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Letter to the Editor

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To the Editor,

The most abundant vitamin D metabolite, 25-hydroxyvitamin D (25(OH)D), is mainly susceptible to hydroxylation on two positions. Hydroxylation on the 1-position by CYP27B1 yields the hormonally active metabolite 1,25-dihydroxyvitamin D (1,25$(OH)_2$D). 25(OH)D may also be hydroxylated on the 24-position by CYP24A1, which returns the seemingly inactive metabolite 24,25-dihydroxyvitamin D (24,25$(OH)_2$D). The amount of circulating 1,25$(OH)_2$D is strictly regulated and independent of circulating 25(OH)D, rarely differs from normal and only requires measurement upon suspicion of specific conditions [1]. On the other hand, the amount of the hormonally inactive metabolite 24,25$(OH)_2$D is dependent on the amount of its predecessor 25(OH)D and the activity of the enzyme responsible for its production. The ratio of 24,25$(OH)_2$D over 25(OH)D is thus indicative for the efficiency of this catabolic clearance by CYP24A1. As such, it can be used to identify disorders caused by enzyme malfunctioning, such as the rare genetic disorder idiopathic infantile hypercalcaemia (IIH). At the heart of this condition is an inactivating mutation in *CYP24A1*, almost completely preventing the inactivation of the active hormone, resulting in the overproduction of 1,25$(OH)_2$D and severe hypercalcaemia [2]. IIH patients present with severely increased 25(OH)D/24,25$(OH)_2$D ratios of >99 [3]. Other genomic mutations leaving some residual enzymatic activity have lower 25(OH)D/24,25$(OH)_2$D ratios, but still transcend values measured in the healthy population [4]. In order to identify deviations in the 25(OH)D/24,25$(OH)_2$D ratio caused by reduced CYP24A1 activity, reference values established in a representative, diverse group of healthy individuals are required. Therefore, the aim of the present study was to establish 24,25$(OH)_2$D and 25(OH)D/24,25$(OH)_2$D reference values in a cohort of 92 healthy individuals (46 women and 46 men, aged 21–71 years), whose characteristics are described in Table 1. Volunteers were recruited by advertisement in a local newspaper with a free house-to-house distribution in the Amsterdam region and by advertisements in the biweekly information bulletin of the Academic Medical Centre of the University of Amsterdam. Subjects were screened for the inclusion and exclusion criteria by telephone and during the intake visit. The inclusion criteria were age between 20 and 70 years and self-reported good health. Exclusion criteria were use of parenteral drugs, use of phenoxybenzamine or tricyclic antidepressants, use of antihypertensives or hospitalization in past 3 months. Samples were drawn between June and September 2008. The Medical Ethics Committee of the Academic Medical Centre in Amsterdam approved the study and informed consent was obtained of all participants. For measurement of 25(OH)D (25$(OH)D_2$ and 25$(OH)D_3$) and 24,25$(OH)_2$D (only 24,25$(OH)_2D_3$), a new LC-MS/MS method was developed on a Vanquish UHPLC

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Table 1: Characteristics of the reference group (n = 92).

	Mean (SD)	Range
Age	44.6 (14.5)	21.2–70.8
Gender (% female)	50	
Height, cm	175.3 (10.4)	154.0–203.0
Body weight, kg	76.4 (13.4)	49.4–110.0
BMI, kg/m^2	24.8 (3.7)	18.0–35.5
Alcohol intake (units/week)[a]	4	0–42
Smokers, %	21	
25OHD, nmol/L[a]	53	9–148

[a]Not normally distributed, therefore median is showed.

system coupled to a TSQ Quantiva tandem quadrupole mass spectrometer (Thermo Scientific). Sample preparation involved precipitation of proteins from 50 µL of sample and 20 µL internal standard solution with 100 µL 50% isopropanol, solid liquid extraction on an Isolute SLE+ 96-well plate (Biotage, Sweden) and derivatization with PTAD. Liquid chromatography was performed on an Acquity UPLC BEH300 C4 (2.1×50 mm, 1.7 µm) maintained at 50 °C, coupled to either an Acquity UPLC BEH C18 (2.1×50 mm, 1.7 µm) or a Phenomenex PFP (2.1×100 mm, 1.7 µm), both maintained at 40 °C. Chromatographic conditions and system 3D setup are presented in Supplementary Figure S1. Quantifier mass transitions were 623.5→298.2 *m/z* for 24,25OH$_2$D$_3$, 607.5→298.2 *m/z* for 25OHD$_3$ and 619.5→298.2 *m/z* for 25OHD$_2$. Parent ions for the internal standards were +6 *m/z* for 24,25OH$_2$D$_3$ and 25(OH)D$_2$ (^{2}H$_6$) and +5 *m/z* for 25(OH)D$_3$ (^{13}C$_5$), daughter ions remained unchanged. For analyte quantification, a six-point calibration curve in 6% BSA ranging from 1.19 to 288.3 nmol/L for 25(OH)D$_3$, from 0.36 to 88 nmol/L for 25(OH)D$_2$ and from 0.12 to 30 nmol/L for 24,25(OH)$_2$D$_3$ was used. The method was validated according to FDA and EMA guidelines and passed all required tests [5, 6]. Samples used for validation were leftover serum or plasma patient samples, not specifically selected based on any criteria. For determination of method accuracy, the NIST Standard Reference Material (SRM) 972a and DEQAS samples 446–450 with NIST target values were used. Full method validation details are summarized in Supplementary Table S1. As the measured 24,25(OH)$_2$D$_3$ and 25(OH)D$_3$ concentrations in the cohort of healthy individuals were not normally distributed, reference values were calculated using the non-parametric percentile method, according to CLSI C28-A3. Reference samples were measured before NIST target values for SRM972a came available [7]. After measurement of SRM 972a and DEQAS samples 446–450 (with a NIST target value) we found a bias of +24% for our method. We have therefore recalculated the earlier obtained reference values for 24,25(OH)$_2$D$_3$ and the 25(OH)D/24,25(OH)$_2$D ratio accordingly. The ratio 25(OH)D/24,25(OH)$_2$D was calculated using 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$. 25(OH)D$_2$ was measured in serum of all the participants but never exceeded 5 nmol/L. No statistically significant difference for 24,25(OH)$_2$D$_3$ or the ratio was observed between men and women (Mann-Whitney test $p = 0.140$ and $p = 0.577$, respectively). Similarly, no change with age was detected ($p = 0.740$). The established reference range is 0.4–8.9 nmol/L for 24,25(OH)2D$_3$ and 10–33 for the 25(OH)D/24,25(OH)$_2$D ratio (Table 2).

Two earlier publications reported similar results (Table 2). Tang et al. determined a reference interval in a population consisting of mainly younger men (all below 32 years of age) [8]. Like us, they chose not to exclude the vitamin D deficient individuals from the reference group. Ketha et al., on the other hand, only included vitamin D-sufficient individuals [3]. We decided to include the apparently healthy individuals with low 25(OH)D levels to avoid omitting a large part of the world's population. Many of the patients we measure in the clinic may be considered vitamin D deficient or insufficient but have a perfectly functioning CYP24A1 enzyme and therefore a healthy 25(OH)D/24,25(OH)$_2$D ratio. To the best of our knowledge, this is the first time reference values for 24,25(OH)$_2$D$_3$ and the 25(OH)D/24,25(OH)$_2$D ratio have been established with a method standardized using the NIST SRM 972a reference material. In conclusion, using a newly developed and standardized LC-MS/MS assay, we have established reference values for the 25(OH)D/24,25(OH)$_2$D ratio in both men and women, aged 20–71 years. They agree with two earlier studies in subpopulations.

Table 2: Determined reference values and those obtained from literature.

Source	24,25(OH)$_2$D$_3$, nmol/L	25(OH)D, nmol/L	25(OH)D/ 24,25(OH)$_2$D$_3$	N	Gender (% female)	Age (range)	Method	Year
Present study	0.4–8.9	12–122	10–33	92	50	20–71	LC-MS/MS	2019
Tang et al. [7]	1.1–13.5	19–126	7–23	1996	25	18–32	LC-MS/MS	2017
Ketha et al. [3]	Unknown	50–200	7–35	91	53	28–86	LC-MS/MS	2016

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