

ORIGINAL ARTICLE

Mutations in *CYP24A1* and Idiopathic Infantile Hypercalcemia

Karl P. Schlingmann, M.D., Martin Kaufmann, Ph.D., Stefanie Weber, M.D., Andrew Irwin, B.Sc., Caroline Goos, Ulrike John, M.D., Joachim Misselwitz, M.D., Günter Klaus, M.D., Eberhard Kuwertz-Bröking, M.D., Henry Fehrenbach, M.D., Anne M. Wingen, M.D., Tülay Güran, M.D., Joost G. Hoenderop, Ph.D., René J. Bindels, Ph.D., David E. Prosser, Ph.D., Glenville Jones, Ph.D., and Martin Konrad, M.D.

ABSTRACT

BACKGROUND

From University Children's Hospital, Muenster (K.P.S., E.K.-B., M. Konrad); University Children's Hospital, Marburg (K.P.S., C.G.); University Children's Hospital, Essen (S.W., A.M.W.); University Children's Hospital, Jena (U.J., J.M.); Kuratorium für Heimdialyse Pediatric Kidney Center, Marburg (G.K.); and Children's Hospital, Memmingen (H.F.) — all in Germany; Queen's University, Kingston, ON, Canada (M. Kaufmann, A.I., D.E.P., G.J.); University Children's Hospital, Istanbul, Turkey (T.G.); and Radboud University, Nijmegen, the Netherlands (J.G.H., R.J.B.). Address reprint requests to Dr. Konrad at the Department of General Pediatrics, University Children's Hospital, Waldeyerstr. 22, D-48149 Muenster, Germany, or at konradma@uni-muenster.de.

Drs. Jones and Konrad contributed equally to this article.

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Vitamin D supplementation for the prevention of rickets is one of the oldest and most effective prophylactic measures in medicine, having virtually eradicated rickets in North America. Given the potentially toxic effects of vitamin D, the recommendations for the optimal dose are still debated, in part owing to the increased incidence of idiopathic infantile hypercalcemia in Britain in the 1950s during a period of high vitamin D supplementation in fortified milk products. We investigated the molecular basis of idiopathic infantile hypercalcemia, which is characterized by severe hypercalcemia, failure to thrive, vomiting, dehydration, and nephrocalcinosis.

METHODS

We used a candidate-gene approach in a cohort of familial cases of typical idiopathic infantile hypercalcemia with suspected autosomal recessive inheritance. Identified mutations in the vitamin D–metabolizing enzyme *CYP24A1* were evaluated with the use of a mammalian expression system.

RESULTS

Sequence analysis of *CYP24A1*, which encodes 25-hydroxyvitamin D 24-hydroxylase, the key enzyme of 1,25-dihydroxyvitamin D₃ degradation, revealed recessive mutations in six affected children. In addition, *CYP24A1* mutations were identified in a second cohort of infants in whom severe hypercalcemia had developed after bolus prophylaxis with vitamin D. Functional characterization revealed a complete loss of function in all *CYP24A1* mutations.

CONCLUSIONS

The presence of *CYP24A1* mutations explains the increased sensitivity to vitamin D in patients with idiopathic infantile hypercalcemia and is a genetic risk factor for the development of symptomatic hypercalcemia that may be triggered by vitamin D prophylaxis in otherwise apparently healthy infants.

VITAMIN D PLAYS A CENTRAL ROLE IN CALCIUM homeostasis and bone metabolism.¹ Vitamin D supplementation or food fortification for the prevention of rickets is advocated routinely for all infants. Although vitamin D is potentially dangerous in very high doses, the margin of safety between the daily requirements of vitamin D and levels that produce toxic effects is considered to be quite large.² However, in the early 1950s, there were reports about a number of infants with unexplained hypercalcemia who presented with failure to thrive, vomiting, dehydration, spikes of fever, and nephrocalcinosis.^{3,4} Laboratory evaluation of these infants revealed severe hypercalcemia and suppressed parathyroid hormone levels. Approximately 200 cases occurred in Great Britain within only 2 years.⁵ Some of the affected children had a complex phenotype that was later identified as the Williams–Beuren syndrome.^{6,7} However, most affected infants did not have syndromic features and were considered to be affected by a milder variant of the syndrome, which was termed idiopathic infantile hypercalcemia or Lightwood type (Online Mendelian Inheritance in Man number, 143880).^{4,8}

Although this disorder was originally considered to be relatively benign, during the acute phase of hypercalcemia, a substantial number of children died.^{9,10} The relation between the epidemic occurrence of idiopathic infantile hypercalcemia and increased doses of vitamin D (up to 4000 IU per day) in infant formula and fortified milk in Great Britain at that time implicated nutritional vitamin D intake in the pathogenesis of this disorder.⁵ However, it was obvious that vitamin D was not the only contributing factor, since most infants receiving this prophylaxis remained unaffected. Therefore, it was proposed that an intrinsic hypersensitivity to vitamin D might be implicated in the pathogenesis.¹¹ It remained unclear whether the underlying defect involved excessive activation of vitamin D or defective inactivation.

During activation, vitamin D first undergoes hydroxylation by 25-hydroxylase (CYP2R1) in the liver, which leads to the formation of 25-hydroxyvitamin D₃.¹² A second hydroxylation by 1 α -hydroxylase (CYP27B1) in the kidney then generates the active form 1,25-dihydroxyvitamin D₃, which exerts its biologic effects by binding to the vitamin D receptor. This active form is inactivated by 24-hydroxylase (CYP24A1), an enzyme

that is responsible for the five-step 24-oxidation pathway from 1,25-dihydroxyvitamin D₃ to calcitroic acid.^{13,14} CYP24A1 can also break down the precursor, 25-hydroxyvitamin D₃, to the inactive metabolite, 24,25-dihydroxyvitamin D₃. The activity of both CYP27B1 and CYP24A1 is predominantly controlled by levels of 1,25-dihydroxyvitamin D₃, serum calcium, and parathyroid hormone. In addition, CYP27B1 is negatively regulated by the concerted action of fibroblast growth factor 23 (FGF23) and *klotho*, a process that closely links vitamin D metabolism to phosphate homeostasis (Fig. 1).¹⁵

Here we describe how inactivating mutations in *CYP24A1* provide a probable molecular basis for idiopathic infantile hypercalcemia, which is inherited as an autosomal recessive trait.

METHODS

PATIENTS

We studied a cohort of six patients from four families with idiopathic infantile hypercalcemia with suspected autosomal recessive inheritance. A second cohort consisted of four patients with suspected vitamin D intoxication in whom severe hypercalcemia had developed after bolus prophylaxis with vitamin D. Data on clinical symptoms and biochemical measures at the time of disease manifestation were collected retrospectively from medical charts. We clinically reevaluated all patients during follow-up and obtained biochemical data. All genetic studies were approved by the ethics committee of the Westfälische Wilhelms University, Muenster. Patients or their parents provided written informed consent.

LABORATORY ANALYSES AND SEQUENCING

We measured levels of serum and urine electrolytes and creatinine in samples obtained from all patients using routine methods. (Detailed descriptions of the analyses of serum parathyroid hormone, 25-hydroxyvitamin D₃, and 1,25-dihydroxyvitamin D₃ are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.) We extracted genomic DNA from the whole blood of affected patients and available family members using standard methods. The entire coding regions and splice sites of *CYP24A1*, *CYP27B1*, *FGF23*, and *KL* (the latter encoding *klotho*) were sequenced from both strands. The presence of newly identified *CYP24A1* sequence variations

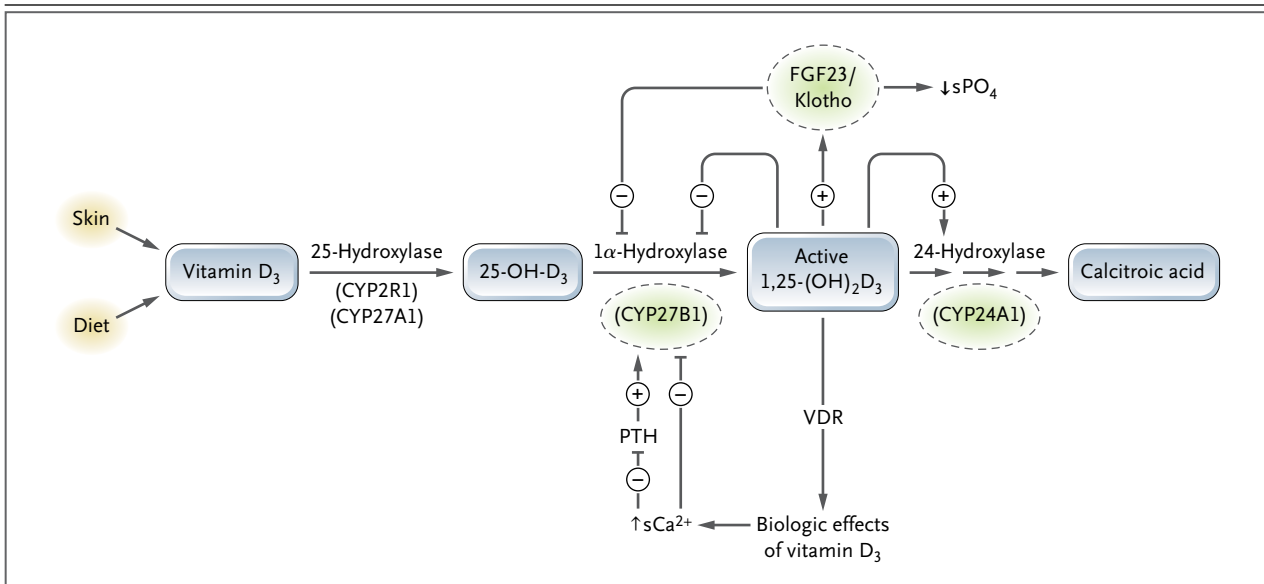


Figure 1. Vitamin D Metabolism with Selected Candidate Genes.

Vitamin D activation involves 25-hydroxylation in the liver, resulting in the formation of 25-hydroxyvitamin D₃ (25-OH-D₃), and 1 α -hydroxylation in the kidney, resulting in the formation of active 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], which exerts its biologic effects by binding to the vitamin D receptor (VDR). Then 1,25-(OH)₂D₃ is catabolized by CYP24A1 through a complex five-step process, commencing with 24-hydroxylation and culminating in the production of water-soluble calcitroic acid. The enzymatic activities of CYP27B1 and CYP24A1 are tightly controlled by the levels of 1,25-(OH)₂D₃, serum calcium, and parathyroid hormone (PTH). In addition, 1,25-(OH)₂D₃ exerts negative feedback on CYP27B1 through the axis of fibroblast growth factor 23 (FGF23) and klotho. The selected candidate genes *CYP27B1*, *CYP24A1*, *FGF23*, and *KL* (encoding klotho) are indicated in green. The abbreviation sCa²⁺ denotes serum ionized calcium, and sPO₄ serum phosphate.

was tested in at least 204 ethnically matched control alleles, whereas the presence of the two previously reported sequence variations R396W (rs114368325) and L409S (rs6068812) was analyzed in 1024 control alleles.

PREPARATION OF PLASMID CONSTRUCTS

Full-length human *CYP24A1*¹⁶⁻¹⁸ was subcloned into a pcDNA5/FRT construct (Invitrogen). Site-directed mutagenesis was conducted with the use of a QuickChange kit (Stratagene). *CYP24A1* mutants were generated (E143del, R159Q, E322K, R396W, L409S, and A475fsX490), and the presence of the desired mutations was confirmed by DNA sequencing.

TRANSFECTION

Human wild-type and mutant *CYP24A1* constructs were transiently or stably transfected into V79-4 Chinese hamster lung fibroblast cells. For stable transfections, a targeted integration method mediated by Flippase recombination enzyme (Flp) was used (Flp-In system, Invitrogen); for transient transfections, *CYP24A1* constructs contain-

ing pcDNA5/FRT were transfected directly into native cells, and enzyme activity was assayed. Experimental details are provided in the Supplementary Appendix.

CELL CULTURE AND ANALYSIS OF CYP24A1 ACTIVITY

Details of cell-culture experiments and analyses of *CYP24A1* activity are provided in the Supplementary Appendix. Transfected cells were incubated in medium containing [3 H]1,25-dihydroxyvitamin D₃. The incubation mediums were extracted and analyzed by high-performance liquid chromatography, as described previously.^{17,18}

RESULTS

CLINICAL FINDINGS

The four index patients in the four families with idiopathic infantile hypercalcemia (Patients 1.1, 2.1, 3.1, and 4.1) presented between the ages of 6 and 8 months with typical symptoms (Fig. 2A). Laboratory evaluation revealed profound hypercalcemia, suppressed intact parathyroid hormone, and hypercalciuria (Table 1). Of note, all four

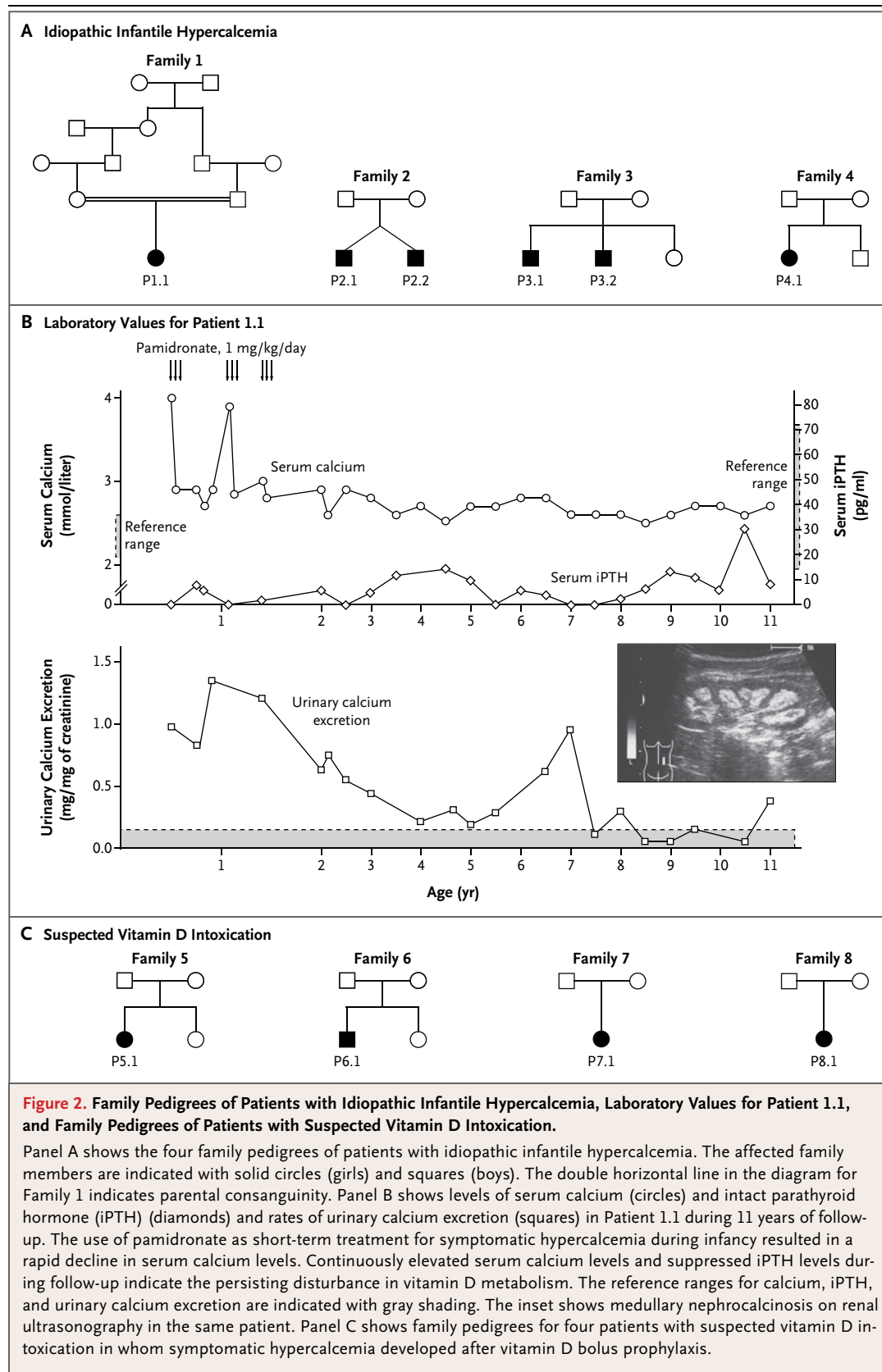


Table 1. Characteristics of the Patients.*				
Variable	Idiopathic Infantile Hypercalcemia			
	Patient 1.1	Patient 2.1	Patient 2.2	Patient 3.1
Clinical data				
Age at presentation	6 mo	6 mo	Asymptomatic; diagnosis during family workup	8 mo
Vitamin D prophylaxis	500 IU per day	500 IU per day	500 IU per day	500 IU per day
Time between bolus and symptoms	NA	NA	NA	NA
Clinical symptoms				
Weight loss or failure to thrive	Yes	Yes	No	Yes
Polyuria or dehydration	Yes	No	No	Yes
Muscular hypotonia or lethargy	Yes	No	No	Yes
Hypercalciuria or nephrocalcinosis	Yes	Yes	Yes	Yes
Laboratory findings†				
At initial presentation				
Serum calcium (mmol/liter)	4.0	4.2	3.7	4.3
Serum intact parathyroid hormone (pg/ml)	<1.0	5	4	<1.0
Serum whole parathyroid hormone (pg/ml)	NA	NA	NA	NA
25-hydroxyvitamin D ₃ (ng/ml)	50	27	27	64
1,25-dihydroxyvitamin D ₃ (pg/ml)	65	57	43	79
At last follow-up				
Duration of follow-up (yr)	10	2	2	7
Serum calcium (mmol/liter)	2.7	2.7	2.6	2.3
Serum intact parathyroid hormone (pg/ml)	8	11	14	5.2
25-hydroxyvitamin D ₃ (ng/ml)	7	21	17	33
1,25-dihydroxyvitamin D ₃ (pg/ml)	37	65	68	34
CYP24A1 mutation	A475fsX490 homozygote	E143del and E151X	E143del and E151X	L409S and R396W

* Laboratory values were obtained at the time of initial presentation and at the last follow-up. To convert the values for calcium to milligrams per deciliter, multiply by 4. To convert the values for 25-hydroxyvitamin D₃ to picomoles per liter, multiply by 2.496. To convert the values for 1,25-dihydroxyvitamin D₃ to picomoles per liter, multiply by 2.6. NA denotes not applicable, and ND not done or not available.

† Normal ranges for laboratory values are as follows: serum intact parathyroid hormone, 14 to 72 pg per milliliter; serum whole parathyroid hormone, 80 to 330 pg per milliliter; 25-hydroxyvitamin D₃, 10 to 65 ng per milliliter; and 1,25-dihydroxyvitamin D₃, 17 to 74 pg per milliliter.

infants had received oral vitamin D supplementation (500 IU per day) from birth. Medullary nephrocalcinosis was seen in all four infants on renal ultrasonography. Short-term treatment included intravenous rehydration and the use of furosemide, glucocorticoids, and pamidronate. Vitamin D prophylaxis was stopped, and a low-calcium diet was initiated. Serum calcium levels normalized within days to weeks. However, as shown in Patient 1.1 as an example, serum calcium levels tended to be continuously elevated during follow-up, whereas intact parathyroid hormone levels remained suppressed (Fig. 2B).

After the diagnosis in the index cases, we evaluated two asymptomatic siblings of index patients. Biochemical analysis in Patient 2.2, the monozygotic twin of Patient 2.1, revealed a similar serum calcium level (3.7 mmol per liter [14.8 mg per deciliter]), a suppressed intact parathyroid hormone level, and hypercalciuria. Medullary nephrocalcinosis was seen on renal ultrasonography. On the basis of the hypercalcemia, Patient 2.2 was treated accordingly. Patient 3.2, the asymptomatic younger brother of Patient 3.1, had serum calcium levels in the upper limit of the normal range during the neonatal period.

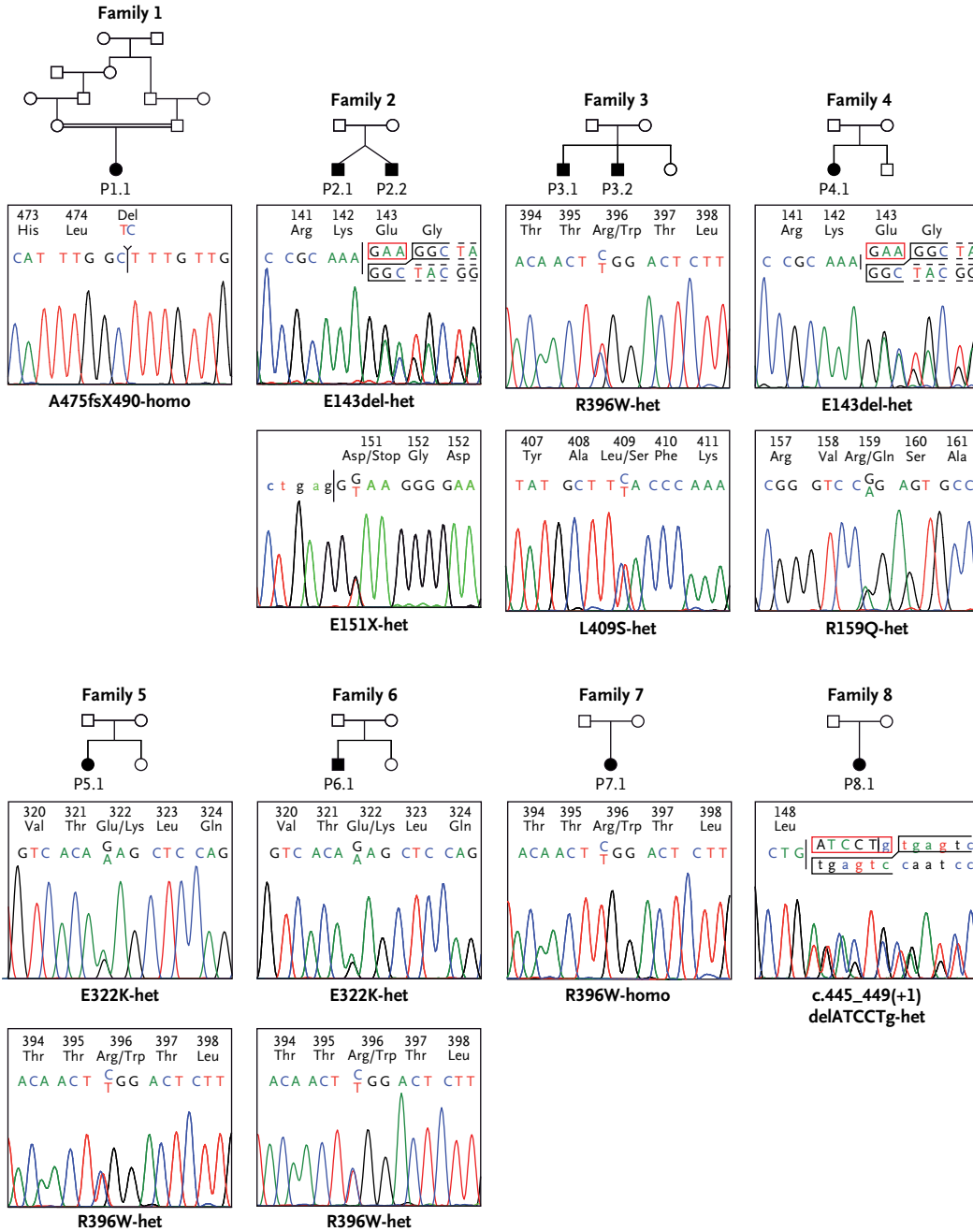
Idiopathic Infantile Hypercalcemia		Suspected Vitamin D Toxicity			
Patient 3.2	Patient 4.1	Patient 5.1	Patient 6.1	Patient 7.1	Patient 8.1
Asymptomatic; diagnosis during family workup	11 mo	7 mo	3.5 mo	7 wk	5 wk
None	500 IU per day	600,000 IU bolus, 3 doses	600,000 IU bolus, 2 doses	600,000 IU bolus, 1 dose	600,000 IU bolus, 1 dose
NA	NA	8 days	17 days	21 days	14 days
No	Yes	No	Yes	No	No
No	Yes	Yes	Yes	Yes	Yes
No	Yes	Yes	Yes	No	Yes
Yes	Yes	Yes	Yes	Yes	Yes
2.4	4.3	3.5	3.5	4.1	5.0
ND	2	NA	NA	NA	NA
NA	NA	350	215	ND	180
33	68	176	ND	178	31
ND	ND	129	ND	ND	ND
1	1	23	23	25	12
2.4	2.8	2.4	2.6	2.5	2.5
1.6	8.9	12	3.8	28	11
33	31	15	21	30	25
47	54	63	87	27	57
L409S and R396W	E143del and R159Q	E322K and R396W	E322K and R396W	R396W homozygote	Complex deletion

Given the history of Patient 3.1, the boys' parents decided against vitamin D prophylaxis for Patient 3.2. The diagnosis was established in Patient 3.2 at 18 months of age after the family's workup. Notably, his serum calcium level at that time was within the normal range, and intact parathyroid hormone levels were suppressed. Medullary hyperechogenicity was seen on renal ultrasonography. No additional treatment was initiated. All patients except for Patient 3.2 had received regular vitamin D₃ supplementation.

The second cohort consisted of four children (Patients 5.1, 6.1, 7.1, and 8.1) with suspected vitamin D toxic effects in whom symptomatic hypercalcemia had developed 1 to 3 weeks after receiving an oral dose of 600,000 IU of vitamin D₂ (Table 1 and Fig. 2C). The administration of

vitamin D given five times during the first 2 years of life (known as pulse therapy) was the preferred mode of prophylaxis in the German Democratic Republic for several decades.¹⁹ All four children received no additional daily vitamin D supplementation. Clinical details regarding Patients 5.1 and 6.1 have been reported previously.²⁰ Serum 25-hydroxyvitamin D₃ levels were increased in Patients 5.1 and 7.1. Serum 1,25-dihydroxyvitamin D₃ was measured only in Patient 5.1 and was elevated. In these patients, levels of whole parathyroid hormone that were measured at presentation were normal; no assay for intact parathyroid hormone was available at that time in East Germany. Treatment with parenteral fluid and glucocorticoids resulted in a rapid normalization of serum calcium levels. The clinical

A CYP24A1 Mutation Analysis



B Sequence Alignment of Vitamin D–Metabolizing Enzymes

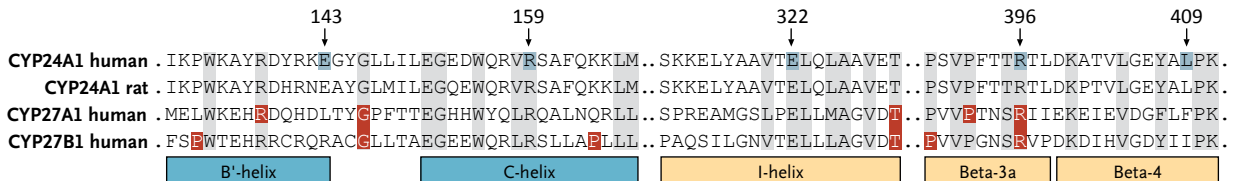


Figure 3 (facing page). CYP24A1 Mutations, Protein Sequence Alignments, and Secondary Structure of CYP24A1.

In Panel A, electropherograms show *CYP24A1* mutations that were identified in Families 1 to 8, with resulting amino acid changes indicated above. In Family 2, Patients 2.1 and 2.2 both had E143del and E151X mutations. In Family 3, Patients 3.1 and 3.2 both had L409S and R396W mutations. In Family 4, the index patient had E143del and R159Q mutations. In Families 5 and 6, the index patients both had E322K and R396W mutations. In Panel B, protein-sequence alignments show the location of mutations in *CYP24A1*. All mutations affect amino acid residues that are highly conserved between species and vitamin D–metabolizing enzymes *CYP24A1*, *CYP27A1*, and *CYP27B1*.¹² Red residues indicate mutations listed in the Online Mendelian Inheritance in Man database and the Human Gene Mutation Database. Light blue residues indicate the newly identified mutations in this study.

course beyond infancy was favorable in all patients, without recurrence of hypercalcemic symptoms. Other causes of hypercalcemia were ruled out in all 10 patients.

MUTATIONAL ANALYSIS

The parental consanguinity (in Family 1) and familial occurrence (in Families 2 and 3) pointed to an inherited basis of idiopathic infantile hypercalcemia. Therefore, we performed a candidate-gene analysis, including the genes of the key enzymes involved in vitamin D metabolism (Fig. 1). Although conventional sequencing of the coding regions of *CYP27B1*, *FGF23*, and *KL* did not reveal pathogenic mutations in patients from both cohorts, the sequence analysis of *CYP24A1* yielded nonsense and missense mutations in the homozygous or compound-heterozygous state in Patients 1.1 to 7.1 (Table 1 and Fig. 3A). Cosegregation analysis was compatible with autosomal recessive inheritance in all families. In Patient 8.1, only one pathogenic mutation was identified, which raised the possibility of a second pathogenic mutation outside the coding region or a heterozygous deletion that was not detected by sequence analysis.

Besides different missense mutations, we identified one premature stop mutation as well as two frameshift mutations leading to truncated *CYP24A1* proteins. Furthermore, we identified an in-frame deletion of E143. All mutations were ruled out in at least 204 control alleles. For the

two mutations, R396W and L409S, that had previously been annotated as putative polymorphisms in the Single Nucleotide Polymorphism Database (dbSNP), we tested a larger sample of 1024 control alleles. Although we did not detect L409S in any control allele, R396W was identified in 4 of the control alleles.

IN VITRO ANALYSIS OF CYP24A1 ACTIVITY

In order to determine the consequence of the identified mutations to human *CYP24A1* function in vitro, we stably and transiently transfected human *CYP24A1* constructs containing the mutations into V79-4 host cells and compared the catabolism of 1,25-dihydroxyvitamin D₃ with wild-type and nontransfected control cells. Transient and stable transfection protocols allowed us to test mutants at both nonsaturating substrate concentrations (0.003 μM for transient transfection and 0.3 μM for stable transfection) and saturating substrate concentrations (0.75 μM for transient transfection and 9.0 μM for stable transfection), respectively, in which stable transfection ensured high and reproducible levels of expression.

We found that 1,25-dihydroxyvitamin D₃ was almost completely metabolized by wild-type *CYP24A1* through the C-24 oxidation pathway intermediates (Fig. 4A) into the water-soluble metabolite, calcitric acid, which was quantified as radioactivity in the aqueous phase (Fig. 4B), as well as the terminal lipid-soluble metabolites, including tetranor-1,23-dihydroxyvitamin D₃ (Fig. 4D). When saturating substrate concentrations were used, almost all the intermediates in the C24-hydroxylation pathway (in addition to 1,23,25-trihydroxyvitamin D₃) were observed with the use of both radioactivity detectors (as measured in millivolts) and photodiode-array detectors (as measured at a wavelength of 265 nm in the ultraviolet spectrum), representing characteristic human *CYP24A1* activity, as reported previously.^{17,18} Under each transfection system and incubation condition used, the *CYP24A1* mutations that were identified in patients with idiopathic infantile hypercalcemia resulted in the ablation of *CYP24A1* catabolic activity (Fig. 4B and Fig. 4E through 4J), reminiscent of similar studies conducted on primary keratinocytes isolated from *CYP24A1* knockout mice.²¹ Only L409S retained small but measurable levels of activity (5.3±0.3%

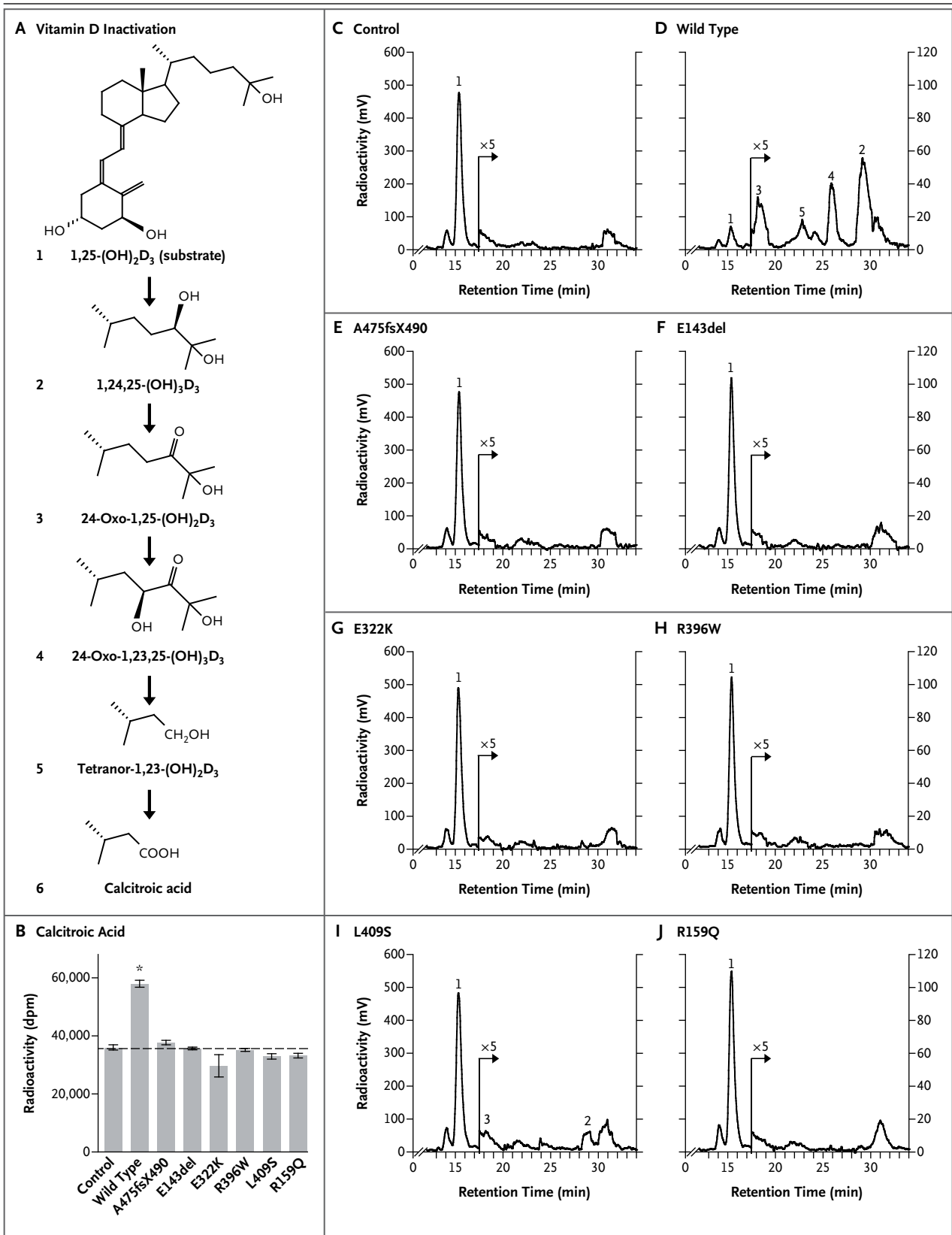


Figure 4 (facing page). Enzyme Activity for Mutant CYP24A1 in Patients with Idiopathic Infantile Hypercalcemia.

In Panel A, 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] is catabolized by human CYP24A1 through a complex five-step process, commencing with C24-hydroxylation and culminating in the water-soluble biliary product calcitroic acid (as shown in the diagram in Fig. 1). The steps in the production of lipid-soluble metabolites in the pathway correspond to the numbered peaks 1 through 5 in the chromatograms. The production of calcitroic acid (step 6) is depicted in Panel B. The bar graph shows water-soluble radioactivity recovered after lipid extraction. The mean net production of calcitroic acid is the fraction of radioactivity above baseline, as indicated by the horizontal dashed line. The I bars represent standard errors from triplicate determinations. The asterisk indicates that the value differs significantly from that of V79-4 control cells ($P < 0.001$ by Student's *t*-test). The results of high-performance liquid chromatography of the lipid-soluble metabolites of 1,25-(OH)₂D₃ in nontransfected control cells (Panel C) and wild-type cells (Panel D) are compared with V79-4 cells that have been stably or transiently transfected with the natural mutations found in patients with idiopathic infantile hypercalcemia (Panels E through J). Radiochromatograms are shown for saturating (9 μM) substrate concentrations. Substrate and metabolite peaks are numbered to correspond with the stages shown in Panel A. The break in the x axis represents a magnification (×5) for better visibility of metabolites with increased polarity. The scale on the right y axis refers to the magnified section of the chromatogram.

of wild-type activity) (Fig. 4I). Data were essentially the same for cells that had been either transiently or stably transfected.

In most studies of engineered mutations at substrate-contact residues in CYP24A1, there have been alterations in regioselectivity or relatively subtle changes in enzyme activity.^{17,18,22,23} In our study, however, the mutations in patients with idiopathic infantile hypercalcemia affected residues of critical structural importance (Fig. 3B, and Fig. S1 in the Supplementary Appendix) and resulted in complete loss of enzyme activity in most cases.

DISCUSSION

In a cohort of infants with idiopathic infantile hypercalcemia, we found loss-of-function mutations in CYP24A1 that appeared to lead to the disease development. CYP24A1 mutations were also detected in a second cohort of patients who presented with clinical symptoms of vitamin D intoxi-

cation 2 to 3 weeks after receiving intermittent high-dose vitamin D prophylaxis. Cosegregation analysis indicated autosomal recessive inheritance. Overexpression of the mutant CYP24A1 enzymes in a eukaryotic cell line revealed a complete loss of function for all identified mutations.

The physiologic importance of CYP24A1 in the catabolism of 1,25-dihydroxyvitamin D₃ and 25-hydroxyvitamin D₃ has already been shown in CYP24A1 knockout (−/−) mice, which have severe hypercalcemia leading to perinatal death in approximately 50% of the animals.^{21,24,25} Long-term vitamin D treatment in CYP24A1^{−/−} mice results in renal calcium deposition compatible with nephrocalcinosis.²⁵ The administration of exogenous 1,25-dihydroxyvitamin D₃ and 25-hydroxyvitamin D₃ to CYP24A1^{−/−} mice leads to a significant increase in 1,25-dihydroxyvitamin D₃ levels, indicating an inability to clear the active vitamin D hormone from the bloodstream.^{21,25} As expected, CYP24A1^{−/−} mice lack 24-hydroxylated vitamin D metabolites.²¹

Our data provide evidence for a crucial biologic role for CYP24A1 in humans. Analyses of vitamin D metabolites in healthy persons who are receiving high doses of vitamin D have shown that in contrast to sharp increases in levels of serum 25-hydroxyvitamin D₃ and its inactive products, serum 1,25-dihydroxyvitamin D₃ levels remain within the normal reference range, indicating tight regulative mechanisms.^{19,26-28} In contrast, patients with idiopathic infantile hypercalcemia have an exaggerated and prolonged increase in levels of active 1,25-dihydroxyvitamin D₃ after receiving prophylactic vitamin D, reflecting the impaired catabolism.^{29,30} Previously reported measurements of serum 24-hydroxylated metabolites in patients with idiopathic infantile hypercalcemia have had inconclusive results.³⁰ However, *in vitro* data that were obtained after the incubation of skin fibroblasts obtained from a patient with idiopathic infantile hypercalcemia with 1,25-dihydroxyvitamin D₃ showed decreased 24-hydroxylated metabolites.³⁰ This result is confirmed by the results of our overexpression studies, which showed a lack of 24-hydroxylated vitamin D metabolites after incubation with [1β-³H]1,25-dihydroxyvitamin D₃, indicating a complete loss of enzyme activity caused by a number of mutations in human CYP24A1.

Surprisingly, we also identified CYP24A1 mutations in four previously healthy children (Fam-

ilies 5 through 8) in whom symptoms of vitamin D intoxication developed after intermittent high-dose vitamin D prophylaxis that was regularly used in East Germany until 1989. Levels of 25-hydroxyvitamin D₃ in these patients, as far as available, were still below values that are generally considered to lead to acute toxic effects (>200 to 240 ng per milliliter).^{27,28} Nevertheless, 1,25-dihydroxyvitamin D₃ levels were found to be elevated in a single patient (Patient 5.1).

The genetic findings in both cohorts pose a critical question regarding the effect of dose and mode of administration of supplemental vitamin D for the manifestation of infantile hypercalcemia. The epidemic of idiopathic infantile hypercalcemia occurred in the United Kingdom in the 1950s after the implementation of an increased dose of vitamin D supplementation (up to 4000 IU per day).^{5,31,32} Concomitantly, less than 10 such cases were reported in the United States, where vitamin D supplementation was approximately 10 to 25% of the dose used in the United Kingdom.³² After the U.K. epidemic, the British Ministry of Health reduced daily allowances of vitamin D to approximately 400 IU, resulting in a significant decline in infantile hypercalcemia.³³ The identification of patients with idiopathic infantile hypercalcemia as an at-risk group may bring a new aspect to the debate concerning vitamin D supplementation.

The strongest argument for the critical role of vitamin D in idiopathic infantile hypercalcemia is the time course in our second cohort, in which clinical symptoms developed rapidly after vitamin D bolus prophylaxis. In the first cohort, all index patients with idiopathic infantile hypercalcemia had received supplementation with 500 IU of vitamin D per day, which is in the range of currently advocated daily vitamin D doses in most Western European countries, Canada, and

the United States.³⁴ Symptomatic hypercalcemia developed in these patients after several months of vitamin D prophylaxis. Of note, two siblings (from Families 2 and 3) remained asymptomatic and were identified only retrospectively by laboratory testing and genetic screening. In this context, Patient 3.2, the brother of Patient 3.1, is of special interest, since despite an uneventful medical history, laboratory analysis showed normocalcemia but suppressed parathyroid hormone levels, and only discrete hyperchogenicity of the medullary pyramids was seen on renal ultrasonography. Importantly, the parents had decided against regular vitamin D prophylaxis in this child because of his brother's illness. This observation supports the hypothesis that a substantial number of genetically affected persons may remain asymptomatic as long as the dose of prophylactic vitamin D is restricted. Such an incomplete penetrance of phenotype is consistent with the reduction of disease incidence after limitation of vitamin D supplementation.

Taken together, our findings indicate that defects in CYP24A1 are causative for idiopathic infantile hypercalcemia and serve as a genetic risk factor for the development of a serious adverse effect of generally advocated vitamin D prophylaxis. There is no doubt about the value and adequacy of daily vitamin D prophylaxis for the prevention of vitamin D deficiency and rickets in infants. Our findings, however, renew the demand for the careful administration of prophylactic vitamin D to avoid vitamin D toxicity.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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