Original Paper

Discordant Clinical Course of Vitamin-D-Hydroxylase (*CYP24A1*) Associated Hypercalcemia in Two Adult Brothers With Nephrocalcinosis

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Key Words

Hypercalciuria • Nephrolithiasis • Kidney stones • Hypervitaminosis • Calcium metabolism

Abstract

Background/Aims: Hypercalcemia can result in nephrocalcinosis/nephrolithiasis and may lead to renal failure. Idiopathic infantile hypercalcemia is caused by mutations of the *CYP24A1* gene, which regulates vitamin D activity. Classically infants present with hypercalcemia. Recently, a number of individuals have been reported with late onset clinical manifestation or late diagnosis in adulthood. All these patients are believed to show hypercalciuria. **Methods:** We report a 24 year old patient of healthy consanguine parents. Genetic analysis was performed by Sanger sequencing of the *CYP24A1* gene in the index patient and targeted exon 2 analysis of all other family members. **Results:** The patient was hospitalized with severe malaise during an acute EBV-infection. He showed hypercalcemia > 3mmol/l and acute, hypovolemic renal failure with profound nephrocalcinosis, but no hypercalciuria. Genetic workup revealed a homozygous loss-of-function mutation p.E143del in the *CYP24A1* gene. His clinically asymptomatic brother showed nephrocalcinosis of lesser degree. Repeatedly, low parathyroid hormone levels were detected in both brothers. **Conclusion:** This family displays the highly variable phenotype of *CYP24A1* biallelic mutation carriers. *CYP24A1* associated disease is an important differential diagnosis for the workup and counseling of infants as well as adults with hypercalcemia since a proper genetic diagnosis may result in therapeutic consequences.

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Introduction

Nephrocalcinosis (NC) is characterized by the deposition of calcium in the kidney parenchyma and tubules, which is usually situated in the medulla and visible on routine clinical imaging, such as ultrasound and computed tomography (CT), and if severe also on conventional abdominal X-ray. In 20% of premature infants, NC is a coincident finding upon abdominal ultrasound [1]. NC is also noted in about 1.7% of autopsies [2] and in approximately 50% of cases this finding can be attributed to malignancy. The etiology of the rest remains mostly unclear, yet clarification of its underlying cause is important, since progressive disease can lead to renal insufficiency and also bears the risk of extrarenal calcification. Histopathologically calcium oxalate (oxalosis; seen in primary and secondary hyperoxaluria) needs to be distinguished from calcium precipitates free of oxalate. Renal calcium deposition as NC and/or recurrent urolithiasis typically occurs in disorders of disturbed calcium or phosphate homeostasis with increased urinary excretion of calcium or phosphate.

Causes for hypercalcemia associated NC can be genetic or acquired. Important examples of the former are Williams-Beuren syndrome (WBS); elastin gene deletions, familial isolated hyperparathyroidism (FIHP), multiple endocrine neoplasia (MEN1) and mutations in the calcium sensing receptor (CASR). Examples of the latter are malignancy, sarcoidosis, drugs (e.g. thiazide diuretics) and vitamin D therapy. Differential diagnosis of disorders associated with hypercalcemia are listed in Table 1.

Clinical workup for NC routinely includes the acid-base status, vitamin D levels, parathyroid hormone (PTH), urinary excretion of calcium, phosphate, oxalate and citrate, as well as the measurement of serum calcium and phosphate. The interpretation of any of these parameters may be hampered by renal insufficiency, as well as dietary influences and a high diurnal variation of urinary excretion parameters. Therefore, careful clinical assessment of extrarenal manifestation, age at onset of symptoms and the family history may pose important clues towards clarification of the disease.

Among the growing list of possible causes of NC, mutations in the cytochrome P450 24-hydroxylase gene (*CYP24A1*, location 20q13.2, OMIM 143880) have been recently recognized as the major cause of autosomal recessive idiopathic infantile hypercalcemia (IHH) [3].

The *CYP24A1* gene encodes the mitochondrial enzyme CYP24A1 which contains a cytochrome P450 domain responsible for inactivation of $1\alpha,25(\text{OH})_2\text{D}_3$ and 25OHD_3 , resulting in $1\alpha,24,25(\text{OH})_3\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$. The CYP24A1 enzyme is inhibited in a feedback mechanism by $1\alpha,24,25(\text{OH})_3\text{D}_3$ and stimulated by $1\alpha,25(\text{OH})_2\text{D}_3$ and PTH [3]. Biallelic mutations of *CYP24A1* cause accumulation of the active form of vitamin D resulting in hypercalcemia (and PTH suppression), increased filtration and renal tubular reabsorption of calcium, and finally deposition of calcium precipitates in the urogenital tract.

In addition to IHH *CYP24A1* mutations are increasingly identified in older patients with a subclinical course or late manifestation of disease which is not always found in relation to vitamin D intake (Table 2).

We report here two discordant brothers bearing a homozygous *CYP24A1* in frame deletion, without apparent hypercalciuria, but with severe nephrocalcinosis, chronic renal failure and serious hypercalcemia in the index patient. Remarkably, despite documented vitamin D supplementation in infancy none of the brothers came to medical attention in childhood. This report emphasizes the importance of a precise molecular diagnosis with regard to proper treatment and counseling (family, life style and diet) in the era of widespread vitamin D supplementation.

Table 1. Differential diagnosis of inherited and acquired disorders associated with hypercalcemia

Disorder	Gene	Inheritance	Hints and hallmarks
Williams-Beuren syndrome	continuous gene deletion syndrome (1.55 Mb including <i>ELN</i> , <i>LIMK1</i> , <i>RFC2</i>); 7q11.23	mostly sporadic	multisystemic developmental disorder with mental retardation, distinctive neuropsychological profile "happy party manner", variable cardiovascular findings (aortic stenosis), abnormalities of renal tract and connective tissue, temporary hypercalcemia and hypercalciuria, NC/UL
(vitamin D induced) infantile hypercalcemia (IHH) , hypercalciuria and nephrocalcinosis (formerly known as idiopathic nonsyndromal infantile hypercalcemia or Lightwood type)	<i>CYP24A1</i> /25 hydroxyvitamin D24-hydroxylase, the key mitochondrial protein for degradation of 1 α ,25(OH) $_2$ D3, the physiologically active form of vitamin D3 ; 20q13.2	ar	increased sensitivity to regular (supplemental) and excess doses of vitamin D resulting in severe symptomatic hypercalcemia (failure to thrive, hypotonia, dehydration, acute renal failure) with hypercalciuria and nephrocalcinosis and/or nephrolithiasis. wide phenotypic spectrum associated with <i>CYP24A1</i> mutations. Usually low/suppressed intact PTH levels and 1 α ,25(OH) $_2$ D3 levels at the upper normal limit
Primary hyperparathyroidism (PHPT): # sporadic PHPT	single parathyroid adenoma , not inherited.	n.a.	prevalence of 3:1000 in the general population with a female-to-male ratio of ca. 3:1 symptoms of hypercalcemia with peak incidence in the sixth decade of life
# MEN1 syndrome-associated PHPT	Menin, 11q13	ad	2%-4% of all PHPT, no sex prevalence with earlier onset two to three decades earlier than sporadic PHPT
# Familial isolated hyperparathyroidism (FIHP)	1. Menin, 11q13 (20%)	ad	Isolated parathyroid adenoma or hyperplasia without other associated endocrinopathies in two or more individuals in one family.
# Hyper-parathyroidism 2 (HRPT2)	2. CASR, 3q21 (10-20%)	ad	inactivating mutations in CaSR hypocalciuric hypercalcemia
# neonatal severe primary hyperparathyroidism (NSHPT)	<i>HRPT2</i> (Parafibromin, 1q31.3; rare)	ad	multiple ossifying fibromas of the mandible and maxilla; recurring pancreatitis,
	CASR, 3q21	ar	very severe hypercalcemia
Metaphyseal chondrodysplasia, Murk Jansen type	<i>PTHRI</i>	ad	severe postnatal short stature, NC normal PTH levels, Hypercalcemia, Hypophosphatemia Hypercalciuria, Hyperphosphaturia
Chronic kidney disease	n.a.	n.a.	Secondary hyperparathyroidism
Granulomatous disease	n.a.	n.a.	Elevated calcitriol levels/no reduction by increased calcium intake, mostly in tuberculosis and sarcoidosis: elevated serum angiotensin converting enzyme, positive tuberculin skin test, pathologic chest radiograph/HRCT
Thyrotoxicosis	n.a.	n.a.	Suppressed TSH, elevated T3/T4
Cancer	n.a.	n.a.	osteolytic metastases (e.g. breast cancer, multiple myeloma, prostate cancer), ectopic secretion of parathyroid-related hormone or PTH (e.g. small cell lung cancer, squamous cell carcinoma, renal cancer, ovarian cancer, non-Hodgkin lymphoma)
Vitamin D/Vitamin A excess	n.a.	n.a.	Intake of dietary supplements
Drugs	n.a.	n.a.	L-thyroxine, estrogen, antiestrogen (e.g. tamoxifen), calcium carbonate (milk alkali syndrome), lithium, thiazide diuretics, teriparatid

ad autosomal dominant, ar autosomal recessive, n.a. not applicable

Table 2. synopsis of currently published CYP24A1 mutations and clinical description

Family patient	onset	NC/UL	HCa, HCu	genotype	Vit D Suppl	publication
1-1	6 mo	NC	HCa; HCu	pA475fs*25/ pA475fs*25	+	Schlingmann, N Engl J Med, 2011 [3]
2-1	6 mo	NC	HCa, HCu	pE143del/pE151*	+	Schlingmann, N Engl J Med, 2011
2-2	asympt	NC	HCa, HCu	pE143del/pE151*	+	Schlingmann, N Engl J Med, 2011
3-1	8 mo	NC	HCa, HCu	pL309S/pR396W	+	Schlingmann, N Engl J Med, 2011
3-2	asympt	NC	HCu	pL309S/pR396W	-	Schlingmann, N Engl J Med, 2011
4-1	11 mo	NC	HCa, HCu	pE143del/pR159Q	+	Schlingmann, N Engl J Med, 2011
5-1	7 mo	NC	HCa, HCu	pE322K/pR396W	+	Schlingmann, N Engl J Med, 2011
6-1	3.5 mo	NC	HCa, HCu	pE322K/pR396W	+	Schlingmann, N Engl J Med, 2011
7-1	2.3 mo	NC	HCa, HCu	pR396W/p.R396W	+	Schlingmann, N Engl J Med, 2011
8-1	1.3 mo	NC	HCa, HCu	c.445_449+1delATCCTG	+	Schlingmann, N Engl J Med, 2011
9-1	10 mo	NC	HCa, HCu	pE143del/ pE143del	+	Dauber, J Clin Endocr Metab, 2012 [11]
10-1	36 mo	NC	HCa, HCu	pE143del/pL148P	- (?)	Nesterova, CJASN, 2013 [9]
11-1	4 mo	NC	HCa, HCu	pR396W/p.R396W	+	Fencl, Eur J Pediatr, 2013 [12]
12-1	5 mo	NC	HCa, HCu	pR396W/p.R396W	+	Skalova, Iran J Kidney Dis, 2013 [13]
13-1	6 mo	NC	HCa, HCu	gross deletion exon 9-11	+	Castanet, J Pediatr, 2013 [5]
13-2	asympt	normal	NA	gross deletion exon 9-11	-	Castanet, J Pediatr, 2013
14-1	5 mo	NC	HCa, HCu	pW134G/pR396W	+	Dinour, Pediatr Nephrol, 2015 [10]
15-1	9 mo	NC	HCa, HCu	pW134G/pE315*	+	Dinour, Pediatr Nephrol, 2015
16-1	5 mo	NC	HCa, HCu	pE143del/ pE143del	+	Dinour, Pediatr Nephrol, 2015
17-1	18 y, dx 35 y	rec UL, NC	HCa, HCu	pE143del/ pE143del	+	Dinour, Pediatr Nephrol, 2015
18-1	19 y	rec UL	HCa, HCu	pE143del/pE143del	-	Streeten, N Engl J Med, 2011 [14]
19-1	44 y	rec UL	HCa, HCu	c.732+1G>A/ c.845-2A>G	?	Tebben, J Clin Endocr Metab, 2012 [8]
20-1	25 y	rec UL	HCa, HCu	pE143del/pL409S	-	Nesterova, CJASN, 2013
21-1	9 y	UL, NC, CKD	HCa, HCu	pE143del/pE143del	-	Dinour, J Urol, 2013 [4]
21-2	asympt, mother	?	HCa	pE143del/pE143del	+	Dinour, J Urol. 2013
22-1	19 y	rec UL	HCa, HCu	pW268*/p.L409S	-	Dinour, J Urol. 2013
22-2	13 y	rec UL, NC	HCa, HCu	pW268*/p.L409S	+	Dinour, J Urol. 2013
23-1	20 y	rec UL, CKD	HCa, HCu	pE143del/pE143del	-	Dinour, Pediatr Nephrol, 2015
24-1	18 y	rec UL	HCa, HCu	pR396W/p.R396W	-	Dinour, Pediatr Nephrol, 2015
25-1	18 y	rec UL	(HCa)HCu	pE143del/ pR396W	-	Wolf, Endocr Pract. 2014 [15]
26-1	3 mo, dx 29y	NC, CKD	HCa, HCu	pW210R/p.W210R	+	Meusburger, Clin Kidney J, 2013 [16]
27-1	20 y	rec UL, CKD	HCa, HCu	pE143del/ pE143del	-	Jacobs, J Clin Endocr Metab, 2014 [17]
28-1	10 y, dx 45y	rec UL, NC	HCa, HCu	pE143del/ pE143del	-	Downen, Kidney Int, 2014 [18]
29-1	45 y	NC	HCa, HCu	p.E469fs*22/ p.P21fs*8	+	Figueres, Am J Kidney Dis, 2015 [19]
30-1	32 y	NC, UL	HCa, HCu	p.L409S/ p.R157W	-	Figueres, Am J Kidney Dis, 2015
31-1	28 d	NC	HCa, HCu	p.R157W/ p.M374T	+	Figueres, Am J Kidney Dis, 2015
32-1	2 mo	NC	HCa, HCu	p.L409S/ p.R396W	+	Figueres, Am J Kidney Dis, 2015
33-1	6 mo	NC	HCa, HCu	p.L409S/ p.L409S	+	Figueres, Am J Kidney Dis, 2015
34-1	2 mo	NC	HCa, HCu	p.R396W/ p.R396Q	+	Figueres, Am J Kidney Dis, 2015
35-1	6 mo	NC	HCa, HCu	p.E143del/ p.L409S	+	Figueres, Am J Kidney Dis, 2015
36-1	24 y	NC, CKD	HCa	pE143del/ pE143del	+	this paper
36-2	asympt	NC	WNL	pE143del/ pE143del	+	this paper

Rec recurrent UL urolithiasis; NC nephrocalcinosis, HCa hypercalcemia, HCu hypercalciuria; mo months; y years; dx diagnosed with xx years; WNL within normal limits, + (late) Vit D supplementation beyond infancy

Material and Methods

Patients

We studied two related patients from a consanguineous family of Eastern Mediterranean origin. All reported individuals gave informed, written consent to genetic analysis and scientific publication under strictly anonymous fashion.

Clinical, imaging and laboratory studies

All studies reported used the routine diagnostic facilities of two hospitals. Genetic analysis was performed by standard Sanger sequencing of the entire CYP24A1 gene in the index patient and targeted exon 2 analysis of all other family members.

Fig. 1. Pedigree and clinical key findings. The index patient (arrow) is homozygous for the *CYP24A1* mutation, as is his 28 year old brother (p.E143del/p.E143del). The parents are heterozygous (WT/p.E143del) for the *CYP24A1* mutation and the third brother is not affected, displaying the wild-type allele homozygously (WT/WT). Both affected brothers show suppressed PTH levels (normal value 15-65 pg/ml), but hypercalcemia is an inconsistent finding. The mildly affected brother shows moderate medullary enhancement on ultrasound, whereas the index patient has profound medullary calcifications.

Results

Patient's history

The 24-year-old index patient presented generally unwell with fever, odynophagia, diarrhea and volume depletion. He appeared agitated, yet was fully orientated and showed no neurological abnormalities. The preclinical medical history was unremarkable, yet two months before admission the patient presented at his general practitioner with mild arterial hypertension, microscopic hematuria and mild proteinuria on dipstick testing, as well as sterile leucocyturia.

Clinical findings and laboratory results

The patient showed renal impairment with a serum creatinine of 2.34 mg/dl and the serum calcium was significantly elevated at 2.92 mmol/l initially, increasing to 3.1 mmol/l on follow up. The abdominal ultrasound showed pronounced signal intense kidneys, most strongly at the renal medulla (Figure 1). The decision was made to admit him for further investigations, because of renal failure of unknown cause with hypercalcemia together with signs of infection.

The extended laboratory workup showed no abnormal findings for c and pANCA, ANA, dsDNA antibodies, angiotensin converting enzyme, Quantiferon test. Urine sediment repeatedly showed abacterial leucocyturia without acanthocytes and any crystals. The serology for Epstein Barr virus was suggestive of reactivated infectious mononucleosis, as suspected cause for the recent infection.

PTH was suppressed and $1\alpha,25(\text{OH})_2\text{D}_3$ was within normal limits (42 ng/l). The patient displayed normal calciuria, and no abnormal excretion of oxalate or citrate (for detailed

Table 3. Laboratory parameters

Parameter	Index patient E143del/E143del; Age 24	Homozygous brother E143del/E143del; Age 28	Standard value
Creatinine	2.34 mg/dl	0.92 mg/dl	<1.2 mg/dl
Estimated GFR	34.4 ml/min	>60 ml/min	>60 ml/min
Urea	72 mg/dl	26 mg/dl	17-43 mg/dl
Calcium	3.1 mmol/l	2.6 mmol/l	2.1-2.7 mmol/l
Phosphate	1.13 mmol/l	0.97 mmol/l	0.81-1.45 mmol/l
Parathyroid hormone (PTH)	4.7 pg/ml	4.6 pg/ml	15-65 pg/ml
PTH related peptide	<1.0 pmol/l	not determined	<4.0 pmol/l
1,25-Dihydroxycholecalciferol	42 ng/l	not determined	18.1-70.6 ng/l
25-Hydroxycholecalciferol	42 ng/ml	not determined	30-70 ng/ml
Urinary oxalate	36.8 mg/24h	34.1 mg/24h	<44 mg/24h
Urinary citrate	275 mg/24h	218 mg/24h	241-531 mg/24h
Urinary calcium	3.5 mmol/24h	2.6 mmol/24h	2.5-7.5mmol/24h
Urinary phosphate	0.32. g/24h	0.24g/24h*	0.6 - 1.2 g/24h

*possibly inappropriate sampling with only 900 ml of urine

Fig. 2. Imaging studies displaying the index patient's nephrocalcinosis. (A) Distinct medullary calcification in conventional abdominal x-ray. (B) Abdominal computed tomography (CT) without contrast medium showing the calcified medullary pyramids.

laboratory parameters see Table 3). He negated the intake of vitamin D and / or calcium containing dietary supplements.

Abdominal x-ray and CT displayed symmetric calcification of the medullary pyramids of both kidneys, typical for Grade III nephrocalcinosis (Figure 2). No solitary kidney stones were found.

Under treatment with intravenous Pamidronate and loop diuretics with fluid load the patient's creatinine slightly improved and the serum calcium moderately decreased. Thus, the patient was dismissed with a serum calcium of 2.5 mmol/l, continuing the next few months with Pamidronate and recommendations for dietary restriction of calcium and vitamin D. Two years after initial presentation abacterial leucocyturia and light proteinuria were persistent and serum creatinine increased to 1.7 mg/dl, which would be classified as chronic kidney disease (CKD) stage 3 (estimated GFR 49 ml/min). Serum calcium was mildly elevated at 2.7 mmol/l.

Family studies and genetic analysis

The consanguineous family history was suspicious for an autosomal recessive disease. The clinical picture with NC, hypercalcemia and suppressed PTH primarily led us to analyze for IHH. Direct Sanger sequencing of the *CYP24A1* gene revealed the previously described [4] c.428_430delAAG (p. E143del) mutation in homozygous state in the index patient and his clinically asymptomatic older brother who demonstrated milder medullary NC (Grade I) and persistently low PTH levels in the absence of hypercalcemia and hypercalciuria (Table 3, Figure 1).

As expected both parents are heterozygous carriers of p.E143del, while the third brother being completely unremarkable with regard to clinical, biochemical and ultrasound findings is homozygous for the wild type allele (Figure 1).

Interestingly, all three brothers are reported to have received prophylactic oral and daily Vitamin D supplementation by the pediatrician during the first year of life.

Discussion

As described above, a reasonably large spectrum of diseases should be considered when encountering patients with hypercalcemia and nephrocalcinosis (NC). Important clues leading to the diagnosis in this case were consanguinity of the parents and consistent suppression of PTH in affected siblings. However, this interesting family shows clearly that the phenotype can be highly variable and thus the diagnosis missed when genetic testing is not performed. First, neither hypercalciuria, nor elevation of 1,25-dihydroxy vitamin D3 were seen in any sibling homozygous for the *CYP24A1* mutation, where the former has also been reported in single cases by others ([4, 5]– see Table 2). Second, hypercalcemia is not a consistent finding, but may be influenced by alimentary factors and other stressors, such as infections and / or acute renal failure. Third, the two affected brothers show greatly differing clinical courses, where the older brother may not have been diagnosed during life-time, if family testing had not been performed. At least in this family, PTH appeared to be the most sensitive laboratory parameter and imaging with ultrasound and / or CT appeared helpful.

In view of the greatly variable phenotype of the disease, it is noteworthy that many patients may experience first clinical complications and diagnosis only in adulthood and not in infancy, as has been initially reported. About 30% of the cases published so far (Table 2) manifest or are diagnosed in adolescence or adulthood. Whether or not any given individual with biallelic inactivation of *CYP24A1* encounters clinical consequences, may depend largely on dietary influences such as calcium and vitamin D intake, but also on other genetic variables, such as calcium receptor sensitivity. However, the type of mutation may also contribute to the variability of disease, in the sense of genotype-phenotype association. The mutation found in the current family p. E143del in exon 2 of the *CYP24A1* gene affects the CYP450 domain and seems to alter the secondary structure of the substrate binding cavity by preventing a structural bond with the amino acid K283. The lost bondage destabilizes the B'/C loop and leads to a reorientation of the B'-helix (see supplemental figure of [3]) and a complete loss-of-function of the enzyme. A number of other mutations have been identified in other families, where most but not all are missense mutations (see Table 2). To what extent these mutations result in variation of enzyme activity and thereby contribute to disease severity is not known to date.

Interestingly, homozygous *Cyp24a1* knockout mice that show elevated $1\alpha,25(\text{OH})_2\text{D}_3$ levels die before weaning [6]. Their homozygous littermates that survive weaning show even 3-fold reduced $1\alpha,25(\text{OH})_2\text{D}_3$ levels compared to wild type mice. Masuda et al. [7] suggest an unknown alternative pathway of Vitamin D metabolism or a reduced renal $1\alpha,25(\text{OH})_2\text{D}_3$ production in these mice. Despite these uncertainties, knowledge of a risk genotype may be valuable in reducing life-time risks by relatively simple dietary restrictions, avoiding high amounts of calcium and Vitamin D. Since the content of one or the other can

be surprisingly high in particular diets, we would emphasize the necessity of a professional dietary consultation.

Furthermore, in severely affected patients, treatment with the cytochrome 3A inhibitor ketoconazole may be beneficial, not only in reducing hypercalciuria and hypercalcemia, but also renal insufficiency [4, 8]. However, frequent and significant liver toxicity may hamper long term use in these patients. Other treatment options may be bisphosphonates, as given to the index patient, or high volume intake with or without the use of loop diuretics. None of these strategies have been tested in a randomized fashion, but this may be worthwhile in the future.

Conclusion

Importantly, the disease termed *idiopathic infantile hypercalcemia* (IHH) may be much more frequent than thought or implicated by its name. Since a significant number of patients manifest late or are diagnosed in adulthood with CYP24A1 associated disease the term IHH should be seen as a misnomer. The allele frequency of inactivating CYP24A1 variants may be unexpectedly high [9]. Thus, this disease should also be suspected in respective non-consanguine families or sporadic cases, since compound heterozygous cases are described [10].

Furthermore, some heterozygous mutations may also result in a similar disease, following an autosomal dominant trait, however this mode of transmission has been an isolated finding so far [8]. Finally, the CYP24A1-associated disease may also result in kidney stones, which is considerably more frequent than IHH. For all these reasons it is pertinent to implement this disease in routine genetic consultations. Besides giving the patient a distinct diagnosis, this approach would improve our knowledge of the disease, the epidemiology and may help us ameliorate the clinical course of this potentially serious condition.

Disclosure Statement

The authors declare no conflict of interest.

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