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Differential diagnosis of vitamin D–related hypercalcemia using serum vitamin D metabolite profiling

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ABSTRACT

Genetic causes of vitamin D–related hypercalcemia are known to involve mutation of 25-hydroxyvitamin D-24-hydroxylase *CYP24A1* or the sodium phosphate co-transporter *SLC34A1*, which result in excessive 1,25-$(OH)_2$D hormonal action. However, at least 20% of idiopathic hypercalcemia (IH) cases remain unresolved. In this case-control study, we used precision vitamin D metabolite profiling based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) of an expanded range of vitamin D metabolites to screen German and French cohorts of hypercalcemia patients, to identify patients with altered vitamin D metabolism where involvement of *CYP24A1* or *SLC34A1* mutation had been ruled out and who possessed normal 25-OH-D_3:24,25-$(OH)_2D_3$ ratios. Profiles were compared to those of hypercalcemia patients with hypervitaminosis D, Williams-Beuren syndrome (WBS), *CYP24A1* mutation, and normal subjects with a range of 25-OH-D levels. We observed that certain IH and WBS patients exhibited a unique profile comprising eightfold to 10-fold higher serum 23,25,26-$(OH)_3D_3$ and 25-OH-D_3-26,23-lactone than normals, as well as very low serum 1,25-$(OH)_2D_3$ (2–5 pg/ml) and elevated 1,24,25-$(OH)_3D_3$, which we interpret implies hypersensitive expression of vitamin D–dependent genes, including CYP24A1, as a general underlying mechanism of hypercalcemia in these patients. Because serum 25-OH-D_3 and 24,25-$(OH)_2D_3$ remained normal, we excluded the possibility that the aberrant profile was caused by hypervitaminosis D, but instead points to an underlying genetic cause that parallels the effect of Williams syndrome transcription factor deficiency in WBS. Furthermore, we observed normalization of serum calcium and vitamin D metabolite profiles at follow-up of an IH patient where 25-OH-D was reduced to 9 ng/ml, suggesting that symptomatic IH may depend on vitamin D nutritional status. Other hypercalcemic patients with complex conditions exhibited distinct vitamin D metabolite profiles. Our work points to the importance of serum vitamin D metabolite profiling in the differential diagnosis of vitamin D–related hypercalcemia that can rationalize expensive genetic testing, and assist healthcare providers in selecting appropriate treatment. © 2021 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: 1,24,25-TRIHYDROXYVITAMIN D_3; 23,25,26-TRIHYDROXYVITAMIN D_3; 25-HYDROXYVITAMIN D_3-26,23-LACTONE; CELL/TISSUE SIGNALING–ENDOCRINE PATHWAYS; CYP24A1; DISEASES AND DISORDERS OF/RELATED TO BONE; HYPERCALCEMIA; LC-MS/MS; NUTRITION; 24,25-DIHYDROXYVITAMIN D; PTH/Vit D/FGF23; SCREENING

Introduction

Vitamin D is metabolized in humans via complex pathways involving multiple metabolites (Figure 1).[1] Dysregulation of vitamin D metabolism results in a number of disease states, such as rickets and hypercalcemia.[2] Although hypercalcemia can arise from a range of factors including calcium alkali syndrome, malignancy, and primary hyperparathyroidism, considerable attention has recently been given to vitamin D–related hypercalcemia,[3] which can result from hypervitaminosis D, but also from autosomal recessive conditions affecting the vitamin D endocrine system, collectively termed idiopathic infantile hypercalcemia (IIH). Hypercalcemia in IIH patients is caused by elevated serum 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D) leading

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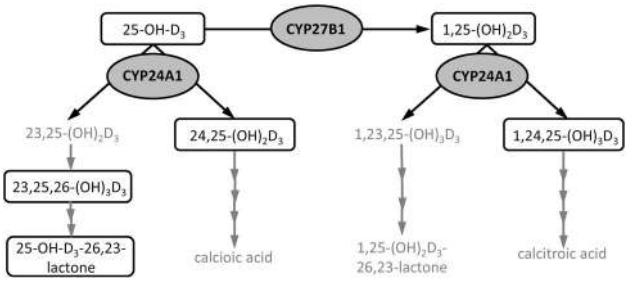

Fig 1. Vitamin D metabolism. Bioactivation of 25-OH-D_3 to 1,25-$(OH)_2D_3$ is catalyzed by CYP27B1. All steps in the complex catabolism of 25-OH-D_3 and 1,25-$(OH)_2D_3$ are mediated by CYP24A1. Catabolic pathways commence with either C23-hydroxylation or C24-hydroxylation, and culminate in either 26,23-lactone products or water-soluble acids. Only the metabolites detected by LC-MS/MS in human serum in this study are shown for simplicity, in black. Other metabolites and pathway steps are shown in gray. Abbreviation: LC-MS/MS, liquid chromatography–tandem mass spectrometry.

to excessive hormonal action; however, there are at least two underlying genetic causes, including: (i) loss-of-function mutations in the 25-hydroxyvitamin D-24-hydroxylase *CYP24A1* (IIH-CYP24A1) resulting in a lack of catabolism of 1,25-$(OH)_2D$[4]; or (ii) loss-of-function mutations in the sodium phosphate co-transporter *SLC34A1* (IIH-SLC34A1) resulting in excessive 1,25-$(OH)_2D$ biosynthesis secondary to chronic phosphate wasting.[5]

Due to the range of nonspecific symptoms in hypercalcemic patients, identification of the underlying cause has remained challenging, with IIH patients initially being confused with Williams-Beuren syndrome (WBS) patients who may present hypercalcemia in infancy but also syndromic association (growth retardation, intellectual disability, facial dysmorphism, great arteries anomaly)[6,7] secondary to a recurrent 1.5-megabase (Mb) deletion on chromosome 7 (7q11.23) including the *ELN* gene. The role of vitamin D metabolism in WBS-associated hypercalcemia remains unclear. Despite attempts to broaden the number of candidate genes used to screen patients with suspected vitamin D–related hypercalcemia, at least 20% of cases remain unexplained; we have termed this idiopathic hypercalcemia (IH). Recently, use of serum vitamin D metabolite profiling based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been used to unequivocally identify IIH-CYP24A1 patients on the basis of elevated 25-hydroxyvitamin D_3:24,25-dihydroxyvtamin D_3 ratios of >80 (25-OH-D:24,25-$(OH)_2D_3$), indicating inappropriately low rates of 24,25-$(OH)_2D_3$ formation by mutated CYP24A1.[8,9] Furthermore, the same methodology has identified characteristic profiles in different physiological states including patients with vitamin D deficiency and dialysis patients,[9,10] as well as in various animal gene knockout models,[11] confirming that 25-OH-D:24,25-$(OH)_2D_3$ ratio is a biomarker of CYP24A1 function and regulation in vivo. We present a case-control study of serum vitamin D metabolites in patients with idiopathic hypercalcemia in comparison to patients with known causes of vitamin D–related hypercalcemia and normal controls with a broad range of 25-OH-D concentrations. We set out to do the following:

1. Broaden our vitamin D metabolite profiling approach to include serum 1,25-$(OH)_2D_3$, 25-OH-D_3-26,23-lactone, 23,25,26-$(OH)_3D_3$, and 1,24,25-$(OH)_3D_3$.
2. Screen a subset of IH patients referred to German and French centers for diseases of calcium and phosphate homeostasis, using vitamin D metabolite profiling where involvement of *CYP24A1* and *SLC34A1* mutation had been excluded.
3. Compare vitamin D metabolite profiles in IH patients with normal subjects with a range of 25-OH-D levels, and patients with known causes of hypercalcemia including hypervitaminosis D and WBS.

We hypothesized that serum vitamin D metabolite profiling based on a broadened range of metabolites can identify dysregulated vitamin D metabolism in certain IH patients possessing normal 25-OH-D_3:24,25-$(OH)_2D_3$ ratios. Our results enable us to propose potential vitamin D–mediated mechanisms of pathogenesis to guide the selection of new candidate genes.

Patients and Methods

Clinical samples

Approximately 180 hypercalcemia patient serum samples were collected as a part of ethically-approved studies on IIH by Schlingmann et al.[4] (German cohort) and Molin et al.[12] (French cohort) from 2010 to 2019. The cohorts include subsets of patients with genetically-confirmed causes of hypercalcemia: IIH-CYP24A1 ($n = 5$), IIH-SLC34A1 ($n = 5$), and WBS (hypercalcemic and non-hypercalcemic, $n = 14$). The cause of hypercalcemia in all other patients is unknown, designated IH, and is the focus of the current study. Samples from patients with hypercalcemia arising from hypervitaminosis D ($n = 2$) were provided by Dr. Joy Wu, Stanford University, and Dr. Joel Finkelstein, Endocrine Unit, Massachusetts General Hospital. For one hypervitaminosis D patient, insufficient volume of serum was available for 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_2D_3$ measurement. All hypercalcemia patients included in the study exhibited total serum calcium >10.6 mg/dl (International System of Units [SI] = 2.6 mmol/L; $\times 0.25$) and parathyroid hormone <20 pg/ml (SI = 2.1 pmol/L, $\times 0.106$), based on inclusion criteria of previous studies on vitamin D–related hypercalcemia based on patients recruited at the same clinical referral centers in Europe.[4,12] Unaffected family members, and specific IH/WBS patients were normocalcemic at certain sampling times. Total serum calcium measurements were based on random, unadjusted values. Parathyroid hormone (PTH) measurements were based on second-generation assays. Certain IH and hypercalcemic WBS patients were normocalcemic at follow-up. Normal controls ($n = 163$) comprised premenopausal and postmenopausal women that were given graded doses of cholecalciferol (or untreated) in order to establish normal patterns of vitamin D metabolism over a broad range of 25-OH-D concentrations; reported by Kaufmann et al.[8] and Gallagher et al.[13–15] All controls, family members, and patients with known causes of hypercalcemia were analyzed for the full range of vitamin D metabolites described in the next section. Only IH patients prescreened for elevated 25-OH-D_3-23,26-lactone and 23,25,26-$(OH)_3D_3$ were selected for full vitamin D metabolite profiling.

Patients with aberrant vitamin D metabolism were grouped according to cause of hypercalcemia where specific diagnoses exist. For idiopathic cases, patients with common patterns of vitamin D metabolites were grouped, and patients with specific diagnoses were included in the groupings if the metabolite

pattern was shared. For example, IH patients with elevated 25-OH-D_3-23,26-lactone and 23,25,26-$(OH)_3D_3$ were grouped with hypercalcemic WBS patients who exhibited the same characteristic. Patients with elevated 25-OH-D_3-23,26-lactone and 23,25,26-$(OH)_3D_3$ were subdivided into those with elevated or suppressed 1,25-$(OH)_2D_3$. Student's *t* test was used to determine the significance of difference in mean metabolite concentrations between groups of hypercalcemia patients with specific diagnoses, or those with similar metabolite patterns, in comparison to normocalcemic controls. To control for dependence of certain vitamin D metabolites on 25-OH-D concentration, comparison of metabolites between groups was facilitated by using vitamin D metabolite ratios, or by comparing patients to subsets of controls with the same 25-OH-D range.

Extraction and LC-MS/MS analysis of vitamin D metabolites

Vitamin D metabolites were extracted from 100 µl of human serum or calibrator using liquid-liquid extraction (LLE) and immunoextraction (IE) as described.[8,9] For LLE, serum was supplemented with deuterated internal standards (d_6-25-OH-D_3, d_3-25-OH-D_2, d_3-3-epi-25-OH-D_3, d_6-24,25-$(OH)_2D_3$, and d_3-25-OH-D_3-26,23-lactone). Then 200 µl of water and 100 µl of 0.1M hydrochloric acid were added, and protein precipitation was completed by adding 150 µl of 0.2M zinc sulfate and 450 µl of methanol, with vortex mixing after each step. After centrifugation, the supernatant was extracted with 1.4 ml of 50% methyl tert butyl ether/hexane.

For IE, 150 µl of serum was supplemented with d_6-1,25-$(OH)_2D_3$, and d_6-1,24,25-$(OH)_2D_3$ extracted using 100 µl anti-1,25-$(OH)_2$D antibody slurry (Immundiagnostik, Manchester, NH, USA) for 2 h using end-over-end rotation.[16] The slurry was isolated by centrifugation and washed with 4 × 400 µl water. Vitamin D metabolites were eluted with 400 µl of ethanol. For both LLE and IE platforms, extracts were evaporated to dryness and derivatized with 4-(2-(6,7-Dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl)1,2,4-triazoline-3,5-dione) (DMEQ-TAD, Wynnewood, PA, USA). Samples were reconstituted in mobile phase enabling either 10-µl injections corresponding to 17 to 35 µl of serum (for LLE) or 35-µl injections corresponding to 110 µl of serum (for IE). Samples were subjected to LC-MS/MS analysis on Waters ACQUITY UPLC/Xevo TQ-S instruments (Waters Corporation, Milford, MA, USA) using both rapid and/or extended chromatographic systems. Mobile phase A and B were composed of methanol or water supplemented with 0.1% formic acid and 2mM ammonium acetate (Fisher, Waltham, MA, USA). Rapid chromatography analysis involved an exponential gradient elution from 65 to 90 mobile phase B over 5 min at a flow rate 400 µl/min on a Waters BEH-phenyl 1.7 µm, 2.1 × 50 mm column (Waters Corporation). For extended chromatography analysis, a linear gradient of 50% to 77% mobile phase B over 18 min was used on a Waters Cortecs C18+ 1.6 or 2.7 µm, 2.1 × 100 mm column (Waters Corporation). All samples prepared by LLE were analyzed by both rapid and extended chromatography methods, whereas samples prepared by IE were analyzed only using the extended method. A limitation of our method was that 23,25,26-$(OH)_3D_3$ (mass to charge ratio [*m/z*] 778 → 468) concentration was estimated based on the recovery of d_6-24,25-$(OH)_2D_3$ using the calibration line for 24,25-$(OH)_2D_3$, because no specific internal standard was available. Lower limits of quantification (LOQ) for vitamin D metabolites detected using LLE are estimated to be 0.01 to 0.05 ng/ml and 1 to 2 pg/ml for 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$, respectively. Interassay coefficients of variation ranged from 4% to 7% for 25-OH-D, 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, and 10% to 15% for 25-OH-D_3-26,23-lactone, 23,25,26-$(OH)_3D_3$, and 1,24,25-$(OH)_3D_3$. Student's *t* test was used to determine significance of changes in vitamin D metabolite levels. Vitamin D metabolites presented in conventional units (ng/ml or pg/ml) can be converted to SI units by multiplying by 2.3 to 2.5 mmol/L or pmol/L, respectively.

Results

Vitamin D metabolite profiling

We employed a combination of IE and LLE to expand the number of vitamin D metabolites that can be quantified by isotope-dilution LC-MS/MS, based on new deuterated internal standards (namely d_3-25-OH-D_3-26,23-lactone and d_6-1,24,25-$(OH)_3D_3$) and derivatization with DMEQ-TAD (Figure 2*A-D*). We observed that 25-OH-D_3-23,26-lactone and 23,25,26-$(OH)_3D_3$ could be detected in human serum using LLE, whereas IE was required for analysis of 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$. We detected 23,25,26-$(OH)_3D_3$ as well as 1,24,25-$(OH)_3D_3$ in the trihydroxyvitamin D fraction of antibody-extracted serum, consistent with findings by groups using the same anti-1,25-$(OH)_2D_3$ antibody to co-extract dihydroxylated vitamin D metabolites as well as 25-OH-D[17] (Figure 2). Given the potential importance of measuring a terminal catabolite of 25-OH-D_3 metabolism, 25-OH-D_3-26,23-lactone, and the initial 1,25-$(OH)_2D_3$ catabolic pathway product, 1,24,25-$(OH)_3D_3$, formed by CYP24A1, we synthesized or generated biologically, novel calibrators and deuterated internal standards d_3-25-OH-D_3-26,23-lactone and d_6-1,24,25-$(OH)_3D_3$ to enable quantitative measurement of these metabolites.[16,18] Unlabeled forms were also available to confirm the identity of peaks observed in serum samples.

Vitamin D metabolite profiles in hypercalcemia patients

Five new cases of CYP24A1 mutation (IIH-CYP24A1) (IH14–IH18; Table 1) exhibited characteristic, elevated 25-OH-D_3:24,25-$(OH)_2D_3$ ratios ($p < .001$), in addition to elevated 1,25-$(OH)_2D_3$ which was consistent with genetic findings. Ratios of 25-OH-D_3:24,25-$(OH)_2D_3$ observed in the current IIH-CYP24A1 patients (mean ratio = 121, $p < .001$) were consistent with previously established cutoffs (ratios >80) using the same analytical methodology based on over 30 IIH-CYP24A1 patients.[10] Patients also exhibited inability to catabolize 1,25-$(OH)_2D_3$ to 1,24,25-$(OH)_3D_3$ and 25-OH-D_3 to 23,25,26-$(OH)_3D_3$, consistent with lack of in vivo CYP24A1 function (Table 1, Figure 2). SLC34A1 patients (Table 1, patients IH19–IH23) have hypercalcemia due to elevated 1,25-$(OH)_2D_3$ as do patients with CYP24A1 mutation ($p < .001$), but the high serum 1,25-$(OH)_2D_3$ in IIH-SLC34A1 arises from excess biosynthesis secondary to phosphate wasting, as opposed to accumulation of this metabolite due to lack of catabolism in IIH-CYP24A1, thus serum 24,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ concentrations remain normal in IIH-SLC34A1 patients. We observed a trend of increasing levels of serum 25-OH-D_3-26,23-lactone and 23,25,26-$(OH)_3D_3$ concentration with increasing levels of 25-OH-D_3 in a series of 163 healthy postmenopausal women given increasing doses of vitamin D_3, as previously observed for 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ (Figures 2 and 3, Table 1). In two patients with hypervitaminosis D (Table 1, IH12 and IH13) who had serum 25-OH-D of 150 to 200 ng/ml, there was a marked increase in 25-OH-D_3-26,-23-lactone and 23,25,26-$(OH)_3D_3$, confirming 25-OH-D_3–dependence

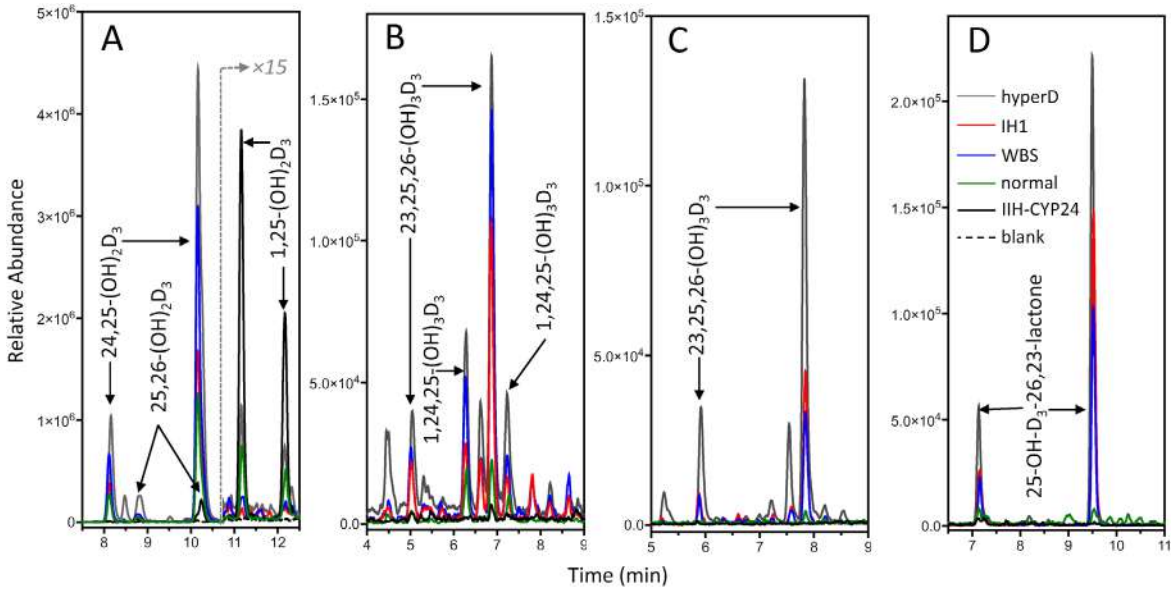

Fig 2. Vitamin D metabolite profiles in patients with vitamin D–related hypercalcemia. LC-MS/MS chromatograms from hypercalcemia patients based on a broadened number of serum vitamin D metabolites including: (*A*) dihydroxylated metabolites including 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ (MRM *m/z* 762 → 468 + 762 → 484) using serum prepared by IE; trihydroxylated metabolites 23,25,26-$(OH)_3D_3$ and 1,24,25-$(OH)_3D_3$ (MRM *m/z* 778 → 468 + 778 → 484) using serum prepared by IE (*B*) or LLE (*C*); and 25-OH-D_3-26,23-lactone MRM *m/z* 774 → 468, which was optimally recovered using LLE (*D*). Two peaks corresponding to 6*S* and 6*R* isomers of DMEQ-TAD adducts for each metabolite are shown. The metabolite 23,25,26-$(OH)_3D_3$ was detectable in both IE and LLE extracts, and is therefore annotated in *B* and *C*. Chromatograms from representative cases of vitamin D–related hypercalcemia and controls: hyperD (IH12), IH1 (unknown cause), WBS (IH5), normal control, IIH-CYP24A1 (IH15), blank (vitamin D–free serum). Abbreviations: DMEQ-TAD, 4-(2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl)1,2,4-triazoline-3,5-dione); hyperD, hypervitaminosis D; IE, immunoextraction; IH, idiopathic hypercalcemia; IIH, idiopathic infantile hypercalcemia; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLE, liquid-liquid extraction; *m/z*, mass to charge ratio; MRM, multiple reaction monitoring; WBS, Williams-Beuren syndrome.

of these two metabolites in vivo (Figures 2 and 3, Table 1). Despite elevated 25-OH-D_3 and 24,25-$(OH)_2D_3$ (as well as normal 1,25-$(OH)_2D_3$) in both cases in absolute terms, the 25-OH-D_3:24,25-$(OH)_2D_3$ ratio remained normal because the hypercalcemia arose from excess vitamin D intake rather than *CYP24A1* mutation. The mechanism of toxicity in hypervitaminosis D patients continues to be debated.[19]

On initial screening, a hypercalcemia patient (IH1) was found to possess normal 25-OH-D_3 and 24,25-$(OH)_2D_3$, but exhibited very low 1,25-$(OH)_2D_3$ (8 pg/ml) by radioimmunoassay (RIA). Further analysis for additional metabolites revealed 10-fold greater 25-OH-D_3-26,23-lactone and 23,25,26-$(OH)_3D_3$ than in normal subjects ($p < .001$), and confirmed the very low serum 1,25-$(OH)_2D_3$, and revealed elevated 1,24,25-$(OH)_3D_3$ relative to 1,25-$(OH)_2D_3$ in this patient (Figures 2 and 3, Table 1). Elevated serum 25-OH-D_3-26,23-lactone and 23,25,26-$(OH)_3D_3$ was not due to hypervitaminosis D because serum 25-OH-D concentration was only 38 ng/ml; thus these metabolites were inappropriately elevated relative to 25-OH-D concentration (Figure 3). This unique profile points to involvement of dysregulated vitamin D homeostasis as a possible cause of hypercalcemia observed in this patient. No specific treatment was recommended in this case, but we presumed cessation of vitamin D supplementation and/or reduction of sun exposure, since the patient was vitamin D–deficient on follow-up with 25-OH-D of 8.9 ng/ml and a slightly elevated 25-OH-D_3:24,25-$(OH)_2D_3$ ratio of 40 (Table 1). Interestingly, all other vitamin D metabolites, as well as serum calcium, had normalized by this time, implying a hypersensitivity to normal vitamin D levels as a general underlying cause, which appeared to resolve when 25-OH-D levels were low. Normocalcemic parents and siblings of IH1 (IH2–IH4) also possessed low 25-OH-D, and slightly elevated 25-OH-D_3:24,25-$(OH)_2D_3$ ratios associated with vitamin D deficiency. Because the hypercalcemia and aberrant vitamin D metabolite profile in IH1 appeared to depend on serum 25-OH-D, analysis of family members, who all had low 25-OH-D, could not confirm our suspicion of genetic origin of this specific type of hypercalcemia.

Three patients with hypercalcemia arising from WBS (Table 1, IH5–IH7) possessed an identical vitamin D metabolite profile to that observed in IH1, characterized by elevated 25-OH-D_3-26,-23-lactone, 23,25,26-$(OH)_3D_3$ and very low 1,25-$(OH)_2D_3$ ($p < .001$). Of interest was WBS patient IH7 who was reassessed while normocalcemic, and was found to have low 25-OH-D_3 and a normal vitamin D metabolite profile, similar to the follow-up assessment of patient IH1. Additionally, IH10 exhibited the same profile as WBS and IH1, but has propionyl coenzyme A (CoA) carboxylase deficiency (propionic academia) where direct involvement of vitamin D metabolism is unknown. We analyzed serum from 11 additional WBS patients who previously exhibited hypercalcemia, but who were sampled during a period of normocalcemia (Table 2, IH24–IH34). The vitamin D metabolite profiles in Table 2 are largely normal, and do not exhibit the aberrant profiles observed in WBS patients with hypercalcemia as well as in patient IH1. The low 25-OH-D levels in half of these patients suggest reduction of dietary vitamin D and/or sun exposure to reduce the risk of hypercalcemia. We identified three further

Table 1. Quantification of serum vitamin D metabolites in hypercalcemia patients

Patient	Disorder	*N*	25-OH-D_3 (ng/ml)	24,25-$(OH)_2D_3$ (ng/ml)	25-OH-D_3: 24,25-$(OH)_2D_3$ (ratio)	23,25,26-$(OH)_3D_3$ (ng/ml)	25-OH-D_3-26,23-lactone (ng/ml)	1,25-$(OH)_2D_3$ (pg/ml)	1,24,25-$(OH)_3D_3$ (pg/ml)
IH1	Unknown	1	38.6	3.68	10.5	0.274	0.871	2.1	22.6
IH1[a]	Unknown	1	8.9	0.22	40.3	<0.010	0.038	36.7	2.2
IH2	(Father)	1	10.7	0.37	28.8	<0.010	0.033	23.7	1.1
IH3	(Mother)	1	6.0	0.14	42.8	<0.010	<0.010	36.9	4.2
IH4	(Brother)	1	16.9	0.46	36.6	<0.010	0.023	53.8	7.6
IH5	Williams Beuren syndrome	1	36.9	3.83	9.6	0.156	0.712	5.1	23.4
IH6	Williams Beuren syndrome	1	32.2	2.33	13.8	0.257	0.955	5.7	11.2
IH7	Williams Beuren syndrome	1	26.0	2.82	9.2	0.044	0.213	5.3	13.3
IH7[a]	Williams Beuren syndrome	1	16.8	0.39	43.0	<0.010	0.035	73.6	13.4
IH8	Pancreatitis-associated hypercalcemia	1	4.4	0.35	16.0	0.045	0.349	57.1	6.9
IH9	Nephrolithiasis	1	18.8	1.14	10.0	0.093	0.667	68.8	8.8
IH10	Propionic acidemia	1	31.1	1.33	23.4	0.052	0.442	3.8	6.7
IH11	Cushings	1	40.3	2.77	14.5	0.084	0.305	134.5	22.3
IH12	Hypervitaminosis D	1	168.0	14.16	11.8	0.447	2.046	–	–
IH13	Hypervitaminosis D	1	200.6	10.14	19.8	0.616	3.219	19.2	95.0
IH14	IIH-CYP24A1	1	18.3	0.18	102.3	<0.010	0.024	65.4	<1.0
IH15	IIH-CYP24A1	1	27.2	0.18	152.0	<0.010	0.067	62.8	<1.0
IH16	IIH-CYP24A1	1	47.9	0.37	130.4	<0.010	0.073	53.2	2.5
IH17	IIH-CYP24A1	1	29.5	0.27	108.1	<0.010	0.025	58.1	<1.0
IH18	IIH-CYP24A1	1	23.6	0.21	113.3	<0.010	0.021	59.9	<1.0
IH19	IIH-SLC34A1	1	29.5	0.52	57.1	<0.010	0.025	96.9	5.5
IH20	IIH-SLC34A1	1	31.8	0.78	40.5	<0.010	0.035	57.8	8.3
IH21	IIH-SLC34A1	1	31.3	1.55	20.1	0.010	0.044	31.8	11.5
IH22	IIH-SLC34A1	1	11.0	0.41	26.6	<0.010	0.032	56.2	7.7
IH23	IIH-SLC34A1	1	45.8	1.88	24.2	0.013	0.054	77.8	19.3
25D>20[b]	Normal	84	35.4 ± 19.4	2.88 ± 1.90	12.8 ± 4.5	0.026 ± 0.024	0.089 ± 0.069	32.0 ± 10.8	9.1 ± 3.8
25D < 20	Normal	79	11.7 ± 5.2	0.47 ± 0.30	31.9 ± 15.9	<0.010	0.038 ± 0.021	36.5 ± 12.6	5.7 ± 3.4
25D-All[c]	Normal	163	24.7 ± 18.9	1.79 ± 1.86	21.4 ± 14.7	0.020 ± 0.020	0.070 ± 0.060	33.8 ± 11.8	7.6 ± 3.9
95% Interval[c]	Normal	163	5.6–70.7	0.15–5.60	7.7–55.1	<0.010–0.076	0.015–0.195	16.2–53.9	2.1–15.5

[a]Serum sample from follow-up.
[b]Normal subjects with [25-OH-D] > 20 ng/ml or < 20 ng/ml.
[c]Mean of all controls, and reference interval based on 5% to 95% percentile limits.

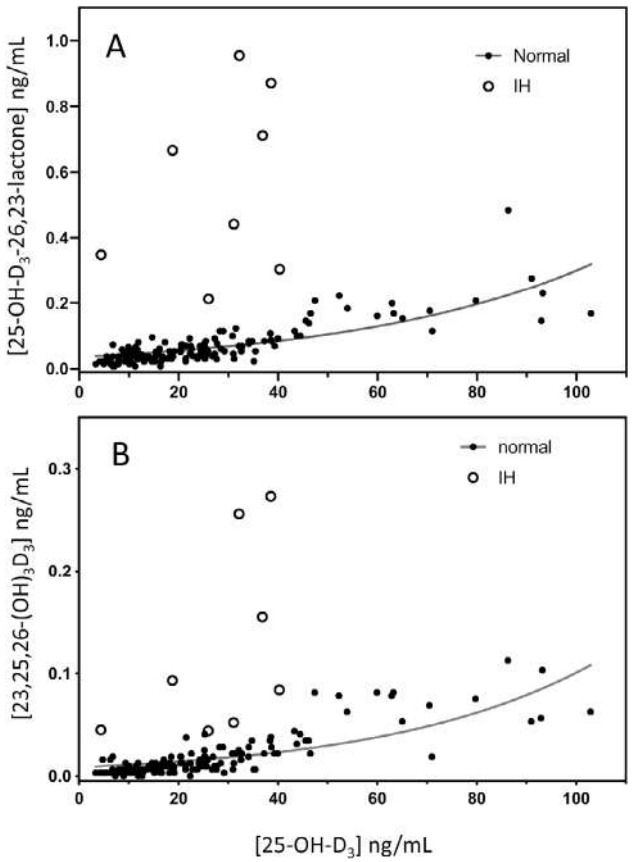

Fig 3. Concentration of 25-OH-D_3-26,23-lactone and 23,25,26-$(OH)_3D_3$ in hypercalcemia patients. Two C23-hydroxylated metabolites of 25-OH-D_3 formed by CYP24A1, (*A*) 25-OH-D_3-26,23-lactone and (*B*) 23,25,26-$(OH)_3D_3$ were determined in normal subjects exhibiting a broad range of 25-OH-D_3 concentrations. Levels of 25-OH-D_3-26,23-lactone (mean, 0.56 ng/ml; range, 0.21–0.96 ng/ml; $p < .001$) and 23,25,26-$(OH)_3D_3$ (mean, 0.12 ng/ml; range, 0.05–0.27 ng/ml, $p < .001$) in IH patients 1, 5–7, and 8–11 are shown in comparison to normals (mean 25-OH-D_3-26,23-lactone <50 ng/ml 25-OH-D = 0.05 ng/ml; mean 23,25,26-$(OH)_3D_3$ <50 ng/ml 25-OH-D = 0.01 ng/ml). Abbreviation: IH, idiopathic hypercalcemia.

hypercalcemia patients (Table 1, IH8, IH9, and IH11) with normal 25-OH-D_3:24,25-$(OH)_2D_3$ ratios where *CYP24A1* and *SLC34A1* mutation had been ruled out—but who had elevated 25-OH-D_3-26,23-lactone and 23,25,26-$(OH)_3D_3$ ($p < .001$). In contrast to IH1, IH10, and the patient with WBS with low 1,25-$(OH)_2D_3$, it appeared that elevated 1,25-$(OH)_2D_3$ was the cause of hypercalcemia. Medical records indicate the presence of additional disease including pancreatitis-associated hypercalcemia (IH8), nephrolithiasis (IH9), and Cushing's syndrome (IH11).

Characterization of the tri-hydroxyvitamin D fraction of the serum vitamin D metabolome

Of importance to this study was unequivocal identification of serum vitamin D metabolites, particularly in the tri-hydroxyvitamin D_3 fraction, which contains at least two components with the same molecular mass. We used a combination of criteria to assign metabolite identities summarized in Table 3, including: co-chromatography with standards, mass spectral properties, distribution of 6*R* and 6*S* DMEQ-TAD adducts, as well as sensitivity to sodium meta-periodate. For example, 23,25,26-$(OH)_3D_3$ and 1,24,25-$(OH)_3D_3$ co-migrated with standards that were well-resolved from each other, as well as 1,23,25-$(OH)_2D_3$, which is present as a very minor peak in human serum. Furthermore, 23,25,26-$(OH)_3D_3$ and 1,24,25-$(OH)_3D_3$ exhibited the typical distribution of 6*R* and 6*S* DMEQ-TAD adducts associated with metabolites of 25-OH-D, as compared with 1α-hydroxylated metabolites. Serum concentrations and optimal recovery from LLE or IE were consistent with catabolic products of 25-OH-D_3 or 1,25-$(OH)_2D_3$, and 23,25,26-$(OH)_3D_3$ concentrations were proportional to its precursor 25-OH-D_3 or downstream pathway product 25-OH-D_3-26,23-lactone. We used two multiple reaction monitoring (MRM) transitions to detect trihydroxylated vitamin D metabolites where *m/z* 778 → 484 is specifically associated with an A-ring hydroxyl group, for which there was no detectable signal for 23,25,26-$(OH)_3D_3$. Due to the presence of vicinal side-chain hydroxyl groups, both 23,25,26-$(OH)_3D_3$ and 1,24,25-$(OH)_3D_3$ were both sensitive to sodium periodate but produced the specific DMEQ-TAD adducts of 26-nor-23-hydroxy-25-keto-vitamin D_3 and 25-nor-24-keto-1-hydroxyvitamin D_3 with molecular ions ($[M + H]^+$) of *m/z* 746 and 702, respectively. Figure 4*A-D* depicts the conversion of 23,25,26-$(OH)_3D_3$ to 26-nor-23-hydroxy-25-keto-vitamin D_3 using a standard and a serum sample from a hypervitaminosis D patient. Our observation was in agreement with the work of Ishizuka et al.[20] who confirmed the presence of serum 23,25,26-$(OH)_3D_3$ in rats given large doses of vitamin D_3.[17] All other metabolites lacking vicinal hydroxyls such as 25-OH-D_3-26,23-lactone remained resistant to cleavage by sodium m-periodate (Table 3).

Discussion

We identified eight hypercalcemic patients with unique vitamin D metabolite profiles associated with WBS, a range of complex medical conditions, and a patient with an unremarkable medical history where the cause of the hypercalcemia is unknown (Tables 1 and 4, Figure 5*A-D*). CYP24A1 attenuates hormonal action of 1,25-$(OH)_2D_3$ by mediating its catabolism via a C24-hydroxylation pathway from 1,24,25-$(OH)_3D_3$ to calcitroic acid; but also catabolizes 25-OH-D_3 via 24,25-OH-D_3 (Figure 1). CYP24A1 also catalyzes a C23-hydroxylation pathway involving metabolites 25-OH-D_3-26,-23-lactone and 23,25,26-$(OH)_3D_3$, which we detected in human serum, despite being more prevalent in opossum and guinea pig.[21] Because *CYP24A1* is the most upregulated gene in response to vitamin D receptor (VDR) transactivation by 1,25-$(OH)_2D$, we conclude that elevated 25-OH-D_3-26,23-lactone, 23,25,26-$(OH)_3D_3$, and rapid clearance of 1,25-$(OH)_2D_3$ via 1,24,25-$(OH)_3D_3$ at normal 25-OH-D concentrations indicates hypersensitive hormonal response of vitamin D–dependent genes (including *CYP24A1*), as a common pathogenic mechanism of hypercalcemia in IH1, IH5–IH7 (WBS), and IH10 (propionic acidemia) (Figure 5*D*). Profiles in IH1, IH5–IH7, and IH10 differ among those observed in IIH-CYP24A1, IIH-SLC34A1, or hypervitaminosis D (Table 4, Figure 5*A-D*), suggesting that vitamin D metabolite profiling can be used in the differential diagnosis of vitamin D–related hypercalcemia.

Why serum 24,25-$(OH)_2D_3$ remained normal and other CYP24A1-dependent catabolites were elevated in certain IH patients is an intriguing question. Proximal C24-hydroxylation pathway products are metabolically unstable, and are catabolized to water-soluble products such as calcioic acid[22] and

Table 2. Quantification of serum vitamin D metabolites in normocalcemic WBS patients

Patient	Disorder	*N*	25-OH-D_3 (ng/ml)	24,25-$(OH)_2D_3$ (ng/ml)	25-OH-D_3:24,25-$(OH)_2D_3$ (ratio)	23,25,26-$(OH)_3D_3$ (ng/ml)	25-OH-D_3-26,23-lactone (ng/ml)	1,25-$(OH)_2D_3$ (pg/ml)	1,24,25-$(OH)_3D_3$ (pg/ml)
IH24	WBS	1	11.9	0.49	24.1	<0.010	0.036	27.1	5.9
IH25	WBS	1	13.9	0.52	26.9	<0.010	0.041	70.3	8.0
IH26	WBS	1	4.0[a]	0.15	27.5	<0.010	0.036	10.4	1.7
IH27	WBS	1	48.7	2.58	18.8	0.032	0.083	69.4	24.1
IH28	WBS	1	14.4	0.58	25.0	<0.010	0.045	52.8	10.0
IH29	WBS	1	35.4	2.93	12.1	0.061	0.169	46.3	20.1
IH30	WBS	1	16.5	0.90	18.5	0.012	0.067	30.7	9.3
IH31	WBS	1	6.9	0.43	15.9	<0.010	0.040	5.0	2.2
IH32	WBS	1	19.7	0.77	25.6	<0.010	0.058	44.6	7.7
IH33	WBS	1	7.5	0.21	36.4	<0.010	0.039	25.8	2.8
IH34	WBS	1	26.2	1.10	23.9	0.014	0.062	54.1	10.3

Abbreviation: WBS, Williams Beuren syndrome.
[a]Patient IH26 had serum 25-OH-D_2 of 32.5 ng/ml for total [25-OH-D] of 36.5 ng/ml.

calcitroic acid[23] via multiple steps. However, the more terminal C23-pathway catabolites detected, such as 25-OH-D_3-26,23-lactone, remain lipid-soluble and accumulate in circulation due to high affinity to vitamin D–binding protein (DBP).[24] Although there is no doubt that reduced serum 24,25-$(OH)_2D_3$ is an effective biomarker of severely impaired CYP24A1 function, we found that 24,25-$(OH)_2D_3$ concentrations unexpectedly remained stable in a series of mouse models with increasing *CYP24A1* expression, but the 25-OH-D_3-26,23-lactone concentration increased proportionally to *CYP24A1* expression.[25] As observed in the mouse models, we propose that 24,25-$(OH)_2D_3$ remained at steady-state concentrations in patients with purportedly increased *CYP24A1* expression; thus, the 24,25-$(OH)_2D_3$ concentration was not elevated because it was being formed and catabolized at an increased rate. Increased formation of 25-OH-D_3-26,23-lactone and 23,25,26-$(OH)_3D_3$ served as more definitive measures of enhanced CYP24A1 function in vivo. Because 23,25-$(OH)_2D_3$ was not detected in C24-hydroxylating species in vivo (such as human or mouse), we could not confirm that this proximal pathway metabolite in the C23-hydroxylation pathway was also in steady-state. Previous in vitro CYP24A1 kinetic analyses revealed that the balance between proximal versus terminal pathway catabolites in either pathway could be altered by manipulating extent of reaction[21] and that intermediates formed by CYP24A1 are released from, and compete to re-access the active site to be metabolized to the downstream metabolite, suggesting that steady-state metabolism of both 24,25-$(OH)_2D_3$ and 23,25-$(OH)_2D_3$ can occur.[26]

Specific details of the pathogenic mechanism of hypercalcemia in IH patients are limited, necessitating continued screening of new candidate genes, and exome sequencing in IH patients. We were unable to assess other physiological parameters, such as intestinal calcium absorption, to support our hypothesis of enhanced vitamin D–dependent gene function in patients IH1, IH5–IH7, and IH10. However, we note at least one other case of a hypercalcemic WBS patient with suppressed 1,25-$(OH)_2D$ where increased dietary calcium absorption was implicated.[27] Although the elevated 25-OH-D_3:24,25-$(OH)_2D_3$ ratio is specific to CYP24A1 mutation whether or not the patient is symptomatic, IH indicated by elevated serum 25-OH-D_3-26,23-lactone and suppressed 1,25-$(OH)_2D$ depends on 25-OH-D status and presence of symptoms at time of sampling, as in patients IH1 and IH7 (WBS), who, at follow-up, were normocalcemic, had low 25-OH-D and otherwise normal profiles—as did a group of normocalcemic WBS patients. Use of our method to explore possible genetic origins of hypercalcemia in patient IH1 was limited, because immediate family members were asymptomatic and possessed low 25-OH-D. We conclude that vitamin D intakes are a possible risk factor for development of hypercalcemia in these cases, as previously observed with IIH-CYP24A1,[28] and that 25-OH-D status should be considered when evaluating the diagnostic potential of our method.

WBS patients lack Williams syndrome transcription factor (WSTF) encoded by the *BAZ1B* gene, involved in VDR-mediated trans-repression of *CYP27B1* and induction of *CYP24A1*. Lack of attenuation of 1,25-$(OH)_2D_3$ biosynthesis, proposed as a possible cause of hypercalcemia in WBS,[27,29] appears inconsistent with the low 1,25-$(OH)_2D_3$ and elevated CYP24A1-dependent metabolites we observed. However, induction of vitamin D–dependent genes (including *CYP24A1*) due to dysregulated 1,25-$(OH)_2D_3$ production rationalizes increased catabolism of 1,25-$(OH)_2D_3$ and 25-OH-D_3. Increased serum calcium in *Baz1b*$^{+/-}$ mice suggest that hypercalcemia in WBS patients is at least partly due to WSTF

Table 3. Characterization of serum vitamin D metabolites produced by CYP24A1 using LC-MS/MS and sodium meta-periodate treatment

Putative identity of metabolite	LC-MS/MS retention time (min)[d]	MRM transition (*m/z*)	Periodate cleavage product retention time (min)[d]	MRM transition (*m/z*)	HPLC retention time (min)
24,25-$(OH)_2D_3$[a,b,c]	8.1, 10.1	762 → 468	7.8, 9.3	702 → 468	16.8
23,25-$(OH)_2D_3$[b,c]	7.9, 10.0	762 → 468	*not cleaved*		14.0
1,24,25-$(OH)_3D_3$[b,c]	6.2, 7.2	778 → 468,484	5.6, 6.6	718 → 468,484	21.2
1,23,25-$(OH)_3D_3$[b,c]	5.7, 7.0	778 → 468,484	*not cleaved*		17.6
23,25,26-$(OH)_3D_3$[a,c]	5.0, 6.9	778 → 468	5.4, 7.3	746 → 468	–
25-OH-D_3-26,23-lactone[a,b,c]	6.1, 8.4	774 → 468	*not cleaved*		19.0

Abbreviations: HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography–tandem mass spectrometry; *m/z*, mass to charge ratio; MRM, multiple reaction monitoring.

[a]Serum.

[b]Synthetic standard.

[c]Cell extract.

[d]Retention time based on extended chromatography method.

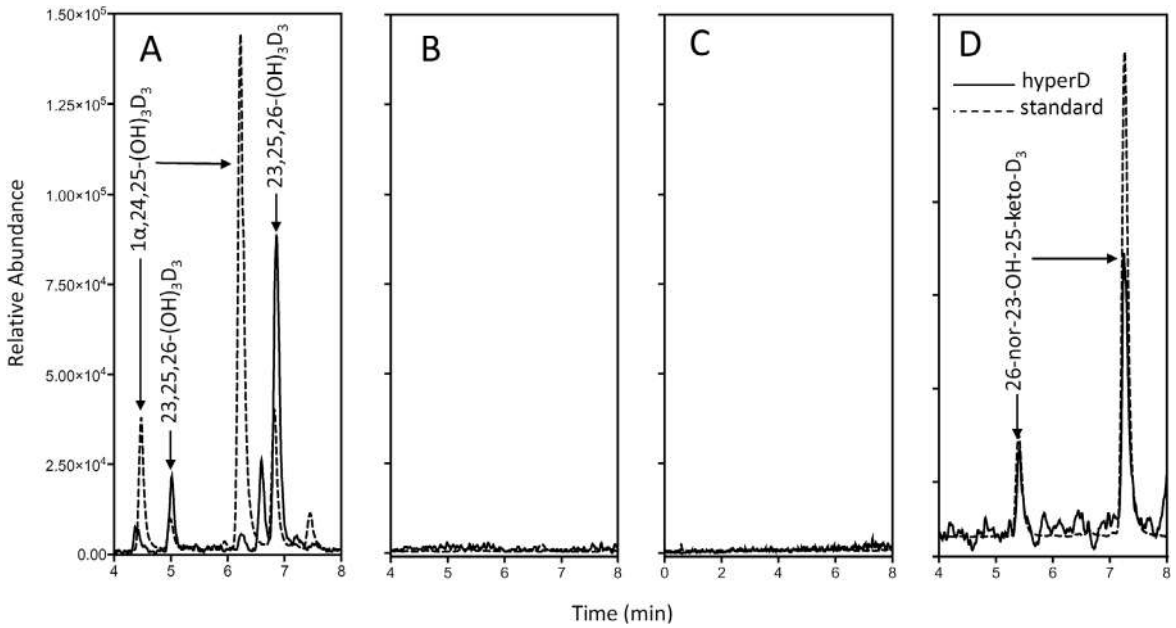

Fig 4. Identification of serum 23,25,26-$(OH)_3D_3$ using LC-MS/MS in conjunction with sodium meta-periodate cleavage. The identity of serum 23,25,26-$(OH)_3D_3$ was confirmed using a number of criteria. (*A*) Comparison of the retention times of trihydroxyvitamin D metabolite peaks in a hypervitaminosis D patient, with an in vitro extract prepared from CYP24A1-expressing cells optimized for C23-hydroxylation, based on the MRM transition *m/z* 778 → 468. The same analysis is shown in *B*, after treatment with periodate. (*C*,*D*) Chromatograms based on the expected mass transition of the periodate cleavage product of 23,25,26-$(OH)_3D_3$, 26-nor-23-hydroxy-25-keto-vitamin D_3 (*m/z* 746 → 468), shown before *C* and after *D* treatment with periodate. Abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; *m/z*, mass to charge ratio; MRM, multiple reaction monitoring.

deletion.[30] In vitro gene expression studies found upregulation of *CYP27B1* and unchanged *CYP24A1* expression upon *BAZ1B* ablation or RNA interference (RNAi) knockdown in MCF7 cells, mouse embryonic fibroblasts, or skin fibroblasts from WBS patients,[30,31] but it remains to be seen how absence of WSTF affects VDR-mediated *CYP24A1* expression specifically in the kidney, which is the main source of circulating vitamin D metabolites.

Excess 1,25-$(OH)_2D$ hormonal action was suspected as a common cause of hypercalcemia in IH8, IH9, and IH11, in whom 25-OH-D_3-26,23-lactone and 23,25,26-$(OH)_3D_3$ appear elevated for this reason. These patients exhibited diverse medical histories including persistent nephrolithiasis, acute pancreatitis-associated hypercalcemia and Cushing's syndrome. Altered vitamin D metabolism has been previously reported in some of these conditions, but mechanistic details remain inconclusive. Consistent with IH8, up to 40% of pancreatitis patients exhibit 25-OH-D < 20 ng/ml,[32] and vitamin D deficiency appears to predict the severity of acute disease.[33] Hypercalcemia is an established risk factor for development of pancreatitis, including cases involving excess serum 1,25-$(OH)_2D_3$, but are more frequently associated with lower 1,25-$(OH)_2D_3$ as compared

Table 4. Vitamin D metabolite profiles in different physiological and hypercalcemic states

Disorder	25-OH-D_3	24, 25-$(OH)_2D_3$	25-OH-D_3:24, 25-$(OH)_2D_3$ ratio	23,25, 26-$(OH)_3D_3$	25-OH-D_3-26, 23-lactone	1,25-$(OH)_2D_3$	1,24,25-$(OH)_3D_3$
Normal>20[a]	→	→	→	→	→	→	→
Normal<20	↓	↓	↑	↓	↓	→	↓
IH1, IH10	→	→	→	↑	↑	↓	↑
WBS[b]	→	→	→	↑	↑	↓	↑
IH8, IH9, IH11	→	→	→	↑	↑	↑	→
Hyper D	↑	↑	→	↑	↑	→	↑
IIH-CYP24A1	↑	↓	↑	↓	↓	↑	↓
IIH-SLC34A1	→	→	→	→	→	↑	→

Abbreviations: Hyper D, hypervitaminosis D; IIH, idiopathic infantile hypercalcemia; IH, idiopathic hypercalcemia; WBS, Williams Beuren syndrome.
[a]Normal subjects with [25-OH-D] > 20 ng/ml or < 20 ng/ml.
[b]WBS with hypercalcemia (IH5–IH7).

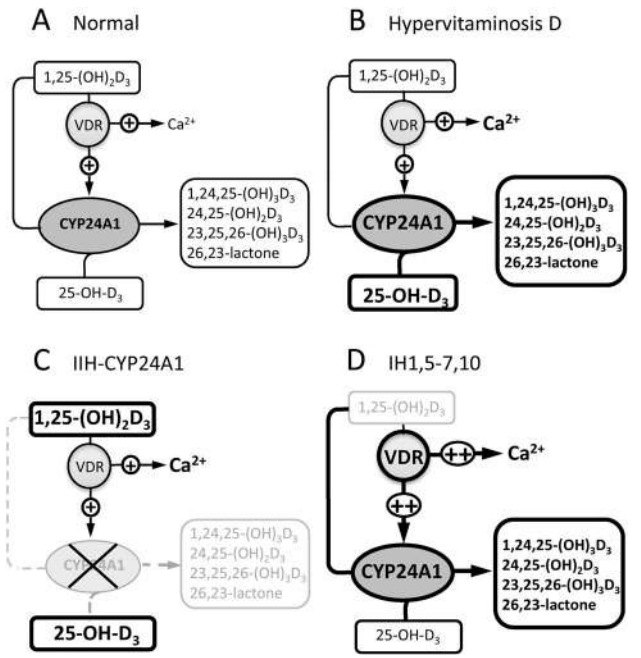

Fig 5. Dysregulated CYP24A1 function in vitamin D–related hypercalcemia. Serum vitamin D metabolite profiles reflect altered CYP24A1 function compared to normal (*A*), arising from dysregulated vitamin D metabolism in hypervitaminosis D (*B*), IIH-CYP24A1 (*C*). (*D*) Summarizes a common vitamin D metabolite profile observed in hypercalcemic WBS patients (IH5–IH7), a patient with propionic acidemia (IH10), and a hypercalcemic patient with an otherwise unremarkable history (IH1). Vitamin D metabolite profile in patients IH8, IH9, and IH11 is not shown, where hypercalcemia is caused by elevated 1,25-$(OH)_2D_3$. Bolded typeface and lines denote proposed increased expression or signal transduction inferred from serum metabolite concentration in hypercalcemia patients compared to normals, whereas gray or dotted lines denote reduced metabolite concentration or protein activity relative to normals. Abbreviation: WBS, Williams-Beuren syndrome.

to normals. One author proposed that local production of excess 1,25-$(OH)_2D_3$ by macrophages in the inflamed pancreas can exacerbate the hypercalcemia and leaks into the circulation, as observed in sarcoidosis.[34] Our observations are consistent with reports of elevated CYP24A1 expression in pancreatic biopsies from chronic pancreatitis patients.[35] There is little consensus on the effect of Cushing's syndrome on vitamin D metabolism, owing to the heterogeneity of underlying causes and extent of glucocorticoid excess in specific cases.[36] In a report of concomitant Cushing's, hypercalcemia, and elevated 1,25-$(OH)_2D_3$, a mesothelioma was found to produce excess 1,25-$(OH)_2D_3$ and adrenocorticotropic hormone (ACTH).[37] Low serum parathyroid hormone-related protein (PTHrp) suggested that the hypercalcemia was caused by 1,25-$(OH)_2D_3$ production by the tumor, which normalized after resection.

Due to study design limitations, we were unable to test our hypothesized mechanism of enhanced vitamin D–dependent gene function at the physiological level in IH patients with low serum 1,25-$(OH)_2D_3$ by measuring intestinal calcium absorption. Exome sequencing and screening of candidate genes, in addition to measurement of physiological parameters will be needed to fully elucidate new causes of vitamin D–related hypercalcemia. Given that only eight patients in our cohort exhibited aberrant vitamin D metabolite profiles suggestive of new mechanisms of vitamin D–related hypercalcemia, the clinical value of broad-based vitamin D metabolite profiling may be limited. The diagnostic value of our method in identifying a subset of IH patients with low 1,25-$(OH)_2D_3$ is also limited by the fact that the patient must be vitamin D sufficient for the characteristic metabolite profile to be observed. Although our LC-MS/MS method is quantitative for most metabolites, owing to the development of niche internal standards such d_3-25-OH-D_3-26,-23-lactone and d_6-1,24,25-$(OH)_3D_3$, we were unable to procure an appropriate internal standard for 23,25,26-$(OH)_3D_3$, rendering our measurement of this metabolite semiquantitative in nature.

First-line treatment of suspected vitamin D–related hypercalcemia involves nonspecific measures including dietary calcium/vitamin D restriction and administration of glucocorticoids. Elucidation of specific causes has spawned the design of more targeted regimens—including correction of hypophosphatemia in IIH-SLC34A1 by phosphate administration[5] and upregulation of alternative CYP3A4-mediated catabolism of 1,25-$(OH)_2D$ in IIH-CYP24A1 by giving rifampin.[38] The rare incidence of a single cause of vitamin D–related hypercalcemia may not warrant routine screening using our method, but our results point to at least five types of abnormal vitamin D metabolite profiles that are useful for secondary screening of hypercalcemic patients, asymptomatic family members, and for evaluating response to treatment. We anticipate that continued improvements to

vitamin D profiling by LC-MS/MS will assist healthcare providers with selecting appropriate treatment for vitamin D–related hypercalcemia, and rationalize expensive genetic testing.

Disclosures

The authors have nothing to disclose.

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Data availability statement

Data available in article.

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