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ARTICLE

Twelve novel *HGD* gene variants identified in 99 alkaptonuria patients: focus on 'black bone disease' in Italy

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Alkaptonuria (AKU) is an autosomal recessive disorder caused by mutations in homogentisate-1,2-dioxygenase (*HGD*) gene leading to the deficiency of HGD enzyme activity. The DevelopAKUre project is underway to test nitisinone as a specific treatment to counteract this derangement of the phenylalanine-tyrosine catabolic pathway. We analysed DNA of 40 AKU patients enrolled for SONIA1, the first study in DevelopAKUre, and of 59 other AKU patients sent to our laboratory for molecular diagnostics. We identified 12 novel DNA variants: one was identified in patients from Brazil (c.557T>A), Slovakia (c.500C>T) and France (c.440T>C), three in patients from India (c.469+6T>C, c.650–85A>G, c.158G>A), and six in patients from Italy (c.742A>G, c.614G>A, c.1057A>C, c.752G>A, c.119A>C, c.926G>T). Thus, the total number of potential AKU-causing variants found in 380 patients reported in the *HGD* mutation database is now 129. Using mCSM and DUET, computational approaches based on the protein 3D structure, the novel missense variants are predicted to affect the activity of the enzyme by three mechanisms: decrease of stability of individual protomers, disruption of protomer-protomer interactions or modification of residues in the region of the active site. We also present an overview of AKU in Italy, where so far about 60 AKU cases are known and DNA analysis has been reported for 34 of them. In this rather small group, 26 different *HGD* variants affecting function were described, indicating rather high heterogeneity. Twelve of these variants seem to be specific for Italy.
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INTRODUCTION

Alkaptonuria (AKU) [OMIM 203500] is caused by deficiency of homogentisate-1,2-dioxygenase (HGD, EC 1.13.11.5).[1] Darkening of the urine upon standing is usually the first sign of AKU, characteristic of homogentisic acid excretion. Homogentisic acid accumulated in the body forms a melanin-like polymer that is deposited in the connective tissues, causing a pathologic pigmentation known as *ochronosis*. In their late 20s, patients start to suffer severe pain due to degenerative ochronotic arthropathy of intervertebral discs and joints, especially in shoulders, hips and knees.

Since November 2012, the DevelopAKUre project has been underway and is focused on clinical testing of nitisinone as a possible treatment for AKU. Its first part, the SONIA1 study, showed that this drug decreases urine homogentisic acid in a dose-dependent manner.[2]

The enzymatic defect in AKU is caused by recessive mutations within the *HGD* gene (HGNC:4892), a single-copy gene that spans 54 363 bp of genomic sequence (3q13.33) and is split into 14 exons and codes for the HGD protomer composed of 445 amino acids.[3,4] The active form of the HGD protein is organised as a hexamer comprising two disc-like trimers.[5] An intricate network of

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non-covalent interactions is required to maintain the spatial structure of the protomer, of the trimer and finally of the hexamer, which can be easily disrupted by variants leading to effects on enzyme function.

AKU has a very low prevalence (1:1 000 000–250 000) in most ethnic groups, but it presents a remarkable allelic heterogeneity—149 different HGD variants have been identified, out of which 116 were reported as mutations and 33 as polymorphisms. All variants are summarised in the HGD mutation database (http://hgddatabase. cvtisr.sk/).[6–8] Some of the reported HGD variants are spread throughout the world, such as c.175delA (p.(Ser59Alafs*52)), one of the first identified AKU mutations, c.899T > G (p.(Val300Gly)).[7] On the other hand, there are variants rather specific for some countries or regions; for example, c.342+1G > A (ivs5+1G > A) for Slovakia and the Czech Republic,[9] c.87+1G > A (ivs2+1G > A) for the gypsy community Narikuravar in India[10] or c.360T > G (p.(Cys120Trp)) for the Dominican Republic.[11]

So far, about 950 AKU patients have been reported in 61 countries worldwide (AKU Society, www.akusociety.org). The highest number of AKU patients, 208, was reported in Slovakia, including 110 children.[12] This country and the Dominican Republic exhibit prevalence of AKU of up to 1:19 000.[13,14] Recently, a high number of AKU cases were also found in Jordan[15] and India,[10] indicating that the overall prevalence of this disease in some countries might be underestimated.

In this report, we present 12 novel variants identified in thr AKU gene, and as most of them were found in the patients from Italy, we summarize data on AKU in this country.

MATERIALS AND METHODS

Patients
We analysed 99 patients from 20 countries for HGD variants. Forty were enrolled in the SONIA1 clinical study, whereas the remaining 59 were sent to our laboratory for routine DNA diagnostics. Diagnoses of AKU were established based on documented elevated homogentisic acid in urine and/or the bluish-black pigmentation in connective tissue (ochronosis). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients included in the study.

Variant identification
All 14 HGD exons in each patient were screened for variants by DNA sequencing as previously described.[8] Variants were reported according to the Human Genome Variation Society (HGVS) nomenclature additions[16] and their description is based on coding DNA Reference Sequence NM_000187.3 (genomic reference sequence NG_011957.1). Exons are numbered like in the study by Granadino et al.[4] Variants and other patient data were deposited in the HGD gene mutation database (http://hgddatabase.cvtisr.sk/),[8] using a specific family/allele code. Variant nomenclature was verified using MUTALYSER Name Checker (https://www.mutalyzer.nl/). In order to make it easier to recognize variants, in the tables, we also indicate their brief names used in AKU scientific community.

Variant verification
For novel missense variants, the conservation of the affected amino acid position between Homo sapiens and Mus musculus, Rattus norvegicus, Danio rerio, Drosophila melanogaster, Arabidopsis thaliana and Aspergillus nidulans was checked using ClustalW2. Where available, carrier status was tested in parents and other relatives, to confirm segregation of the variant with the disease. PolyPhen-2 (Polymorphism Phenotyping v2)[17] and SNAP (Screening for non-acceptable polymorphisms)[18] were used to predict the possible effect of amino acid substitutions on the structure and function of the human HGD protein (NP_000178.2). These tools were selected as they use information on 3D structure of the protein and may be more reliable for HGD variants, as was shown before.[19]

We also used mCSM[20] and DUET[21] in order to predict the effects of variants on a structural basis. These latter approaches are novel machine-learning algorithms that use the three dimensional structure in order to predict quantitatively the effects of point variants on protein stability and protein-protein and protein-nucleic acid affinities. Two crystal structures of human HGD were used in this analysis (PDB code 1EY2 and 1EYB[5]). The effect of the variants was assessed in the context of the molecular interactions of the wild-type residue, and mCSM and DUET were used to predict the effects of the variants on protomer and hexamer thermal stability, and mCSM-PPI[20] to predict the effects of the variants on the affinity of the protomers to interact with each other. To examine Lys353, a loop missing in the HGD crystal structures (residues 348–355) was modelled and minimised using ModLoop[22] and Schrodinger.[23]

The effect of the splicing variants was predicted using Splice Site Prediction by Neural Network (SSPNN) (http://www.fruitfly.org/seq_tools/splice.html) and Human Splicing Finder (HSF).[24]

RESULTS

Novel HGD gene variants
Genomic sequencing in 40 SONIA1 AKU patients identified 20 different variants, and variants on both alleles were identified in all of them (Supplementary table 1). Two HGD variants were novel (Table 1): c.158G > A (p.(Arg53Gln)) present in the homozygous state in one patient of Indian origin; and c.500C > T (p.(Tyr167Ile)) identified in one copy in a Slovak AKU patient.

In the remaining 59 AKU cases, 32 different HGD variants affecting protein function were found, including 10 novel (Table 1, Supplementary table 1).

In all 99 patients, all exons were sequenced in order to exclude the presence of other potentially pathogenic variant.

Nine of the tested patients come from Jordan, three of whom were reported before (patients 2,5,8; Supplementary table 1).[25] Seven of these cases carried the homozygous variant c.365C > T (p.(Ala122Val)) in exon 6, indicating a founder effect (Figure 1, Supplementary table 1). Two siblings carried also c.16–1G > A (ivs1-1G > A) in the heterozygous state (Figure 1). We did not confirm the presence of variants in exons 11, 13 and 10 published in patient 2, 5, and 8 before.[25]

In one patient from Israel, a recently reported genomic deletion of exon 2 (c.16-272_c.87+305del) including flanking intronic sequences was identified (Supplementary table 1).[26]

In two patients, we were not able to identify any HGD gene variant affecting function, and in two other cases, only one variant was found. All of these cases were of Italian origin (Supplementary table 1).

In summary, including the novel variants, the total number of different HGD function affecting variants reported in the HGD mutation database is now 129 across 380 patients.

Missense variant verification
ClustalW2 showed that all novel missense variants affected conserved amino acid position (data not shown). Moreover, at least one of the prediction programs PolyPhen-2 or SNAP indicated a pathogenic effect of all but one novel amino acid substitutions on the structure and function of the human HGD protein (Table 1). In addition, mCSM and DUET provided a structural understanding behind the inactivation of HGD activity. The variants are spread throughout the structure: three are located along protomer interaction interfaces (Arg53, Tyr40, Gly309), several are buried in the core of the protomers (Phe147, Thr167, Met186, Gly205, Lys248, Gly251) and one lies on a solvent-exposed loop (Lys353). The structural analysis of the identified variants allowed their classification based on the predicted effects into three classes: (i) those that alter the active site, reducing activity; (ii) those that destabilize the protein, reducing activity; and (iii) those that prevent formation of the homohexamer, disrupting activity.

Table 1 Novel *HGD* gene variants identified in 99 AKU patients

Exon/ intron	Brief variant name	Variant code in HGD mutation database	DNA change	Predicted protein change	Type of change	PolyPhen2 prediction (pph2_prob)	SNAP predictions (RI, Expected Accuracy)	monomer_ DUET (ΔΔG) Kcal/mol	mCSM-PPI (ΔΔG) Kcal/mol	Mechanism of Action	Country of origin
3	R53Q	AKU_00152	c.158G>A	p.(Arg53Gln)	missense	probably damaging (0.996)	non-neutral (1, 63%)	0.245	−3.348	Destabilises PPI	Italy
3	Y40S	AKU_00160	c.119A>C	p.(Tyr40Ser)	missense	probably damaging (0.999)	non-neutral (3, 78%)	−1.355	−3.471	Destabilises protomer and PPI	Italy
7	F147S	AKU_00155	c.440T>C	p.(Phe147Ser)	missense	probably damaging (0.999)	non-neutral (2, 70%)	−3.606	−0.135	Destabilises protomer	France
7i	ivs7+6T>C	AKU_00159	c.469+6T>C	p.(Arg145Serfs*22)	splicing (DS)	n.a.	n.a.	n.a.	n.a.	n.a.	India
8	T167I	AKU_00161	c.500C>T	p.(Thr167Ile)	missense	probably damaging (1.00)	non-neutral (3, 78%)	0.208	−0.521	Destabilises PPI	Slovakia
9	M186K	AKU_00149	c.557T>A	p.(Met186Lys)	missense	possibly damaging (0.447)	non-neutral (1, 63%)	−1.518	−0.563	Destabilises protomer and PPI	Brazil
9	G205D	AKU_00153	c.614G>A	p.(Gly205Asp)	missense	benign (0.08)	neutral (3, 78%)	−1.55	−0.141	Destabilises protomer	Italy
9i	ivs9−85A>G	AKU_00162	c.650−85A>G	p.(Gly217_Ala218ins28)	splicing? (new AS)	n.a.	n.a.	n.a.	n.a.	n.a.	India
10	K248E	AKU_00151	c.742A>G	p.(Lys248Glu)	missense	probably damaging (1.00)	non-neutral (4, 82%)	−1.955	−0.146	Destabilises protomer	Italy
10	G251D	AKU_00156	c.752G>A	p.(Gly251Asp)	missense	probably damaging (1.00)	non-neutral (3, 78%)	−2.64	−1.518	Destabilises protomer	Italy
12	G309V	AKU_00163	c.926G>T	p.(Gly309Val)	missense	probably damaging (1.00)	non-neutral (2, 70%)	−0.17	−1.11	Destabilises PPI	Italy
13	K353Q	AKU_00154	c.1057A>C	p.(Lys353Gln)	missense	probably damaging (0.996)	neutral (3, 78%)	0.357	0.121	Potential change in active site/activity	Turkey

The predicted changes of the missense variants upon protomer stability and protein-protein affinity from DUET and mCSM-PPI, respectively, as well as the proposed mechanism leading to loss of activity are shown. PolyPhen2 and SNAP predictions of the effect of the missense variants on the function of the HGD protein are also indicated. Variants were described based on coding DNA Reference Sequence NM_000187.3 (genomic reference sequence NG_011957.1). In order to make it easier to recognize variants, we also indicate their brief names used in the AKU scientific community.
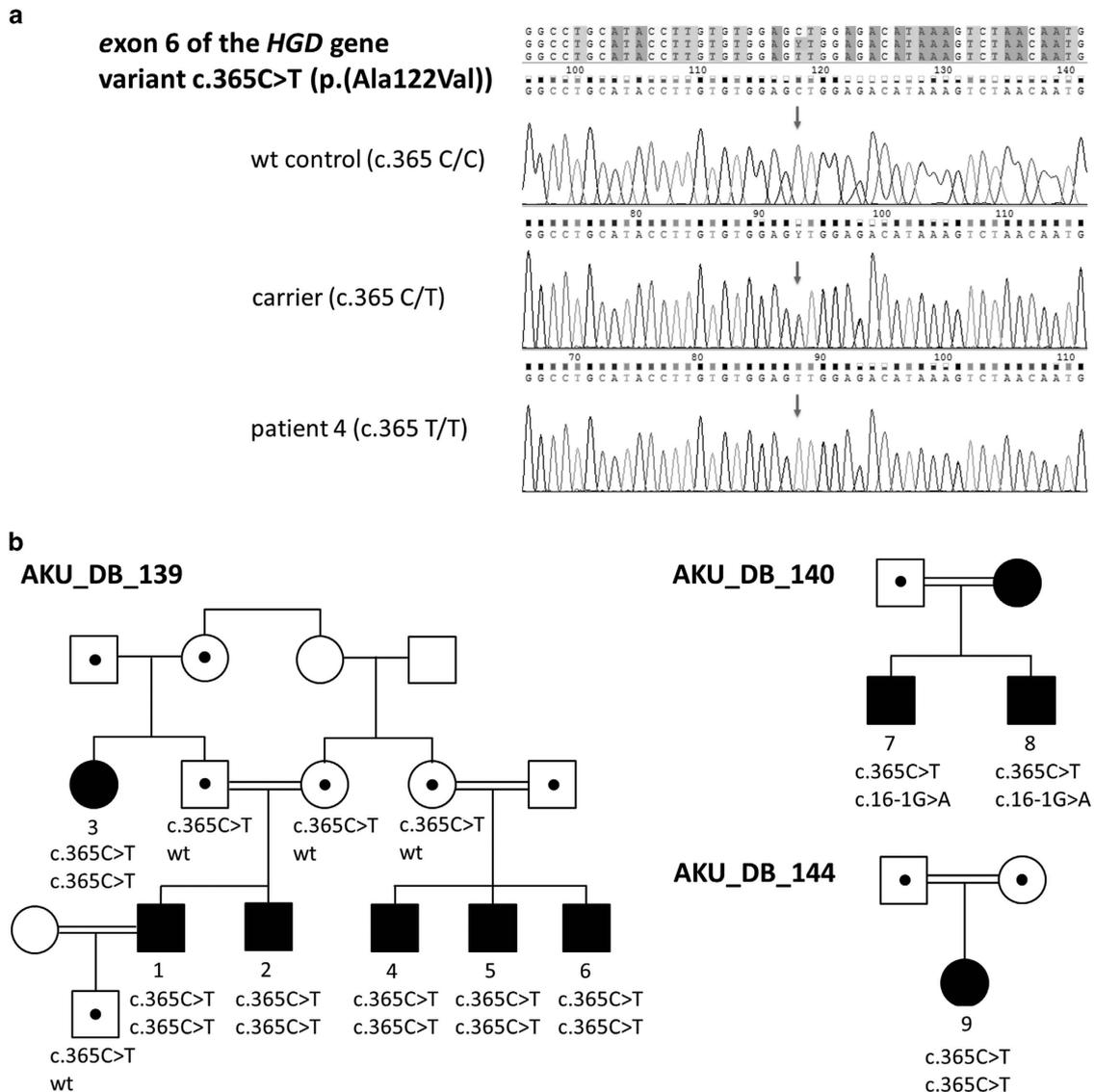
Figure 1 c.365C>T (p.(Ala122Val)) founder variant identified in Jordanian families. (**a**) Chromatogram showing exon 6 of *HGD* gene sequence. Nucleotide c.365 is indicated by red arrow. Wild-type sequence is in the upper panel, heterozygous carrier in the middle and the patient homozygous for c.365C>T variant in the lower one. (**b**) Pedigrees of consanguineous Jordanian families AKU_DB_139, AKU_DB_140 and AKU_DB_144 tested for *HGD* variants. All families are included in the *HGD* mutation database, under the code indicated. DNA was analysed in individuals where mutation status is indicated. In the family AKU_DB_140, a splicing variant c.16-1G>A in intron 1 was found in heterozygous state. wt indicates a wild-type allele in carriers. The numbers under analysed patients correspond to the patient's numbers in Supplementary table 1. The full colour version of this figure is available at *European Journal of Human Genetics* online.

The first class of variants are predicted to affect HGD activity through direct alteration of the active site. The novel variant c.1056A>C (p.(Lys353Gln)) is located on a loop at the mouth of the active site (Supplementary Figure 1). This loop is disordered in the human crystal structure, reflecting its flexibility and possible role in regulating access to the active site. Consistent with its solvent-exposed, flexible nature, the c.1056A>C variant is predicted by mCSM and DUET to have minimal effect on either stability or hexamer formation. However, the loss of the positive charge will alter potential interactions, and it is likely that this variant may alter the flexibility of the loop and consequently catalytic activity. Molecular dynamics simulations using Desmond suggests that the mutant loop indeed has greater flexibility, showing an increase in average RMSDs for the backbone of approximately 20% (data not shown).

The second group of variants affect the production of active HGD enzyme through destabilization of the protomer structure. These variants typically perturb the local secondary structure by the introduction of energetically unfavourable changes, disrupting the interactions made by the wild-type residues (Supplementary Figure 2). Examples of the disruptive nature of these variants include: change of Phe147 to serine would likely disrupt a series of strong intramolecular hydrophobic and pi-pi interactions, strongly destabilising the protomer; the variant c.742A>G (p.(Lys248Glu)) would add an intramolecular ionic repulsive force; mutation of Thr168 to isoleucine would expose a destabilising free main chain NH and would sterically hinder formation of the protomer; and Gly205 and Gly251 are both buried glycines that adopt a positive phi angle in the crystal structures that would usually be energetically unfavourable for most non-glycine amino acids.

The mutations in the third group are at interfaces between protomers and are likely to affect the enzyme activity by lowering stability of the symmetrical homohexameric structure. Both Arg53 and Tyr40 make strong intermolecular interactions between protomers, with Arg53 forming an ionic/hydrogen bond network and Tyr40 making a series of strong hydrophobic and pi-pi interactions (Supplementary Figure 3). Mutation of Arg53 to glutamine, p.(Arg53Gln), was not predicted by mCSM or DUET to affect protomer stability, however, mCSM-PPI predicted the mutation to highly destabilise the formation of the hexamer, because of the loss of the interactions made by the arginine. Mutation of Tyr40 to serine, introducing a polar amino acid residue in the middle of a series of hydrophobic interactions is predicted to be highly destabilising of both protomer and hexamer. Gly309 is sitting along one of the protomer-protomer interfaces with a positive phi angle and mutation to a valine would disrupt the local secondary structure and this interaction.

Splicing variants

We also identified two novel mutations potentially affecting mRNA splicing (Table 1). One is a variant c.469+6T>C in intron 7, identified in the homozygous state in a patient from India. This change is predicted to weaken acceptor splice site by both SSPNN and HSF (data not shown).

The second variant, located 85 nucleotides upstream from the intron 9 acceptor splice site (c.650−85A>G), was found in the homozygous state in a patient from India (Supplementary table 1). No other change was found in this individual by sequencing or by the genomic PCR designed to uncover exon 2 genomic deletion. This substitution was predicted by HSF to potentially activate a cryptic splice site at this location, indicated by improved splice site score (+58.01%, data not shown). This site might compete with the natural intron 9-acceptor splice site. However, no cDNA of the patient was available for analysis in order to prove the splicing effect of this change.

AKU in Italy

So far, about 60 AKU cases are known (aimAKU, www.aimaku.it) and the results of mutation analysis are now reported for 34 families,[27,28] *HGD* mutation database, present work).

It is remarkable that in 68 AKU chromosomes identified in Italy and published in *HGD* mutation database, 26 different *HGD* variants were found, indicating extremely high allelic heterogeneity (Table 2). Twelve of these variants seem to be specific for Italy (Table 2). However, for four Italian patients, only one variant has been identified so far (two in our study: P56 (c.4697+2T>C/?), P46 (c.752G>A/?) (Supplementary table 1) and two reported before (c.650−56G>A/?, c.31_32delGGinsATT/?).[29] In other three Italian probands no variant was identified at all (one in our study P22, and two previously reported, including P21 also analysed by us (Supplementary table 1)[27].

DISCUSSION

We describe 12 novel variants identified in 99 AKU patients from 20 countries, increasing to 129 the total number of HGD function-affecting variants described so far in 380 patients with this rare disease, as reported in the *HGD* mutation database (http://hgddatabase.cvtisr.sk/).

The local effect of the novel missense variants was assessed in the context of the molecular interactions of the wild-type residue using mCSM[20] and DUET.[21] For all variants, disruptive effects were predicted, with change resulting in destabilisation of the protomer, reduced protein-protein affinity or altered activity. With the exception

of c.614G>A (p.(Gly205Asp)), a pathogenic effect of amino acid substitutions on the structure and function of the human HGD protein was also predicted by PolyPhen-2 or SNAP.

The effect of potential splicing-affecting variant c.650−85A>G was tested by SSPNN and HSF, and a potential activation of cryptic splice site in intron 9 was predicted. If this novel site is used, 84 bases can be included into *HGD* transcript between exons 9 and 10, causing insertion of 28 amino acids into HGD protein sequence (c.649_650ins GAAAGCCTTTCTCTTCATGCAACCATGGGCATCTTT CCTATG TTTTGGAAGTTTCTAAAAGACTTTTGGGTTACTGTTTTCTAG, p.(Gly217_Ala218ins LysProPheSerSerCysAsnHisGlyHisLeuSerTyrVal LeuGluValSerLysArgLeuLeuGlyTyrCysPheLeuGly). The insertion is located on a loop that sits at the interface between two different protomers. There is no room in this region to accommodate a significant change, and so, this mutation would be very disruptive to hexamer formation, as it would interfere and block two separate protomer-protomer interactions. Interestingly, polymorphism c.650−86A>G was described by Vilboux *et al.*[19] Unfortunately, the cDNA of the patient is not available for analysis in order to confirm the splicing effect. We plan to perform MLPA analysis in this patient, in order to test for a presence of possible larger deletions.

AKU generally has low prevalence worldwide, with the notable exceptions of Slovakia[30,31] and the Dominican Republic.[13] Although a clear founder effect was observed in the Dominican republic,[11] in Slovakia, already 13 different *HGD* variants affecting function are reported in 121 AKU chromosomes, though most of the patients in this country come from a previously genetically isolated region.[7–9,32–35] The most frequent variant found in Slovakia is p.Gly161Arg, present in 45% of all AKU alleles (*HGD* mutation database).

Recently, several new AKU cases were also reported in southern Jordan,[15] in villages characterised by a high level of consanguineous marriages, thus a founder effect is expected. Surprisingly, five different variants were reported in them.[15,25] We re-analysed three patients from this study, however, we were not able to confirm results reported before. None of the variants reported by Al-Sbou[25] was found in the DNA samples from the patients. Instead, we show a clear founder effect in Jordan, where most of the patients carried the c.365C>T (p.(Ala122Val)) missense variant in exon 6.

As the majority of novel mutations originate from Italy, we focus on a brief overview on AKU in this country, where about 60 patients have been identified so far. This was possible through early call-for-patients at relevant National Congresses[28] and, more recently, with the important contribution of the Italian AKU patient Association (aimAKU). Even though epidemiological estimates specific to Italy are not available, on the basis of the commonly held figures of disease incidence worldwide (1:250 000–1:1 000 000 live births) in a population of about 60 million inhabitants with a birth rate of 9/1000, 0.5–2 new cases per year are expected. As AKU does not generally reduce the lifespan of affected individuals, 40–160 cases are predicted. Therefore, it would seem that the number of ascertained patients fall well within the expected range of AKU prevalence, which means that a good sensitisation work has been made in this country. Including present data, there are 34 Italian families reported in the *HGD* mutation database and previously published.[7,27–29,32,33] In this rather small group, 26 different AKU-causing mutations were described, indicating high heterogeneity. Moreover, 12 of these mutations seem to be specific for Italy.

In four Italian patients, so far, only one mutation has been identified, and in three patients, mutations have not been discovered at all. Altogether, for 8 AKU patients reported in the database, no

Table 2 AKU variants identified in Italian AKU patients

Exon/ intron	Brief variant name	DNA change	Predicted protein change	Number of AKU chr.	% out of 68 chr.	Country in which variant was found	Patient reference
00	unknown	c.(?)	p.(?)	10	14.7%		27,28,29, Dr Biolcati, Dr Santucci, Dr Porfirio
02	**G11fs (F10fs)**	**c.31_32delGGinsATT**	**p.(Gly11Ilefs*3)**	**3**	**4.4%**	**Italy**	**Dr Santucci**
03	**Y40S**	**c.119A>C**	**p.(Tyr40Ser)**	**2**	**2.9%**	**Italy**	**Dr Biolcati**
03	L44F	c.130C>T	p.(Leu44Phe)	1	1.5%	Italy, Algeria	Dr Biolcati
03	R53Q	c.158G>A	p.(Arg53Gln)	2	2.9%	Italy, India	Dr Biolcati
03	S59fs (R58fs)	c.175delA	p.(Ser59Alafs*52)	2	2.9%	UAE, Finland, Slovakia, Turkey, India, USA, La Reunion, UK	Dr Biolcati
04	**W60X**	**c.179G>A**	**p.(Trp60*)**	**2**	**2.9%**	**Italy**	**7**
07	D153fs (G152fs)	c.457dupG	p.(Asp153Glyfs*26)	2	2.9%	Slovakia, France, USA, Italy, Macedonia	28
07i	ivs7+2T>C	c.469+2T>C	p.(Arg145Serfs*22)	1	1.5%	France, USA, UK, Italy	Dr Biolcati
07i	**ivs7+5G>A**	**c.469+5G>A**	**p.(Arg145Serfs*22)**	**5**	**7.4%**	**Italy**	**28**
08	G161R	c.481G>A	p.(Gly161Arg)	3	4.4%	Slovakia, USA, UK, France, Italy, Czech republic, Poland, Germany, Hungary, France/Serbia	Dr Biolcati
09	**G198D**	**c.593G>A**	**p.(Gly198Asp)**	**2**	**2.9%**	**Italy**	**27**
09	**G205D**	**c.614G>A**	**p.(Gly205Asp)**	**1**	**1.5%**	**Italy**	**Dr Porfirio**
09i	**ivs9–17G>A**	**c.650–17G>A**	**p.(?)**	**1**	**1.5%**	**Italy**	**29**
09i	ivs9–56G>A	c.650–56G>A	p.(?)	2	2.9%	Italy, Italy/France	29
10	F227S	c.680T>C	p.(Phe227Ser)	2	2.9%	Spain,Italy	Dr Porfirio
10	P230S	c.688C>T	p.(Pro230Ser)	4	5.9%	Spain, Turkey, Slovakia, Canary Island, USA, Italy, France	Dr Porfirio
10	**K248E**	**c.742A>G**	**p.(Lys248Glu)**	**2**	**2.9%**	**Italy**	**Dr Biolcati**
10	**K248R**	**c.743A>G**	**p.(Lys248Arg)**	**1**	**1.5%**	**Italy**	**28**
10	**G251D**	**c.752G>A**	**p.(Gly251Asp)**	**1**	**1.5%**	**Italy**	**Dr Santucci**
11	G270R	c.808G>A	p.(Gly270Arg)	2	2.9%	Italy, Slovakia, France/Armenia, Brasil, USA, Dominican republic, Turkey, UK	28
12	**G309V**	**c.926G>T**	**p.(Gly309Val)**	**2**	**2.9%**	**Italy**	**Dr Santucci**
12	V324fs	c.970dupG	p.(Val324Glyfs*3)	1	1.5%	Italy, USA	Dr Biolcati
13	**K353Q**	**c.1057A>C**	**p.(Lys353Gln)**	**1**	**1.5%**	**Italy**	**Dr Porfirio**
13	G360R	c.1078G>C	p.(Gly360Arg)	3	4.4%	Italy, USA, USA/India, Australia	28
13	M368V	c.1102A>G	p.(Met368Val)	5	7.4%	France/Armenia, Spain, The Netherlands, France, Finland, Portugal, Germany, Slovakia, USA, Switzerland/Belgium, Italy, France	Dr Porfirio
14	E401Q	c.1201G>C	p.(Glu401Gln)	5	7.4%	Italy, USA	27

Variants are described based on coding DNA Reference Sequence NM_000187.3 (genomic reference sequence NG_011957.1). In order to make it easier to recognize variants, we also indicate their brief names used in AKU scientific community. Variants so far identified only in Italy are shown in bold.

mutations were identified, and 22 with only one mutation were found. Whether deep intronic mutations or large deletions encompassing one or more exons are occurring in these cases remains to be investigated. Indeed, recently, the first large deletion of exon 2 including intronic sequences was reported in one case from Lebanon,[26] that was also identified in our study in two siblings from Israel. This might indicate that this type of mutations needs to be also considered in other cases where genomic sequencing does not lead to mutation identification.

For a patient to display AKU symptoms, a loss of more than 99% of the enzymatic activity is required.[36] So far, observed absence of a clear correlation between genotype and phenotype may be explained by the variability in residual HGD enzymatic activities caused by different mutations.[19] Owing to the complex hexameric structure of the *HGD* enzyme, genotype-phenotype correlation studies are even more complicated when in the patients two different mutations are to be considered. In addition, an effective AKU severity scoring system, AKUSSI, has been developed only recently.[37] One of the planned outcomes of the DevelopAKUre project will be detailed clinical characterisations of AKU patients, including mutations, various biochemical parameters as well as AKUSSI. We believe that the tools utilised here for studying the effects of HGD mutations identified in AKU patients provide an important basis for understanding and predicting genotype-phenotype correlations in AKU, the first described inborn error of metabolism.[38]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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