015) 636–645.
[58] I.H. de Boer, M.C. Sachs, M. Chonchol, J. Himmelfarb, A.N. Hoofnagle, J.H. Ix, R. A. Kremsdorf, Y.S. Lin, R. Mehrotra, C. Robinson-Cohen, D.S. Siscovick, M. W. Steffes, K.E. Thummel, R.P. Tracy, Z.C. Wang, B. Kestenbaum, Estimated GFR and circulating 24,25-dihydroxyvitamin D_3 concentration: a participant-level analysis of 5 cohort studies and clinical trials, Am. J. Kidney Dis. 64 (2014) 187–197.
[59] S. Hsu, L.R. Zelnick, Y.S. Lin, C.M. Best, B.R. Kestenbaum, K.E. Thummel, A. N. Hoofnagle, I.H. de Boer, Validation of the 24,25-dihydroxyvitamin D_3 to 25-hydroxyvitamin D_3 ratio as a biomarker of 25-hydroxyvitamin D_3 clearance, J. Steroid Biochem. Mol. Biol. 217 (2022).
[60] M. Kaufmann, N. Morse, B.J. Molloy, D.P. Cooper, K.P. Schlingmann, A. Molin, M. L. Kottler, J.C. Gallagher, L. Armas, G. Jones, Improved screening test for idiopathic infantile hypercalcemia confirms residual levels of serum 24,25-$(OH)_2D_3$ in affected patients, J. Bone Miner. Res. 32 (2017) 1589–1596.
[61] J.E. Camara, S.A. Wise, A.N. Hoofnagle, E.L. Williams, G.D. Carter, J. Jones, C. Q. Burdette, G. Hahm, F. Nalin, A.J. Kuszak, J. Merkel, R.A. Durazo-Arvizu, P. Lukas, E. Cavalier, C. Popp, C. Beckert, J. Schultess, G. Van Slooten, C. Tourneur, C. Pease, R. Kaul, A. Villarreal, F. Ivison, R. Fischer, J.M.W. van den Ouweland, C. S. Ho, E.W.K. Law, J.N. Simard, R. Gonthier, B. Holmquist, M.C. Batista, H. Pham, A. Bennett, S. Meadows, L. Cox, E. Jansen, D.A. Khan, K. Robyak, M.H. Creer, M. Kilbane, P.J. Twomey, J. Freeman, N. Parker, J.Y. Yuan, R. Fitzgerald, S. Mushtaq, M.W. Clarke, N. Breen, C. Simpson, C.T. Sempos, Assessment of serum total 25-hydroxyvitamin D assay commutability of Standard Reference Materials and College of American Pathologists Accuracy-Based Vitamin D (ABVD) Scheme and Vitamin D External Quality Assessment Scheme (DEQAS) materials: vitamin D Standardization Program (VDSP) commutability study 2, Anal. Bioanal. Chem. 413 (2021) 5067–5084.
[62] C.T. Sempos, J.M. Betz, J.E. Camara, G.D. Carter, E. Cavalier, M.W. Clarke, K. G. Dowling, R.A. Durazo-Arvizu, A.N. Hoofnagle, A. Liu, K.W. Phinney, K. Sarafin, S.A. Wise, P.M. Coates, General steps to standardize the laboratory measurement of serum total 25-hydroxyvitamin D, J. AOAC Int. 100 (2017) 1230–1233.