Review

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***CYP24A1* and *SLC34A1* genetic defects associated with idiopathic infantile hypercalcemia: from genotype to phenotype**

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Abstract: Loss of function mutations in the *CYP24A1* gene, involved in vitamin D catabolism and in calcium homeostasis, are known to be the genetic drivers of both idiopathic infantile hypercalcemia (IIH) and adult renal stone disease. Recently, also defects in the *SLC34A1* gene, encoding for the renal sodium-phosphate transporter NaPi-IIa, were associated with the disease. IIH typically affects infants and pediatric patients with a syndrome characterized by severe hypercalcemia, hypercalciuria, suppressed parathyroid hormone level and nephrolithiasis. In *SLC34A1* mutated carriers, hypophosphatemia is also a typical biochemical tract. IIH may also persist undiagnosed into adulthood, causing an increased risk of nephrocalcinosis and renal complication. To note, a clinical heterogeneity characterizes IIH manifestation, principally due to the controversial gene-dose effect and, to the strong influence of environmental factors. The present review is aimed to provide an overview of the current molecular findings on the IIH disorder, giving a comprehensive description of the association between genotype and biochemical and clinical phenotype of the affected patients. We also underline that patients may benefit from

genetic testing into a targeted diagnostic and therapeutic workflow.

Keywords: calcium; *CYP24A1*; idiopathic infantile hypercalcemia; phosphate; *SLC34A1*; vitamin D.

Introduction

The idiopathic infantile hypercalcemia (IIH) is a rare genetic cause of nephrolithiasis (NL) typically occurring in pediatric subjects with an estimated incidence of 1:33,000 live birth [1]. The etiology has remained undefined for a long time, until the inactivation of cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*) was identified as the molecular basis [2]. Recently, also the loss of function genetic variants in the solute carrier family 34 member 1 (*SLC34A1*) gene, coding for the NaPi-IIa cotransporter, were ascertained as an additional pathogenic mechanism [3], widening the batch of the IIH genetic cause. The molecular heterogeneity allows the differentiation of two IIH forms, the IIH type 1 (OMIM 143880) caused by *CYP24A1* genetic variants and the IIH type 2 (OMIM 616963) associated with *SLC34A1* impairments. Both genetic defects lead to a shared pathological phenotype characterized by inappropriate increment of vitamin D active metabolites with persistent hypercalcemia, hypercalciuria and suppressed parathyroid hormone (PTH) [4]. These typical biochemical alterations lead to abnormal renal calcium (Ca) depositions with the consequent higher risk of NL and nephrocalcinosis (NC). *SLC34A1* genetic variants carriers also manifest hypophosphatemia and renal phosphate (Pi) wasting. Despite the term “infantile”, the disease is not only confined to infancy as it may also affect subjects in later childhood and adulthood.

The challenge related to the correct IIH diagnosis lies in several pitfalls. Particularly, the peculiar

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genotype-phenotype relationship along with the strong influence of microenvironment triggering factors lead to a pattern of different clinical manifestations. Moreover, *SLC34A1* pathogenic variants are associated with overlapping conditions in addition to IIH, including hypophosphatemic nephrolithiasis with osteoporosis, type 1 (NPHLOP1, OMIM ID 612286) and Fanconi renal tubular syndrome, type 2 (FRTS2, OMIM ID 613388).

Although IIH is a rare condition, the associated health effects can become severe if not promptly identified. Understanding of the IIH physiology is mandatory for the correct diagnosis and treatment. In this regard, the molecular evaluation has relevant and practical consequences in patients' care, giving the opportunity of targeted personalized therapies.

In the last decade, IIH assumed an emerging role among the genetic causes of pediatric-onset NL/NC, with an increment of case reports in the literature. The present paper provides an overview of the biological and pathological relevance of *CYP24A1* and *SLC34A1* defects in IIH. We focus on the genetic aspects of the disease, giving a comprehensive update on the mutations regarding both

genes, with reference to computational models and *in vitro* mutagenesis studies supporting their functional characterization.

Biological relevance of *CYP24A1* and *SLC34A1*

CYP24A1 role in vitamin D catabolism

CYP24A1 is a mitochondrial inner membrane P450 enzyme involved in the catabolism of vitamin D. It is expressed in many tissues including kidney, bone, skin and in the intestine [5, 6]. In the vitamin D metabolic pathway (Figure 1), the *CYP24A1* enzyme catalyzes the conversion of the vitamin D biological active form $1,25(\text{OH})_2\text{D}_3$ into metabolites targeted for excretion. Particularly, the $1,25(\text{OH})_2\text{D}_3$ is inactivated by *CYP24A1* through multiple hydroxylation steps at C-23 or C-24, resulting in the $1,25(\text{OH})_2\text{D}_3$ -26,23 lactone and in the biliary catabolite

Figure 1: Overview of vitamin D₃ pathway and physiological role of *CYP24A1*.

Diagram shows the main steps of vitamin D₃ metabolism, the enzymes involved in the metabolic pathway and the regulatory mechanisms. PTH, parathyroid hormone; FGF23, fibroblast growth factor-23.

calcitroic acid, respectively. Also, the $25(\text{OH})\text{D}_3$ metabolite is transformed by *CYP24A1* into $24,25(\text{OH})\text{D}_3$, which will be further converted to the water-soluble calcitroic acid [7, 8]. The enzymatic activity of *CYP24A1* is strongly regulated by several biochemical and genetic factors [9]. Firstly, the hypocalcemic condition results in the inhibition of *Cyp24A1* expression through the destabilization and suppression of its mRNA turnover [10]. Several papers demonstrated how PTH downregulates *CYP24A1* expression and enzymatic activity [11–13]. This regulatory effect results from both transcription and post-transcription modulation. Although PTH modestly increases *CYP24A1* promoter activity [14], it also showed an important post-transcriptional effect on *CYP24A1* mRNA stability, promoting its degradation [15–17]. To date, the specific PTH response elements in the *CYP24A1* gene are unknown and many aspects of the regulatory mechanism remain to be elucidated [17]. Notably, the PTH inhibitory effect was not observed in extra-renal sites such as the intestine and epidermis [12, 18]. The opposite effect is mediated by the $1,25(\text{OH})_2\text{D}_3$ that regulates its own biosynthesis through a positive feedback on *Cyp24a1*, stimulating its gene expression [19]. In addition, the fibroblast growth factor 23 (FGF23), together with its co-receptor α -Klotho, positively modulates *CYP24A1* activity in response to hypercalcemia and hyperphosphatemia [10, 20]. The crucial role of *CYP24A1* in the modulation of the $1,25(\text{OH})_2\text{D}_3$ intracellular concentration and half-life in the bloodstream, was widely investigated. A higher degree of impairment of $1,25(\text{OH})_2\text{D}_3$ and $25(\text{OH})\text{D}_3$ pharmacokinetics was observed in *Cyp24a1*-null mice compared to heterozygotes *Cyp24a1*^{+/-} [5]. Particularly, loss of function mutations in the *CYP24A1* gene result in the increase of both $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ levels which enhance intestinal Ca absorption and bone reabsorption [21]. The consequent hypercalcemic phenotype is the major cause of IIH manifestations.

***SLC34A1* (NaPi-IIa) role in renal phosphate handling**

Body Pi levels are under a strong physiological control mediated by the intestinal uptake, the renal reabsorption/excretion and the exchange between extracellular sites and bones [22]. In the human kidney, free-Pi is filtered in the glomerulus and subsequently reabsorbed in the range of 65%–70% along the nephron [23]. A group of transmembrane Na^+ -coupled Pi cotransporters (NaPi-II), belonging to the solute carrier family 34 (*SLC34*), plays a central role in Pi handling [24]. Particularly, the isoform

NaPi-IIa (*SLC34A1*), expressed in the apical brush-border membrane (BBM) of the proximal tube [25], plays the most important role in the conservation of filtered Pi from primary urine, being responsible for 60%–70% of this in rats and mice [23, 26]. The renal Pi handling via NaPi-IIa is influenced by several regulatory mechanisms that adjust BBM cotransports expression to the physiological need [22, 27, 28]. Particularly, the NaPi-IIa is regulated by vitamin D and PTH, together with other non-classical mineral metabolism components, such as the phosphatonins FGF23 and α -klotho [29]. $1,25(\text{OH})_2\text{D}_3$ modulates both intestinal and renal Pi handling upregulating the NaPi-II cotransporters expression [23, 30, 31]. However, conflicting findings surrounding the direct effects of $1,25(\text{OH})_2\text{D}_3$ on Pi renal reabsorption have been reported [30, 32]. In vitamin D-deficient rats, the expression of NaPi-IIa cotransporter in juxtamedullary kidney cortex is significantly reduced and the administration of $1,25(\text{OH})_2\text{D}_3$ leads to normalization of NaPi-IIa expression and Pi uptake [30]. Also, in vitamin D receptor (Vdr)-null growing mice, Kaneko et al. observed a reduced renal Pi reabsorption with hypophosphatemia [33]. However, in this mouse model, a rescue diet completely normalized the expression of the renal *Slc34a1* cotransporter together with the renal Pi reabsorption. This observation suggested that the alteration in Pi homeostasis observed in Vdr-null mice could not be directly related to the loss of vitamin D signaling. On the contrary, it could be the result of high PTH levels [33]. PTH promotes Pi renal excretion stimulating NaPi-IIa endocytosis from the apical membrane of the proximal tubule cells [28, 34]. Like PTH, a high Pi diet also induces down-regulation of NaPi-IIa probably through a similar mechanism, even if this process was not deeply studied [35]. FGF23 is an additional regulatory element, which is produced within the bone in response to high vitamin D levels. In the kidney, FGF23 acts as phosphaturic factor inducing the down-regulation of NaPi-IIa expression, in cooperation with the cofactor α -klotho [22, 34]. Taking into account the complex cross-talk between the regulatory mechanisms of both Ca and Pi metabolism pathways, it is not surprising as the impairment of NaPi-IIa activity also leads to relevant consequences in Ca mineral balance. IIH infants carrying *SLC34A1* inactivating mutations show a persistent condition of hypophosphatemia. Experimental data from animal studies demonstrated that FGF23 serum concentration directly varies with Pi serum concentration [36]. This correlation was also observed in human studies, although with some controversial results [27]. The hypophosphatemia itself, together with the suppressed concentration of FGF23, leads to the stimulation

of *CYP27B1* and simultaneous inhibition of *CYP24A1* activity [36, 37]. The resulting increment of $1,25(\text{OH})_2\text{D}_3$ levels leads to the hypercalcemic phenotype [3].

Pathological relevance of *CYP24A1* and *SLC34A1* defects

The first clinical reports of IIH were described in the 1950s in the UK, when an epidemic peak of IIH was observed after the introduction of an increased prophylactic dose of vitamin D in infant formula and milk products [38]. After halving of the vitamin D dose, a concomitant decrease of the incidence was observed. In the 1970s, a similar phenomenon was reported in Poland [39] and in Eastern Germany [40]. In the same years, a genetic basis related to defective mechanisms of vitamin D inactivation was proposed as one of the possible cause of the disease [41–43]. Sixty years later, inactivating mutations accounted in homozygosis or in compound heterozygosis within the *CYP24A1* gene were identified as the specific IIH genetic cause [2]. Moreover, in 2016 Schlingmann and coworkers described a subgroup of IIH patients without *CYP24A1* mutations showing idiopathic renal Pi wasting together with the other IIH typical biochemical traits. In these patients, the disturbances observed in Ca homeostasis resulted as the consequence of the primary defect in Pi metabolism caused by *SLC34A1* genetic variants. In this cohort, as opposite to *CYP24A1*-mutated subjects, the clinical and laboratory findings also persisted after the discontinuation of vitamin D prophylaxis, while a rapid response to Pi supplementation was registered [3]. These findings supporting the role of *SLC34A1* as alternative genetic driver of IIH, emphasize the molecular heterogeneity of the disease and open an additional way to increase the number of patients in which a definitive molecular diagnosis could be reached.

IIH is a rare condition mainly characterized by severe hypercalcemia with hypercalciuria, suppressed PTH and deposits of Ca in the kidney (NL/NC), with a predisposition to renal complications. Typical manifestations of IIH include also polyuria with dehydration, vomiting and constipation [2]. Anorexia and weight loss are reported, together with the difficulty in healthy eating acquisition [44]. Affected babies could also exhibit muscular hypotonia and lethargy, with an inability to grow [45]. Delay in the development of mental and movement abilities were also reported [46]. IIH is not restricted to infancy and its clinical manifestations may appear later into adult life. In this case, the sub-clinical long-term hypercalcemia/

hypercalciuria causes kidney stones disease in adulthood with a more severe damage of kidney function [47–49]. Affected adults may also develop extra-renal manifestations, including Ca deposits in the joints [50] and in the cornea, a low bone mineral density and osteoporosis, principally related to the increase of the osteoclast activity [51–53].

CYP24A1 pathological role: the peculiar genotype-phenotype correlation

The association between *CYP24A1* defects and Ca homeostasis impairment with hypercalcemia and kidney stones is widely supported by experimental studies. The pathological phenotype observed in mouse model with homozygotes loss of function mutations in *Cyp24a1* includes hypercalciuria, abnormal kidney morphology and dilated renal tubules, together with bone mineralization defects [19]. In a recent paper, Jing et al. defined *CYP24A1* as a novel candidate gene for kidney disease pathogenesis [54]. Moreover, this enzyme deficiency is estimated to characterize 4%–20% of all Ca kidney stone forming patients [53]. IIH caused by homozygous or compound heterozygous variants in the *CYP24A1* gene shows an autosomal recessive inheritance. However, high variability in the disease expression emerges as a result of genetic and environmental cross-talk. Clinical and biochemical phenotypes are featured by a gene-dose effect: subjects with bi-allelic genetic variants show a more severe phenotype compared to mono-allelic carriers, with the latter being asymptomatic or borderline [55, 56]. Moreover, in the heterozygote individuals, the chronic and latent abnormalities of vitamin D metabolism seems more likely to predispose to long-term clinical events such as renal stones [57]. In some cases, this clinical outcome variability was suggestive of a dominant inheritance with reduced penetrance [58]. In addition, differences in the effects of mono-allelic haplo-insufficiency were also observed with respect to the age of onset: heterozygote infants typically show higher $1,25(\text{OH})_2\text{D}_3$ and lower PTH levels than adults, suggesting that the prematurity in the renal function could influence the tolerance to vitamin D [59]. In addition to the genetic background, the IIH manifestations and the phenotypic differences between infants and adult carriers rely on many environmental factors like diet, lifestyle, vitamin D intake and activity of the other vitamin D metabolism enzymes [60]. Sunlight exposure and vitamin D supplementation therapy were identified as the two main environmental triggering factors [61, 62]. In particular, Schlingmann et al. described *CYP24A1*-mutated asymptomatic children

who rapidly manifested the IIH phenotype only after the administration of a high dose of prophylactic vitamin D [2]. The higher intake of Ca and vitamin D is a common practice not only in infant diets but also during pregnancy and lactation, in order to preserve maternal, fetal and child health. During the gestational period, adaptive mechanisms naturally occur in order to meet fetal needs [63, 64]. In women carriers of *CYP24A1* defects, these physiological changes, coupled with the pharmacological treatment, could unmask an underlying IIH phenotype and lead to gestational maternal hypercalcemia, with possible hypertension and fetal demise [62, 65]. With regard to the exposure to sunlight, a significant seasonal variation was observed in IIH patients with a trend to higher calcemia and calciuria, as well as lower PTH levels, from April to September (at greater exposure) as compared to the winter months [52]. Taken together, these findings support the conclusion that the dose and as well as the type of vitamin D administration may drastically modulate adverse effects. Thus, *CYP24A1*-deficient subjects are recommended to carefully observe their vitamin D intake or to completely restrict its intake. Consequently, the IIH diagnosis via the identification of *CYP24A1* mutations carriers is also useful for the appropriate vitamin D supplementation scheme. Both protection and/or mild exposure to the sun are also suggested to prevent the worsening of clinical and biochemical manifestations [52].

***SLC34A1* pathological role: heterogeneity of clinical phenotype**

The pathophysiology underlying NC/NL in carriers of *SLC34A1* genetic variants is based on the primary loss of Pi in the urine. The Pi wasting and the suppression of FGF23 lead to an inappropriate $1,25(\text{OH})_2\text{D}_3$ synthesis via the inhibition of *CYP24A1* catabolic activity and, on the other hand, via the stimulation of *CYP27B1*. The consequent status of hypercalcemia, hypercalciuria and hyperphosphaturia promotes the formation of Ca-Pi crystals and renal calcifications [66]. Mutations in the *SLC34A1* gene were widely associated with kidney stones phenotypes. In particular, *SLC34A1* was annotated among the relevant genes for idiopathic Ca stones in several human GWASs and replicated candidate-gene studies [67, 68]. Extensive studies proved the pathological role of both complete deletion and loss of function genetic variants in the *SLC34A1* gene [69, 70]. Homozygous knockout (KO) mice exhibit hypophosphatemia and renal Pi wasting with an adaptive increase of the serum $1,25(\text{OH})_2\text{D}_3$ and associated hypercalcemia and hypercalciuria, together with renal Ca/Pi

depositions with an apatitic mineral phase [71, 72]. Additionally, a phenotype with hypophosphatemia, hypercalcemia and NL was observed in mice carriers of point mutations inactivating the *Slc34a1* gene [73]. As described for *CYP24A1*, autosomal recessive inheritance is associated with *SLC34A1* genetic variants, with a phenotype variability related to mono- and bi-allelic defects. Heterozygotes *Slc34a1*^{+/-} mice are partially able to maintain normal serum Pi levels with a modest increase in urinary Pi excretion. These findings suggest that kidneys of *Slc34a1*^{+/-} mice are able to react properly to the increase of renal Pi loss and, to the resulting modification of Pi homeostasis [69]. Schlingmann et al. evaluated *Slc34a1* KO mice fed with diets characterized by variable content of vitamin D and Pi. A low Pi diet led to severe hypophosphatemia only in the KO mice whereas wild type (WT) animals remained normophosphatemic. Furthermore, the high vitamin D diet resulted in its overload that is not compensated in the KO animals. In a lowPi/highD diet, representative of infants with breast milk alimentation and vitamin D fortification, only the KO mice showed the IIH manifestations due to their lack of FGF23 regulatory activity. Furthermore, avoiding the vitamin D intake does not lead to the normalization of laboratory findings as it was obtained only after the Pi load [3, 70]. As discussed before, the association of IIH with *SLC34A1* defects was discovered recently [3]. Until then, genetic variants in this gene were known to give several clinical phenotypes that overlap with IIH symptoms mainly related to hypophosphatemia [74]. The first condition associated with heterozygote genetic variants in the *SLC34A1* gene is NPHLOP1. Common manifestations are persistent idiopathic hypophosphatemia with Pi wasting, hypercalciuria, bone demineralization and kidney stones [75]. However, this autosomal dominant form was not confirmed in subsequent studies [76–78]. An autosomal recessive form related to genetic variants in the *SLC34A1* gene is FRTS2, described for the first time in Israeli-Arab patients [79]. Particularly, Tieder et al. described two adolescent siblings with severe hypophosphatemic rickets and osteopenia, severe hypercalciuria without renal tubular acidosis and significantly elevated serum levels of $1,25(\text{OH})_2\text{D}_3$. Clinical reassessment of these patients after two decades also revealed proximal tubulopathy, normocalcemia, normocalciuria, renal Pi wasting with hypophosphatemic bone disease and stage II–III chronic kidney disease (CKD) [78]. However, the occurrence of FRTS2 in the molecular context of *SLC34A1* genetic variants is unclear [22]. Despite that there is phenotypic similarity between humans FRTS2 and mice models with NaPi-IIa disruption, there are several unexplained differences between the two species, including

the absence of: (a) clear FRTS2 phenotype with classic rickets in the affected mice and (b) renal calcification in the affected humans [78]. To date, no additional patients with NaPi-IIa disruption and FRTS2 have been reported. Additionally, several case reports described IIH with NC in patients affected by Sotos syndrome. This condition is caused by a heterozygous microdeletion on chromosome 5q35 removing both the nuclear receptor binding SET domain protein 1 (*NSD1*) and the *SLC34A1* genes. Together with the typical manifestations of Sotos syndrome related to the *NSD1* gene deletion, the mineral disorders observed were linked to NaPi-IIa haploinsufficiency [80].

Due to the phenotype variability in homozygotes carriers and to the debate regarding the impact of heterozygotes genetic variants, the clinical effects of NaPi-IIa impairments in humans show some controversial aspects. To date, pathogenic alleles of *SLC34A1* seem to contribute to both autosomal dominant and autosomal recessive renal stone disease, suggesting the importance of other factors that can influence the clinical features of the disease [74].

Genetic variant spectrum of *CYP24A1* and *SLC34A1*

All the genetic variants accounted in *CYP24A1* and *SLC34A1* genes and associated with IIH are shown in Tables 1 and 2, respectively. The present variants are reported and described in the following databases: (1) Leiden Open Variant (<http://www.lovd.nl/3.0/home>) [95]; (2) Human Gene Mutation (<http://www.hgmd.cf.ac.uk/ac/index.php>) [96] and (3) ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) [97]. Through the MEDLINE consultation, all potentially relevant primary papers (last released Dec 2018) were examined. Furthermore, the reference list was double checked for additional publications. Recommendations of the Human Genome Variation Society [98] were followed for the description of variants and a checking of the correct nomenclature was performed using Mutalyzer 2.0.26 tool (<http://lovd.nl/mutalyzer/>) [99]. In particular, for each genetic variant we herein provide details including:

1. The exon/intron involved, followed by the genomic and coding position (GRCh38/hg19) based on the reference sequences NG_008334.1 and NM_000782.4 for the *CYP24A1* gene and NG_016223.1 and NM_003052.4 for the *SLC34A1* gene. Nucleotide numbering at coding sequence starts with c.1 at the “A” of the “ATG” start codon that corresponds to the genomic position

g.5399 for *CYP24A1* and g.6312 for *SLC34A1*, according to the mentioned reference sequences.

2. The type of genetic variant, reported as: missense, nonsense, frameshift, in-frame deletion/duplication, splice site mutation, large deletion.
3. The effect predicted at the protein level is reported as referred to the *CYP24A1* NP_000773.2 and *SLC34A1* NP_003043.3 accession numbers, with the indication of the predicted protein domain involved, according to the published structural information [100, 101].
4. Annotations details for the Reference SNP (rs) ID number obtained from Database of Single Nucleotide Polymorphism (dbSNP) (<https://www.ncbi.nlm.nih.gov/projects/SNP/>) [102].
5. Data supporting the pathological significance of the mutations obtained from the published functional studies based on *in silico* prediction algorithms and/or *in vitro* testing. For those variants with unknown functional impact, we performed a prediction of the pathogenicity using the following *in silico* tools: PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) [103], MutationTaster (<http://www.mutationtaster.org/>) [104] and SIFT algorithm (<http://sift.jcvi.org/>) [105]. The 3' and 5' splice sites analysis was achieved using Human Splicing Finder v3.1 tool (<http://www.umd.be/HSF3/>) [106].

A comprehensive description of each variant encompassing all molecular, biochemical and clinical details is available for both *CYP24A1* and *SLC34A1* genes in Supplementary Tables 1 and 2, respectively. Additionally, graphical linear maps of the pathogenic variants spanning the genes are depicted in Figure 2. Notably, the IIH case reports with definitive genetic diagnosis were more frequently associated with *CYP24A1* defects compared to *SLC34A1*. The limited number of pathogenic variants reported is mainly due to the very recent discovery of the gene-disease association, since the first evidence was published in 2011 for *CYP24A1* and 2016 for *SLC34A1* [2, 3]. Additionally, the IIH can be considered an under-diagnosed condition due to the high variability of its clinical manifestations together with the lack of genetic analysis routinely performed.

CYP24A1 pathogenic variants in IIH

A total of 41 pathogenic variants were identified for the *CYP24A1* gene. The majority of the protein domains results as affected by one or more deleterious amino acid changes (Figure 3). Overall, the most common types of genetic variants are missense (23/41), followed by

Table 1: Loss-of-function genetic variants of CYP24A1.

Exon/ intron	Genomic position (NG_008334.1)	Transcript position (NM_000782.4)	Type of mutation	Protein change (NP_000773.2)	CYP24A1 domain	rs ID number	In vitro testing	In silico analysis	Ref
1	g.5460delC	c.62delC	Frameshift	p.(Pro21ArgfsTer8)	MTS	rs774432244	N/A	Deleterius [52]	[52]
1	g.5505delC	c.107delC	Frameshift	p.(Pro36LeufsTer11)	MTS – α A region	N/A	N/A	Deleterius (#) [MT]	[81]
1	g.5645C>G	c.247C>G	Missense	p.(His83Asp)	α A	N/A	N/A	Deleterius [44]	[44]
2	g.6020T>G	c.400T>G	Missense	p.(Trp134Gly)	α B'	N/A	Reduced activity [SDM] [65]	–	[65]
2	g.6048_6050delAAG	c.428_430delAAG	In-frame deletion	p.(Glu143del)	α B' – α C region	rs777676129	LoF [SDM] [2]	–	[2]
2	g.6063T>C	c.443T>C	Missense	p.(Leu148Pro)	α B' – α C region	rs139763321	LoF [FIB] [53]	Deleterius [53, 82]	[53]
2	g.6065_6070delATCCTG	c.445_449+1delATCCTG	Splice site	p.(Ile149GlyfsTer31)	α B' – α C region	N/A	N/A	Deleterius (#) [MT]	[2]
3	g.7309G>T	c.451G>T	Nonsense	p.(Glu151Ter)	α B' – α C region	rs387907323	N/A	Deleterius (#) [MT]	[2]
3	g.7322G>A	c.464G>A	Nonsense	p.(Trp155Ter)	α C	N/A	N/A	Deleterius (#) [MT]	[59]
3	g.7327C>T	c.469C>T	Missense	p.(Arg157Trp)	α C	rs35873579	N/A	Deleterius [53, 59]	[52]
3	g.7334G>A	c.476G>A	Missense	p.(Arg159Gln)	α C	rs387907322	LoF [SDM] [2]	–	[2]
4	g.9374T>C	c.628T>C	Missense	p.(Trp210Arg)	α E	N/A	N/A	Deleterius [83]	[83]
5	g.13164T>A	c.660T>A	Nonsense	p.(Tyr220Ter)	α E	N/A	N/A	Deleterius (#) [MT]	[82]
5	g.13171A>T	c.667A>T	Nonsense	p.(Arg223Ter)	α E – α F region	rs367995295	N/A	Deleterius (#) [MT]	[51]
IVS5	g.13237G>A	c.732+1G>A	Splice site	–	–	rs374088730	N/A	Deleterius [53, 58]	[58]
6	g.14440T>A	c.758T>A	Missense	p.(Met253Lys)	α F – α G region	N/A	N/A	Deleterius [59]	[59]
6	g.14486G>A	c.804G>A	Nonsense	p.(Trp268Ter)	α G	rs778693884	N/A	Deleterius [60]	[60]
6	g.14505T>C	c.823T>C	Missense	p.(Trp275Arg)	α G	N/A	N/A	Deleterius (#) [PP-2; SIFT; MT]	[84]
IVS6	g.16114A>G	c.845-2A>G	Splice site	–	–	rs778754341	N/A	Deleterius [53, 58]	[58]
7	g.16214G>T	c.943G>T	Nonsense	p.(Glu315Ter)	α I	N/A	N/A	Deleterius (#) [MT]	[65]
7	g.16235G>A	c.964G>A	Missense	p.(Glu322Lys)	α I	rs387907324	LoF [SDM] [2]	–	[2]
7	g.16236G>A	c.965A>C	Missense	p.(Glu322Ala)	α I	N/A	LoF [SDM] [59]	Deleterius [59]	[59]
7	g.16251C>T	c.980C>T	Missense	p.(Ala327Val)	α I	N/A	N/A	Deleterius (#) [PP-2; SIFT; MT]	[59]
7	g.16260C>T	c.989C>T	Missense	p.(Thr330Met)	α I	rs552310427	LoF [SDM] [59]	Deleterius [59]	[59]
8	g.19863_19870delCAGTCTAA	c.999_1006delCAGTCTAA	Frameshift	p.(Ser334ValfsTer9)	α I	rs770035617	N/A	Deleterius [62]	[62]
8	g.19867dupC	c.1003dupC	Frameshift	p.(Leu335ProfsTer11)	α I	N/A	N/A	Deleterius (#) [MT]	[59]
8	g.19985T>C	c.1121T>C	Missense	p.(Met374Thr)	α J – α K region	rs6022990	N/A	Deleterius [53] ^a	[52]
8	g.20002T>C	c.1138T>C	Missense	p.(Cys380Arg)	α K	N/A	LoF [SDM] [59]	Deleterius [59]	[59]
8	g.20011G>C	c.1147G>C	Missense	p.(Glu383Gln)	α K	rs777011420	N/A	Deleterius [82, 85]	[85]
IVS8	g.20022G>A	c.1157+1G>A	Splice site	–	–	N/A	N/A	Deleterius (#) [HSF; MT]	[81]
9	g.20822C>T	c.1166C>T	Missense	p.(Pro389Leu)	α K – β 1-4 loop	rs778432343	N/A	Deleterius [59]	[59]
9	g.20842C>T	c.1186C>T	Missense	p.(Arg396Trp)	β 1-4	rs114368325	LoF [SDM] [2]	Deleterius [82]	[2]

Table 1 (continued)

Exon/ intron	Genomic position (NG_008334.1)	Transcript position (NM_000782.4)	Type of mutation	Protein change (NP_000773.2)	<i>CYP24A1</i> domain	rs ID number	<i>In vitro</i> testing	<i>In silico</i> analysis	Ref
9	g.20843G>A	c.1187G>A	Missense	p.(Arg396Gln)	β1-4	rs143934667	LoF [SDM] [53]	Deleterius [53, 59]	[52]
9	g.20862delA	c.1206delA	Frameshift	p.(Val403PhefsTer15)	β2-1	N/A	N/A	Deleterius (#) [MT]	[59]
9	g.20882T>C	c.1226T>C	Missense	p.(Leu409Ser)	β2-2	rs6068812	Reduced activity [SDM] [2]	–	[2]
9–12	–	–	Large deletion	–	–	N/A	N/A	–	[86] ^b
10	g.21471C>T	c.1315C>T	Missense	p.(Arg439Cys)	αK''	rs374292194	LoF [SDM] [59]	Deleterius [59]	[59]
10	g.21522G>C	c.1366G>C	Missense	p.(Gly456Arg)	CYS loop	rs748429181	N/A	Deleterius [59]	[59]
10	g.21562_21563delAG	c.1406_1407delAG	Frameshift	p.(Glu469AlafsTer22)	αL	N/A	N/A	Deleterius [52]	[52]
10	g.21582_21583delCT	c.1426_1427delCT	Frameshift	p.(Cys477LeufsTer14)	αL	rs876657376	LoF [SDM] [2]	–	[2]
11	g.21762C>T	c.1508C>T	Missense	p.(Pro503Leu)	β4-2	rs766440228	N/A	Deleterius [82]	[82]

All the mutations in *CYP24A1* associated with IIH phenotype are reported. In the functional description, a “#” symbol means that the analysis was performed in this study using *in silico* predictive tools. In the remaining cases, the specific reference studies are indicated. ^aConflicting interpretation of pathogenicity, see text. ^bBreakpoint characterization: chr20:52763705_52774628 [87]. MTS, mitochondrial targeting sequence; LoF, loss of function; SDM, site-directed mutagenesis; FIB, activity measured in fibroblast; SIFT, Sorts Intolerant From Tolerant tool; PP-2, Polymorphism Phenotyping v2; HSF, Human Splicing Finder; MT, Mutation Taster. Applied ranking scores for each program are available in Supplementary Table 1. N/A, not available; dash (–), not needed.

the frameshift and in-frame deletions (7/41), nonsense (5/41), splice site changes (4/41), frameshift duplications (1/41) and large deletions (1/41). Relevant details about the effect of the genetic variations on the enzyme activity were obtained through *in vitro* site-direct mutagenesis (SDM) analysis and computational model via web-based tools [109]. Genetic variants within *CYP24A1* can affect the enzyme structure and catalytic activity, through several mechanisms, including: (a) the alteration of both the substrate (e.g. p.Leu409Ser; p.Leu148Pro) and the heme (e.g. p.Arg159Gln; p.Arg396Trp; p.Ser334ValfsTer9) binding; (b) the substrate access (e.g. p.Glu143del) and the product egress; (c) the disruption of ferredoxin cofactor binding (e.g. p.Glu383Gln) and d) the protein folding (e.g. p.Glu322Lys) [2, 53, 82, 110]. In particular, the c.476G>A (p.Arg159Gln) and the c.1186C>T (p.Arg396Trp) variants influence the heme binding site due to the destruction of the hydrogen bond between the heme propionate group and the arginine residues [110]. Consequently, both the protein structure stability and the catalytic proprieties are drastically impaired [109]. Instead, the binding of the 1,25(OH)₂D₃ substrate is influenced by the c.1226T>C (p.Leu409Ser) mutation, causing the decrease of enzymatic activity [53]. Notably, the leucine 409 does not directly contribute to the substrate-binding pocket or to the substrate access channel and both the WT Leu and the alternative serine residues have similar volumes [111]. The functional effect of this amino acid substitution is probably related to the conformational change of an α-helix into a β-turn [109]. The amino acid residue Leu148 directly interacts with the biochemical substrate where the substitution c.443T>C (p.Leu148Pro) leads to a decrease of the enzyme activity of about 25%–50% [53]. Computational analysis demonstrated that this genetic variant determines the change of a small coil with an impaired protein stability [109]. The c.964G>A (p.Glu322Lys) substitution abolishes an important hydrogen bond between the I-helix and the B'/C loop backbone, altering their orientation and affecting the correct protein folding and stability [112]. Moreover, Masuda et al. demonstrated that the substitutions of the residues Trp134 and Leu148, together with Met246 and Ile131, affect the substrate orientation into the enzyme channel and lead to the decrease of the enzymatic activity [113]. Among the *CYP24A1* genetic variants reported in the Table 1, the small deletion c.428_430delAAG (p.Glu143del) is one of the most common in IIH patients, being that it has been described in many case reports [2, 48, 51, 53, 59, 60, 65, 82, 85, 112]. This variant represents a mutational *hot spot* of the *CYP24A1* gene, together with the c.1226T>C

Table 2: Loss-of-function genetic variants of SLC34A1.

Exon/ intron	Genomic position (NG_016223.1)	Transcript position (NM_003052.4)	Type of mutation	Protein change (NP_003043.3)	SLC34A1 domain	rs ID number	In vitro testing	In silico analysis	Ref
(2–12)	–	c.1-?_1416+?del	Large deletion	p.?	–	N/A	N/A	–	[88]
4	g.6803_6823del21	c.272_292del21	In frame deletion	p.(Val91_Ala97del)	N-terminal	rs876661296	Partial LoF [SDM] [3]	Deleterious [74]	[3] ^a
5	g.7062G>T	c.458G>T	Missense	p.(Gly153Val)	HP1	rs769409705	LoF [SDM] [3]	–	[3]
5	g.7062G>C	c.458G>C	Missense	p.(Gly153Ala)	HP1	rs769409705	LoF [SDM] [3]	–	[3]
5	g.7064_7084dup	c.460_480dup	In frame duplication	p.(Ile154_Val160dup)	HP1	N/A	LoF [SDM] [89]	–	[89] ^b
5	g.7068T>C	c.464T>C	Missense	p.(Leu155Pro)	HP1	N/A	LoF [SDM] [3]	–	[3]
6	g.8356_8357del	c.557_558del	Frameshift	p.(Pro186Hisfs*26)	TM2	N/A	N/A	Deleterious (#) [MT]	[3]
6	g.8443G>A	c.644G>A	Missense	p.(Arg215Gln)	TM2	N/A	LoF [SDM] [90]	Deleterious [90]	[3]
IVS6	g.8444G>A	c.644+1G>A	Splice site	–	–	rs201304511	N/A	Deleterious (#) [HSF; MT]	[3]
7	g.8797T>C	c.682T>C	Missense	p.(Trp228Arg)	TM3	N/A	N/A	Deleterious [91]	[91]
8	g.8920G>A	c.914G>A	Nonsense	p.(Trp305Ter)	ECL	N/A	N/A	Deleterious (#) [MT]	[3]
9	g.14333T>G	c.1006T>G	Missense	p.(Cys336Gly)	ECL	rs876661338	LoF [SDM] [3]	–	[3]
IVS9	g.14334G>A	c.1006+1G>A	Splice site	–	–	rs200095793	N/A	Deleterious (#) [HSF; MT]	[3]
IVS9	g.14336_14339del	c.1006+3_1006+6del	Splice site	–	–	N/A	N/A	Deleterious (#) [HSF; MT]	[3]
10	g.14765G>A	c.1174G>A	Missense	p.(Asp392Asn)	–	N/A	N/A	Deleterious [88]	[88]
11	g.17332G>C	c.1204G>C	Missense	p.(Gly402Arg)	–	rs3761311751	N/A	Deleterious [92]	[93] ^c
11	g.17351T>A	c.1223T>A	Missense	p.(Val408Glu)	HP2	N/A	LoF [SDM] [3]	–	[3]
IVS12	g.17649G>A	c.1416+5G>A	Splice site	–	–	N/A	N/A	Deleterious (#) [HSF; MT]	[3]
13	g.18361_18362del	c.1425_1426del	Frameshift	p.(Cys476SerfsTer128)	Loop 5–6	N/A	LoF [SDM] [3]	–	[3]
13	g.18398T>C	c.1462T>C	Missense	p.(Trp488Arg)	TM6	N/A	LoF [SDM] [3]	–	[3]
13	g.18420G>A	c.1484G>A	Missense	p.(Arg495His)	–	rs188409125	LoF [SDM] [94]	Deleterious [88]	[88] ^d
13	g.18550G>A	c.1614G>A	Nonsense	p.(Trp538Ter)	N/A	N/A	N/A	Deleterious (#) [MT]	[3]
13	g.18645C>A	c.1708C>A	Missense	p.(Pro570Thr)	–	rs144700897	N/A	Deleterious [88]	[88]

All the mutations in SLC34A1 associated with IIH phenotype are reported. In the functional description, a “#” symbol means that the analysis was performed in this study using *in silico* predictive tools. In the remaining cases, the specific reference studies are indicated. ^aThis variant was previously reported by Lapointe et al. in hypercalciuric stone-forming patients [76]. ^bThis variant was previously reported by Magen et al. in association with FRTS2 [78]. ^cThis variant was previously reported by Daga et al. [92]. ^dThis variant was previously reported by Rajagopal et al. [94]. HP, hairpin; TM, transmembrane domain; ECL, extracellular Loop; LoF, loss of function; SDM, site-directed mutagenesis; FIB, activity measured in fibroblast; SIFT, Sorts Intolerant From Tolerant tool; PP-2, Polymorphism Phenotyping v2; HSF, Human Splicing Finder; MT, Mutation Taster. Applied ranking scores for each program are available in Supplementary Table 2. N/A, not available; dash (–), not needed.

Figure 2: Distribution of IIH-causing mutations in the context of *CYP24A1* and *SLC34A1* gene structures.

For both *CYP24A1* (A) and *SLC34A1* (B), the Figure shows the linear maps of the gene and the location of the pathogenic mutations. Each rectangle represents a single exon, whereas the horizontal lines represent intronic regions. Missense (blue), non-sense (orange), frameshift (red) and in-frame indels (gray) are shown on the top, whereas splice-site mutations (purple) are shown below. The figures were obtained by custom tracks feature of the web tool ProteinPaint [107].

(p.Leu409Ser) in the French population [59] and the c.1186C>T (p.Arg396Trp) in Italians [82]. The deletion of this highly conserved amino acid has a great impact on the enzyme function, destabilizing the B'/C loop and causing a reorientation of the B'-helix. The consequent impairment of the substrate binding cavity shape causes a drastic reduction of enzyme activity *in vitro* [2]. Also *in vivo* biochemical functional data provide strong evidence about the impact of this mutation in the IIH development [114]. Interestingly, the genetic variant c.823T>C (p.Trp275Arg) was retrospectively identified in a patient presenting with hypercalcemia, high 1,25(OH)₂D level and elevated 25(OH)D/24,25(OH)₂D ratio, providing

the impairment of *CYP24A1* catabolic activity [84]. The c.1121T>C (p.Met374Thr) variant has conflicting interpretations. Molin et al. described this variant as a polymorphism rather than a mutation, considering its unusually high MAF (=0.0083) and the corresponding measurable *CYP24A1* *in vitro* activity [59]. Despite these findings, the variant is described as causative of IIH in compound heterozygosis with the c.469C>T (p.Arg157Trp) [52] and as a pathogenic by *in silico* predictive algorithms [53, 59]. Finally, we highlight as the *CYP24A1* missense variant NM_000782.4: c.735G>A (NP_000773.2: p.Met245Ile) is not reported in the Table 1 due to its lack of association with a clear IIH phenotype. This variant

Figure 3: The crystal structure of rat *CYP24A1*.

Figure shows the crystal structure of rat *CYP24A1* (PDB ID: 3K9V) [100] created with Protein Workshop 4.2.0 tool [108]. The *CYP24A1* structure are colored according to a chain color ramp from N-terminal (purple) to C-terminal (red).

within exon 6 of the *CYP24A1* gene involves the highly conserved amino acid residue occurring in the polar region of the F helix: it is annotated with a very low MAF (=0.00045). Gigante et al. detected this genetic variant in homozygote status in an asymptomatic child during genetic screening [82]. This Italian proband (14 years old) showed biochemical findings within the normal range: nevertheless, the putative role of this variant in a late onset manifestation of the disease or in a latent phenotype was hypothesized. The authors also underlined that this asymptomatic child never received vitamin D supplementation. As reported by other authors, vitamin D supplementation can unmask a latent IIH condition due to the incomplete penetrance of genetic background [2]. Functional *in silico* analysis of p.Met245Ile proved a loss of enzymatic activity [82]. Additionally, the Met245 residue plays a relevant role in the catalysis, as evidenced by rat site-directed mutants that showed changes in binding affinity for the $1,25(\text{OH})_2\text{D}_3$ substrate along with an enhancement in the $25(\text{OH})\text{D}_3$ ones [115]. However, definitive *in vitro* experiments for the specific p.Met245Ile variant are necessary to establish its biological significance.

SLC34A1 pathogenic variants in IIH

The spectrum of *SLC34A1* genetic alterations in IIH comprises truncating as well as missense and splice site variants. According to the predicted NaPi-IIa structure model [101], most of these mutations appear to be located within transmembrane helices (TMs) domains. Several genetic changes were functionally tested *in vitro*, in heterologous expression systems, especially in *Xenopus* oocytes or in opossum kidney (OK) cells. As indicated in Table 1, altogether these experiments showed a reduced function of the mutated transporters, mainly due to trafficking defects. Interestingly, the small in-frame deletion c.272_292del21 (p.Val91_Ala97del) was reported for the first time by Lapointe et al. in hypercalciuric stone-forming patients. Despite the authors defined a lack of association between NaPi-IIa alterations and renal Pi handling, we would underline as only heterozygote patients were analyzed in this study [76]. The same variant was successively detected in compound heterozygosis with the c.1534C>T (p.Arg512Cys) in an infant with kidney stones and primary hyperoxaluria [116]. Schlingmann et al. described the same variant in homozygote IIH carriers [3]. The pathological relevance of the c.272_292del21 (p.Val91_Ala97del) mutation was also supported by paper regarding an infant with Sotos syndrome and clinical IIH. This patient carried both the typical Sotos micro-deletion involving the *SLC34A1* gene on one allele and the p.Val91_Ala97del variant on the other allele [80]. Particularly, the deletion is located in the N-terminus of the NaPi-IIa protein and probably its occurrence arose from a DNA strand slippage due to the pairing between homologous sequences of 5 bp flanking the deleted region [76]. *In vitro* functional analysis of p.Val91_Ala97del mutant showed an unclear transport defect, with a Pi transport activity comparable with the WT [3] or lower than WT [76]. The transfection of EGFP-tagged into OK cells demonstrated a partial retention of the protein, with an expression in both the intracellular compartment and plasma membrane. However, all patients carrying this deletion presented with a clinical phenotype similar to those with other IIH pathogenic variants, proving its pathogenicity role in the disease occurrence. Additionally, the c.458G>T/C (p.Gly153Val/Ala), c.464T>C (p.Leu155Pro), c.1006T>G (p.Cys336Gly), c.1223T>A (p.Val408Glu), c.1425_1426del (p.Cys476Serfs*128) and c.1462T>C (p.Trp488Arg) variants were tested *in vitro* as loss of function mutations due to a disturbed trafficking and a complete intracellular retention of the NaPi-IIa [3]. The c.644G>A (p.Arg215Gln) variant is predicted to be located in the first structural repeat unit (RU) of the protein, particularly the amino acid

substitution affects a charged residue of the TM2 domain, altering the helical packing and/or the mobility of the transporter. Moreover, *in vitro* analysis confirmed a reduction of Pi intake transport of the mutated transporter and an impaired trafficking to the cellular membrane, with only a marginal effect on the transport behavior [90, 101]. Recently, in a large study analysis of target tubulopathy genes, Ashton et al. described the c.1484G>A (p.Arg495His) variant in an IIH patient [88]. This variant was previously identified in two Argentinean siblings (2 years old and 10 years old) with not persistent hypercalcemia, hypercalciuria, hypophosphatemia, low PTH level and NC [94]. This amino acid substitution resides in a domain essential for PTH-mediated endocytosis of NaPi-IIa and *in vitro* Pi uptake experiments showed a reduction in the amount of Pi transported [94]. The genetic variant c.1204G>C (p.Gly402Arg) was reported for the first time by Daga et al. in an Egyptian patient affected by nephrocalcinosis [92]. In a recent paper, the same variant was described in a case report of prenatal hyperechogenic kidneys corresponding to postnatal nephrocalcinosis [93]. The c.460_480dup (p.Ile154_Val160dup) variant was reported by Demir et al. in two infants of Israeli and Turkish descent, presenting with IIH and early-onset partial proximal tubular impairment [89]. This *SLC34A1* genetic variant was previously reported in association with FRTS2 and CKD in the same families' older members [78]. Either the intra-familial phenotypic heterogeneity or an age-dependent disease progression, observed between homozygotes adult and infant carriers, is still pending further investigations. Of interest, several *SLC34A1* mutations are annotated with a high minor allele frequency (Supplementary Table 2). It is currently unknown why *SLC34A1* mutations have

accumulated such a high frequency in the general population [117]. This finding reinforces the debate surrounding *SLC34A1* defects and suggests the strong influence of environment as well as other physiological factors in the development of a clear pathogenic phenotype.

SLC34A1 pathogenic variants in other phenotypes

Due to the mentioned controversial aspects related to the clinical heterogeneity that characterizes NaPi-IIa defects, another two different phenotypes, in addition to IIH, can be mentioned. An overview of the published pathological variants accounted in the *SLC34A1* gene associated with these additional phenotypes is reported in Table 3. In the first clinical description of the NPHLOP1 condition, Priè et al. evaluated two heterozygote children, carrying the missense variants c.142_143delGCinsTT (p.Ala48Phe) and c.439G>A (p.Val147Met). Functional analysis of these protein alterations confirmed an impaired NaPi-IIa transport activity [75]. On the other hand, the molecular finding of *SLC34A1* mutations in the clinical setting of the FRTS2 was achieved during the reassessment of two patients previously evaluated at the clinical level by Tieder et al. [79]: 12 years later, these patients were found to be homozygote carriers of the *SLC34A1* variant c.460_480dup (p.Ile154_Val160dup) [78]. Furthermore, many case reports described *SLC34A1*-mutated subjects in the clinical context of hypophosphatemia with Pi wasting and kidney stones. Compound heterozygosis in *SLC34A1* was also described in an infant with NC and primary hyperoxaluria carrying the two missense variants c.271_291del

Table 3: Loss-of-function variants of *SLC34A1* associated to non-IIH phenotype.

Exon/intron	Genomic position (NG_016223.1)	Transcript position (NM_003052.4)	Protein change (NP_003043.3)	Clinical phenotype	Ref
3	g.6589_6590delinsTT	c.142_143delinsTT	p.(Ala48Phe)	NPHLOP1	[75]
5	g.7002C>T	c.398C>T	p.(Ala133Val)	HCu, hPi, NL	[85]
5	g.7041C>T	c.437C>T	p.(Pro146Leu)	HPi, NL	[85]
5	g.7043G>A	c.439G>A	p.(Val147Met)	NPHLOP1	[75]
5	g.7064_7084dup	c.460_480dup	p.(Ile154_Val160dup)	FRTS2	[78]
6	g.8335T>C	c.536T>C	p.(Leu179Pro)	NC	[92]
12	g.17576G>A	c.1348G>A	p.(Gly450Ser)	HPi, HCu, NL	[85]
12	g.17595T>A	c.1367T>A	p.(Ile456Asn)	HCu, NL	[85]
13	g.18402A>G	c.1466A>G	p.(Tyr489Cys)	NL, low PTH, hPi	[118]
13	g.18470C>T	c.1534C>T	p.(Arg512Cys)	NC	[116]
13	g.18660C>T	c.1724C>T	p.(Thr575Ile)	NL, Hca	[92]

All the mutations in *SLC34A1* associated with other than IIH phenotype are reported. NPHLOP1, hypophosphatemic nephrolithiasis with osteoporosis, type 1; FRTS2, Fanconi renotubular syndrome, type 2; HCa, hypercalcemia; HCu, hypercalciuria; hPi, hypophosphatemia; NL, nephrolithiasis; NC, nephrocalcinosis; PTH, parathyroid hormone.

(p.Val91_Ala97del) and c.1534C>T (p.Arg512Cys) [116]. *SLC34A1* was also associated with recurrent kidney stones disease in a large Icelandic genome analysis consortium where the missense variant c.1466A>G (p.Tyr489Cys) was associated with hypophosphatemia, NL and low PTH level [118]. Successively, Braun et al. described, in a cohort of 143 pediatric individuals, several heterozygote variants in the *SLC34A1* gene associated with clinical features of NL, hypercalciuria and hypophosphatemia. Despite their apparent dominant effect, this paper did not provide any definitive explanation about the pathogenic role of each allele variant [85]. In this regard, Dinour et al. described clinical evidence of NC and kidney failure only in the homozygous carriers belonging to different members of Israeli-Arab families [90]. Moreover, Daga et al. recently evaluated a cohort of early-onset NL/NC and described both mono- and bi-allelic variants in the *SLC34A1* gene: the author reported two cases of recessive transmission associated with the compound heterozygosis of the c.644+1G>A splice site variant with the c.1204G>C (p.Gly402Arg) mutation or the homozygote c.1724C>T (p.Thr575Ile) missense change, respectively. Other two missense variants were described in heterozygosis, the c.398C>T (p.Ala133Val) and the c.536T>C (p.Leu179Pro). Notably, the individuals harboring heterozygotes variants exhibited NC, hypercalcemia, hypercalciuria and hypophosphatemia consistent with renal Pi wasting. On the other hand, affected individuals carrying the dominant mutations showed NC and hypercalciuria without hypercalcemia. The authors highlighted that a dichotomy based on the age of onset could characterize *SLC34A1* genetic variants: recessive cases are typically reported in infancy (median age: 0.25 years) whereas dominant cases tend to present later in childhood (median age: 3.5 years) [92]. Recently, Fearn et al. reported two patients with metabolic acidosis, hyperphosphaturia and renal stones: this clinical phenotype occurring in both dominant and recessive patterns. Overall, an unclear pathogenic role of mono- and bi-allelic loss of function mutations in *SLC34A1* genes has emerged [74].

Clinical and diagnostic relevance

IIH is an uncommon disorder and a high degree of misdiagnosis can occur. The genotyping of *CYP24A1* and *SLC34A1* genes is considered the definitive step in the diagnosis of this condition and, consequently, all patients with a suspected IIH phenotype should be addressed to the molecular testing. A genetic basis of IIH should be particularly taken into account in the case of early-onset, familial prevalence,

familial consanguinity and a history of multiple or recurrent kidney stones [85]. Across the availability of molecular high-throughput approaches, the mutation assay could be evaluated not only as the final step into a diagnostic workflow but also as the viable and cost-effective screening tool [116, 119]. At present, genetic testing is recommended but not widely accessible to all patients and clinicians, due to both the lack of available genetic counseling and to the need of specialized laboratories. The efficient identification of both infantile and adult forms of *CYP24A1* or *SLC34A1* deficiency has a great impact on the patient's management in terms of appropriate treatment and diet modification. In IIH patients, the source of vitamin D, such as supplementation therapy, is removed and a low-Ca diet is introduced. In the case of defective NaPi-IIa, Pi supplementation is the targeted therapeutic approach, aimed at restoring the serum Pi levels and at normalizing vitamin D and Ca metabolism [3, 66, 91]. In this sense, early and correct molecular diagnosis may be crucial for the setting of the optimal feedback therapy in response to the primary genetic defect [81]. The pharmacological treatments that can be administrated in IIH patients are mainly focused on the avoidance of long-lasting and recurrent hypercalcemic episodes [120]. Several therapeutic approaches can be applied, principally focused on the decrease of intestinal Ca absorption, the promotion of renal Ca excretion, the reduction of Ca release from bone and the inhibition of the *CYP27B1* activity [121]. Common treatments include the administration of steroids, bisphosphonates, calcitonin and glucocorticoids that can be given if the symptomatic hypercalcemia persists despite hydration and diuretics [21]. Bisphosphonates inhibit osteoclast activity and osteocytes apoptosis, indirectly stimulating the osteoblast function [122]. Administration of this drug has been described as a successful treatment in an infant with severe hypercalcemia due to a *CYP24A1* genetic variant, however, a long-term evaluation and more extensive clinical trials are necessary to understand the beneficial and side effects of this therapy in *CYP24A1* mutation carriers [123]. On the contrary, long-term steroids administration seems not to be an efficient therapy for IIH, as in some patients with *CYP24A1* genetic variants the hypercalcemia persists after pharmacological treatment [124]. A successful use of theazole agents is reported as a specific targeted pharmacological therapy of IIH caused by *CYP24A1* loss of function. This class of drugs, primarily antifungal agents, is specifically used in vitamin D-mediated hypercalcemia as general inhibitors of cytochrome P450-enzymes. These drugs are heterocyclic ring compounds classified as imidazoles (e.g. ketoconazole) or triazoles (e.g. fluconazole), containing two or three nitrogen atoms in theazole ring,

respectively. In particular, ketoconazole was successfully used in patients with *CYP24A1* impairment showing an effective normalization of biochemical parameters [53, 58, 125, 126]. However, long-term and high-dose treatment ketoconazole may be associated with several adverse effects such as renal and hepatic toxicity and a protracted administration is not always advisable. Fluconazole, however, shows a lower inhibitory activity, is well tolerated due to reduced side effects and, consequently, it should be considered as an alternative treatment [127]. Another therapeutic approach proposed in IIH treatment foresees the induction of the *CYP3A4* activity through administration of rifampin. *CYP3A4* is a P450 enzyme that inactivates many drugs and may represent an alternative pathway for the inactivation of the active vitamin D metabolites [128]. Notably, the long-term monitoring through dietary modification and biochemical follow-up represents a crucial issue for avoiding relapses and acute exacerbation of the disease. Generally, while hypercalcemia resolves during follow-up, persistent hypercalciuria is a common finding [129]: as a consequence, the main goal of long-term evaluation is the prevention of renal deterioration and its associated complications.

Conclusions and future prospects

The recent association between *CYP24A1* and *SLC34A1* variants with IIH syndrome have piqued the interest of laboratory professionals, geneticists and clinicians, paving the way for a progressive growth of literature case reports. If acquired conditions are the main causes of hypercalcemia in adults, inherited disorders and vitamin D intoxications are the most prevalent in infancy and require more attention in the diagnostic evaluation. Notably, in IIH patients the clinical outcome could also be triggered by the administration of vitamin D. The public belief about the health benefits of high levels of vitamin D, together with the prophylactic measures aimed at the prevention of rickets, has led to a common use of supplementation schemes. IIH infants receiving high doses of vitamin D are more vulnerable to toxicity: therefore, a tailored dosage regimen is required in the view of personalized medicine. In this scenario, the relevance of genetic testing is emerging dramatically. In early-onset NC/NL subjects and before prescribing vitamin D supplementation therapy, molecular analysis should be routinely taken into account in both adults and infants to rule out abnormalities in Ca and Pi metabolism. The molecular evaluation allows the definitive diagnosis of IIH also suggesting the specific

IIH subtype. This has led to a significant improvement of medical evaluations, with practical consequences in patients' care in terms of tailored treatments. The introduction of the genetic testing for *CYP24A1* and *SLC34A1* offers a valuable tool to fully understand the vitamin D and Pi metabolic pathways of each patient. Moreover, clinicians would mainly benefit from these results in the forthcoming monitoring of infants as well as adult patients in the context of personalized and precision medicine. Finally, the possibility to reduce the risk of future chronic manifestations represents an important aspect of lifestyle quality of patients and could reflect on a wider scale the efficiency of public health.

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