University of Dundee

University of Dundee

Analytical performance specifications for the measurement uncertainty of 24,25-dihydroxyvitamin D examinations

Cavalier, Etienne; Fraser, Callum G.; Bhattoa, Harjit Pal; Heijboer, Annemieke C.; Makris, Konstantinos; Vasikaran, Samuel

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):
Cavalier, E., Fraser, C. G., Bhattoa, H. P., Heijboer, A. C., Makris, K., Vasikaran, S., Huyghebaert, L., Peeters, S., Le Goff, C., Herrmann, M., Carobene, A., & IFCC-IOF Committee for Bone Metabolism (2023). Analytical performance specifications for the measurement uncertainty of 24,25-dihydroxyvitamin D examinations. *Clinical Chemistry and Laboratory Medicine*. https://doi.org/10.1515/cclm-2023-0176

Etienne Cavalier*, Callum G. Fraser, Harjit Pal Bhattoa, Annemieke C. Heijboer, Konstantinos Makris, Samuel Vasikaran, Loreen Huyghebaert, Stéphanie Peeters, Caroline Le Goff and Markus Herrmann, Anna Carobene and the IFCC-IOF Committee for Bone Metabolism[a]

Analytical performance specifications for the measurement uncertainty of 24,25-dihydroxyvitamin D examinations

Abstract

Objectives: The exploration of the metabolites in the degradation pathways of vitamin D (VTD) has gained importance in recent years and simultaneous quantitation of twenty-five-hydroxy vitamin D (25(OH)D) mass concentration together with 24,25-dihydroxyvitamin D (24,25(OH)2D) has been proposed as a newer approach to define VTD deficiency. Yet, no data are available on 24,25(OH)2D biological variation (BV). In this study, we evaluated 24,25(OH)2D's BV on the European Biological Variation Study (EuBIVAS) cohort samples to determine if analytical performance specifications (APS) for 24,25(OH)2D could be generated.

Methods: Six European laboratories recruited 91 healthy participants. 25(OH)D and 24,25(OH)2D concentrations in K_3-EDTA plasma were examined weekly for up to 10 weeks in duplicate with a validated LC-MS/MS method. The Vitamin D Metabolite Ratio (24,25(OH)2D divided by 25(OH)D × 100) was also calculated at each time point.

Results: Linear regression of the mean 24,25(OH)2D concentrations at each blood collection showed participants were not in steady state. Variations of 24,25(OH)2D over time were significantly positively associated with the slopes of 25(OH)D concentrations over time and the concentration of 25(OH)D of the participant at inclusion, and negatively associated with body mass index (BMI), but not with age, gender, or location of the participant. The variation of the 24,25(OH)2D concentration in participants over a 10 weeks period was 34.6%. Methods that would detect a significant change linked to the natural production of 24,25(OH)2D over this period at p<0.05 would need a relative measurement uncertainty (u%)<14.9% while at p<0.01, relative measurement uncertainty should be <10.5%.

Conclusions: We have defined for the first time APS for 24,25(OH)2D examinations. According to the growing interest in this metabolite, several laboratories and manufacturers might aim to develop specific methods for its

---

Etienne Cavalier, Callum G. Fraser and Anna Carobene contributed equally to this work.

[a]IFCC-IOF Committee for Bone Metabolism: Etienne Cavalier, Harjit Pal Bhattoa, Annemieke Heijboer, Giovanni Lombardi, Samuel Vasikaran, Vincent Delatour, Karen Phinney, Catharine Sturgeon, Hubert Vesper, Markus Herrmann, Jean-Paul Cristol, Edgard Delvin, Lee Sang-Kuk, Masakazu Miura, Richard Pikner, Niklas Rye Jørgensen, Aylin Sepici Dinçel, Galina Zemstovskaja, Kristina Åkesson, Olivier Bruyère, Richard Eastell, Joseph Foldes, Patrick Garnero, John Kanis, Nancy Lane, Michaël McClung, Stuart Silverman.

*Corresponding author: Professor Etienne Cavalier, Department of Clinical Chemistry, University of Liège, CHU de Liège, Centre de Recherche Intégré sur les Médicaments (CIRM), Domaine du Sart-Tilman, 4000 Liège, Belgium, Phone: +32 4 3668822, E-mail: Etienne.cavalier@chuliege.be. https://orcid.org/0000-0003-0947-2226
Callum G. Fraser, Centre for Research into Cancer Prevention and Screening, University of Dundee, Ninewells Hospital and Medical School, Dundee, Scotland. https://orcid.org/0000-0002-1333-7994

Harjit Pal Bhattoa, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary. https://orcid.org/0000-0002-4909-0065

Annemieke C. Heijboer, Department of Clinical Chemistry, Endocrine Laboratory, Amsterdam Gastroenterology & Metabolism, Vrije Universiteit Amsterdam and University of Amsterdam, Amsterdam UMC, Amsterdam, The Netherlands

Konstantinos Makris, Clinical Biochemistry Department, KAT General Hospital, Athens, Greece

Samuel Vasikaran, PathWest Laboratory Medicine, Fiona Stanley Hospital, Murdoch, WA, Australia

Loreen Huyghebaert, Stéphanie Peeters and Caroline Le Goff, Department of Clinical Chemistry, University of Liege, CHU de Liege, CIRM, Liege, Belgium

Markus Herrmann, Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria

Anna Carobene, Laboratory Medicine, IRCCS San Raffaele Scientific Institute, Milan, Italy

---

determination. The results presented in this paper are thus necessary prerequisites for the validation of such methods.

Keywords: 24,25(OH)2D; 25(OH)-vitamin D; analytical performance specifications biological variation; measurement uncertainty; vitamin D; Vitamin D Metabolite Ratio (VMR).

Introduction

The exploration of the metabolites in the degradation pathways of vitamin D (VTD) has gained importance in recent years. In humans, 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)2D) are metabolized into 24,25-dihydroxyvitamin D (24,25(OH)2D) and 1,24,25 trihydroxy vitamin D (1,24,25(OH)3D) by the CYP 24A1 gene which instructs for a 24-hydroxylase enzyme, mutations of which lead to severe idiopathic infantile hypercalcemia [1] or hypercalcemia [2]. Simultaneous quantitation of 25(OH) D and 24,25(OH)2D has been proposed as a newer approach to define vitamin D deficiency [3].

To date, 24,25(OH)2D can only be quantitated by liquid chromatography coupled with mass spectrometers in tandem (LC-MS/MS). A candidate reference method has been published [4] and standards from National Institute of Standards and Technology (NIST) are available, as well as an external quality assessment scheme (DEQAS) proposing certified values for 24,25(OH)2D. We have recently shown a very good concordance between two different LC-MS/MS methods for 24,25(OH)2D measurement [5]. Nevertheless, no data are available on the components of biological variation (BV) of this metabolite, which is a necessary prerequisite to set analytical performance specifications (APS) [6]. In a recent paper, we used samples generated in the European Biological Variation Study (EuBIVAS) [7] to set APS for 25(OH)D [8]. Our results showed that the traditional approach to the generation and application of data on BV was not applicable to 25(OH)D since random variation around a fixed homeostatic setting-point of 25(OH)D concentration over time was not found. However, we proposed another approach to determine APS for 25(OH)D examinations based on measurement uncertainty and physiological variation of 25(OH)D concentrations over time.

In this study, we aimed to evaluate the BV of 24,25(OH)2D on the same EuBIVAS samples as used for 25(OH)D to assess if APS for this metabolite of interest could be generated.

Materials and methods

Biological variation data derived from the EuBIVAS

The EuBIVAS study involved six European laboratories located in Milan (Italy, 45.47°N, 9.19°E), Padua (Italy, 45.41°N, 11.87°E), Bergen (Norway, 60.39°N, 5.33°E), Madrid (Spain, 40.42°N, 3.70°W), Assen (The Netherlands, 52.99°N, 6.6°E), and Istanbul (Turkey, 41.01°N, 28.97°E) [7]. At the beginning of the study, 105 subjects were recruited. Three subjects were not included in the final cohort after application of the inclusion/exclusion criteria at the first collection, and five people withdrew during the study for personal reasons. The final study population for sample collection consisted of 97 presumed healthy volunteers, among which six were excluded for various reasons (see [7] for more details). Among these 91 subjects, sufficient sample volume was available to examine 24,25(OH)2D in 80 of them (33 males and 47 females; age range: 21–69 years).

The participants completed an enrolment questionnaire to provide information on their lifestyle and presumed health status, which was further verified by a set of commonly performed laboratory tests during each collection. One potential participant was taking vitamin D supplementation and was therefore excluded. All laboratories followed the same protocol for the pre-examination phase. Fasting blood samples were drawn by venepuncture weekly for 10 consecutive weeks (April–June 2015) on a set day (Tuesday–Friday) and, at the same time (e.g., between 08.00 and 10.00 at each weekly visit) by the same phlebotomist at most visits, further minimising variation. Seventy-seven participants completed all 10 collections, 10 participants completed nine collections, two participants completed eight collections, and two participants completed seven collections. The K_3-EDTA plasma samples collected by each laboratory were sent frozen on dry ice to San Raffaele Hospital, Milan, Italy. The samples were stored in a –80 °C freezer until they were shipped on dry ice to the CHU de Liège, Belgium, where 24,25(OH)2D concentrations were examined in 2019.

The EuBIVAS protocol was approved by the Institutional Ethical Review Board of San Raffaele Hospital in agreement with the World Medical Association Declaration of Helsinki (protocol number: WG-BV project #001, 50/INT 2014) and by the Ethical Board/Regional Ethics Committee for each laboratory. All participants signed a written consent form.

Analytical methods

We measured 24,25(OH)2D and 25(OH)D concentrations with a LC-MS/MS method that we developed in-house [10]. This method was recently compared with another and gave similar results [5]. The CHU de Liège laboratory participates in the DEQAS survey for 24,25(OH)2D and 25(OH)D and the method is recognized as traceable to the Centers for Disease Control and Prevention (CDC) reference method according to the Vitamin D Standardization-Certification Program (VDSCP) [9]. Internal quality control materials (two concentrations) were measured at the beginning and end of each run. The limit of quantitation (LoQ) for 24,25(OH)2D was set at 0.26 µg/L.

Data analysis

Samples donated from each participant were measured in duplicate within the same run on a single day. To evaluate the general trend in the overall 24,25(OH)2D over the study period, we calculated the regression of the mean of the duplicate measurements from every blood collection 1,2 … 10 vs. the blood draw number (1–10). Subjects were considered in steady state if the 95% confidence intervals (CI) of the slope of the regression line included zero.

We also calculated the Vitamin D Metabolite Ratio (VMR) ratio as being the ratio of 24,25(OH)2D divided by 25(OH)D × 100 [10].

We calculated the relative measurement uncertainty (u%) [11] necessary to observe a significant change of 24,25(OH)2D concentrations

over the 10 weeks period with a defined probability according to the approach that we previously published [8] as Change over 10 weeks = $2^{1/2} \times u\% \times Z$ with Z=1.645 and Z=2.326 for an unidirectional change probability of 95 and 99%, respectively.

Data analyses were performed using Microsoft Excel 2010 and Medcalc (Ostend, Belgium).

Results

Two of the 80 participants had 24,25(OH)2D concentrations that were below the LoQ at the time of enrolment and remained undetectable throughout the 10 weeks study period. The 25(OH)D concentrations of these participants were 5.2 and 5.0 µg/L, and they were both from Istanbul. The overall mean 24,25(OH)2D concentration in the remaining

78 subjects was 1.52 µg/L 95% confidence interval (CI): (1.47–1.57 µg/L) and the mean analytical coefficient of variation (CV_A) determined from duplicate tests was 4.8% (95% CI: 4.5–5.1%) (Table 1).

A linear regression of the mean of the duplicate concentrations from each blood collection against the number of collections revealed that the participants were not in a steady state with respect to 24,25(OH)2D concentrations, since the 95% confidence interval for the slope did not contain zero. Figure 1 demonstrates that, regardless of the geographic location of the participants, 24,25(OH)2D concentrations tended to increase linearly with sampling time during the 2015 European spring. According to the Cochran test [12], this set of data could not be considered homogeneous, which is a prerequisite for the estimation of within-subject (CV_I) and between-subject (CV_G) BV.

Table 1: Mean 24,25-dihydroxyvitamin D (24,25(OH)2D) concentration in the European Biological Variation Study (EuBIVAS) cohort.

|		Number of individuals	Total number of results	Mean number of samples/individual	Mean number of replicates/sample	Mean concentration (95% CI)	CV_A, % (95% CI)[a]
24.25(OH)2D, µg/L	All	78	1,510	9.68	1.93	1.52 (1.47–1.57)	4.8 (4.5–5.1)
	Men	33	635	9.62	1.99	1.15 (1.11–1.19)	
	Women	45	875	9.72	1.92	1.79 (1.72–1.87)	

[a]Analytical variation (CV_A) estimates were based on CV-ANOVA of duplicate analyses of all study samples.
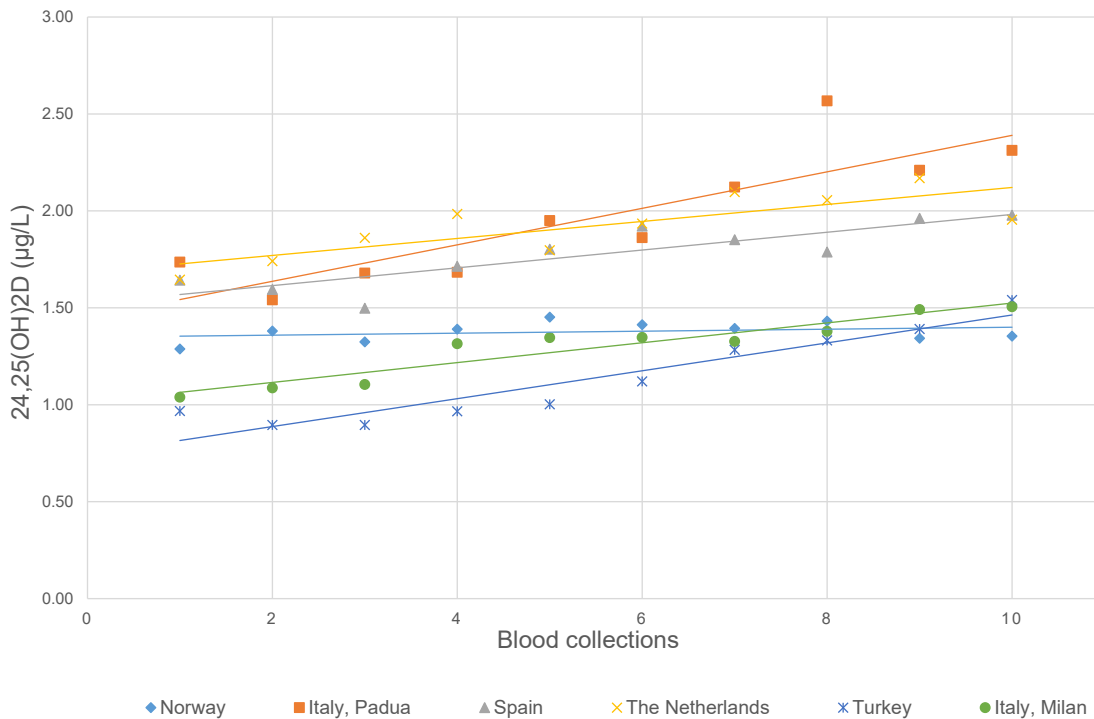
Figure 1: Evolution of 24,25(OH)2D over a 10 weeks period in ostensibly healthy participants from six different European cities during spring 2015.

Figure 1 demonstrates that the mean 24,25(OH)2D concentration for the groups of participants varied geographically (i.e., the intercepts varied) and increased at slightly different rates for each location (i.e., the slopes differed). However, there was no significant association between location and the changes in the slopes of the individual participants of 24,25(OH)2D over time. Indeed, a multiple regression analysis revealed that the variations of 24,25(OH)2D over time were significantly positively associated with the slopes of 25(OH)D concentrations over time ($p < 0.0001$) and the concentration of 25(OH)D of the participant at inclusion ($p = 0.02$), and negatively associated with Body Mass Index (BMI) ($p = 0.03$), but not with age, gender, or location of the participant ($p > 0.05$). 66% of the variation of 24,25(OH)2D over time could be explained by the variations of 25(OH) over time, the concentration of 25(OH)D at inclusion, and BMI, according to a model of backward regression.

The mean weekly variation of 24,25(OH)2D concentration over time (± relative standard measurement uncertainty) was $3.3 \pm 1.8\%$. In consequence, a reference method procedure aiming at detecting such a variation should present a $u < 3.3\%$. But from a clinical perspective, there is no need to consider measuring 24,25(OH)D in such a short period as a week. Indeed, it seems to be good clinical practice to verify an inappropriately low 24,25(OH)2D in a hypercalcaemic patient presenting with renal calculus recurrence and low parathyroid hormone concentrations after a physiological sunshine exposure or a low dose vitamin D supplementation before going for expensive genetic testing. Figure 2 shows the
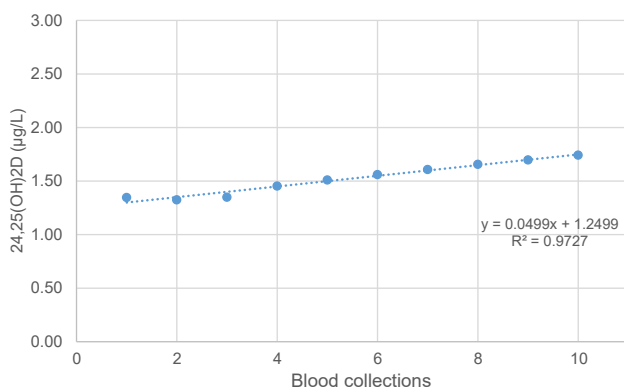
Figure 2: Regression of the 24,25(OH)2D mean weekly concentrations vs. the study week.

regression of the 24,25(OH)2D mean weekly concentrations vs. the study week. This regression shows that the physiological variation of the 24,25(OH)2D concentration in participants over a 10 weeks period was 34.6%. Hence, a method that would detect a significant change linked to the natural production of 24,25(OH)2D when the individuals are exposed to spring sunshine over a 10 weeks period at $p < 0.05$ would need a $u\%$ derived from this equation: $34.6\% = 2^{1/2} \times u\% \times 1.645$, namely a $u < 14.9\%$. If it was deemed clinically necessary to be more certain that an increase had occurred, for example, at $p < 0.01$, the appropriate Z-score should be 2.326 so that the APS for the measurement uncertainty would be $< 10.5\%$, since the formula would become $34.6\% = 2^{1/2} \times u\% \times 2.326$.

The mean VMR for the 78 participants was 7.5 (95% CI: 7.4–7.6), and the p 2.5 and p 97.5 (CI) values were 3.4 (3.1–3.6) and 12.8 (12.5–13.3), respectively (Table 2). Figure 3 depicts the regression equation for the VMR over the 10 donations. The slope of the regression was significantly different from zero, as the 95% confidence interval did not fully encompass this value (0.0391; 95% CI: 0.0060–0.07230). The VMR increased by 4.8% over the preceding 10 weeks.

Discussion

The degradation pathway of vitamin D has gained considerable interest in clinical practice and the VMR, that is the Vitamin D Metabolite Ratio (24,25(OH)2D/25(OH)D multiplied by 100), has been proposed as a biomarker of vitamin D sufficiency which could replace serum 25(OH)D alone as an interesting alternative to define vitamin D deficiency [3, 13]. To the best of our knowledge, this is the first report presenting the natural evolution of 24,25(OH)2D and VMR over time in a healthy population. EuBIVAS has so far delivered BV estimates and APS related for 81 different measurands [14], the results of most are summarized in [15]. The EuBIVAS approach presents a number of benefits. Firstly, it is sufficiently powered to enable subgroup analysis. This has allowed gender specific data and for some measurands also menopausal age specific data [14], which until this point have been unavailable for many measurands. Secondly, the BV estimates have been obtained based on current best-practice recommendations for study design [14]. Moreover,

Table 2: Vitamin D Metabolite Ratio (VMR – [24,25-dihydroxyvitamin D divided by 25 hydroxy vitamin D] × 100) observed in the EuBIVAS cohort.

		Number of individuals	Total number of results	Mean value (95% CI)	0.025 fractile (95% CI)	0.975 fractile (95% CI)
VMR	All	78	1,488	7.5 (7.4–7.6)	3.4 (3.1–3.6)	12.8 (12.5–13.3)
	Men	33	635	7.0 (6.8–7.1)	3.1[a]	11.7[a]
	Women	45	866	7.9 (7.7–8.1)	3.6[a]	13.2[a]

[a]Insufficient number of samples to calculate the 95% confidence intervals (CI).
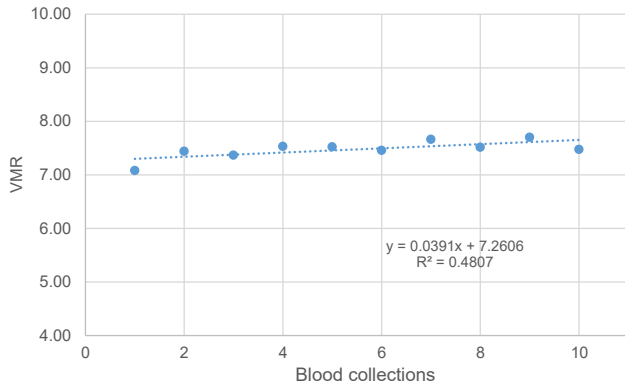
Figure 3: Regression of the metabolite ratio (VMR) (24,25(OH)2D/25(OH)D × 100) mean weekly concentrations vs. the study week.

the absence of clear differences between groups from Turkey, Norway, The Netherlands, Spain, and Italy, demonstrated also by the Principal Component Analysis, an unsupervised machine learning approach [16], confirms that the obtained data are internationally transportable across healthcare systems and that they can be used to deliver APS for systems to be used internationally.

Our results show that, compared to the natural change of 25(OH)D over the same period, as we have documented [8], the change of 24.25(OH)2D was very similar (31.6 vs. 34.6%, respectively), which leads to minimal increase of the VMR over time. The parallel evolution of the two metabolites is clinically interesting. Indeed, our data show that any change in 25(OH)D is mirrored by a change in 24,25(OH)2D leading to an equilibrium between the moieties. Hence, the ratio seems to be stable in the individuals who presented 24,25(OH)2D concentrations higher than the analytical LoQ. Interestingly, a more prescriptive analysis of our results showed that eight subjects with low 25(OH)D concentrations at inclusion to the study presented 24,25(OH)2D concentrations initially lower than the LoQ. Among six of these participants, 24,25(OH)2D became measurable when 25(OH)D concentrations increased over time (range: 2–9 weeks). The 25(OH)D concentrations of these participants at inclusion ranged from 5.8 to 14.5 µg/L (mean: 9.9 µg/L). Once it was possible to calculate the VMR, it did not significantly change over time. Finally, in two participants, 25(OH)D concentration did not increase over time, neither did their 24,25(OH)2D. Future studies would be interesting to provide results on the variation of the VMR over a longer period of time like a year period.

From other important perspectives, our results show that the traditional approaches to the generation and application of data on BV was neither applicable to

24,25(OH)2D, as it was similarly the case for 25(OH)D, since the participants were clearly not in steady-state with regard to 24,25(OH)2D concentrations over time. We thus used the approach based on measurement uncertainty to define APS for 24,25(OH)2D based on the physiological variation of this vitamin D metabolite. Our results show that a reference method procedure (RMP), which should be able to detect a weekly variation of the metabolite, should have a $u < 3.3\%$. In the development of their RMP for 24,25(OH)2D, Tai et al. achieved an expanded uncertainty of $<2.8\%$ [4], which is totally consistent with the proposals we have documented here. The mean increase of 24,25(OH)2D over a 10 weeks period was 34.6%. Accordingly, a routine method which would aim at detecting an increase of 24,25(OH)2D over 10 weeks would require $u < 14.9$ and $<10.5\%$ respectively for a 95 or 99% probability of detection. Interestingly, when we validated a routine LC-MS/MS method for 24,25(OH)2D [10] we found that the u ranged from 5.3 to 11.6% (for concentrations ranging from 1.4 to 2.7 µg/L).

In conclusion, for the first time, we have defined APS for 24,25(OH)2D examinations. According to the growing interest in this metabolite, several laboratories and manufacturers might aim to develop specific methods for its determination. The results presented in this paper are thus necessary prerequisites for the validation of such methods.

Author contributions: Conceptualization, Etienne Cavalier, Callum G Fraser, Anna Carobene; Data curation, Pierre Lukas Loreen Huyghebaert, Stéphanie Peeters, Caroline Le Goff; Formal analysis, Etienne Cavalier, Callum G Fraser and Anna Carobene; Investigation, Etienne Cavalier; Methodology, Callum G Fraser and Anna Carobene; Supervision, Etienne Cavalier; Writing – original draft, Etienne Cavalier, Callum G Fraser and Anna Carobene; Writing – review & editing, Markus Herrmann, Harjit P Bhattoa, Annemieke C Heijboer, Konstantinos Makris and Samuel Vasikaran. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.
Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: The EuBIVAS protocol was approved by the Institutional Ethical Review board of San Raffaele Hospital in agreement with the World Medical Association Declaration of Helsinki (protocol number: WG-BV project

#001, 50/INT 2014) and by the Ethical Board/Regional Ethics Committee for each laboratory. All participants signed a written consent form.

References

1. Schlingmann KP, Kaufmann M, Weber S, Irwin A, Goos C, John U, et al. Mutations in CYP24A1 and idiopathic infantile hypercalcemia. N Engl J Med 2011;365:410–21.
2. Molin A, Baudoin R, Kaufmann M, Souberbielle JC, Ryckewaert A, Vantyghem MC, et al. CYP24A1 mutations in a cohort of hypercalcemic patients: evidence for a recessive trait. J Clin Endocrinol Metab 2015; 100:E1343–52.
3. Cavalier E, Huyghebaert L, Rousselle O, Bekaert AC, Kovacs S, Vranken L, et al. 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 2019;58:197–201.
4. Tai SSC, Nelson MA. Candidate reference measurement procedure for the determination of (24 R),25-dihydroxyvitamin D3 in human serum using isotope-dilution liquid chromatography–tandem mass spectrometry. Anal Chem 2015;87:7964–70.
5. Zelzer S, Le Goff C, Peeters S, Calaprice C, Meinitzer A, Enko D, et al. Comparison of two LC-MS/MS methods for the quantification of 24,25-dihydroxyvitamin D3 in patients and external quality assurance samples. Clin Chem Lab Med 2022;60:74–81.
6. Sandberg S, Fraser CG, Horvath AR, Jansen R, Jones G, Oosterhuis W, et al. Defining analytical performance specifications: consensus statement from the 1st Strategic Conference of the European Federation of Clinical Chemistry and Laboratory Medicine. Clin Chem Lab Med 2015;53:833–5.
7. Carobene A, Strollo M, Jonker N, Barla G, Bartlett WA, Sandberg S, et al. Sample collections from healthy volunteers for biological variation estimates' update: a new project undertaken by the Working Group on Biological Variation established by the European Federation of Clinical

Chemistry and Laboratory Medicine. Clin Chem Lab Med 2016;54: 1599–608.
8. Cavalier E, Fraser CG, Bhattoa HP, Heijboer AC, Makris K, Ulmer CZ, et al. Analytical performance specifications for 25-hydroxyvitamin D examinations. Nutrients 2021;13. https://doi.org/10.3390/ nu13020431.
9. Sempos CT, Betz JM, Camara JE, Carter GD, Cavalier E, Clarke MW, et al. General steps to standardize the laboratory measurement of serum total 25-hydroxyvitamin D. J AOAC Int 2017;100. https://doi.org/10. 5740/jaoacint.17-0259.
10. Fabregat-Cabello N, Farre-Segura J, Huyghebaert L, Peeters S, Le Goff C, Souberbielle JC, et al. A fast and simple method for simultaneous measurements of 25(OH)D, 24,25(OH)2D and the Vitamin D Metabolite Ratio (VMR) in serum samples by LC-MS/MS. Clin Chim Acta 2017;473: 116–23.
11. Farrance I, Frenkel R. Uncertainty of measurement: a review of the rules for calculating uncertainty components through functional relationships. Clin Biochem Rev 2012;33:49–75.
12. Cochran WG. The distribution of the largest of a set of estimated variances as a fraction of their total. Ann Hum Genet 1941;11: 47–52.
13. Berg AH, Powe CE, Evans MK, Wenger J, Ortiz G, Zonderman AB, et al. 24,25-Dihydroxyvitamin D3 and vitamin D status of community-dwelling black and white Americans. Clin Chem 2015;61:877–84.
14. Sandberg S, Carobene A, Bartlett B, Coskun A, Fernandez-Calle P, Jonker N, et al. Biological variation: recent development and future challenges. Clin Chem Lab Med 2022. https://doi.org/10.1515/cclm-2022-1255.
15. Carobene A, Aarsand AK, Bartlett WA, Coskun A, Diaz-Garzon J, Fernandez-calle P, et al. The European Biological Variation study (EuBIVAS): a summary report. Clin Chem Lab Med 2021;60: 505–17.
16. Carobene A, Campagner A, Uccheddu C, Banfi G, Vidali M, Cabitza F. The multicenter European Biological Variation Study (EuBIVAS): a new glance provided by the Principal Component Analysis (PCA), a machine learning unsupervised algorithms, based on the basic metabolic panel linked measurands. Clin Chem Lab Med 2021;59:556–68.