LC-MS/MS for Identifying Patients with CYP24A1 Mutations

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BACKGROUND: Patients have been described with loss-of-function CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1) mutations that cause a high ratio of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D [25(OH)D/24,25(OH)2D], increased serum 1,25-dihydroxyvitamin D, and resulting hypercalcaemia, hypercalciuria and nephrolithiasis. A 25(OH)D/24,25(OH)2D ratio that can identify patients who are candidates for confirmatory CYP24A1 genetic testing would be valuable. We validated an LC-MS/MS assay for 24,25(OH)2D(D3 and D2) and determined a 25(OH)D/24,25(OH)2D cutoff to identify candidates for confirmatory genetic testing.

METHODS: After addition of isotope-labeled internal standard, serum samples were extracted by solid-phase extraction, derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione, and quantified by LC-MS/MS. We measured 25(OH)D/24,25(OH)2D in 91 healthy patients and 34 patients with clinically suspected CYP24A1-mediated hypercalcaemia.

RESULTS: The limits of detection and quantification were 0.03 (0.2) and 0.1 (0.24) nmol/L, respectively, for 24,25(OH)2D3, and 0.1 (0.23) and 0.5 (1.16) nmol/L for 24,25(OH)2D2. Intra- and interassay imprecision was 4%–15% across the analytical measurement range of 0.1–25 ng/mL (0.2–60 nmol/L). No interference was observed with 25(OH)D and 1,25(OH)2D. 25(OH)D/24,25(OH)2D of 7–35 was observed in healthy patients, whereas in 2 patients with CYP24A1 mutations, 25(OH)D/24,25(OH)2D was significantly increased (99–467; P < 0.001). A 25(OH)D/24,25(OH)2D ratio ≥99 identified patients who were candidates for CYP24A1 genetic testing.


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Vitamin D is essential for healthy calcium and phosphate homeostasis (1). Vitamin D3 is formed in the skin by the UV light–mediated photolysis of 7-dehydrocholesterol (1, 2), whereas vitamin D2 is obtained through the diet (1). The metabolism of both vitamin D metabolites is identical in mammals. Vitamin D is converted in the liver by 25-hydroxylase to 25-hydroxyvitamin D [25(OH)D]3 (3, 4). Depending on calcium and phosphorus demands, 25(OH)D3 is metabolized by 25(OH)D-1α-hydroxylase to its bioactive form, 1,25-dihydroxyvitamin D [1,25(OH)2D]3 or by 25(OH)D-24-hydroxylase to an inactive metabolite, 24R,25-dihydroxyvitamin D [24,25(OH)2D]3 (1, 5–9).

Vitamin D–related disorders are diagnosed by measuring serum concentrations of total (D2 + D3) 25(OH)D or 1,25(OH)2D (10, 11). Inactivating mutations of CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1) cause hypercalcaemia, hypercalciuria, and increased 1,25(OH)2D concentrations (12–20). In studies describing LC-MS/MS assays for 24,25(OH)2D quantification, the relationship between 25(OH)D and 24,25(OH)2D has been used as a nutritional marker for assessment of optimum vitamin D supplementation (21). Mutations that cause reduced or complete loss of 24-hydroxylase function result in low or undetectable serum 24,25(OH)2D or increased 25(OH)D/24,25(OH)2D ratio. A ratio that could identify candidates for confirmatory genetic testing has not been defined.

Loss-of-function mutations in CYP24A1 have been identified as the underlying cause of hypercalcaemia previously considered to be idiopathic. Several groups have identified patients with similar mutations in the 24-
Genotype Phenotype Correlation in Vitamin D Metabolism

In the majority of studies, the hydroxylase gene (12, 13, 15, 16, 18–20). Nonetheless, the population frequency of CYP24A1 mutations is currently not known, and a 25(OH)D/24,25(OH)2D value that could identify patients who are candidates for CYP24A1 mutation testing would be clinically valuable. Here we have developed an LC-MS/MS assay for 24,25(OH)2D quantification, established a 25(OH)D/24,25(OH)2D reference interval, and established a diagnostic cutoff for 25(OH)D/24,25(OH)2D that can identify patients in whom genetic testing for CYP24A1 mutations is warranted.

**Methods**

**PARTICIPANTS AND SAMPLES**
The Mayo Clinic Institutional Review Board approved these studies. For determination of reference intervals, we used serum samples from 91 healthy individuals on whom 25(OH)D testing was ordered. There was sufficient clinical information available for all of the patients to confirm that none of them had any bone- or kidney-related conditions or were on medications that can affect bone/mineral metabolism. Each individual was screened by a detailed chart review, and none of the individuals from whom samples were obtained were taking drugs known to affect mineral metabolism, such as thiazide diuretics, corticosteroids, anticonvulsant drugs, or bisphosphonates. Patients with kidney stones were excluded. No individual had other kidney, gastrointestinal, or bone diseases known to affect vitamin D metabolism. Study participants were not taking vitamin D or calcium supplements, or if they were, were taking ≤1000 mg calcium per day and ≤1000 IU vitamin D3 or vitamin D2 per day. We collected samples between May and August 2012 and recorded serum concentrations of calcium, creatinine, albumin, and alkaline phosphatase in the reference group. We measured serum 25(OH)D/24,25(OH)2D in patients who underwent evaluation of hypercalcemia at our institution and in whom other causes of hypercalcemia had been ruled out.

**MEASUREMENT OF 24,25(OH)2D3 AND 24,25(OH)2D2 BY LC-MS/MS**

We prepared a 10-μg/mL solution of 24R,25(OH)2D3 and 24R,25(OH)2D2 (Medical Isotopes) in 100% ethanol. Calibrator concentrations were 0, 0.1, 0.5, 1, 5, 10, and 25 ng/mL [0, 0.2, 1.2, 2.4, 12.0, 24.0, and 60.0 nmol/L for 24,25(OH)2D3, and 0, 0.2, 1.2, 2.3, 11.7, 23.3, and 58.3 nmol/L for 24,25(OH)2D2]; concentrations of QC samples (prepared in stripped serum) were 0.6, 5.5, and 14 ng/mL [1.4, 13.2, and 33.6 nmol/L for 24,25(OH)2D3; 1.4, 12.8, and 32.7 nmol/L for 24,25(OH)2D2]. For 500 μL serum, we added 50 μL [1.25 ng (0.003 nmol)] deuterated 24,25(OH)2D3 and deuterated 24,25(OH)2D2 (Toronto Research Chemicals), followed by 500 μL hydrochloric acid (0.2 N) 15 min later. After mixing, we applied the sample to a Bond-Elut (C18, 250 mg, 6 mL) cartridge (Varian Instruments), which was washed once with 2 mL of 70:30 methanol:water (1.4 mL methanol:0.6 mL water) and once with 2 mL of 90:10 hexane:methylene chloride (1.8 mL hexane:0.2 mL methylene chloride). Vitamin D metabolites were eluted with 2 mL of 90:10 hexane:isopropanol (1.8 mL hexane:0.2 mL isopropanol). Eluents were dried and derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) [Sigma; 250 μg/mL (285 μmol/L) solution in acetonitrile].

The derivatized vitamin D metabolites were separated by liquid chromatography at a flow rate of 0.25 mL/min on an Agilent XDB-C8, 2.1 × 50-mm column over 7.25 min with a methanol-H2O-ammonium formate (1 mM) linear gradient (60%–95%). Mass spectrometry was performed in the multiple reaction monitoring mode on an AB Sciex 5500 mass spectrometer with Analyst 1.6.2 software (AB Sciex) for data acquisition and analysis. The MS/MS conditions for measuring 24,25(OH)2D are described in Table 1.

**OTHER ASSAYS**

We measured concentrations of serum calcium and PTH with standard clinical assays, and 25(OH)D (22) and 1,25(OH)2D (22, 23) with LC-MS/MS assays. For

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**Table 1.** Mass spectrometer settings for 24,25(OH)2D3 and 24,25(OH)2D2 LC-MS/MS assay.

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*a Q1 indicates the masses of the PTAD adduct with 24,25(OH)2D3 or 24,25(OH)2D2.
25(OH)D quantification, we added 2.5 ng internal standard \([d6-25](OH)D_3\) and \([d3-25](OH)D_2]\) to a 100-μL serum sample in a 96-well plate, and the sample was equilibrated on an orbital shaker for 15 min at room temperature. We added 100 μL acetone, followed by 450 μL ethyl acetate. After the solutions had settled, we used an automated liquid handling system to transfer the organic top layer to a different sample plate. The extraction was repeated 4 times. The samples were then subjected to derivatization by different triazine-dione compounds (22). The reaction mixtures in the 96-well plates were dried under a N\(_2\) stream, and the residue was reconstituted in 150 μL of 50:50 methanol:water mixture. The CV across the analytical measurement range of 25(OH)D was 3.7%–15.2%.

**24,25(OH)\(_2\)D ASSAY VALIDATION**

We determined the limit of detection (LOD), limit of quantification (LOQ), lowest analyte concentration with interassay imprecision of <20% CV, recovery of exogenous spiked 24,25(OH)\(_2\)D\(_3\) and 24,25(OH)\(_2\)D\(_2\), and intraassay and interassay imprecision for low, medium, and high controls. We measured intraassay imprecision with 3 concentrations of QC pools. Each pool was assayed 20 times, and the CV was calculated. We measured interassay imprecision by assaying 3 concentrations of QC pools each 20 times over 20 days, and the CV was calculated. We assessed recovery with 5 different serum samples spiked with varying concentrations of 24,25(OH)\(_2\)D\(_3\) and 24,25(OH)\(_2\)D\(_2\) to cover the analytical measurement range \([24,25](OH)D_3\): neat serum concentrations, 0.35–3.40 ng/mL; spike concentrations, 4.8–9.1 ng/mL; 24,25(OH)\(_2\)D\(_2\): neat serum concentrations, 1.40–5.02 ng/mL; spike concentrations, 4.8–9.1 ng/mL. Percent recovery was calculated as the measured concentration/expected concentration × 100.

Linearity across the analytical measurement range was determined as follows. We prepared 5 pools of admixtures by mixing high- and low-concentration samples in 75:25, 50:50, and 25:75 ratios. Each pool was assayed 3 times, and the recovery was calculated as above. Recovery of 90%–110% was considered acceptable. We assessed dilutional linearity by serially diluting a high QC sample with the 0-ng/mL standard. Serial dilutions up to 1:16 were assessed. The LOD was determined by assaying a 0-ng/mL standard 20 times in 1 day. We used limit of the blank = mean blank + 1.645 (SD of the blank) and LOD = limit of the blank + 1.645 (SD of the low concentration sample) (CLSI EP17-A13).

Carryover, interference, ion suppression, and stability studies were performed. For the assessment of ion suppression, a syringe pump was connected to the column effluent, and a solution of PTAD adducts of 24,25(OH)\(_2\)D\(_2\) and \(D_3\) was infused directly into the electrospray source until a constant response was obtained. Then a stripped serum blank and a pooled serum control were extracted and prepared after complete sample workup was injected onto the column to observe the effect of matrix suppression on the response for the continuously infused 24,25(OH)\(_2\)D\(_2\) and \(D_3\). We assessed carryover by examining the recovery of a low QC sample injected and quantified after analysis of a sample spiked with 50 ng/mL 24,25(OH)\(_2\)D\(_3\) and 24,25(OH)\(_2\)D\(_2\). Sample stability was determined over 7 days. Ten sample pools were prepared, aliquoted, and assayed every day over 7 days, and the percentage difference from day 0 was calculated. Room temperature, refrigerated, and freeze–thaw cycle stability was determined. Nonparametric data analysis was performed (JMP, version 10.0, SAS) to establish a reference interval for 25(OH)D/24,25(OH)\(_2\)D by use of the 2.5th to 97.5th percentiles for the healthy population.

**Results**

The Q1/Q3 ion pairs used to quantify PTAD-derivatized 24,25(OH)\(_2\)D\(_3\)-d6, 24,25(OH)\(_2\)D\(_3\), 24,25(OH)\(_2\)D\(_2\)-d3, and 24,25(OH)\(_2\)D\(_2\) were \(m/z\) 580.35/298.20, 574.35/298.20, 589.35/298.20, and 586.35/298.20, respectively (Table 1). The LODs for 24,25(OH)\(_2\)D\(_3\) and 24,25(OH)\(_2\)D\(_2\) were 0.03 ng/mL (0.2 nmol/L) and 0.1 ng/mL (0.23 nmol/L); the corresponding limits of quantification were 0.1 ng/mL (0.2 nmol/L) and 0.5 ng/mL (1.2 nmol/L). On the basis of the LOQ and the highest calibrators used, the analytical measurement range for undiluted samples was set at 0.1–25 ng/mL (0.2–60 nmol/L) for 24,25(OH)\(_2\)D\(_3\) and 0.5–25 ng/mL (1.2–58.3 nmol/L) for 24,25(OH)\(_2\)D\(_2\). Across this range, intraassay imprecision was CV 3.1%–6.2% for 24,25(OH)\(_2\)D\(_3\) and 11.7%–14.8% for 24,25(OH)\(_2\)D\(_2\). The corresponding interassay values were 45.1%–8.3% and 3.0%–10.1%. Recovery of exogenous 24,25(OH)\(_2\)D\(_3\) and 24,25(OH)\(_2\)D\(_2\) spiked into samples was 94%–100% and 90%–94%, respectively (Table 1). 24,25(OH)\(_2\)D\(_3\) showed very low cross-reactivity (0.6%) with the spiked 25(OH)D\(_3\) and 24,25(OH)\(_2\)D\(_2\) showed 4% cross-reactivity. We observed <5% signal suppression for both 24,25(OH)\(_2\)D\(_2\) and \(D_3\).

The characteristics of the individuals whose samples were included to determine the reference interval are shown in Table 3. The 2.5th to 97.5th percentiles from nonparametric analysis for 25(OH)D/24,25(OH)\(_2\)D in healthy individuals was 7–35 (mean, 12.5; SD, 4). In these individuals, serum 24,25(OH)\(_2\)D\(_3\) concentrations correlated with 25(OH)D\(_3\) concentrations of 7–60 ng/mL (17.5–150 nmol/L): 24,25(OH)D\(_3\) = 0.10 × 25(OH)D\(_3\) − 0.32; \(r^2 = 0.75; n = 91\). The relationship between 25(OH)D and 25(OH)D/24,25(OH)\(_2\)D in healthy patients and patients with confirmed CYP24A1 mutations is shown in Fig. 1. Biochemical parameters of the patients who were found to have CYP24A1 muta-
tions are shown in Table 4. Among the patients who were referred for 24,25(OH)2D assessment from our clinical practice (n = 34), 2 (patients 1 and 2 in Table 4) were found to have CYP24A1 mutations. Patient 1 was found to have previously undescribed mutations (p.S334V, frame shift, and p.R396W, missense mutation). Patient 2 was found to have a previously described deletional mutation, p.E143Del, and a novel missense mutation, p.R396W. The p.E143Del deletional mutation was previously described by Nesterova et al. (14). In both patients, other common causes of hypercalcemia such as primary hyperparathyroidism were excluded before referral for 24,25(OH)2D assessment.

Discussion

LC-MS/MS ASSAY FOR 24,25(OH)2D QUANTIFICATION

Our assay used specific deuterated internal standards for 24,25(OH)2D3 and 24,25(OH)2D2. Before the deuterated internal standards for 24,25(OH)2D3 were available, we observed higher LOQ and imprecision for 24,25(OH)2D3 during the validation process. In our assay, the 2 diastereomers of 24,25(OH)2D2-PTAD and 24,25(OH)2D3-PTAD were chromatographically separated. During our validation studies, we observed interference from 25,26(OH)2D with the major isomer of the 24,25(OH)D3-PTAD (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue1). Therefore, the minor isomer for 24,25(OH)2D3-PTAD was used for validation.

One of the first reports describing an LC-MS/MS method for 24,25(OH)2D quantification showed a linear relationship between 25(OH)D and 24,25(OH)2D. We observed a similar linear relationship in the 25(OH)D concentration range of 10–60 ng/mL. Serum 25(OH)D/24,25(OH)2D is a measure of 24-hydroxylase functional status in an individual and is potentially useful for assessing optimal supplementation (21). The use of 25(OH)D/24,25(OH)2D as a biomarker of optimal response to vitamin D supplementation has been limited to research studies (24), and its use in routine clinical practice is currently limited. In contrast to others (15), we did not observe any residual signal in the chromatographic retention region in the patient samples with CYP24A1 mutations.

DIAGNOSTIC CUTOFF FOR 25(OH)D/24,25(OH)2D

So far, there seems to be no correlation between a specific mutation and 25(OH)D/24,25(OH)2D, degree of PTH suppression, or 1,25(OH)2D increase. For our reference range study in healthy individuals,
25(OH)D/24,25(OH)2D was within a narrow range (7–35) over a wide range of 25(OH)D values. We observed an increase in 25(OH)D/24,25(OH)2D in patients with 25(OH)D <20 ng/mL (Fig. 1A, inset). Our observation was similar to that described by Kauffmann et al. (15). Despite an increase in 25(OH)D/24,25(OH)2D at 25(OH)D <20 ng/mL, the ratio in patients affected with deleterious CYP24A1 mutations was significantly higher (P < 0.001), with almost no overlap between unaffected and affected populations (Table 4). Nevertheless, 25(OH)D/24,25(OH)2D ratios should be interpreted with caution in 25(OH)D-deficient patients, because very low 25(OH)D concentrations can limit the conversion to the inactive 24-hydroxylated metabolite.

An evident clinical use of 24,25(OH)2D measurement is for identifying patients with CYP24A1 mutations. In our institution, on physician request, we analyzed 25(OH)D/24,25(OH)2D in 34 patients with hypercalcemia and high clinical suspicion of an underlying genetic cause. Other causes of hypercalcemia (hyperparathyroidism or hypervitaminosis D) were ruled out before recommending 24,25(OH)2D testing. 25(OH)D/24,25(OH)2D measurement in 2 of these patients was suggestive of reduced 24-hydroxylase activity. Patients 1 and 2 (Table 4) were found to have increased 25(OH)D/24,25(OH)2D ratios (336 and 467, respectively). On the basis of this biochemical information, confirmatory genetic testing was recommended, and these patients were found to have pathogenic CYP24A1 mutations. Our results, along with the data from published reports, show that a 25(OH)D/24,25(OH)2D cutoff of ≥99 identifies patients with CYP24A1 mutations. The biochemical phenotypes expected in patients with PTH-mediated, excess vitamin D–mediated hypercalcemia or hypercalcemia due to CYP24A1 mutations are shown in Fig. 1B. Pathogenic CYP24A1 mutations, although rare, should be considered in the differential diagnosis of hypercalcemia. This is particularly important in cases where other etiologies of hypercalcemia (such as primary hyperparathyroidism) have been ruled out (Fig. 1B).

![Fig. 1. (A), Association between 25(OH)D/24,25(OH)2D in healthy individuals (●) and patients with CYP24A1 mutations (▲). The inset shows that although 25(OH)D/24,25(OH)2D at 25(OH)D <20 ng/mL is higher, it distinguishes between unaffected and affected individuals. (B), Expected biochemical profiles in PTH, vitamin D excess, and CYP24A1-associated hypercalcemia. The ↑ indicates abnormally high value above normal reference interval; the ↓ indicates abnormally low value below normal reference interval; the hyphen (-) indicates normal values compared to reference interval.](image-url)
Increased 1,25(OH)\(_2\)D has been observed in hypercalcific kidney stone formation. Increased 1,25(OH)\(_2\)D concentrations are responsible for the hypercalcific stones described by Kaufmann et al. and Schlingmann et al. (12, 13). Whether 25(OH)\(_2\)D/24,25(OH)\(_2\)D biochemical screening will be useful in clinical management of hypercalcific stone formation is still in question. However, patients with hypercalcific stones who exhibit increases in serum 1,25(OH)\(_2\)D and suppressed PTH are excellent candidates for 25(OH)\(_2\)D/24,25(OH)\(_2\)D testing, since their biochemical phenotype would be similar to that observed in pathogenic CYP24A1 mutations (Fig. 1B).

We recently evaluated an infant with hypercalcemia associated with vitamin D toxicity. The patient had increased serum 25(OH)\(_2\)D concentrations with a healthy 25(OH)\(_2\)D/24,25(OH)\(_2\)D ratio. Thus, the serum 25(OH)\(_2\)D/24,25(OH)\(_2\)D ratio could be useful in such cases to differentiate between iatrogenic and genetic causes of hypercalcemia associated with alterations in vitamin D physiology. The biochemical phenotype expected in a patient with vitamin D toxicity–associated hypercalcemia is shown in Fig. 1B.

Because of strong dependence of substrate [25(OH)\(_2\)D] concentrations, concomitant measurement of 25(OH)\(_2\)D by the same method is necessary. This is an important consideration for clinicians and clinical chemists who recommend this testing, because the absolute value of serum 24,25(OH)\(_2\)D alone in the reference range (1–3 ng/mL) can be misleading. For example, in a hypothetical patient with 24,25(OH)\(_2\)D 1 ng/mL and 25(OH)\(_2\)D 15 ng/mL, the patient would have a healthy 25(OH)\(_2\)D/24,25(OH)\(_2\)D, whereas in a patient with 24,25(OH)\(_2\)D 1 ng/mL and 25(OH)\(_2\)D 85 ng/mL, a deficiency in 24-hydroxylase function is likely. This point is also apparent from patients described in the literature who, despite seemingly “normal” serum concentrations of 25(OH)\(_2\)D and 24,25(OH)\(_2\)D, have a ratio that depicts impaired 24-hydroxylase functional status. This is especially relevant in certain populations such as African Americans, who have been shown to have low concentrations of both 25(OH)\(_2\)D and 24,25(OH)\(_2\)D but have equivalent ratios of the 2 metabolites in comparison to whites (25).

One of the limitations of our study is the paucity of data in patients with kidney disease. Vitamin D metabolism is impaired in patients with kidney disease, leading to reduced production of 1,25(OH)\(_2\)D and 24,25(OH)\(_2\)D. Bosworth et al. have demonstrated that patients with kidney disease have decreased concentrations of 24,25(OH)\(_2\)D and increased 24,25(OH)\(_2\)D/25(OH)\(_2\)D ratios (26). These patients may also develop unexplained hypercalcemia, and thus an overlap between the biochemical signs of CYP24A1 deficiency vs renal insufficiency may be observed. Because our population did not include patients with kidney disease, further studies are needed to define how 25(OH)\(_2\)D/24,25(OH)\(_2\)D compares between patients with CYP24A1 deficiency and patients with kidney disease, and whether a different diagnostic threshold is appropriate for patients with kidney disease. This will improve the diagnostic accuracy of 25(OH)\(_2\)D/24,25(OH)\(_2\)D for identifying patients with CYP24A1 mutations and healthy or impaired renal function.

In summary, our results show that increased 25(OH)\(_2\)D/24,25(OH)\(_2\)D supports the diagnosis of reduced CYP24A1 activity in patients with mutations of the CYP24A1 gene (12, 13). 25(OH)\(_2\)D/24,25(OH)\(_2\)D ≥99 in a patient with hypercalcemia of unknown etiology warrants genetic confirmation. Since the description of patients with CYP24A1 mutations independently by 2 groups (12, 13), the availability of LC-MS/MS assays for 24,25(OH)\(_2\)D quantification has enabled diagnosis of more cases. The increase in the number of reported cases in the last few years (14, 16, 19) suggests that CYP24A1-associated hypercalcemia is more prevalent than originally thought. Measurement of 25(OH)\(_2\)D/24,25(OH)\(_2\)D should be considered a part of the clinical workup in

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\(^{a}\) Patients were genetically confirmed to have pathogenic CYP24A1 mutations.
\(^{b}\) Tebben et al (12) described the splice junction mutation, which is predicted to cause a premature stop codon and a complete loss of 24-hydroxylase function.
\(^{c}\) Nesterova et al. (14).
\(^{d}\) Patients described in Schlingmann et al. (13), and 24,25(OH)\(_2\)D quantification described by Kaufmann et al. (15).
patients with hypercalcemia of otherwise unknown etiology.

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