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Ciclo XXVII

PRIMARY HYPEROXALURIAS IN ITALY AND EUROPE

Settori scientifico disciplinari di afferenza: MED/03, MED/38

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Chapter 1

General introduction and aims

Primary hyperoxalurias (PH) are a group of rare autosomal-recessive diseases, which were first described in the 1920s, but only recognized as a genetic entity in the 1960s. The primary hyperoxalurias are characterized by a wide clinical and genetic heterogeneity; the clinical spectrum encompasses patients with severe nephrocalcinosis in the first year of age and progression to diffuse multisystem oxalosis, adult patients with recurrent urolithiasis, and even individuals still asymptomatic in late age. A proportion of cases are responsive to vitamin B6 supplementation, but the only effective treatment is liver- or combined kidney/liver transplantation. Liver transplantation is an invasive and problematic procedure; thus, approaches aimed at a deeper knowledge of genotype/phenotype correlations as well as at the development of new treatment strategies, appear to be desirable .

From the genetic point of view, three types of Primary Hyperoxalurias are currently known: type 1 due to *AGXT* gene mutations, type 2 caused by *GRHPR* mutations and type 3, related to *HOGA1*.

Although its genetic basis is known, it is still underdiagnosed even in developed countries, and the knowledge of genotype-phenotype correlations is still inadequate to support treatment choices.

The Italian study of PH1 was started in the ‘80s by Antonio Amoroso for Genetics, in collaboration with Franco Linari, Martino Marangella, Licia Peruzzi and several other clinicians all over Italy. More recently, the genetic diagnosis and the task of maintaining the Italian Registry of PH1 was transferred to our Unit at the San Luigi Hospital in Orbassano.

In 2008 we joined OXALEurope, the European Hyperoxaluria Consortium (www.OxalEurope.org) in order to favour a continuous exchange of data and procedures with other European experts involved in research and care of primary hyperoxaluria. This collaboration has made possible to set up a common database of a cohort of more than 500 primary hyperoxaluria patients, currently the largest at international level.

Aim of the PhD project was to refine the current knowledge on genotype/phenotype correlations, to better define the therapeutic strategy in patients and to extend the molecular analysis to HOGA1 gene, a recently described gene responsible for PH type 3.

Chapter 2

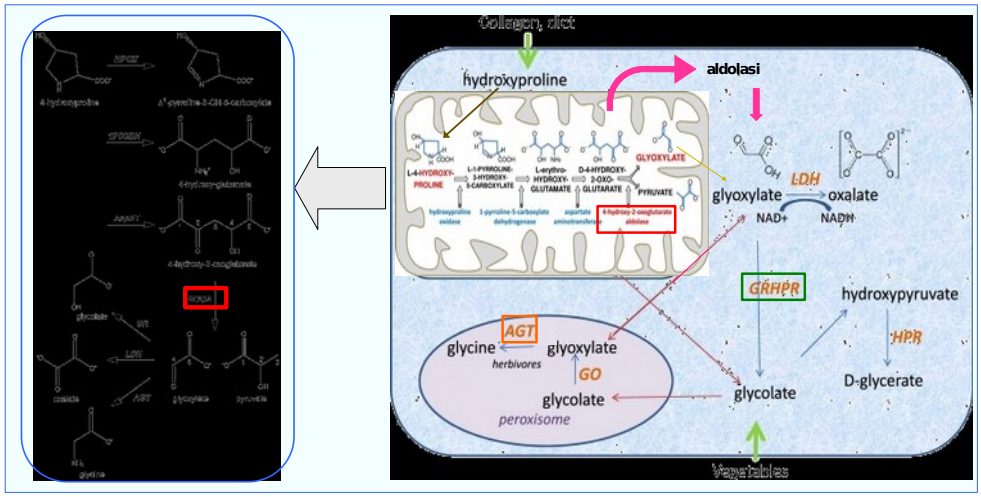
Primary Hyperoxalurias

2.1- Primary Hyperoxaluria type 1

Primary Hyperoxaluria type 1 (PH1) is estimated to occur in 1:120,000 live births in Europe . Its prevalence of ranges from 1:1,000,000 to 3:1,000,000 depending on the population studied . PH1 is estimated to account for less than 1% of pediatric cases of end-stage renal failure in Europe .

In the 1980s Danpure et al. identified the responsible enzyme of PH1: a liver peroxisomal enzyme, the alanine:glyoxylate aminotransferase (AGT) , which catalyzes the conversion of glyoxylate to glycine. When AGT activity is absent, glyoxylate is converted to oxalate, which forms insoluble calcium salts (oxalate cannot be metabolized in mammals).

Figure 2.1: The Metabolic Pathways of Oxalate Production in Humans, modified from Danpure et al., 2004



As all aminotransferases, AGT is a pyridoxal phosphate-dependent enzyme. The protein (392aa) has a homodimeric 86kDa structure , that was solved by X-ray crystallography by Zhang et al. in 2003. Figure 2.2 shows a three-dimensional representation of the AGT dimer where the mitochondrial targeting sequences (pink), the N-terminal alpha helical stretch (green), the PLP cofactor (orange) and the sites of most frequent mutations (blue) are highlighted.

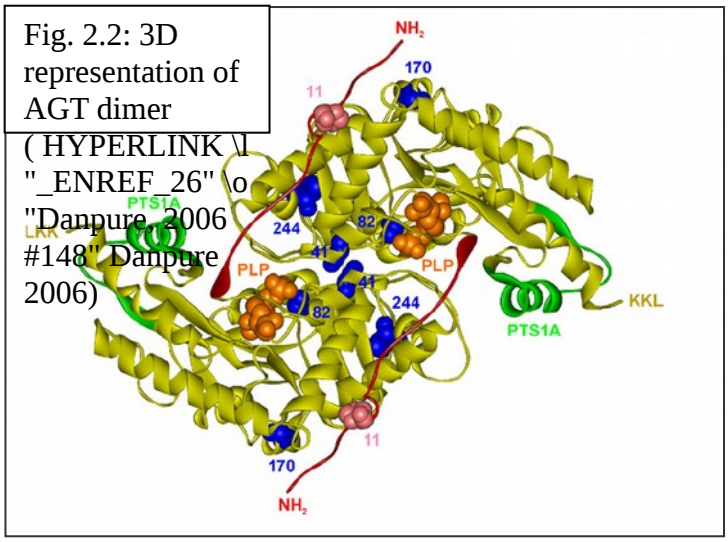


Fig. 2.2: 3D representation of AGT dimer (HYPERLINK \l "_ENREF_26" \o "Danpure, 2006 #148" Danpure 2006)

The protein is synthesized in the hepatocite ribosomes; the monomers fold and rapidly dimerize in the cytosol. The dimerization allows the peroxisome import and the conversion in a fully active state. The first 20 residues make an N-terminal extension that wraps over the surface of the cognate subunit. The next 260 residues form the catalytic domain that contains most of the active site and the dimerization interface. The C-terminal of about 110 residues contains the peroxisomal targeting information. Each subunit binds one pyridoxal-phosphate molecule (PLP), which forms a Schiff base with Lys209. Monomeric AGT has a vastly reduced catalytic activity, is unstable and rapidly aggregates and is degraded .

AGT is encoded by the human alanine-gloxylate-aminotransferase (AGXT) gene, located on chromosome 2q37.3 (11 exons, NM_000030.2 http://www.ncbi.nlm.nih.gov/). (Figure 2.3).

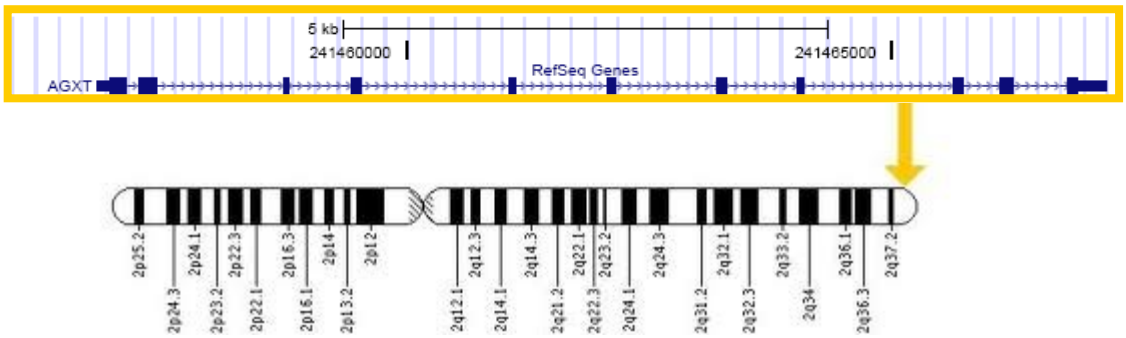


Figure 2.3: AGXT location.

Normal human AGT exists as two polymorphic variants: the ‘‘Major’’, more common, and the ‘‘minor’’, less common, allele. Major allele has a 80% frequency among individuals of

European origin and the minor one a 20% frequency in Europeans, 2% in Japanese, 3% in South African Blacks .

The minor allele is characterized by several SNPs in linkage disequilibrium among themselves, notably p.Pro11Leu, p.Ile340Met and a 74-bp duplication in intron 1 (c.165+14_+88del74). The only variant of functional significance is the p.Pro11Leu aminoacid substitution at the N-terminal, which is thought to permit formation of an α-helical conformation similar to that found in proteins normally targeted to the mitochondria . In the absence of a disease-causing mutation, this mitochondrial targeting of the minor allele is of scarce or no clinical significance. However, when in cis with some AGXT mutations, such as p.Gly170Arg, it has a synergic effect and results in mistargeting of the protein to the mitocondria (Fig. 2.4).

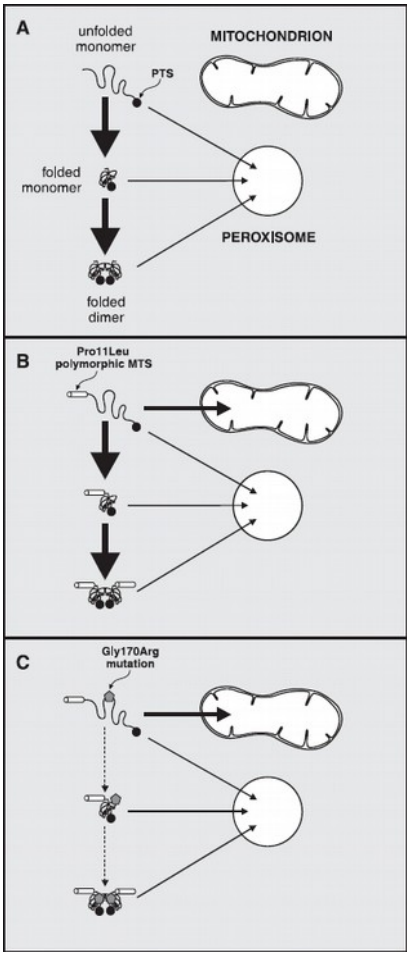


Figure 2.4:
A: AGT encoded by the major AGXT allele folds and dimerizes very rapidly.
B: AGT encoded by the minor AGXT allele: despite the greater import potential to mitochondria that to peroxisomes, most AGT is prevented from being targeted to mitochondria by its still faster rate of folding and dimerization. Therefore, most AGT are imported into peroxisomes and only a small proportion (5%) into mitochondria.
C: PH1 mutant AGT containing both the Pro11Leu polymorphism and the p.Gly170Arg mutation: the double substitution inhibits folding and/or dimerization.
Therefore, newly synthesized AGT spends a longer time in a conformation compatible with the mitochondrial import machinery. Most of the AGT is targeted to mitochondria..

2.2- Primary Hyperoxaluria type 2

Primary Hyperoxaluria type 2 (PH2, OMIM# 604296) accounts for 5 to 17% of PH cases.

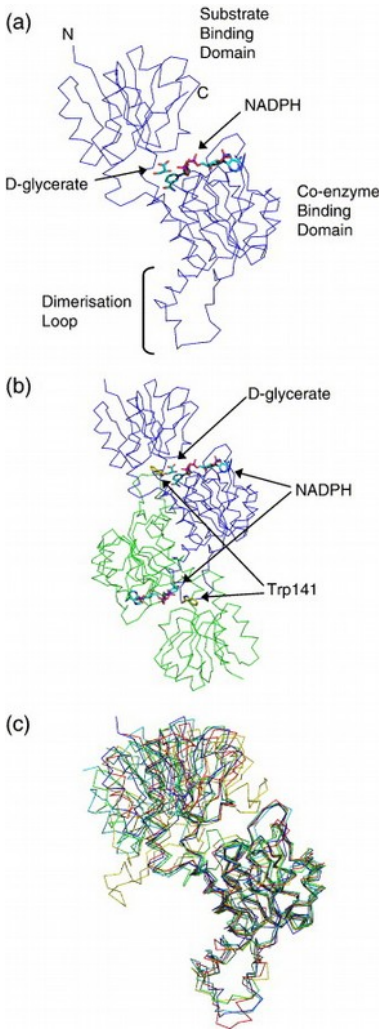
Infantile oxalosis and systemic oxalosis are not reported in PH2 patients, although ESRD occurs in approximately 20% . The low prevalence of PH2 does not allow genotype-phenotype correlations at the present time.

PH2 is caused by deficiency of the ubiquitous enzyme glyoxylate-reductase/hydroxypyruvate-reductase (GR/HPR), a bifunctional enzyme that catalyzes the reduction of glyoxylate and hydroxypyruvate (Figure 2.4). The lack of GR leads to oxalate accumulation, as a result of impaired glyoxylate conversion to glycolate. Excess oxalate is then degraded by LDH.

Lack of HPR action is responsible for hydroxypyruvate accumulation, which is in turn converted into L-glicerate. Therefore, the elevation of urinary L-glyceric acid is the biochemical marker of PH2 . The diagnosis is confirmed by the finding of two mutated *GRHPR* alleles.

The normal GR/HPR enzyme is a homodimer (Figure 2.5) with a large coenzyme-binding domain (residues 107-298) and a smaller substrate-binding domain (5-106 and 299-328) .

Figure 2.5: Overall structure of the human GRHPR. (a) C^{α} trace of a single subunit, subunit A shown in blue; co-enzyme and substrate in cyan and labeled. The amino and carboxyl termini are labeled N and C, respectively. (b) C^{α} trace of a GRHPR dimer; subunit A is shown in blue, and subunit B in green. The Trp141 side-chain is shown in stick representation in yellow and is labeled; and the co-enzyme and substrate are in cyan and also labeled. (c) Overlay of a single subunit of GRHPR (subunit A; blue) with CtBP (red), D-LDH (green), FDH (yellow) and bacterial D-GDH (cyan) .

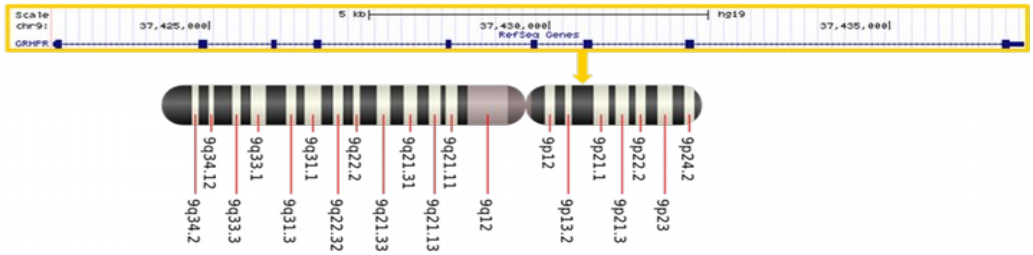


A prominent extended helix-loop region wraps around the other subunit (dimerization loop, residues 123-149). The apex of this loop contains a tryptophan residue at position 141 and the residue from one subunit is projected into the active site of the other subunit and contributes to substrate

specificity. The protein is found primarily in the cytosol although some immunoreactivity has been found in mitochondria.

All the missense mutations described to date result in proteins with no catalytic activity .

Figure 2.6: *GRHPR* gene (9p11.2; 9 exons).



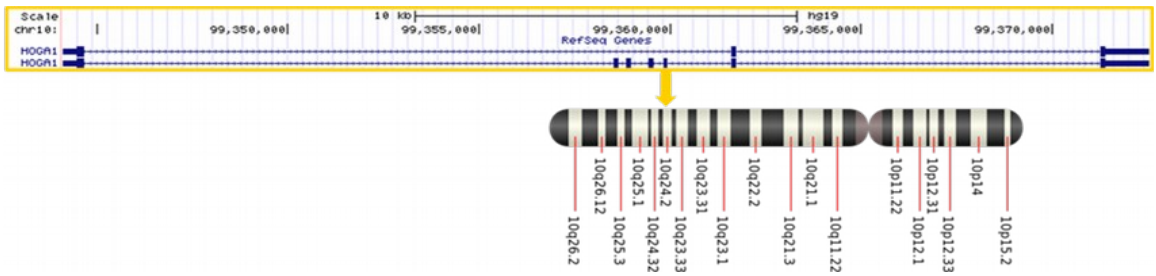
2.3- Primary Hyperoxaluria type 3

Primary hyperoxaluria type 3 (PH3, OMIM#613616) was identified in 2010 and arises from homozygous or compound heterozygous mutations in the HOGA1 gene (formerly DHDPSL) .

No data on PH3 prevalence are available. A German study reported a 30% prevalence of PH3 among patient with a clinical diagnosis of non PH1-nonPH2 .

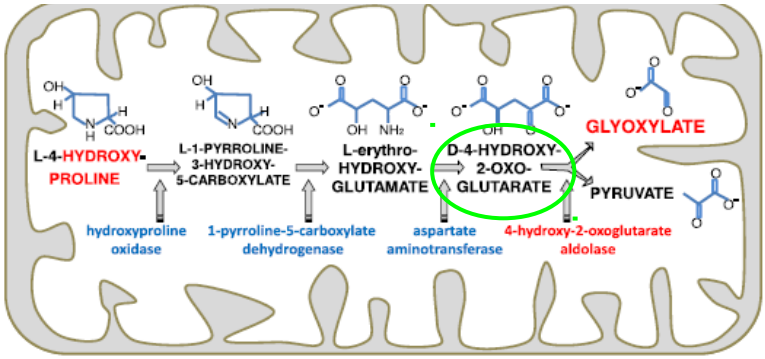
The *HOGA1* gene (10q24.2) spans 7 exons (327aa); two HOGA1 transcripts have been described: one transcript is composed by all 7 exons and the second one, shorter, lacks the central part .

Figure 2.7: *HOGA1* gene (10q24.2).



HOGA1 encodes the 4-hydroxy-2-oxoglutarate aldolase enzyme, responsible for the final step of mitochondrial hydroxyproline metabolism (Figure 2.8) from 4-hydroxy-2-oxoglutarate to glyoxylate and pyruvate .

Figure 2.8: The metabolic pathway of Hydroxyproline degradation in humans .



The enzymatic block leads to an increase of the 4-hydroxy-2-

oxoglutarate (HOG) substrate, which can be found in elevated levels in the urine, plasma, and liver of PH 3 patients .

The HOGA enzyme is a homotetramer (two dimers). Each monomer is composed by a TIM barrel domain (residues 26 - 259) and a three-helical bundle at the C-terminus (residues 260 - 327). The active site is located at the C-terminal end of the TIM barrel domain near the monomer-monomer interface of the ‘‘tight’’ dimer (colored different shades of orange and gray in Figure 2.9). Critical residues for the active site function (Tyr 168, Lys 196) and for catalysis (Ser77, Asn78, Ser198) were identified. *HOGA1* mutations currently described in PH3 patients disrupt the active site (e.g. Arg70Pro, Arg97Cys, Pro190Leu, Thr280Ile, Gln287Val) or the tetramerization (Cys257Gly) .

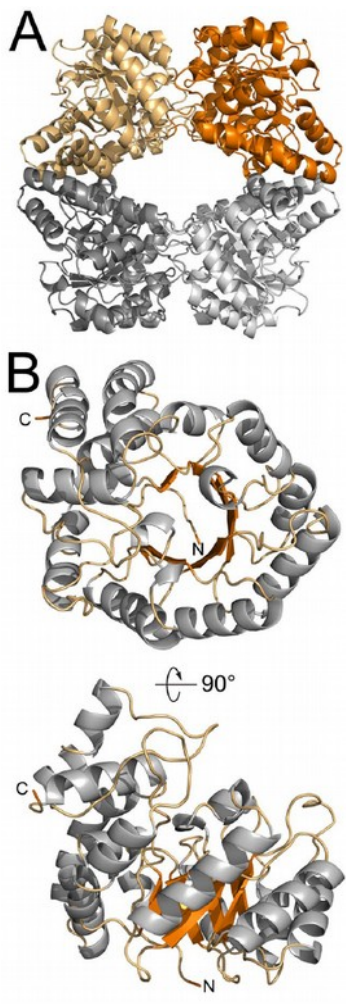
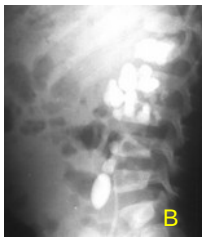


Figure 2.9: Structure of human HOGA. (A) Homo-tetrameric organization of hHOGA. The tetramer consists of a dimer of dimers (orange and gray). (B) The hHOGA monomer. Two orthogonal views illustrate the overall architecture, TIM barrel fold, and C-terminal helical bundle. Coloring is as follows: gray, a-helices; orange, b-strands; lightorange, loop regions .

The precise mechanisms underlying the hyperoxaluria are still not fully understood and different models have been proposed. The leakage of HOG from the mitochondrial compartment

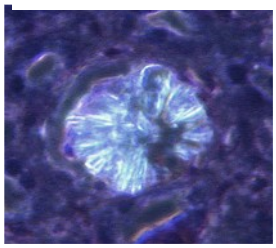
into the cytosol, where a yet-to-be identified aldolase will convert HOG again into the highly reactive oxalate precursor glyoxylate has been proposed. Another factor that may significantly contribute to increased oxalate generation could be the recently reported inhibition of mitochondrial GRHPR by excess of HOG .

From a clinical perspective, PH3 is also unique: clinical remission is observed in the majority of patients, without a parallel biochemical remission. Moreover, a peculiar biochemical aspect of PH3 is the presence of a marked hypercalciuria. Early onset of calcium-oxalate urolithiasis that becomes more and more quiescent with age, the presence of intermittent hypercalciuria (never seen in PH 1 and 2), and the absence of marked nephrocalcinosis and renal insufficiency seem to be characteristics of PH 3 . Although several studies encompassing about 50 patients confirmed the absence of CKD/ESRD, this finding should still be considered preliminary .



2.4- Symptoms

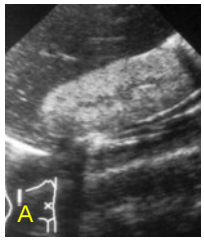
Oxalate crystal



Oxalate is mainly eliminated via the kidneys, but urine easily supersaturates. Calcium oxalate accumulation in the kidneys and other organs leads to end-stage renal disease (ESRD) and systemic oxalosis.

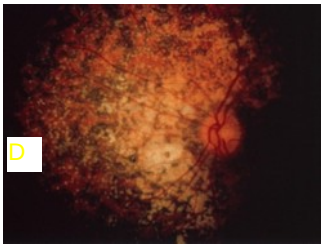
The presentation of PH is variable, with reported age at onset of symptoms ranging from birth to 57 years : these characteristics are responsible for an under-diagnosis of this disease, as demonstrated by the high proportion (up to 30%) of late diagnosis in advanced renal failure or after failure of kidney transplantation. Usually the first symptoms are related to the presence of renal stones : colic pain, hematuria, dysuria, recurrent urinary tract infections and, in some cases, renal failure.

Oxalosis is the deposition of oxalate in a variety of tissues and it occurs when the Glomerular Filtration Rate (GFR) is less than 60mL/min/1.73m^2 and plasma oxalate concentration exceeds 20uM .



Main tissues involved in systemic oxalosis are :

- kidney and urinary tract (Figure A and B)
- bone and bone marrow (Figure C): bone is the largest repository for excess oxalate. Deposition may lead to pain, multiple fractures, arthropathy, osteosclerosis pancytopenia and erythropoietin-resistant anemia
- Eye (Figure D): retinopathy, optic atrophy
- Teeth: root reabsorption, pulp exposure, dental pain
- Nerves: peripheral neuropathy (axonal degeneration and segmental demyelination), brain and meninges. .
- Heart and vessels: conduction block, myocarditis, cardioembolic stroke, vasospasm, livedo reticularis, vessel calcification, peripheral gangrene
- *Calcinosis cutis*



- Hepatosplenomegaly

2.5- Diagnosis

The first confirmation of PH is given by biochemical testing : the routine test is based on the measurement of oxalate in 24-hour urine collection, or in plasma for patients in ESRD. Normal urinary oxalate excretion is less than 0.46 mmol/1.73 m2/24h.

In PH1 the urinary oxalate excretion tends to be higher (2.14±1.29 mmol per 1.73m2 per day) than in PH2 (1.46±0.49mmol per 1.73m2 per day).

Oxalate urinary excretion differs between males and females and is not stabilized until ages 14 to 18 years (Table 2.1).

Table 2.1: Urinary Oxalate levels diagnostic for PH1 in children

Age	**Urinary oxalate [umol/mmol $_{cr}$]**
< 6 months	> 370
6 months - 2 years	> 260
2 – 5 years	> 140
5 - 12 years	> 80

Approximately 66% of individuals with PH1 also have a high concentration of glycolic acids in plasma and urine (hyperglycolic aciduria) . Moreover, an elevated plasma glycerate level may be helpful for distinguishing PH1 from PH2 (Table 2.2).

Table 2.2 Normal Values for Urinary Oxalate, Glycolate, and L-Glycerate Excretion in 24-Hour Urine and Spot Urine Samples. Adapted from . [1]Values are laboratory and method dependent. [2]To prevent alkaline conversion of ascorbate to oxalate in urine, the sample must be strongly acidified to stabilize ascorbate and minimize formation of

calcium crystals .

Parameter	Age	Normal Values [1]
		In 24-hour urine sample
Urinary oxalate excretion [2]	All ages	<0.50 mmol (<45 mg)/1.73 m2/day
Urinary glycolate excretion		<0.50 mmol (<45 mg)/1.73 m2/day
Urinary l-glyceric acid excretion		<5 μmol/L
		In spot urine sample
Spot urinary oxalate-to-creatinine molar ratio [2]	0-6 mos	<325-360 mmol/mol
	7-24 mos	<132-174 mmol/mol
	2-5 yrs	<98-101 mmol/mol
	5-14 yrs	<70-82 mmol/mol
	>16 yrs	<40 mmol/mol
Spot urinary glycolate-to-creatinine molar ratio	0-6 mos	<363-425 mmol/mol
	7-24 mos	<245-293 mmol/mol
	2-5 yrs	<191-229 mmol/mol
	5-14 yrs	<166-186 mmol/mol
	>16 yrs	<99-125 mmol/mol
Spot urinary l-glycerate-to-creatinine molar ratio	< 5 yrs	14-205 mmol/mol
	5-14 yrs	23-138 mmol/mol
	>16 yrs	<138 mmol/mol

In patients with renal insufficiency plasma oxalate and gycolate measurement is preferable. Normal ranges are defined for the specific assay used and are affected by the degree of renal dysfunction and the dialysis (Table 2.3).

Table 2.3: Oxalate and glycolate levels diagnostic for PH1 in ESRD

Renal insufficiency (GFR<60ml/min/1,73m^2)	Plasma oxalate >60 μmol/l	Plasma glycolate>10 μmol/l

Assay of AGT catalytic activity in liver biopsy was considered the gold standard for diagnosis. Now, it has been largely replaced by molecular genetic testing, which is easier to perform and less dangerous for the patient.

Molecular genetic testing of the three genes on blood DNA is now used for confirmation of the clinical diagnosis.
The identification of mutation carriers makes a presymptomatic diagnosis possible and opens the opportunity to address mutation carriers to an appropriate genetic counseling.

2.6- Treatment

Basically, the conservative treatment of PH has not improved over the last 20 years, despite fundamental insights regarding molecular mechanisms and pathophysiology of the disease.

Reduction of calcium oxalate supersaturation. The general therapies for nephrolithiasis benefit all individuals with PH: large volumes of fluid (2-3 L/m^2/24 hours) at regular intervals over the entire day/night prevent calcium oxalate supersaturation . Supplementation of dietary calcium with 300 mg calcium at each meal significantly decreases urinary calcium oxalate without altering calcium excretion . Drugs such as thiazides and potassium citrate or neutral orthophosphates can decrease urinary calcium excretion and inhibit stone formation, respectively .

Enzyme reactivation therapy in PH1. AGT is a pyridoxal-phosphate (PLP)-dependent enzyme. It is well known that specific genotypes (e.g. p.Gly170Arg, p.Ile244Thr and p.Phe153Ile respond to treatment with pyridoxine (vitamin B6, precursor to PLP). Response to treatment is defined by a greater than 30% reduction in plasma oxalate concentration or normalization of urinary oxalate excretion within three months of therapy .

Dialysis. Standard dialysis protocols are not sufficient to reverse, nor even stabilize an affected individual's oxalate burden. Intensified dialysis protocols can be more effective but this intensity of care is difficult to maintain over the long term .

Organ transplantation. Isolated kidney transplantation restores oxalate excretion to "normal" and could be used in all the three types of PH . Isolated kidney transplantation in PH1 is used in individuals with full pyridoxine responsiveness or very slow progression of their disease .

Liver transplant is used in PH1, since it can restore the AGT function (AGT is a liver-specific enzyme). Currently, "preemptive" liver transplantation is used before end-stage renal disease (restores enzyme activity, decreases ongoing oxalate synthesis). Combined liver-kidney transplantation, either concurrent or sequential is used in ESRD patients .

Therapies Under Investigation

- Oxalate-degrading bacteria. *Oxalobacter formigenes*, a normal component of the intestinal flora, is able to degrade intestinal oxalate , thus reducing the amount of oxalate available for intestinal absorption . *O. formigenes* is also thought to stimulate secretion of endogenous oxalate into the intestine for its own metabolic use .

During the past three decades it has been proved that the intestine plays an important role in oxalate homeostasis and that *Oxalobacter formigenes* can regulate oxalic acid absorption in the gut .

Two pilot trials with orally administered *Oxalobacter formigenes* in PH patients demonstrated a reduction of urine or plasma oxalate levels . A multicenter Phase III trial is ongoing in PH patients with EGFR > 40ml/min to determine the efficacy of this bacterium in reducing oxalate excretion (ClinicalTrials.gov Identifier: NCT02012985). One of our Italian patients is enrolled in this study. Another open-label study is beginning, aimed to evaluate plasma oxalate reduction in PH patients already in ESRD (ClinicalTrials.gov Identifier: NCT02000219).

- Hepatocyte transplantation**.** Repopulation of the liver with normal or genetically corrected hepatocytes could correct the disease and it is less invasive than liver transplantation. However, host cells must be ablated as they would continue to produce oxalate and the donor hepatocytes would then require a growth advantage to achieve repopulation. The effectiveness of this approach has been demonstrated in a mouse model of PH1 .

- Gene therapy. Salido et al. demonstrated that hepatic expression of human AGT by adenoviral vector-mediated gene transfer in Agxt-/- mice normalized urinary oxalate excretion and prevented oxalate crystalluria.

- Chemical chaperones**.** The majority of the most common alleles associated to PH1 affect protein folding and stability, suggesting that most PH1 patients may show defective AGT function due to alterations in its protein homeostasis. Salido et al delineated important checkpoints in AGT protein homeostasis, such as the stability of the apo-proteins and the

recognition of folding intermediates by molecular chaperones that might be specifically targeted to restore AGT function in PH1 patients. They showed that several PH1 mutations of the minor haplotype strongly interact with Hsc70 chaperones, and they previously demonstrated that they can correct the mitocondrial mistargeting (in p.G170R mutation) and prevent the aggregation induced by the p.I244T mutation . Hsp70, Hsp60 and Hsp90 chaperone systems are known to cooperate in assisting protein folding and they could be potential targets for correction of the folding defects displayed by PH1 mutants .

- Substrate reduction therapy through RNA-interference. A US company (Dycerna) is developing a RNA-interference based technique that has been shown efficient in AGXT knockout mice and in PH1 monkey model. The first trial in human is expected to begin by June 2015.

2.7- Differential Diagnosis

PH1 accounts for 70-80% of PH cases, while PH2 and PH3 are rarer.

Other subtypes of primary hyperoxaluria termed **non PH1/2 or atypical PH** are increasingly reported, since 10% of patients with a phenotype compatible with PH do not show mutations in the known genes. The pathophysiology of atypical PH is unknown, making therapy and counseling extremely difficult.

- PH must be differentiated from secondary forms of hyperoxaluria where a different underlying disorder causes increased absorption of dietary oxalic acid. Diseases affecting the small bowel, such as celiac disease, Crohn's disease and short bowel syndrome can be associated with hyperoxaluria. The precipitation of enteric calcium by non-absorbed free fatty acids leads to loss of normal inhibition in oxalate reabsorption from the gut, increasing plasma oxalate concentration by increasing paracellular and transcellular transport. Delivery of excess fatty acids and bile salts to the colon also injures the mucosa and increases oxalate absorption . Gastric bypass procedures used in the treatment of obesity have been associated with increased oxalate absorption, pronounced hyperoxaluria, and increased risk of kidney stone formation . The [$^{13}C_2$]oxalate absorption test has proven a valuable tool for differentiation the two conditions.

- Dent disease is a rare genetic renal tubular disease characterized by manifestations of proximal tubule dysfunction (low-molecular-weight proteinuria and hypercalciuria), nephrolithiasis, nephrocalcinosis, and progressive renal failure. Differential diagnosis is based on the presence of hypercalciuria and low-molecular-weight proteinuria in Dent disease.

- Hyperoxaluria has been described in total parenteral nutrition in premature infants and adults and in peroxisomal disorders .

Chapter 3

The Italian database

3.1 - General description of Italian Cases

Since the beginning of PH molecular diagnosis in Italy efforts were made to collect the clinical data of the Italian PH patients. In the last few years an Italian PH database was created to systematically register genetic and phenotypic data for each PH patient. Phenotypic data were collected at onset and at follow up. All the data were transferred to the European data base in 2013.

At the end of 2014 the Italian database includes 80 PH1 patients (28 females and 52 males) and 2 PH2 patient. Median age of the cohort is 22 years old (I-III quartile: 11-35,3). Thirteen patients (16%) have consanguineous parents (for 16 patients (20%) this information is not available). In 30 cases a familial history of PH is reported.

Symptoms at onset are mainly nephrolithiasis (NL - 42 patients – 52,5%) and nephrocalcinosis (NC- 26 patients – 35,2%), as generally reported in PH patients. Three patients were diagnosed at family screening before symptoms development. Median age at onset is age 4 years (0.5-11,5), median age at diagnosis is 11 years old (4-30).
Biochemical measurements at diagnosis were not available for 11 patients. For the other patients it was available the plasma or the urinary oxalate dosage (plasma dosage is more difficult, so it is usually preferred the urinary dosage. Urine dosage is not feasible in ESRF patients). Median plasma oxalate at diagnosis is 130 umol/L (24-187; data not available in 23 patients) and median urinary oxalate is 230 uM/mM creat/24h (151-435,5; data not available in 41 patients). Glycolate dosage is no longer available, so median value were calculated among few patients: median plasma glycolate, available for 27 patients, is 183umol/L(125,5- 366,0) and median urinary glycolate, available for 20 patients, was 319uM/mM creat/24h (184,5-463,0)

Liver biopsy for AGT activity measurement was available for 35 patients (liver biopsy is now substitute by genetic testing, that is more reliable and less invasive). Mean residual AGT activity was negligible in 27 patients and only in 8/35 (22,9%) samples a partial AGT activity was detected (mean activity: 39±13% of the normal).

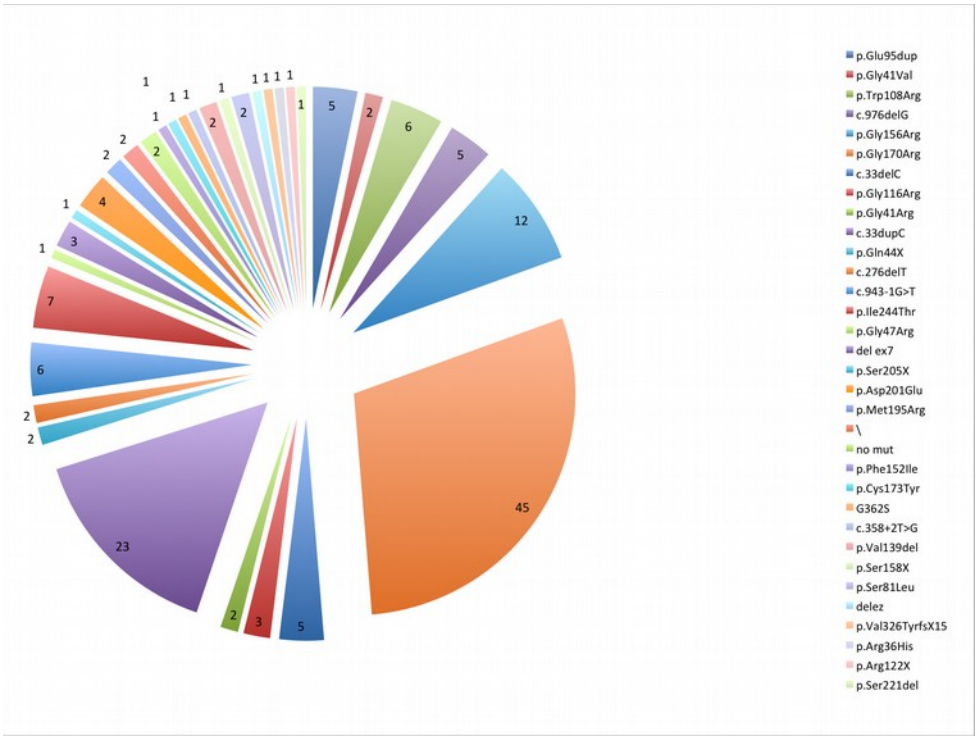
At the end of 2014, 55 patients have had ESRF. Median age at ESRD is 14 years of age (4-31).

Ten patients had a kidney-only transplant and, among them, 2 had a second kidney-only transplant. In other 6 cases the second kidney transplant was combined with liver transplant. Median age at first kidney transplant was 18 years old (10-35).

In 17 patients a combined kidney-liver transplant was performed and in one a sequential kidney-liver transplant. Median age at kidney-liver transplant was 13 years old (4-27). In 2 cases a second kidney transplant was performed after the combined kidney-liver. One patient underwent two combined kidney-liver transplants. In one case a preemptive liver transplant was performed.

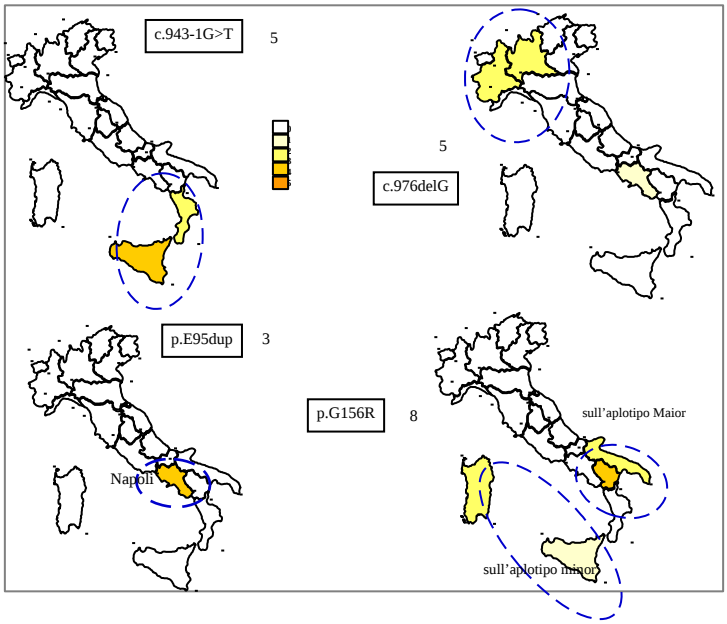
Some recurrent mutations are present (Figure 3.1). As reported in literature, also in the Italian cohort the most common mutation is p.Gly170Arg (28% of the alleles), in 14 patients in homozygosis, in 11 in heterozygosis with a missense mutation and in 4 with a null mutation.

Figure 3.1: AGXT mutations in the Italian cohort



Some mutations are recurrent among not related patients belonging to the same geographic area. Therefore, for these mutations we were able to recognize a founder effect (Figure 3.2).

Figure 3.2: mutation distribution



3.3 - HOGA1 and GRHPR genes analysis

In order to increase the detection rate of mutations in PH patients referred to our Centre we set up the analysis of the entire AGXT (including the promoter), of GRHPR and HOGA1 genes.

HOGA1 and GRHPR analysis is performed by direct sequencing the 5' and 3' UTR, the coding sequence and exons-introns junctions of isoform 1 (HOGA1 RefSeq: NM_138413.3; GRHPR RefSeq: NM_ 012203.1).

Amplimers	Length (bp)	Forward Primer	Reverse Primer
Exon 1	672	**CTGGCTTCAGAGCTGGGAAT**	AGCTGTCCAGGGATAGTCTGG TAC
Exons 2+3	811	**ATGGTTGAGCCACTGCATC**	CACGACATTGGGAGGCTTA
Exons 4+5	744	**AGGTGGCCCTGTAGTGAAGC**	ACTCAGAAACCTGGCCACG
Exon 6	417	**ACAGAGCATGCCTTTGATGTAG**	GAGAGAACATCCCAGTGTG
Exon 7	400	ACTTCAATGTTCTGAAAGTGACAGA	**AGGCAGCCTTTCACAGG**

Table 3.1: *HOGA1* PCR: amplimers length and forward and reverse primers sequences; sequencing primers in bold.

Amplimers	Length (bp)	Forward Primer	Reverse Primer
Exon 1	308	**TCCCTCTCGCGAAGCCACA**	GGCCACAAGGCCCAAACG
Exon 2	375	**CCATCAGAGGCCAGGATTC**	CTGTGCCAGAGCAAGCTATC
Exon 3	410	AGGCCCTGAGGTGAACCC	**CTGAATGGCCGAGGGATATG**
Exon 4	278	**TGGAAATGTCCCAGCAGTAGA**	CTCCTAACCTCTAGATCCGC
Exon 5	252	**GACCTGGAGGGTGGGTTTAT**	AAACCACGCTGTGAGAGCC
Exon 6	260	**CTGTGCTGATGAAAAGGGTCT**	CAAACTGGGCACAGATAGGC
Exon 7	287	CGCCATCTGGTTGTCCCTAG	**CACCAACAACCCACGGG**
Exon 8	274	**TTCATTCTGTAGGTTGTTCTGCC**	CTCAAAAACACTGGTCCTGTC
Exon 9	257	GCTGAAGGCTGCTGAACC	**GATCCAAATCAAGCCTGGG**

Table 3.2: *GRHPR* PCR: amplimers length and forward and reverse primers sequences; sequencing primers in bold.

Twenty-four patients with high clinical suspicion of PH, but negative for *AGXT* mutations type 1 patients referred to our Unit were selected on the basis of their on clinical characteristics (presence of nephrolithiasis and/or nephrocalcinosis, age at onset < 15 years), biochemical parameters (oxalemia > 80 mmol/L, urinary oxalate and glycolate

levels as shown in tab.1, reduction of renal function) and absence of identifiable cause of secondary hyperoxaluria (intestinal malabsorption, inflammatory bowel diseases, etc.). Nine non-PH1 patients were excluded from this study because of a different diagnosis made after *AGXT* testing (four patients were affected with secondary forms of hyperoxaluria, three by kidney malformations, one received a diagnosis of Dent syndrome and one of Pseudo-Bartter syndrome). Fifteen probands were thus included.

All patients or their legal representatives gave informed consent for the present genetic analysis.

The 15 patients analysed (six males - 40% and nine females - 60%) have clinical characteristic comparable with the PH1 cohort: mean age at onset of symptoms is 5,3 years (range: 2 months - 32 years), 74% have nephrolithiasis, 33% nephrocalcinosis and one patient is affected by both (Table 3.3). Renal function was normal in nine; three had already progressed to ESRD (data not available for three patients).

Mean oxaluria calculated on values of eight patients was 103.86 mMol/mMol creatinine (range: 55-209) and 0.92 mMol/24h (normal range: 0.51 - 1.78). In the patients in ESRD state the mean serum oxalate was 15.8 µmol/L (normal range: 1 - 65.2).

According to recent guidelines , even if the *AGXT* genetic test resulted negative these findings supported the suspicion of PH, and prompted for further molecular analysis.

Table 3.3: patient analysed in the study

ID	gender	Age at last FU	Age at onset of sympt.	ESRD at last FU	Urinary oxalate uMol/mMol crea	Urinary Calcium mEq/L	Urinary Calcium Ca/crea	Plasma oxalate uMol/L	Clinical presentation
1	M	44 y	32 y	Yes				80	Bilateral nephrolithiasis
2	F	13 y	9 y	No	70			5,6	Nephrolithiasis
3	M	46 y	3 y	Yes				16	nephrolithiasis
4	M	4 y							nephrocalcinosis
5	F	6 y	6 mo	Yes	106			4	nephrolithiasis, ematuria, urinary tract infection

6	M	4 mo	2 mo	No	366	1,0	5,6		Nefrocalcinosi
7	F	16 y	9 y	No	86	2,1	1,2		nephrolithiasis, urinary tract infection
8	F	3 y	4 mo	No	115			10	nephrolithiasis, nephrocalcinosis, hematuria
9	F	5 y	2 y	No	180				nephrolithiasis
10	M	2 y	6 y	No	205				Bilateral nephrolithiasis, hematuria,
11	M	37 y	7 y	Yes					Nephrolithiasis
12	F	3 y	1 y		190	8,2		6	nephrolithiasis, hematuria
13	M	2 y	14 mo	No	170	2,1	1	9	Nephrocalcinosis and Nephrolithiasis
14	F	13 y	9 y	No		0,8	0,2		nephrolithiasis, urinary tract infection
15	M	1 y	2 mo						nephrocalcinosis, urinary tract infection

The GRHPR and HOGA1 analysis in the 15 patients give a normal result. Heterozygous SNP are reported in the following table

	GRHPR		HOGA1	
ID	**Result**	**Heterozygous SNP**	**Result**	**Heterozygous SNP**
1	WT	rs10973332:C>A	WT	rs11817730:A>G rs7078003:C>T rs2297644:C>T
2	WT	-	WT	-
3	WT	rs35891798:G/- rs10644334:CT(8_9)	WT	-
4	WT	rs10973332:C>A rs10644334:CT(8_9)	WT	rs11527932:G>T
5	WT	rs10644334:CT(8_9)	WT	-
6	WT	rs10644334:CT(8_9)	**UV**	-
7	WT	-	WT	rs11817730:A>G rs7078003:C>T rs297644:C>T rs12261752:A>C
8	WT	-	WT	rs75929214:C>T rs12261752:C>A
9	WT	rs35891798:G/- rs76299266:G>A	WT	rs12261752:C>A
10	WT	rs10644334:CT(8_9)	WT	rs75929214:C>T rs2297643:A>G rs12261752:C>A
11	WT	-	WT	rs2297643:A>G rs115279832:G>T rs12261752:C>A
12	WT	rs35891798:G/- rs10644334:CT(8_9)	WT	-
13	WT	-	WT	rs75929214:C>T
14	WT	-	WT	rs75929214:C>T rs74155508:G>C
15	WT	-	WT	rs11817730:A>G rs75929214:C>T rs7078003:C>T rs2297644:C>T

Patient n°6 is homozygous for the *HOGA1* variant c.341-81delT in intron 2 (Fig. 3.3). The presence of *HOGA1* variant was validated by bidirectional resequencing of an independent PCR product and described according the Human Genome Variation Society guidelines.

A)

AGAGGATGGGAAGGAGTTRGTCAGCTGTGTCTCTTGCAGGCTTCGTGGTCCAGGGCTCCA esone 2

ATGGYGARTTTCCTTTCCTGACCAGCAGTGAGCGCCTCGAGGTGGTGAGCCGTGTGCGCC

AGGCCATGCCCAAGAACAGGCTCCTGCTAGCTGGCTCCGGATGCSAGTGTGAGCCAGAAT

intron 2

GCCCTGGGCCCTGSGGGTGGGTGGATGTGCAGGATCCAGGCTCCTAGGCCCTAGCTTGGG

TCCTGTCTCCTTGTTCTGCYTCTTCTGGGACATGCTGAGGCACCTTCGCTTGGCCCAGTG

TCCTGGTCCAGGCCTCCTTCTGCCTGCTCTCACCTCTCTCCTTCCTCTGGCAGCCACTCA exon 3

AGCCACAGTKGAGATGAYCGTCAGCATGGCCCAGGTCGGGGCTGACGCRGCCATGGTGGT

GACCCCTTGCTACTATCGTGGCCGCATGAGCAGYGYGGCCCTCATTCACCRCTACACYAA

GGTGTGTGTGAGGCCTGAGACCAAGAGGAGGCTCTGCCCAGGGAGAGGAGATAAGGGAAG

intron 3

B)

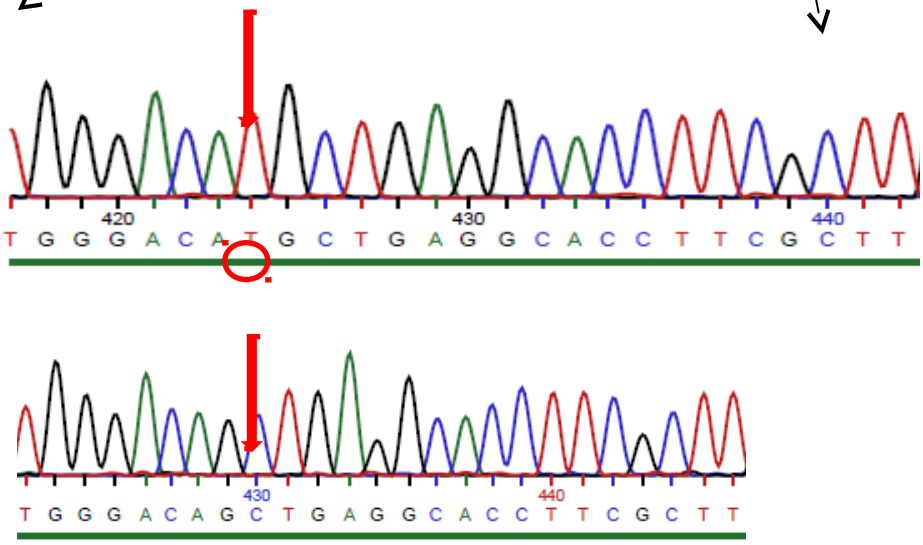


Figure 3.3: c.341-81delT variant in HOGA1 intron 2

- HOGA1 sequence: exon 2, intron 2, exon 3. Common SNP are highlighted in green
- Chromatograms of the wild-type (top) and c.341-81delT deletion (bottom).

The c.341-81delT variant isn't reported in literature in PH patients nor in the 1000Genomes populations (http://www.1000genomes.org).

Intron 2 sequence is conserved through primates but not in other species (http://genome.ucsc.edu/cgi-bin/hgc) (Fig. 3.4).

Functional enzyme analysis is not possible: the patient died at 4 months of life because of respiratory failure.

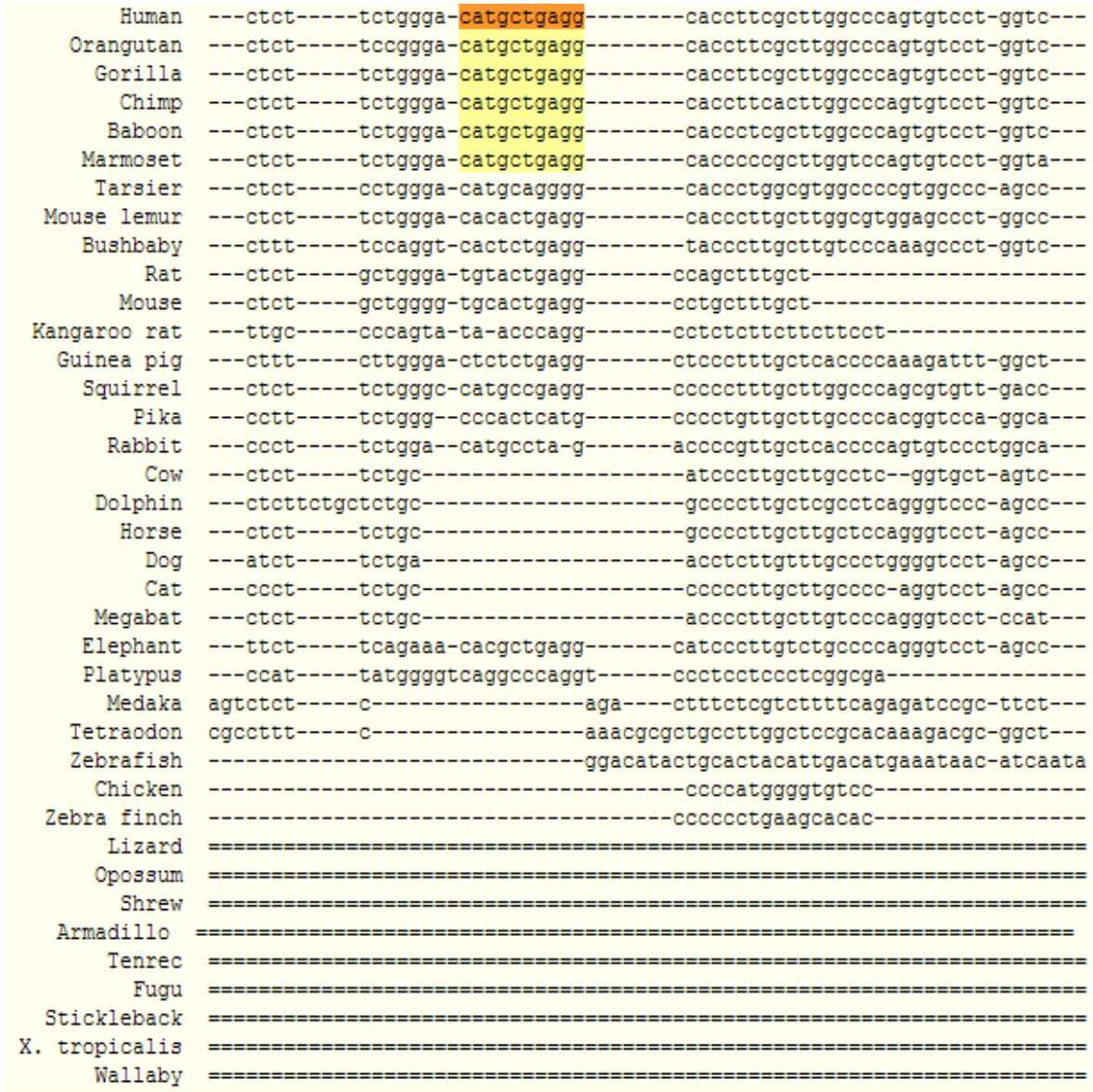


Figure 3.4: HOGA1 Intron 2 evolutionary conservation.

In silico analyses postulate that the T deletion in c.341-81 generates an acceptor splice site (AG in c.341-79), possibly recognized by the spliceosome; moreover, the upstream sequence, rich in T and C, is similar to the 3' consensus sequence (Fig. 3.5).

AGGCCATGCCCAAGAACAGGCTCCTGCTAGCTGGCTCCGGATGCCAGTGTGAGCCAGAAT
GCCCTGGGCCCTGGGGGTGGGTGGATGTGCAGGATCCAGGCTCCTAGGCCCTAGCTTGGG
TCCTGTCTCCTTGTTCTGCCTCTTCTGGGACATGCTGAGGCACCTTCGCTTGGCCCAGTG
TCCTGGTCCAGGCCTCCTTCTGCCTGCTCTCACC**TCTCTCCTTCCTCT**GGCAG**CCACTCA**

Figure 3.5: in black: HOGA1 intron 2 sequence; in pink: part of exons 2 e 3. c.341-81 is highlighted in red, the putative new acceptor splice site in yellow, in green: normal donor

and acceptor splice site. In light blue: the normal consensus sequence, in grey the C-T reach sequence, similar to the normal consensus sequence

The following table summarizes the probability scores of the new splice site recognition.

Software	Range values	Score	Probability
SSF	0-100	78,17	78,17%
Max Ent	0-16	7,95	49,7%
NN Splice	0-15	< 0,4	< 2,6%
Gene Splicer	0-15	6,59	43,9%
HSF	0-100	82,5	82,5%

Four out of five software predict a possible damaging role of the variant; the alternative splicing is predicted to produce a transcript 79 bp longer (r.340_341ins341-79_341-1), which includes part of intron 2. This could lead to a shift in the reading frame, responsible for the production of a truncated protein (premature stop codon at the beginning of exon 3: p.Thr115Glufs*43) (Fig.3.6).

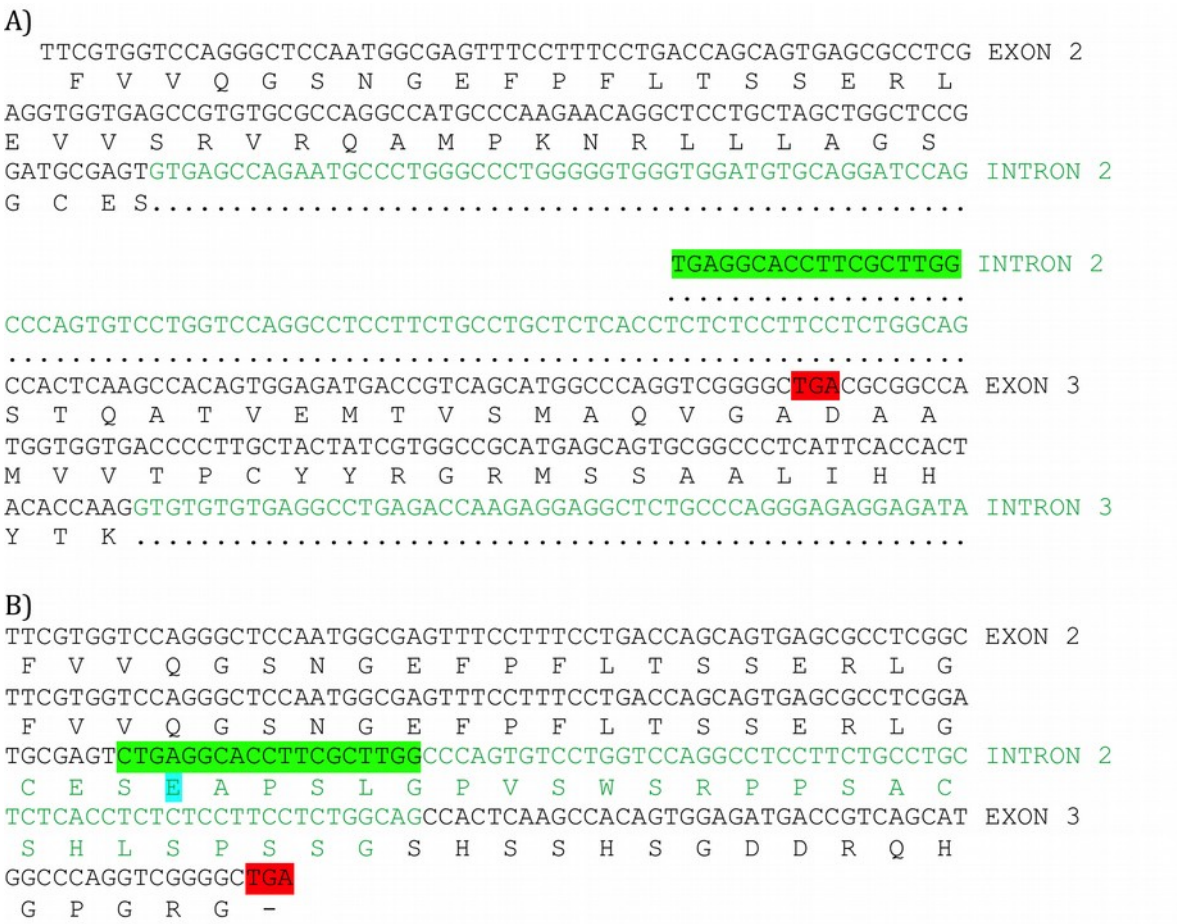


Figure 3.6: A) Wild type nucleotidic and aminoacidic sequence;

B) nucleotidic and aminoacidic sequence with the frameshift; in green: translated part of intron 2, in red: stop codon.

The c.341-81delT variant isn't predicted to alter HOGA isoforms 2 and 3, because these isoforms didn't includes exons 2 and 3 translation. These two isoforms are likely not functional.

In order to clarify the role of the c.341-81delT variant we decided to set up an in vitro approach, using a previously described technique (Cavalieri S. et al, Eur J Hum Genet 2012). With the collaboration of Alessandra Cuccurullo, BS and Prof. Brusco a minigene assay was set up to test the effect of the HOGA1 c.341-81delT variant on splicing.

We amplified a 449-bp genomic fragment from Patient 6 and a wild type control, using the following primers Forward: 5'-ACTGGAATTCGTGGGTGGATGTGCAGGAT-3'; Reverse: 5'-GGTCAGGTAAGCCTCCCAAT-3'. PCR products were cloned into a TA-vector (pGEM-T Easy Vector - Promega, Madison, WI, USA) (Fig.5) and transformed in DH5a bacterial cells, following the manufacturer's recommendations (RBC Bioscience, Chung Ho City, Taiwan).

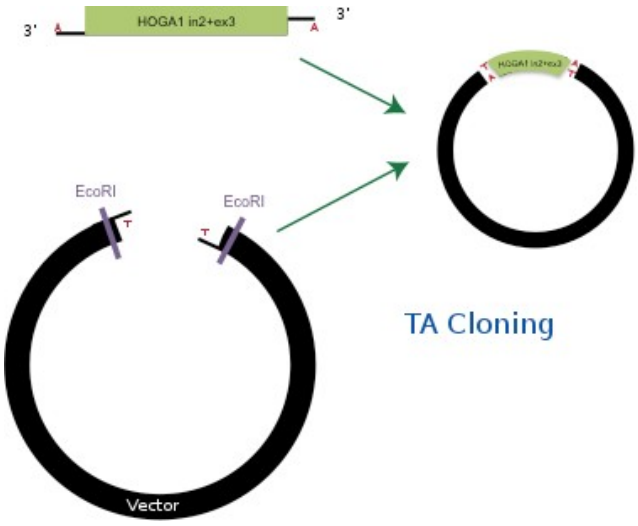


Figure 3.7: scheme of the TA vector.

Plasmids containing the wild type or the variant sequence were extracted using the PureYield plasmid system (Promega) and sequence checked. After preparative digestion with EcoRI restriction endonuclease, inserts were sub-cloned into a pSPL3 exon-trapping vector (Life Technologies, Paisley, UK) (Fig. 3.8). The insertion orientation was checked

by PCR using a Forward primer designed on pSPL3 together with each of the above mentioned PCR primers; we selected the plasmids with the correct orientation (amplified with the PCR reverse primer).

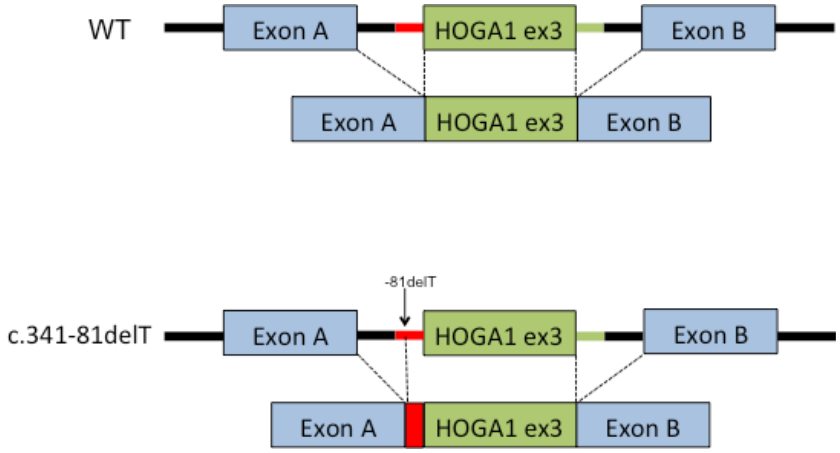


Figure 3.8: sPSL3 expected splicing. Fragments of the human HOGA1 gene containing exon 3 were cloned into the splicing vector pSPL3 generating plasmids pSPL3-HOGA1-Exon3-WT and pSPL3-HOGA1-delTExon3. The blue boxes mark pSPL3 exons A and B (derived from the B-globin gene) with the adjacent black lines depicting the flanking intronic sequence of the pSPL3 exons. The green box marks HOGA1 exon 3. The red box represents the 79bp insertion expected from the generation of a novel splice-site by the c.341-81delT variant. The interrupted black lines mark the spliced out intronic sequences.

pSPL3 plasmids containing the wild-type or the variant sequence were extracted using PureYield Plasmid Midiprep System (Promega), and transfected into COS and HEK293 cells using the TurboFect kit (Fermentas, Vilnius, Lithuania). After 24h, total RNA was extracted and retrotranscribed with the Cells-To-CT kit (Life Technologies). The cDNA was sequenced using PCR vector primers (Fw: 5'-TCTGAGTCACCTGGACAACC-3'; Rv: 5'-ATCTCAGTGGTATTTGTGAGC-3'). The expected final products were (Fig. 3.9):
1- a 263bp band resulting from the complete skipping of the inserted exon, due to the higher power of the vector splicing sites.
2- a 391bp band, resulting from the canonical splicing of *HOGA1* exon 3
3- a 471bp band, resulting from the alternative splicing generated by the deletion of T in c.341-81

1) Vector exons A+B (263bp)
TCTGAGTCACCTGGACAACCTCAAAGGCACCTTTGCTAAGCTGAGTGAACTGCACTGTGACAAGCT
GCACGTGCTCTAGAGTCGACCCAGCAACCTGGAGATCTCCCGAGGGGACCCGACAGGCCCGAAGGA
ATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTTCGACCAATTCACTCCTCAGGTGC
AGGCTGCCTATCAGAAGGTGGTGGCTGGTGTGGCCAATGCCCTG**GCTCACAAATACCACTGAGAT**
2) WT (391bp)

TCTGAGTCACCTGGACAACCTCAAAGGCACCTTTGCTAAGCTGAGTGAACTGCACTGTGACAAGCT
GCACGTGCTCTAGAGTCGACCCAGCACCACTCAAGCCACAGTGGAGATGACCGTCAGCATGGCCCA
GGTCGGGGCTGACGCGGCCATGGTGGTGACCCCTTGCTACTATCGTGGCCGCATGAGCAGTGCGGC
CCTCATTCACCACTACACCAAGACCTGGAGATCTCCCGAGGGGACCCGACAGGCCCGAAGGAATAG
AAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTTCGACCAATTCACTCCTCAGGTGCAGGC
TGCCTATCAGAAGGTGGTGGCTGGTGTGGCCAATGCCCTG**GCTCACAAATACCACTGAGAT**

3) delT (471bp)

TCTGAGTCACCTGGACAACCTCAAAGGCACCTTTGCTAAGCTGAGTGAACTGCACTGTGACAAGCT
GCACGTGCTCTAGAGTCGACCCAGCACTGAGGCACCTTCGCTTGGCCCAGTGTCCTGGTCCAGGCC
TCCTTCTGCCTGCTCTCACCTCTCTCCTTCCTCTGGCAGCCACTCAAGCCACAGTGGAGATGACCG
TCAGCATGGCCCAGGTCGGGGCTGACGCGGCCATGGTGGTGACCCCTTGCTACTATCGTGGCCGCA
TGAGCAGTGCGGCCCTCATTCACCACTACACCAAGACCTGGAGATCTCCCGAGGGGACCCGACAGG
CCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTTCGACCAATTCACTC
CTCAGGTGCAGGCTGCCTATCAGAAGGTGGTGGCTGGTGTGGCCAATGCCCTG**GCTCACAAATACC**
ACTGAGAT

Figure 3.9: sequences of the expected products. Vector primers are highlighted in bold. The insertion point between the vector exons A and B is highlighted in blue. HOGA1 ex 3 is highlighted in green; in red the 79bp insertion expected from the generation of a novel splice-site by the c.341-81delT variant.

Electrophoresis of cDNA products was performed on a 2% agarose gel and demonstrated the presence of two bands at 263bp and 391bp; the expected band at 471bp wasn't present. These showed no differences between post-transfection wild type and deleted spliced products (Fig. 3.10)

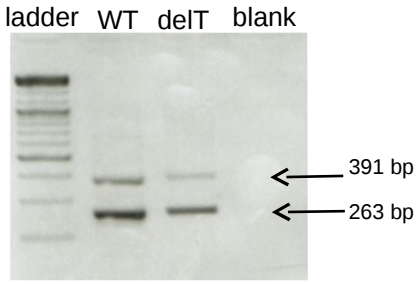


Figure 3.10: 2% agarose gel electrophoresis of the wild type and delT products.

3.3 - AGXT promoter analysis

Sato et al described a functional AGXT promoter (with TATA boxes and INR initiator elements) in the 5'-flanking region of human AGXT.

The analysis was set up by Sanger sequencing and primers were designed on the sequence of chr2:241806700-241808285 (NCBI built GRCh37, hg19). Four amplimers were designed.

Amplimers	Length (bp)	Forward Primer	Reverse Primer
Amplimer 1	426	**TGAGCATGGCAGAAAAGGAA**	TCCTCCCTCCTGAGGCTGTAC
Amplimer 2	395	**AAGTACCTCAAACAGGTCTCAA**	ATTTTCTCTCTAAATCCCGCTACC
Amplimer 3	542	**TTTGATTGGCAACTGACTGAAGA**	ACGTCCAGCCCCTCTTATTCT
Amplimer 4	486	CAGGGTCAGCAAACCACAGT	**TGGCCAAAGCCTGGAATAT**

Table 3.4: *AGXT* promoter PCR: amplimers length and forward and reverse primers sequences; sequencing primers in bold.

The genomic sequence was analysed in 4 amplimers as shown below:

AAAGGGAAAAACGTGACCTACCCCCCTGAACTCCCTTTCAGCAGCAAGTAGTCAGGCGCACTGGAC
ACCCCTCTTCACTGTGCCGTTTCCCCTTTCTTGAGACCCCAACAGGAAGCAGGTGGACATGAGCAT
GGCAGAAAAGGAAGGGTCAAAGATTTCACCAAGATATTTGTCAGAGGGAAAATGAGGACAGCAAAG
ATCACCTGGAGACCATCAAGCAGGCCCCAGGTGGGGTGGGTGGAGGGGAGCCTCTTTATTTGAGAA
ATGTAGAATGAATTAGACTTCCCTATTATCTAAAGTTGGCACCTGGTTCCAGGCCTCTTTTCAACC
TAAAACTTATACATAACTAGAATTTCTATACATCTCTGGAATGCAGCATGCTGAAACTCACTGTGC
AACCCCTGCCTTGTGTGTGTCTGTATACCATGCAGGTTTGCTTGCTAAATTGAGCCTGTAAACCAA
AAAGTACCTCAAACAGGTCTCAATTAATTTAGAAAGTTTTTGTTGCCAAGGCTAAGGATGCACCCG
TGGTACAGCCTCAGGAGGGAGGACATCCTTGCCAAGCTCAAGGGTGCACCCCTGACCACAAGGGCC
CAAGGTGGTCGGGGCACAGCTTGCTTTTATACATTTTAGGGAGATATGAGGCATCAATCAATACAT
GGGAGATTTACATTGGTTCCATCTGGAAGGGCGGGACAACTTGAATTGGGGGGCTTCCAGGTCATA
GGTAGATTTAAACATATTTTGATTGGCAACTGACTGAAGAATTATTATCAGCAGAAAGGAGTGTCT
GGGTTATGAAATAGGGGTTGCAGGGAGCAGATGATGCCTTCAGGTAGCGGGATTTAGAGAGAAAAT
AGATGGTAAATGCTTCCTATCAGACTTAAGGTCTATGTCGATGTTAATGCTGGGGGTTATGATGTG
GCATGGCCTGAACGTCTTCCGGGTTAAAGTTTAGAGCACCCTGGCGGACAGGGAACCCCATTCAGA
TGGTTGCTGGGGGGCCTTCGAATTTTATTTTTGGTTTACAAGCCTTCCCATCAGCTGCGCCAGGGT
CAGCAAACCACAGTCCATGGGCCCAATCTGGCCTGCCACCTGCTTTTTGTAAATAAAGTTTTATAG
GAACACGCCCACACCTCTGGCAGCTGCTTTGCAAGCACGCAGCAGGGTTGAGTTGTTGCAACATAG
ACCACAGGGCCTGCCAAGCCTCAATTATCTGTGCTCCGACCCTTTAAGAAACACTTCTCTCACCCC
TGAGCTAAGCAGAATAAGAGGGGCTGGACGTGCAGGACTCAGAGTGGGAGCGAGGAGGGCTGGGGT
GAGGACAGCTTTGTCACACTCTGTTTTTCTGTCACTCAGCCCTGGGGGCTCCCTCTGCCTGAGGGC
TTCGTCCCCTGCTGCCTGGCCGTGACCCGCAGCACAAGCACAGATAAGCCTCAGGGAACAAAAGGC

AGGGCTGCCA**CGGAAGCCCATCCACCAATCCTCACCTCTCACCTCTGTGTCCGCCCTGCTGGGAAA**
TATTCCAGGCTTTGGCCAAGGCCAGTGCAGCCCCAGGTTCCCGAGCGGCAGGTTGGGTGCGGACCA
TG

DNA sequence of chr2:241806700-241808285 (NCBI built GRCh37, hg19); the 5'UTR is highlighted in bold; in purple the ATG. The four primer pairs are highlighted in grey, red, green and yellow. In light blue

Sequencing of the *AGXT* promoter in the above mentioned 15 patients revealed in two of them two heterozygous novel variants: c.-647C>T and c.-424C>T. Neither variant was reported in dbSNP141, 1000Genomes (http://www.1000genomes.org), EVS (Exome variant server). We considered these variants not to be pathogenic since they do not lie in any known regulatory site. The validity of this conclusion is limited by the scarce available literature on the *AGXT* promoter that precludes comparison with more studies.

Chapter 4

Primary Hyperoxaluria in Europe

4.1- The OxalEurope Group

OXALEurope, the European Hyperoxaluria Consortium was founded in 2008 (www.OxalEurope.org) in order to bring together expert European clinicians and scientists involved in patient care and the study of the molecular pathology of primary hyperoxaluria.

The aims of the present consortium are:
- To set up a database of primary hyperoxaluria patients to allow study of the natural history of the diseases
- Empower research and retrieve patients through a network of experts
- Assessment of best treatment options

Currently, 17 University Hospitals or research institutes from 8 European countries (France, Germany, Italy, The Netherlands, Poland, Spain, Sweden and the United Kingdom) are members of the Consortium, which has its base at the University Hospital of Cologne, Germany.

The first step was the development of the European PH registry, which is the basis for research projects and could be a fundamental resource to feed the international liaison PH database, in which the European and the US based Mayo Clinic Hyperoxaluria database may join.
OxalEurope members obtained IRB approval for participation.

4.2- The European Registry

This is the largest database of PH1 patients available currently, through pan-European collaboration of centers of expertise for PH, some of which serve as diagnostic referral centers for other non-European countries.
The earliest diagnosis dates from 1980 and the database was frozen on 31 December 2010 for analysis. It comprises clinical, genetic and biochemical information from 526 patients who fulfilled the following diagnostic criteria of PH1:
a) mutations on both AGXT alleles;
b) reduced AGT catalytic activity;
c) hyperoxaluria with hyperglycolic aciduria as defined by age-related reference ranges ;
d) 'other' category included plasma oxalate >100 umol/L; raised plasma glycolate ; a single mutant allele with associated hyperoxaluria and clinical symptoms; or presence of abundant oxalate crystals in a renal biopsy.

The results of the preliminary analysis on the OxalEurope database were published by Mandrile et al. .
Patients and methods
Median follow-up was 11.5 years (I-III quartiles: 4.2-20.2). Family screening identified 74 patients (14%) with a median time to follow-up of 12 years (I-III quartiles: 6.9-18.6). Complete genotype data was available for 410 patients. Urinary oxalate results were not included due to difficulties harmonizing submitted data. Time to follow-up was defined by the interval from date of symptoms to date of last clinical visit or date of death. Renal survival was calculated by the Kaplan-Meier method in a multivariate analysis adjusted for age and gender, with patient data censored at last follow up.
Pyridoxine responsiveness was recorded according to the clinician's report.
More than 140 AGXT mutations have been documented , resulting in a vast number of defective enzyme phenotypes; the "Primary hyperoxaluria mutation database" is available at:www.uclh.nhs.uk/OurServices/ServiceA-Z/PATH/PATHBIOMED/CBIO/Pages/Phmdatabase.aspx

Mutations are spread along all the exons and most mutations are found in a wide range of ethnic groups. In the genotype-phenotype analysis we grouped genotypes in three classes according to the expected effect on the protein product (Figure 4.1):
1. p.Gly170Arg (G170R): associated with mitochondrial mistargeting when in cis with the minor allele
2. Other missense (M): missense and in frame ins/del that are unlikely to cause complete

lack of protein production.
3. Null (N): nonsense, frameshift and splice-site mutations, assumed to produce no protein product.

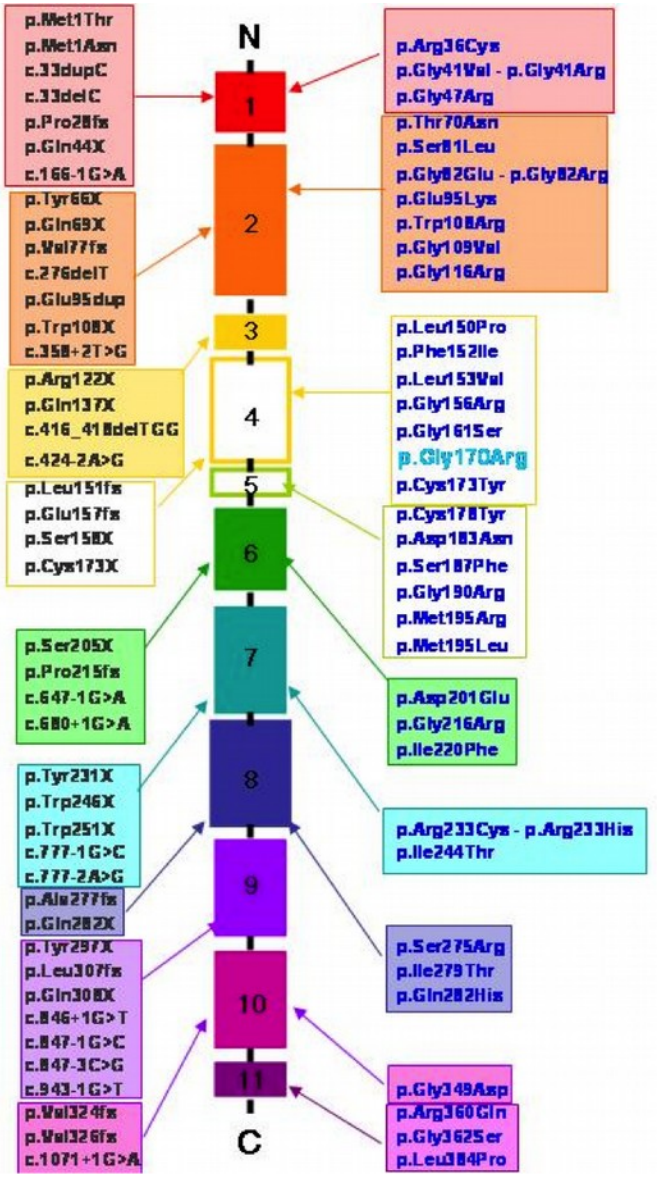


Figure 4.1: distribution of AGXT mutations; blue character indicates Missense mutations, black null mutations, light blue mistargetting mutation.

The effect of different genotype classes on time to ESRD was estimated using Cox proportional hazards models with adjustment for gender. Proportional hazard assumption was checked using the Grambsh and Therneau test. Continuous variables were expressed as median and I-III quartiles as measure of variability; categorical variables were expressed as percentages and absolute numbers. F Test was used to evaluate clinical variables among genotype groups. Median times at ESRD and at symptoms were computed for each

genotype using the method of Kaplan-Meier and differences were assessed by the log-rank test.

All statistical testing was conducted at the 0.05 level. PASW Statistics (version 20) and R (version 2.13) statistical packages were used for analysis.

Clinical characteristics

The most frequent symptom at onset was urolithiasis: nephrocalcinosis and/or urolithiasis occurred in 70% of patients (Table 4.1). Infantile onset of symptoms (<1 year) occurred in 29% (36.6% with ESRD at diagnosis) whereas 15% of patients experienced the first manifestation in adulthood (>18y). Median age at diagnosis was 8y (interquartile range: 2.6-22.2) and 28% of patients were diagnosed beyond 18y. The clinical characteristics of OxalEurope patients differ from those of the US registry data : the median age of first symptoms was 3.9y compared with 5.5y; median age at diagnosis was slightly younger (8.1y compared to 10y). While urolithiasis was the most common presenting symptom, it was less common overall than in the US study where it accounted for 90% of presenting symptoms. We analyzed PH1 patients exclusively, contrary to the US study that included all types of PH, and may explain the different age of onset of symptoms between the studies, whereas the relatively low number of patients presenting with kidney stones or nephrocalcinosis may be due to the relatively high number of patients found by family screening.

ESRD at diagnosis was present in 195 of 451 OxalEurope patients (43%, data not available for 75 patients) at a median age of 31.2y (range: 11.5–40). Of patients diagnosed in adulthood, 70% were in ESRD at the time of diagnosis, versus 29% of patients diagnosed in childhood. These data indicate a considerable diagnostic delay; overall, 43% of patients presented in ESRD in contrast to 20% reported in the US study although the median ages of US patients were slightly lower (23y vs 31.2y in this study). ESRD was also the presenting feature in 30% of children. Late diagnosis may depend on several factors. Firstly, detection of hyperoxaluria by metabolic analysis is less common in adult than in pediatric practice. Secondly, some patients may only experience occasional stone passage that may not be recognized as a symptom of primary hyperoxaluria. Thirdly, delayed diagnosis may be due to cumbersome biochemical procedures (collection of urine, shipment of specimen) and the multisystem character of PH1 in advanced disease.

Table 4.1. Clinical characteristics of PH1 patients and relatives in the OxalEurope cohort. Number of patients/number of available data are given in brackets. For continuous variables: I quartile / median value / III quartile; range of values in brackets (last column).

		N	Range (years)
Gender:			
males	57%	(300/526)	
females	43%	(226/526)	
Diagnosis based on:			
Genetic and enzyme analysis	37.2%	(196/526)	
Genetic analysis alone	47.2%	(248/526)	
Enzyme analysis alone	8.2%	(43/526)	
Urine analysis alone (oxalate, glycolate)	0.9%	(5/526)	
Other (e.g renal biopsy, high plasma oxalate)	6.5%	(34/526)	
Symptoms:			
asymptomatic	4%	(19/526)	
urolithiasis	32%	(171/526)	
nephrocalcinosis	17%	(88/526)	
urolithiasis and nephrocalcinosis	21%	(111/526)	
other (urinary tract infection, hematuria, failure to thrive)	8%	(46/526)	
recurrence post renal transplantation	3%	(14/526)	
unknown	15%	(77/526)	
Age at first symptoms:	0.7 3.9 9.3	(calculated on 446 pts)	(0-66)
in patients presenting with urolithiasis	1.9 4.6 11.7	(calculated on 262 pts)	(0-66)
in patients presenting post renal transplantation	6.8 15.0 27.0	(calculated on 12 pts)	(6-45.4)
Symptoms < 1 year	29%	(131/446)	
Age at diagnosis	2.6 8.1 22.2	(calculated on 456 pts)	(0-72)
Diagnosis ≥ 18years	28%	(128/456)	
ESRD at diagnosis	43%	(195/451)	
ESRD at follow up	58%	(267/458)	
Death	14%	(67/477)	
Age at death	2.35 15.5 33.9	(data missing for 3 pts)	(0.3-73.8)
Relatives (74 patients)			
Age of first symptoms in relatives	0.3 3.9 7.1	(calculated on 58 pts)	(0.3-7.1)
Age of diagnosis in relatives	1.6 6.8 16.0	(calculated on 72 pts)	(1.59-16)
Age at ESRD in relatives	11.5 23.0 34.4	(calculated on 22 pts)	(11.5-34.4)

The cumulative patient survival was 95, 93, 85 and 74% at 5, 10, 30 and 50 years of age respectively. Sixty-seven (14%) patients died. Sixty-four (96%) were in ESRD.
Among the 256 patients with preserved renal function at diagnosis (56%), 74 developed

ESRD thereafter (29%). At last follow-up, 267 patients were in ESRD (58%), with data on renal function missing for 67 patients (Table 1).
Progression to ESRD was slightly, but not significantly, earlier in males than in females (log-rank test p-value: 0.155; mean age at ESRD in males: 24.3; I-III quartile: 20.3-31.3; females: 27.2; I-III quartile: 23.5-33.5).

Clinical features in 74 patients diagnosed by family screening

Median age at diagnosis was 6.8y (I-III quartile: 1.6-16). At diagnosis, 18% of these patients were asymptomatic; renal manifestations included urolithiasis 31%, nephrocalcinosis 12%, or both 24%, other symptoms 8% (recurrence post renal transplantation 1%), unknown 5%. ESRD at diagnosis occurred in 16 % vs 43% of index cases. At the end of follow-up, ESRD had occurred in 34% vs 58% of index patients. Overall, median age at ESRD in the relatives was 23y (I-III quartile: 11.5-34.4). Median ages at onset of symptoms, at diagnosis and at ESRD were not statistically different from index cases (p 0.78, 0.78 and 0.71 respectively).

Genotype-phenotype correlations

Our data confirmed the majority of known geno-phenotype associations, but grouping AGXT mutations in three genotype classes allowed us to found a difference, not previously described, in clinical outcome with respect to onset of ESRD associated with different genetic subclasses.
Not surprisingly, the most common pathological variant in our cohort was the G170R mutation accounting for approx 40% of alleles, with patients homozygous or compound heterozygous having a more favorable outcome particularly in terms of pyridoxine responsiveness, consistent with results of previous studies .

Phenotype correlation of AGXT mutation classes

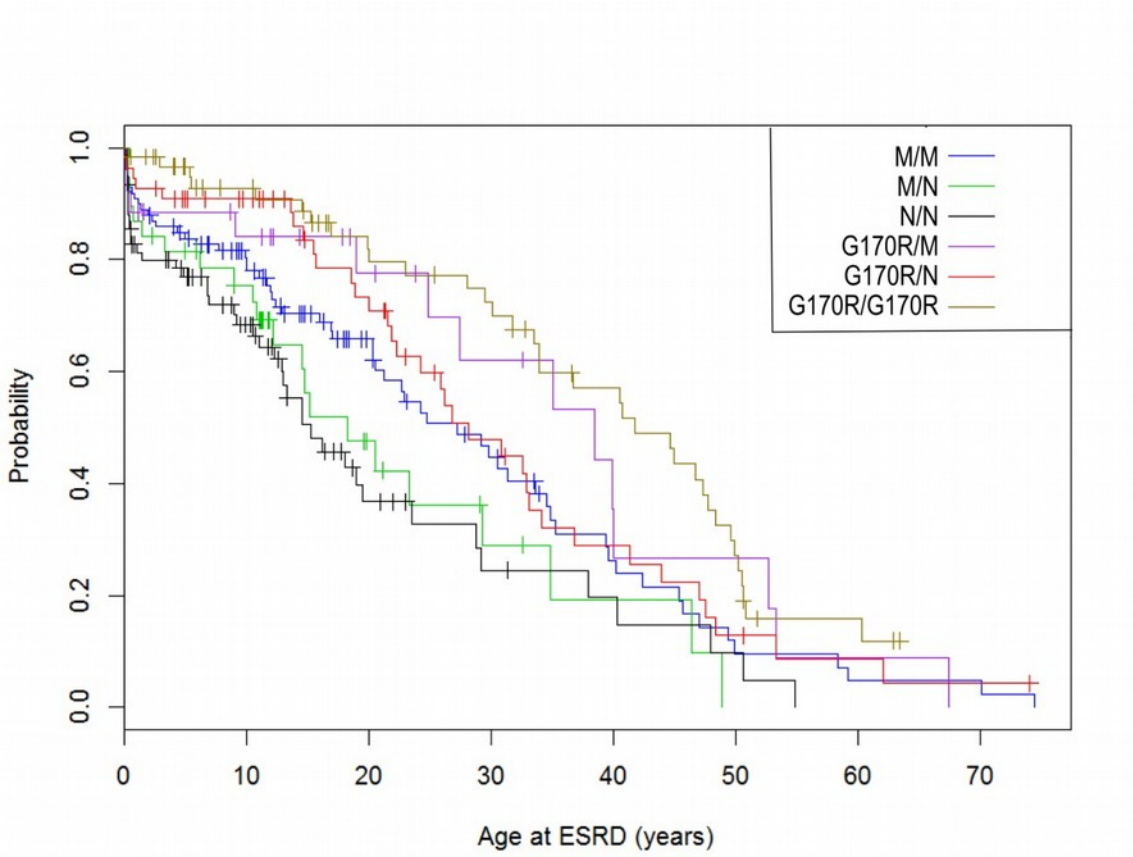
Among the clinical variables considered, age at onset of symptoms, age at ESRD and AGT activity in liver biopsy were significantly different among the genotypes (Table 4.2).
Onset of symptoms occurred later in G170R homozygotes (median: 6y) and was intermediate in G170R/N compound heterozygotes (median: 4y) compared with the lowest values of N/N homozygotes (median: 3y) and M/N heterozygotes (median: 2y) (Table 4.2).

Table 4.2. (following page) Main clinical features for each AGXT mutation class genotypes. Number of patients given in brackets. For continuous variables: I quartile / median value / III quartile. p-value is calculated among all groups; all statistical testing was conducted at the 0.05 level. Tests used: 1Pearson test; 2Kruskal-Wallis test

		G170R/G170R (71)	G170R/M (32)	M/M (116)	G170R/N (61)	M/N (42)	N/N (88)	p
Gender:	F	48% (34)	28% (9)	41% (47)	33% (20)	52% (22)	43% (38)	0.176[1]
	M	52% (37)	72% (23)	59% (69)	67% (41)	48% (20)	57% (50)	
Age at diagnosis		6.4 29.9 46.1	2.1 8.4 25.7	1.8 7.7 17.9	4.3 16.1 34.7	1.8 6.0 16.7	1.0 5.7 11.2	<0.001[2]
Age at onset of symptoms		0.9 6.2 22.1	0.6 3.5 10.27	0.9 3.5 7.8	1.6 4.5 12.4	0.5 2.0 5.3	0.5 3.0 5.6	0.023[2]
Symptoms:	Asymptomatic	8% (5)	0% (0)	6% (6)	0% (0)	0% (0)	8% (6)	0.02[1]
	Urolithiasis	48% (30)	37% (10)	36% (36)	57% (31)	31% (11)	26% (19)	
	Nephrocalcinosis	11% (7)	19% (5)	16% (16)	17% (9)	19% (7)	28% (21)	
	Urolithiasis AND nephrocalcinosis	23% (14)	26% (7)	32% (32)	9% (5)	28% (10)	26% (19)	
	Other (urinary tract infection, hematuria, failure to thr ive)	10% (6)	15% (4)	6% (6)	13% (7)	14% (5)	8% (6)	
	Recurrence post renal transplantation	0% (0)	4% (1)	3% (3)	4% (2)	8% (3)	4% (3)	
Age at ESRD		19.9 33.9 47.5	11.5 31.2 39.9	3.4 16.9 34.1	15.5 25.1 33.8	1.9 11.5 19.9	0.4 9.9 17.6	<0.001[2]
ESRD at diagnosis		49% (30)	35% (9)	41% (40)	52% (28)	46% (18)	42% (32)	0.622[1]
ESRD developed during follow-up		56% (35)	54% (14)	55% (56)	62% (34)	57% (23)	57% (43)	0.976[1]
AGT in liver (% activity)		12.7 33.0 43.0	6.2 13.0 18.0	0.0 3.3 9.0	5.4 6.5 16.0	0.0 2.3 7.3	0.0 3.6 6.9	<0.001[2]

Pyridoxine	No response	40% (19)	47% (9)	43% (29)	52% (24)	67% (18)	54% (27)	0.104[1]
	Response	60% (28)	47% (9)	54% (36)	43% (20)	33% (9)	36% (18)	
	Not treated	0% (0)	5% (1)	3% (2)	4% (2)	0% (0)	10% (5)	

Figure 4.2: censored Kaplan-Meier curves for age at onset of ESRD for each genotype class. Data was available for 355 patients, log-rank test p-value: <0.001.



Renal survival by censored Kaplan-Meyer analysis (Fig. 4.2) showed that having at least one G170R allele was also associated with a slower progression to ESRD (33.9 years in G170R/G170R, 31 years in G170R/M and 25 years in G170R/N heterozygotes) compared with N/N homozygotes (9.9 years). M/N heterozygotes (11.5 years) and M/M homozygotes (16.9 years) had intermediate values; such stratification of the ESRD risk was confirmed by gender-adjusted Hazard ratios (Fig. 4.3). However, 5/70 G170R homozygotes progressed to ESRD early (before 10 y), possibly reflecting suboptimal clinical management, presence of unidentified comorbidity, environmental factors, other possible genetic modifiers, or a deletion/gross rearrangement of the second allele that may remain undetected if only the index case is analyzed.

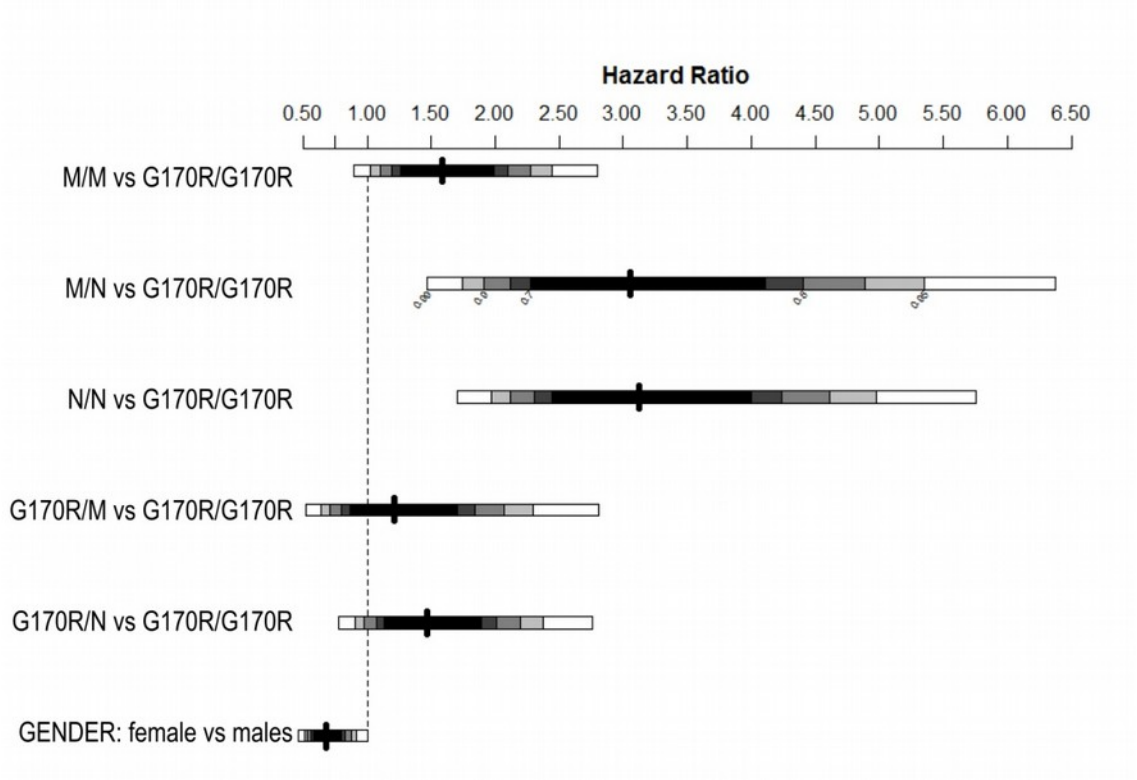


Figure 4.3 Hazard Ratios of age at onset of ESRD adjusted for gender (estimated with Cox Model). Reference group is G170R homozygotes. The following confidence levels are all shown: 0.9, 0.95, and 0.99, using different tone of grey (from darkest to lightest).

Null mutations were generally associated with a poor prognosis, in accordance with the complete lack of AGT protein. Nevertheless, in 14/88 N/N homozygous patients, renal function was preserved to at least 16 years; seven of these developing ESRD after 20 years of age. Some N/N patients were even reported to be pyridoxine-responsive although it was not clear whether the degree of response was as currently defined . However, in some of these cases pyridoxine may work on other possible glyoxylate-using enzymes.

In the present study M/M homozygotes had a better outcome than the N/N and G170R/M heterozygosity was associated with a better outcome than G170R/N, likely due to residual enzymatic activity of the M allele. The same holds true comparing M/N with N/N (table 2, fig 2 and 3). This risk stratification indicates that at least some missense mutations do retain significant enzymatic function. In an attempt to highlight the effects of individual missense mutations, we ordered the patients carrying the same mutation according to their median age at onset and at ESRD, and compared the rank positions in the M/M, G170R/M

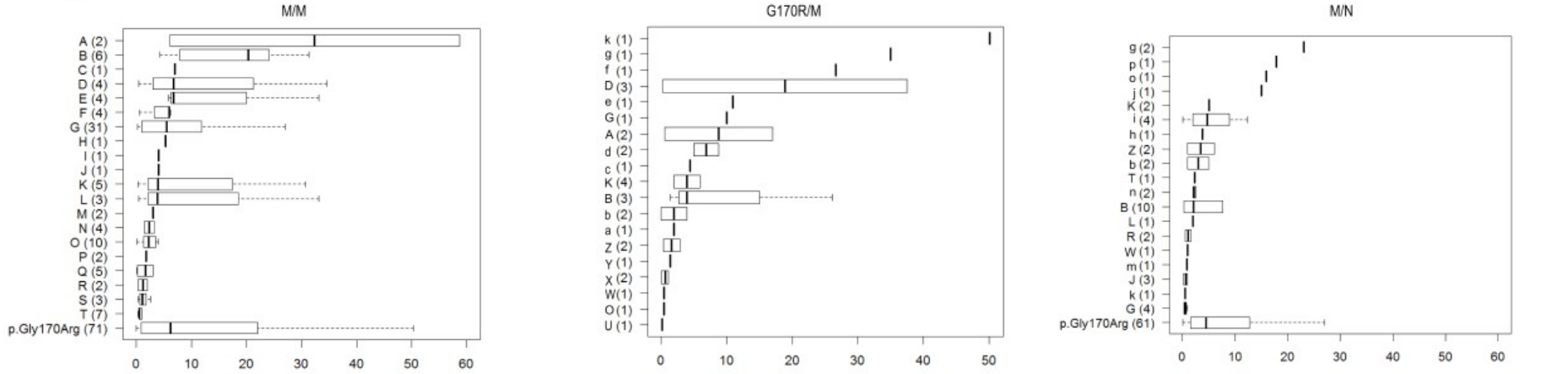
M/N series (Fig. 4.4). For both outcomes, the rank position of most mutations was similar in the three series: indeed, it can be noted on simple inspection that mutations associated with early progression to ESRD (e.g. p.Gly116Arg) are recurrent in the different series and the same holds true for mutations ranked in the late progression (e.g. p.Gly47Arg). The similarity of the ranking position is statistically significant (age at onset: p=0.02, age at ESRD: p=0.008). This similarity of rank order of the missense mutations among the three series provides suggestive evidence of their graded residual activity, sometimes well correlating with AGT activity in liver and in vitro findings. Our findings will require confirmation as the registry expands. The observed differences in age of onset and of ESRD could not be ascribed to previously known reasons, as for the majority of mutations *in vitro* activity was reported to be very low (http://www.uclh.nhs.uk/phmd).

Figure 4.4**.** Ranking of patient subgroups carrying the same missense mutation ordered according to their median a) age at onset and b) age at ESRD either in homozygous state (left panels), compound heterozygous state with G170R (middle panels) or compound heterozygous with a null mutation (right panel). The number of patients with the relevant mutation is given in parentheses.

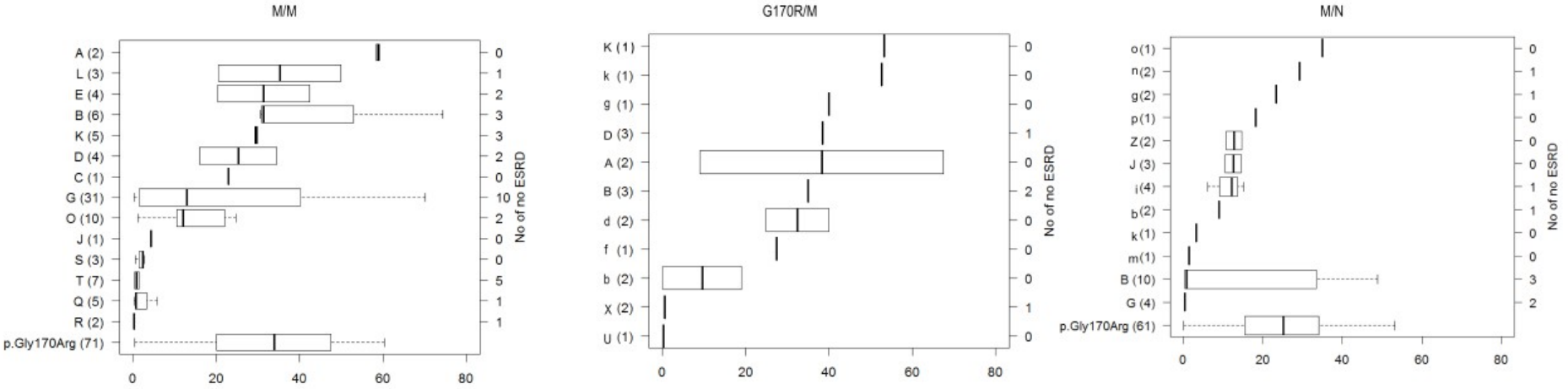
Missense mutations are coded as follow:

Code	Mutation name	One letter code
A	p.Arg36Cys	R36C
B	p.Phe152Ile	F152I
C	p.Leu384Pro	L384P
D	p.Gly156Arg	G156R
E	p.Gly82Arg	G82R
F	p.Leu101Pro	L101P
G	p.Ile244Thr	I244T
H	p.Ala368Thr	A368T
I	p.Arg233His	R233H
J	p.Trp108Arg	W108R
K	p.Gly41Arg	G41R
L	p.Gly190Arg	G190R
M	p.Glu274Asp	E274D
N	p.Gly350Asp	G350D
O	p.Met195Arg	M195R
P	p.Ser218Leu	S218L
Q	p.Gly82Glu	G82E
R	p.Arg360Gln	R360Q
S	p.Cys178Tyr	C178Y
T	p.Asp201Glu	D201E
U	p.Glu95dup	E95dup
V	p.Met195Leu	M195L
W	p.Arg233Cys	R233C
X	p.Cys173Tyr	C173Y
Y	p.Ser275Arg	S275R
Z	p.Ser81Leu	S81L
a	p.Ile220Phe	I220F
b	p.Gly116Arg	F116R
c	p.Gly362Ser	G362S
d	p.Gly109Val	G109V
e	p.Thr70Asn	T70N
f	p.Leu153Val	L153V
g	p.Gly47Arg	G47R
h	p.Leu150Pro	L150P
i	p.Gly161Ser	G161S
j	p.Ser205Leu	S205L
k	p.Ile279Thr	I279T
m	p.Gly216Arg	G216R
n	p.Gln282His	Q282H
o	p.Gly41Val	G41V
p	p.Asp183Asn	D183N

a) Age at onset of symptoms



b) Age at ESRD



In addition to G170R, several other mutations that were found in significant numbers were considered in either homozygous or compound heterozygous states:

p.Ile244Thr. The 31 homozygotes displayed a wide age range at onset (median: 5.8y, range: 0-49) and of ESRD (median: 12y, range: 0-70). All four p.Ile244Thr/N compound heterozygotes had a neonatal onset, one developed ESRD in the first months of life, while three, all under 5y, still retain renal function. Eight of the nine patients who were pyridoxine responsive had preserved renal function (mean age at last follow up: 6.7y, range: 1-17), whereas only three of the seven non-responsive patients had preserved renal function (median age at last follow up: 5y, range: 1-38).

The wide range of age of onset and of ESRD in p.Ile244Thr homozygous patients could be due to the differences highlighted in functional studies, where it has been demonstrated that the mutation retains significant activity when expressed on the major haplotype, whereas in association with the minor haplotype it negatively influences AGT stability .

p.Phe152Ile is also associated with a wide age range at onset of symptoms in the six homozygotes (median 20y; range: 10 - 24), in the three compound heterozygotes with G170R (median: 14.5y; range: 1.5-26) and in the ten with a Null mutation (median: 0.2y; range: 2-4.5). Three homozygotes developed ESRD after age 30y while the other three, older than 16y, still have preserved renal function (mean age: 24y). This is similar to the finding in p.Phe152Ile/G170R heterozygotes (age at ESRD: 36y in two patients and one, age 12y, with preserved renal function). Overall, there was a much later onset of renal impairment in this group.

The p.Phe152Ile AGT mutant forms aggregates and needs to be continuously supplied with pyridoxine to remain in a catalytically competent form . When p.Phe152Ile is associated with the minor allele mitochondrial mistargeting occurs. In accordance with these functional studies , we observed a relatively mild disease associated with this mutation.

p.Met195Arg. The ten homozygotes had early onset of symptoms (median: 2y, range: 0-3) but similar age at ESRD compared to other mutations (median: 16y, range: 9-22) and two patients have preserved renal function at 20y and 16y.

p.Asp201Glu. Five out of seven homozygous patients presented early (median age at onset: 0.5y, range: 0.5-2.7) but retained kidney function at last follow up (median age at last

follow up: 13y, range: 4-15 y). The two other homozygotes, with neonatal onset of symptoms, developed early ESRD (3 months and 1 year respectively). The single heterozygous p.Asp201Glu/N patient has preserved kidney function at last follow up (10y).

p.Ser81Leu is associated with an earlier onset of ESRD when in compound heterozygosis with a Null mutation (two patients with ESRD at 10 and 14 y) than with a p.G170R mutation (2 patients older than 20 y with preserved renal function). Our clinical findings support a loss of function for p.Ser81Leu, predicted by in silico analysis to result from impaired pyridoxine binding.

p.Arg36Cys. The two homozygous patients and two p.Arg36Cys/G170R heterozygotes display a wide range of onset ages (3, 6, 17, 58 years) but late ESRD (the two homozygotes at 58y; one p.Arg36Cys/G170R at 67y and the other one with preserved renal function). No compound heterozygotes with a Null mutation were present in the database. The better clinical outcome in all three patients well correlates with the partial catalytic activity shown in the *in vitro* studies, especially on the major allele .

Most, but not all of the missense mutations have now been expressed *in vitro* and the majority have little activity when expressed on the minor allele (http://www.uclh.nhs.uk/phmd and) but some have significant activity on the major allele. Unfortunately the polymorphism status for many of our patients was unknown and therefore in most cases it was not possible to assess whether the polymorphic background contributed to a milder phenotype.

The partial discordance between early functional studies and our clinical findings could be related to limitations of *in vitro* studies and suggests that new techniques are needed to better explore the folding dynamics of AGT and the molecular basis of the disease . The cellular assay first described by Danpure and colleagues and recently updated is expected to contribute to a more accurate assessment of the residual function of different mutations.

Chapter 5
The Telethon project

In collaboration with Prof. Borri-Voltattorni's group (University of Verona) we developed a Telethon project (GGP10092), aiming to improve the clinical management of patients affected by Primary Hyperoxaluria Type I (PH1) by exploiting the knowledge on the genotype-enzymatic phenotype-clinical phenotype relationships to develop new specific and non-invasive treatment strategies.

Although PH1 is characterized by a significant variability in terms of enzymatic phenotype, the majority of the pathogenic variants are believed to share both structural and functional defects, as mainly revealed by data on AGT activity and expression level in crude cellular extracts. However, the knowledge of the defects of the AGT variants at a protein level is still poor. Therefore, in the Telethon project several AGT mutations were studied in vitro .

In an attempt to shed light on the mechanisms leading to PH1, we focused our attention on the molecular defects underlying AGT deficiency in compound heterozygous patients. Like in other recessive diseases, many PH1 patients are compound heterozygous expressing two different AGT alleles. In these patients, interallelic complementation (IC) phenomena could occur, leading to a phenotype less severe (positive IC) or a more severe (negative IC) phenotype than that of the homozygous counterparts. IC effects arise from the combination of monomers bearing different mutations yielding heterodimeric species with functional and/or structural properties different from the average of those of parental homodimers. Until now, only "single protein" studies have been undertaken to investigate the molecular pathogenesis of PH1, and the possible interplay between two different pathogenic mutations at clinical and enzyme level has never been analysed. We started from the clinical data on two PH1 patients, one hemizygous for the S81L mutation associated with the major allele, and the other compound heterozygous for the S81L mutation on the major

allele and the most common mutation in Caucasian patients, i.e. the G170R associated with the minor allele (See Appendix 1).

In order to develop new treatment strategies, less invasive and effective for all the patients, our planning act followed two approaches, the first based on enzyme enhancement and the second based on enzyme administration.

Studies performed in the last years, along with the results obtained during this project, have highlighted that in many cases PH1 can be considered a misfolding disease. In fact, many mutations induce subtle conformational changes leading to a variety of downstream effects including an increase in the aggregation propensity, a decreased stability of the dimeric structure and an enhanced susceptibility to intracellular degradation. One of the approaches currently under investigation to treat misfolding diseases is the enzyme enhancement therapy, which is based on the use of pharmacological chaperones. Pharmacological chaperones are small molecules able to specifically bind a mutated protein and to shift the equilibrium toward the attainment of the native structure. In case of diseases caused by enzymatic deficits, molecules acting as pharmacological chaperones are competitive inhibitors, which specifically bind the enzyme active site and are able to dissociate in the presence of the physiological substrate. Thus, in an attempt to identify molecules acting as pharmacological chaperones for AGT variants showing folding defects, we focused our attention on aminoxyacetic acid (AOA) a known competitive inhibitor of AGT. We studied the interaction of AOA with AGT-Ma, AGT-Mi and the pathogenic variants G41R-Ma (characterized by an increased intraperoxisomal aggregation propensity), G170R-Mi (the most common variants associated with mitochondrial mistargeting) and I244T-Mi (characterized by a partial mitochondrial mistargeting and an increased aggregation propensity). Moreover, we analysed the effects of the presence of AOA in a well established disease model based on CHO-GO cells stably expressing AGT-Ma, AGT-Mi or the pathogenic variants G41R-Ma, G170R-Mi and I244T-Mi. The results of our investigations allowed us to conclude that:

- AOA behaves as a competitive slow, tight-binding inhibitor of the analysed enzymatic species

- AOA is able to act as a pharmacological chaperone for the pathogenic variants because it decreases the aggregation propensity of G41R-Ma and promote the peroxisomal localization of G170R-Mi and I244T-Mi. These results provide the proof-of-principle that an enzyme enhancement therapy for PH1 could be possible and constitute the base for the identification of molecules more specific for AGT. In this regard, large scale in silico screening of libraries of commercially available compounds are ongoing.

Since PH1 originates from the deficit of a single enzyme, the opportunity to restore the catalytic pool of the hepatocytes by administering exogenous enzyme is an intriguing perspective. However, two of the major issues for the development of an enzyme administration therapy is the obtainment of large amounts of pure protein from eukaryotic sources and the intracellular delivery of the exogenous protein. During our project, we setup a protocol to purify recombinant AGT from CHO cells stably expressing the AGXT gene. We obtained about 1 mg of pure protein from 1L of cellular culture. In order to obtain an AGT able to cross the plasma membrane, a dual approach was used: the construction of a fusion protein between AGT and the Tat peptide exploiting the membrane crossing capabilities of the Tat moiety, and the conjugation of AGT with a polymeric nanocarrier made of PEGylated-poliglutammic acid (PEG-PGA) able to deliver the functional enzyme across the plasma membrane. We found that neither the fusion with the Tat peptide nor the conjugation with PGA-PEG significantly affect the spectroscopic and kinetic properties of AGT. Both strategies proved to be effective in transducing active AGT into CHO-GO cells in a time-dependent and concentration-dependent manner. Moreover, the transduction of CHO-GO cells with Tat-AGT or with PEG-PGA-AGT is able to restore the ability of the cells to detoxify intraperoxisomal glyoxylate. An important question still remains open concerning the achievement of the correct peroxisomal localization of transduced AGT.

Thus, both Tat-AGT and PEG-PGA-AGT conjugates possess the basic requisites for the development of a therapy based on the intracellular delivery of functional enzyme, thus possibly rescuing for the enzymatic deficit of PH1. It should be considered that the two approaches present different strength. The Tat-AGT fusion protein is a more simple solution but the presence of the Tat tag does not confer any protection against the possible degradation of AGT in the bloodstream and has the major shortcoming of the possible

induction of an immune response for long-lasting treatments. On the other hand, the deployment of a polymeric carrier conjugated to AGT adds a layer of control over the stability of the conjugate and its targeting properties. In fact, the polymer moiety is known to increase the stability of proteins in physiological fluids and it can be modified ad hoc to direct the protein to a particular tissue. Thus, PEG-PGA-AGT should be regarded as the preferred strategy to be implemented for the development of an enzyme administration therapy for PH1.

Finally, it should be taken into account that CHO-GO cells only represent an in vitro disease model and that the physiological localization of AGT are hepatic cells. Thus, our future goals will be to test the ability of the fusion protein and the conjugates both to be internalized in cellular models of hepatic cells, such as HepG2, and to localize in the liver of a mouse disease model.

Chapter 6

General discussion and conclusions

Primary Hyperoxaluria is a disease with a broad range of clinical symptoms and the molecular mechanisms leading to the disease are not fully described.

In this PhD thesis some contributions were made to the general understanding of this disease, both as regards the genotype-phenotype correlations and as functional characterization of AGXT mutations.

A grey zone of cases in which suspicions based on clinical and biochemical findings are not confirmed by genetic testing is recognized in the diagnostic practice of most rare genetic diseases and in PH1 as well. To clarify the inconclusive diagnoses in our historical series of cases referred for genetic diagnosis of PH we selected 15 patients with primary oxaluria, *AGXT* mutation-negative, to address to the analysis of the *AGXT*-promoter and the additional known genes *GRHPR* and *HOGA1*. Our analyses did not provide evidence of pathogenic mutations of these genes in any of the 15 patients.
In two patients we identified two novel heterozygous *AGXT*-promoter variants, c.-647C>T and c.-424C>T. We considered these variants not to be pathogenic since they do not lie in any known regulatory site. The validity of this conclusion is limited by the scarce available literature on the *AGXT* promoter that precludes comparison with more studies.
In one patient we detected a novel homozygous *HOGA1* variant in intron 2 (c.341-81delT), not reported in literature. The sequence of intron 2 is highly conserved among primates but not in other species (http://genome.ucsc.edu/cgi-bin/hgc), and thus it can be hypothesized to play a role for HOGA expression and activity in primates, including humans. Neither RNA nor HOGA enzymological studies were possible for lack of biological samples of the patient; thus, to evaluate the effect of the variant we set up a minigene assay, that in contrast to *in silico* predictions, did not reveal any difference between the product of wild type and c.341-81delT variant constructs. A possible explanation is that the power of the

splicing site may be weaker than the canonical splicing site, so it could not be revealed by the simple minigene protocol in COS-7 and HEK293T cells. Despite these limitations, this report could be a useful base for further functional studies of this variant.

Since oxalate metabolism is complex and not fully described, it is possible that other genes are presently uncovered and even a multiallelic PH inheritance has been suggested. Among PH candidate genes, the three human lactate dehydrogenases (*LDHA*, *LDHB*, *LDHC*) have an important role in excessive oxalate production: LDH is able to convert glyoxylate in oxalate, glyoxylate in glycolate and hydroxypyruvate in L-glycerate, using NAD(H) as cofactor. In the future, next generation sequencing could be a useful tool to both discover novel genes involved in glyoxalate metabolism and PH phenotype modifiers.

Thanks to the collaboration with Prof. Borri-Voltattorni's group, we were able, for the first time, to understand the effects of the S81L mutation on the major allele and the G170R on the minor allele under homozygous and heterozygous conditions. Our investigation was not limited to the AGT activity of the purified recombinant forms of S81L-Ma, G170R-Mi and S81L-Ma/G170R-Mi and to the specific activity upon expression or coexpression of the S81L and G170R variants in CHO cells, but was extended to their expression level, coenzyme binding affinity, subcellular localization and functionality. This broad spectrum analysis resulted to be useful considering the multiplicity of the molecular mechanisms accounting for AGT deficiency. The results gave new significant insights into the PH1 mechanism, as we report the molecular basis of the pathogenicity of the S81L mutation on the major allele and the interplay between this mutation and the G170R mutation on the minor allele. When expressed individually the S81L-Ma and G170R-Mi variants show distinct features. As already reported and confirmed in this study, when only G170R-Mi allele is expressed, AGT retains catalytic activity and PLP binding affinity, but it is almost completely redirected to mitochondria . On the other hand, we reported that when the S81L-Ma allele (lacking the putative MTS) is expressed, AGT shows a decreased catalytic activity, is almost completely present in the apo form, and is mainly peroxisomal. Thus, while the pathogenicity of the G170R mutation is related to AGT mistargeting, that of the S81L mutation is linked to the reduction of PLP binding affinity. We were able to recreate the naturally occurring mutations S81L and G170R in AGT identified in compound

heterozygous patient and to isolate and characterize the heterodimer in the purified recombinant form. This allowed us to establish that it exhibits a considerable k_{cat} value and, in agreement with its $K_{D(PLP)}$ value, it is present in the cell mainly in the holo form. More importantly, it shows a peroxisomal localization. The peroxisome-to-mitochondrion mistargeting of AGT in PH1 is due to the combined effects of (i) the generation of a MTS resulting from the P11L polymorphism, and (ii) the presence of monomeric AGT. Since in the heterodimer G170R-Mi/S81L-Ma only the first condition occurs, even if limited to one subunit, its peroxisomal localization is not surprising. It can be also speculated that in cells expressing both S81L-Ma and G170R-Mi the rate of the formation of the heterodimer is faster than that of the import of G170R-Mi monomers in mitochondria. Moreover, although cells expressing both G170R-Mi and S81L-Ma show a reduced expression level, they display an ability to detoxify glyoxylate greater than that of cells expressing homodimer S81L-Ma and equal to those expressing homodimers G170R-Mi. In the first case, this could be interpreted on the basis of the significant amount of protein present in the holo-form. In the second case, the detoxification ability could be ascribed to the proper subcellular localization of the heterodimer.

All together, these results indicate that the association between the G170R and S81L monomers leads to a global positive IC between these mutations. The clinical data reported in this study appear to agree with our biochemical data: the G170R/S81L compound heterozygous patient had a late onset of ESRD compared with the one functionally hemizygous for the S81L mutation who only expresses the S81L-Ma variant.

This work constitutes a valid example of an approach useful to understand the enzymatic phenotype in compound heterozygous PH1 patients, with potential applications to the study of the interaction between other pathogenic mutations. Besides providing a better knowledge of the disease pathogenesis, the results obtained could allow to develop proper therapeutic treatments, as the hybrid protein could have defects different from those of the parental enzymes.

The genotype-phenotype studies were made possible by the collaboration with several European Centres, all being part of the OxalEurope group. The decision to assemble a common database allowed the formation of the largest PH1 registry worldwide. Thanks to the high number of patients and the use of a standardized form for data collection it

promises not only a detailed description of PH1 across European populations, but also a unprecedented possibilities for drawing genotype-phenotype comparisons and defining shared guidelines for diagnosis and treatment. In addition, in the next years it may become the instrumental basis to address a variety of issues of clinical and genetic interest such as the epidemiology of PH, the mutational spectrum, treatment regimen, disease progression, inter-population comparisons and many others.

As regards the clinical findings, it is relevant to note that the disease is largely diagnosed in later stages, frequently after renal failure or even after a renal transplant, which is soon damaged by oxalate deposition. As early conservative treatment may delay renal damage, particularly in pyridoxine responsive patients, metabolic screening for all children at first presentation of a kidney stone or nephrocalcinosis, and for all adults with recurrent stone disease, might be a most important recommendation.

As for transplant strategies is not jet possible to define strong recommendation, further analyses will be performed.

As for genotype-phenotype analysis, we decided to classify mutations in classes, similarly to what is usually done for Cystic Fibrosis , in order to limit the number of comparisons that would otherwise have limited the statistical strength even of the large number of patients of the OxalEurope registry.

Some correlations already suggested are confirmed: mistargeting mutation has a higher AGT activity, later age at onset and at ESRD, better response to pyridoxine and lower levels of urinary oxalate. Moreover, even if the disease eventually leads to ESRD, genotypes with a significant residual enzymatic activity are on average associated with a longer disease free period.

However a minority of patients, five in OxalEurope cohort, had an early progression to ESRD. These patients are shown in the figure 7.1 and are one German with ESRD at 1 year, one Dutch with ESRF at 3 years, a German and a French with ESRD at 5 years and a German with ESRD at 10 years.

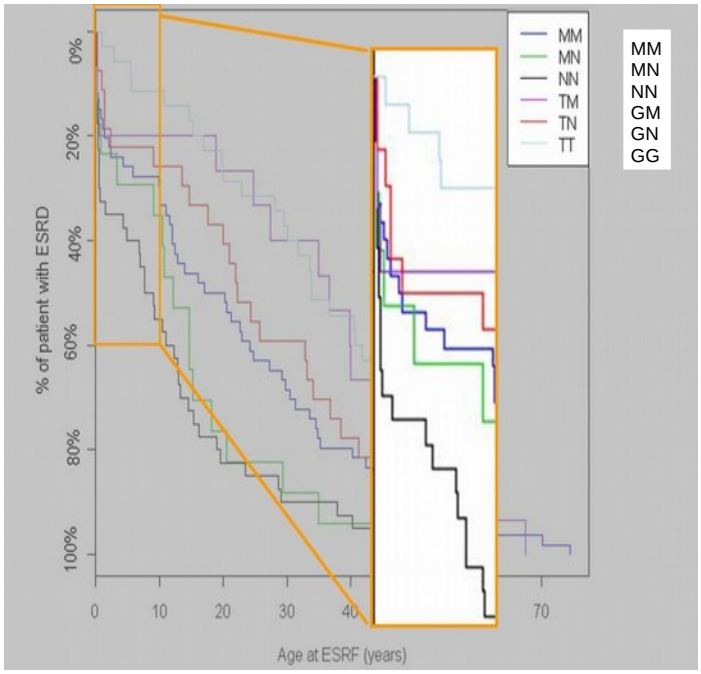


Figure 7.1: focus on patients with a progression to ESRD within 10 years of age.

This finding deserves a further check of the real genotype, in particular testing for large gene deletions, in order to evaluate if a hemizygosis is present, that could explain the worse clinical course because these patients would be re-classified as a heterozygotes G170R/N.

A late age at onset of ESRD in some patients homozygous for a null mutation and an early onset in some G170R homozygotes has already been reported and ascribed to other factors different from AGXT genotype .

Having at least one allele with a mistargeting mutation is sufficient to confer a better course to the disease, probably thanks to a significant level of residual enzyme addressed to the peroxysome.

On the contrary, some missense mutations are frequently associated with an early onset of ESRD, probably depending on the structural alteration induced by the mutation.

In conclusion, this study of an unprecedented number of PH1 patients showed geno-phenotype associations that have not been previously described. These findings may have important implications for the management of PH1 patients and underline the necessity of

genotyping PH patients, including unambiguous assignment of minor/major allele status. We also identified considerable diagnostic delay in this disease, which may partly explain the overall adverse outcome in some patients. A limitation of this first report is the absence of some clinical data and the lack of systematic nationwide data collection, accounting for the inability to provide precise prevalence and incidence estimates. Moreover, pyridoxine sensitivity was not formally tested as is now recommended . Notwithstanding these limitations, this report shows that the OxalEurope database, with establishment of regular data input, will represent an invaluable resource for delineating genotype-phenotype and other clinically relevant issues in PH. Moreover, the database will provide the backbone to design future therapies and conduct prospective clinical trials in PH ultimately providing personalized medicine to this devastating metabolic disease.

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Appendix 1

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S81L and G170R mutations causing Primary Hyperoxaluria type I in homozygosis and heterozygosis: an example of positive interallelic complementation

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Primary Hyperoxaluria type I (PH1) is a rare disease due to the deficit of peroxisomal alanine:glyoxylate aminotransferase (AGT), a homodimeric pyridoxal-5′-phosphate (PLP) enzyme present in humans as major (Ma) and minor (Mi) allele. PH1-causing mutations are mostly missense identified in both homozygous and compound heterozygous patients. Until now, the pathogenesis of PH1 has been only studied by approaches mimicking homozygous patients, whereas the molecular aspects of the genotype-enzymatic-clinical phenotype relationship in compound heterozygous patients are completely unknown. Here, for the first time, we elucidate the enzymatic phenotype linked to the S81L mutation on AGT-Ma, relative to a PLP-binding residue, and how it changes when the most common mutation G170R on AGT-Mi, known to cause AGT mistargeting without affecting the enzyme functionality, is present in the second allele. By using a bicistronic eukaryotic expression vector, we demonstrate that (i) S81L-Ma is mainly in its apo-form and has a significant peroxisomal localization and (ii) S81L and G170R monomers interact giving rise to the G170R-Mi/S81L-Ma holo-form, which is imported into peroxisomes and exhibits an enhanced functionality with respect to the parental enzymes. These data, integrated with the biochemical features of the heterodimer and the homodimeric counterparts in their purified recombinant form, (i) highlight the molecular basis of the pathogenicity of S81L-Ma and (ii) provide evidence for a positive interallelic complementation between the S81L and G170R monomers. Our study represents a valid approach to investigate the molecular pathogenesis of PH1 in compound heterozygous patients.

INTRODUCTION

Liver peroxisomal alanine:glyoxylate aminotransferase (AGT) is a homodimeric pyridoxal-5′-phosphate (PLP)-dependent enzyme, which catalyses the conversion of L-alanine and glyoxylate to pyruvate and glycine, respectively. AGT deficiency is responsible for Primary Hyperoxaluria type I (PH1) (MIM 259900), a rare autosomal recessive disease with an estimated prevalence of 1–3 per million population in Europe (1,2). In PH1 patients, glyoxylate accumulation and its conversion to the metabolic end product oxalate lead to calcium oxalate (CaOx) supersaturation and precipitation as CaOx stones. This condition manifests as urolithiasis and/or nephrocalcinosis, and, if untreated, as systemic oxalosis with CaOx deposition in many organs (3).

The *AGXT* gene encoding AGT is present in human population as two haplotypes, the 'major' (encoding AGT-Ma) and the 'minor' (encoding AGT-Mi), the latter characterized by a 74-bp duplication in intron 1 and two point mutations causing the P11L and I340M amino acid substitutions (4). Most of the mutations associated with PH1 (>150) are missense and

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concern residues spread over the entire 3D structure of the enzyme (5). Biochemical, bioinformatic and cell biology studies have revealed that pathogenic mutations can either alter the AGT catalytic machinery (6–9) and/or undermine the stability of the folded conformation (10–14) leading, in most cases, to protein aggregation and/or mitochondrial mistargeting (15). Moreover, some mutations have only an impact on the apo-form of the protein (12,16,17).

Like in other recessive diseases, a substantial share of PH1 patients are compound heterozygous expressing two different AGT alleles. In these patients, interallelic complementation (IC) phenomena could occur, leading to a phenotype less severe (positive IC) or a more severe (negative IC) than that of the homozygous counterparts. Interallelic complementation effects arise from the combination of monomers bearing different mutations yielding heterodimeric species with functional and/or structural properties different from the average of those of parental homodimers. Until now, only 'single protein' studies have been undertaken to investigate the molecular pathogenesis of PH1, and the possible interplay between two different pathogenic mutations at clinical and enzyme level has never been analysed. Taking into account that many PH1 patients are compound heterozygous, the investigation of their heterozygous status is highly desirable and will possibly further expand the phenotype spectrum of the disease.

Here, we started from the clinical data on two PH1 patients, one hemizygous for the S81L mutation associated with the major allele and the other compound heterozygous for the S81L mutation on the major allele and the most common mutation in Caucasian patients, i.e. the G170R associated with the minor allele. It is known that the G170R mutation does not affect the kinetic and coenzyme binding properties of AGT (17) but causes a folding defect leading to an erroneous targeting to mitochondria, where the enzyme cannot perform glyoxylate detoxification (18). The S81L pathogenic mutation has been firstly identified by Williams *et al.* (19). Ser81 forms a hydrogen bond with the phosphate group of PLP and, although the molecular defect of the S81L-Ma variant is unknown, the mutation to Leu has been predicted to mainly affect the coenzyme binding affinity (20). We used a bicistronic eukaryotic expression vector and a dual-vector prokaryotic expression strategy to study for the first time not only the consequences of the S81L mutation on AGT but also the interplay at molecular and cellular level between the G170R and the S81L mutations. By investigating the effects of each mutation and those of their combination on the expression level, specific activity, subcellular localization, functionality as well as on the coenzyme binding affinity and the kinetic and spectroscopic properties of AGT, we were able to (i) elucidate the molecular defects of the S81L-Ma variant, (ii) mimic in mammalian cells the natural heteroallelic state occurring in compound heterozygous patients bearing the S81L and the G170R mutations, (iii) isolate and characterize the G170R-Mi/S81L-Ma heterodimer and (iv) provide evidence for a global positive IC effect between the G170R and S81L mutations.

RESULTS

Clinical cases

Patient 1 is a 30-year-old Albanian male, affected by nephrolithiasis since age 3. PH1 was diagnosed at age 24 (AGT activity in liver biopsy reduced to 23% of normal). At diagnosis, urography showed atrophy of the right kidney and multiple stones in the left one. Serum creatinin was 1.18 mg/dl, creatinin clearance 86 ml/min/1.73 mq, urinary oxalate 1.3 mmol/24 h, urinary glycolate 830 μmol/24 h, plasma oxalate 8 μmol/l and plasma glycolate 5 μmol/l. He had a good pyridoxine response (600 mg/die): urinary oxalate decreased to 0.480 mmol/24 h and plasma oxalate to 0.8 μmol/l. At age 32, serum creatinin was 1.5 mg/dl and urinary oxalate 0.35 mmol/die; no systemic oxalosis was present. *AGXT* gene sequencing demonstrated compound heterozygosis for S81L and G170R mutations (on major and minor haplotype, respectively). No kidney disease was reported in the family, nor consanguinity among parents.

Patient 2 is a 15-year-old Serbian female; at 11 months, she presented failure to thrive, anorexia and recurrent kidney stones; at that time, urinary oxalate was 242 mmol/24 h. End-stage renal disease (ESRD) occurred at age 11 (serum creatinin 1744 μmol/l). Plasma oxalate was 110 μmol/l. At that time, abdominal ultrasound showed nephrocalcinosis and kidney volume reduction. She presented growth retardation (height: $<10^{\circ}$ centile; weight: $3-10^{\circ}$ centile), painful bone attacks and leg deformity (genu varum) owing to systemic oxalosis. At age 15, she developed hyperparathyroidism, mild left ventricular hypertrophy and heart dilatation and non-specific central irritation at electroencephalogram (no seizures referred). No formal pyridoxine testing was performed. She underwent a combined kidney–liver transplant at age 15. *AGXT* gene sequencing demonstrated compound heterozygosis for S81L and S205X mutations (both on major haplotype). Thus, this patient can be considered 'functionally hemizygous' for the non-null mutation, because it only expresses the allele bearing the S81L mutation. No kidney disease was reported in the family, nor consanguinity among parents.

Based on these data, which indicate a different severity of the two cases, we decided to investigate the enzymatic phenotype associated with the S81L mutation on the major allele as well as the occurrence of possible IC effects between the S81L and the G170R mutation on the minor allele. To this aim, considering the great variety of the molecular mechanisms leading to AGT deficiency in PH1, we studied the consequences of each mutation alone and of their combination on (i) the protein expression level, the specific activity, the subcellular localization and the functionality upon expression in a eukaryotic cellular system and (ii) the coenzyme binding affinity and the spectroscopic and kinetic features of purified variants in the homodimeric and heterodimeric state.

S81L and G170R mutations in homozygous and heterozygous conditions: effect on the expression level and the specific activity of AGT

We investigated the cellular effects of the S81L mutation on AGT-Ma both in conditions mimicking a homozygous patient and in conditions mimicking a compound heterozygous patient bearing the G170R mutation on AGT-Mi in the second allele. To this aim, we constructed a series of pIRES vectors that allowed us to express under the same transcript two copies of the AGT cDNA bearing the analysed polymorphic and pathogenic mutations in different combinations (Fig. 1). To distinguish between the first and the second gene product, we

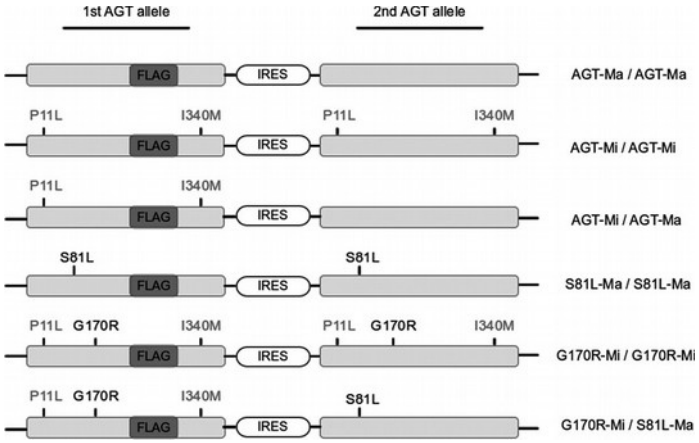

Figure 1. Graphical sketch of the bicistronic pIRES constructs expressing the AGT variants. The first ORF is bearing the FLAG sequence. Polymorphic and pathogenic mutations in each construct are marked grey and black, respectively.

introduced a FLAG tag on the surface portion of the loop 251–266, exactly between the residues 254 and 255 of the first gene product. The chosen site, away from both the C-terminal peroxisomal targeting sequence and the active site, is predicted to accommodate the tag without generating significant steric hindrance. As shown in Figure 1, in the constructs encoding two different enzymatic species, the FLAG tag was inserted on the background of the minor allele. This allowed us to detect only the product of the second allele by an antibody specific for AGT-Ma. Each vector was transduced in CHO-GO cells, which stably express glycolate oxidase (GO) and represent a widely used cellular model for PH1 (21). Preliminary transient transfection experiments showed that the presence of the FLAG tag did not significantly affect the AGT expression level, the enzymatic activity and the intracellular trafficking. Thus, CHO-GO clones stably expressing each of the constructs were selected, and the levels of expressed AGT were analysed by western blot. A double band responsive to the anti-AGT antibody, absent in the parental cells, is present in all the clones (Fig. 2A and B). The band at the higher molecular weight is also responsive to the anti-FLAG antibody, thus confirming that the clones express both AGT alleles. To investigate whether the coexpression of the two allelic forms actually generates heterodimeric species, we immunoprecipitated stably transfected CHO-GO cells expressing AGT-Mi/AGT-Mi, G170R-Mi/G170R-Mi, AGT-Mi/AGT-Ma and G170R-Mi/S81L-Ma with an anti-FLAG antibody and we detected the precipitated proteins by western blot using an antibody specific for AGT-Ma (Fig. 3). The anti-FLAG antibody pulled down a protein that reacted with the anti-AGT-Ma antibody only in the AGT-Mi/AGT-Ma and G170R-Mi/S81L-Ma clones. This indicates the formation of heterodimeric species in these samples, because the FLAG tag is present on the first allele, whereas the AGT-Ma epitope in these constructs is encoded by the second allele (Fig. 1). These data validate our experimental model for the study of the S81L and G170R mutations in heterozygous state and the evaluation of possible IC effects at cellular level. However, these results should be interpreted with caution considering that the relative proportions of homodimeric and heterodimeric species cannot be measured under these experimental conditions.

In line with previous data (15), the expression level of AGT-Mi/AGT-Mi, AGT-Mi/AGT-Ma and G170R-Mi/G170R-Mi was about 75, 90 and 46%, respectively, that of AGT-Ma/AGT-Ma. Moreover, while the S81L mutation did not significantly reduce the expression level of AGT-Ma/AGT-Ma, cells expressing the G170R-Mi/S81L-Ma construct showed an AGT expression level equal to ∼30% that of cells expressing AGT-Mi/AGT-Ma (Fig. 2A and B). Similar results were obtained in different clones expressing the G170R-Mi/S81L-Ma construct, and real-time PCR experiments did not reveal differences between the clones at transcription level. Thus, the reduced AGT level in G170R-Mi/S81L-Ma transfectants should originate from a defect at the protein level leading to an increased aggregation and/or degradation propensity of the heterodimeric form with respect to the homodimers G170R-Mi and S81L-Ma. In this regard, we found that in cells expressing the G170R-Mi/S81L-Ma construct, (i) the ratio of soluble/insoluble AGT does not significantly differ from that of cells expressing the AGT-Mi/AGT-Ma construct, and (ii) no aggregates of AGT can be revealed upon cross-linking with bis-N-succinimidyl-(pentaethyleneglyocol) ester. Thus, it can be suggested that the low AGT expression level of the clone G170R-Mi/S81L-Ma could arise from an increased susceptibility to degradation of the heterodimeric species with respect to the homodimers G170R-Mi and S81L-Ma (see below).

We then measured the specific activity of each clone in the absence and presence of PLP in the assay mixture, representing the amount of the holoenzyme and of the total active protein (apoenzyme plus holoenzyme), respectively (Fig. 2C). In the

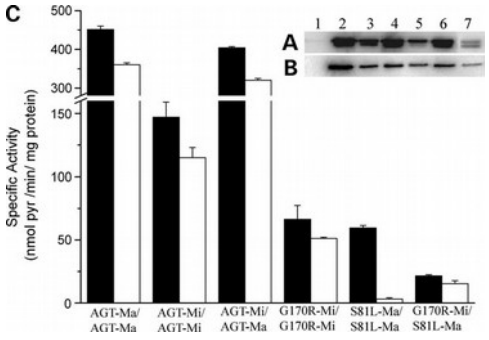

Figure 2. Western blot and specific activity analyses of CHO-GO cell clones expressing the AGT variants. Four micrograms of cell lysate was immunoblotted with (**A**) anti-AGT from rabbit and anti-rabbit HRP-conjugated antibodies and (**B**) mouse anti-FLAG and anti-mouse HRP-conjugated antibodies. Lanes: 1. CHO-GO; 2. AGT-Ma/AGT-Ma; 3. AGT-Mi/AGT-Mi; 4. AGT-Mi/AGT-Ma; 5. G170R-Mi/G170R-Mi; 6. S81L-Ma/S81L-Ma; 7. G170R-Mi/S81L-Ma. (**C**) Hundred micrograms of cell lysate were incubated with 0.5 M L-alanine and 10 mM glyoxylate at 25°C in 100 mM KP, pH 7.4, in the presence (black) or in the absence (white) of 200 μM PLP. Data are representative of three independent experiments. Bar graphs represent the mean ± SD.

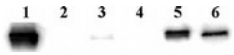

Figure 3. Identification of the presence of heterodimeric forms of AGT by immunoprecipitation. Three hundred micrograms of cell lysate were immunoprecipitated using an anti-FLAG antibody and detected by western blot using rabbit anti-AGT-Ma and anti-rabbit HRP-conjugated antibodies. Lanes: 1. purified AGT-Ma control non-immunoprecipitated; 2. CHO-GO parental cells; 3. AGT-Mi/AGT-Mi; 4. G170R-Mi/G170R-Mi; 5. AGT-Mi/AGT-Ma; 6. G170R-Mi/S81L-Ma.

presence of PLP, the specific activity of AGT-Mi/AGT-Mi and AGT-Mi/AGT-Ma was reduced by ∼35 and 20% with respect to that of AGT-Ma/AGT-Ma, whereas that of G170R-Mi/G170R-Mi is reduced by ∼70% with respect to that of AGT-Mi/AGT-Mi, in line with previous data on cellular lysates (15). Moreover, the specific activity of S81L-Ma/S81L-Ma was ∼20% that of AGT-Ma/AGT-Ma, whereas that of G170R-Mi/S81L-Ma was ∼4% that of AGT-Mi/AGT-Ma. As compared with the activity in the presence of added coenzyme, the specific activity of AGT-Ma/AGT-Ma, AGT-Mi/AGT-Mi, AGT-Mi/AGT-Ma and G170R-Mi/G170R-Mi measured in the absence of PLP decreased by 25–30% and that of G170R-Mi/S81L-Ma decreased by 40%, thus indicating that in these clones, AGT is in large amount present as holoenzyme. In contrast, the specific activity of S81L-Ma/S81L-Ma decreased by 95% in the absence of PLP in the assay mixture, thus revealing that this variant in the cell is almost entirely present as apoenzyme.

Overall, the data obtained in the CHO-GO cellular model indicate that (i) the S81L-Ma variant is almost entirely present as apoenzyme in the cell and its reduced specific activity is not ascribable to a reduced expression level and (ii) when the S81L-Ma and the G170R-Mi variants are co-expressed in the same cell, the formed heterodimeric species are mainly present as holoenzymes, but the total AGT expression level is lower than that of cells expressing homodimeric S81L-Ma or G170R-Mi.

Table 1. Kinetic parameters and $K_{D(PLP)}$ values of the AGT variants

Enzyme	$K_{D(PLP)}$ (μM)	k_{cat}/s^{-1}	$K_{m\ L\text{-}ala}$ (mM)	$K_{m\ glyoxylate}$ (mM)
AGT-Ma (6)	0.27 ± 0.03	45 ± 2	31 ± 4	0.23 ± 0.05
AGT-Mi (10)	0.26 ± 0.02	37 ± 5	28 ± 2	0.22 ± 0.01
G170R-Mi (17)	0.4 ± 0.1	34 ± 3	36 ± 2	0.4 ± 0.1
S81L-Ma	17 ± 3	13.9 ± 0.9	40 ± 8	0.16 ± 0.02
G170R-Mi/S81L-Ma	1.2 ± 0.1	18.3 ± 0.6	45 ± 3	0.18 ± 0.02

S81L and G170R mutations in homozygous and heterozygous conditions: effect on the structural and the functional properties of AGT

Although the data obtained in CHO-GO cells confirm the pathogenicity of the mutations, they do not allow the identification of the molecular mechanisms(s) by which the S81L mutation, both in homozygosis and in heterozygosis with the G170R mutation, generates the AGT malfunction. To gain insights into this issue, the homodimer S81L-Ma and the heterodimer G170R-Mi/S81L-Ma were expressed in *Escherichia coli*, purified and characterized. Their spectroscopic and kinetic properties were compared with those of AGT-Ma, AGT-Mi and G170R-Mi, previously studied (17). Absorbance, visible and near-UV CD spectra of S81L-Ma and G170R-Mi/S81L-Ma in the holo-form were similar to each other and to the corresponding ones of AGT-Ma, AGT-Mi and G170R-Mi. Nevertheless, while no significant changes were observed in 1-anilino naphthalene sulphonic acid (ANS) emission fluorescence for the holo-forms of AGT-Ma, AGT-Mi, S81L-Ma and G170R-Mi, an about 10-fold increase in ANS emission fluorescence intensity in the holo-heterodimer G170R-Mi/S81L-Ma was observed (Supplementary Material, Fig. S4). This indicates that the heterodimeric species displays an increased exposure of hydrophobic patches on its surface, thus suggesting that the formation of the heterodimer causes a conformational change with respect to the homodimeric species. Following this view, we can speculate that, as found for other proteins that fold in the cell cytosol (22), the increased exposure of hydrophobic surfaces would possibly induce the proteasomal degradation of the heterodimer by triggering the cellular quality control machinery.

The effect of the S81L mutation on the PLP binding affinity of the homodimer and the heterodimer was also investigated. As expected, considering that Ser81 is hydrogen-bonded with the PLP phosphate, the S81L-Ma homodimer displayed a ∼70-fold increase in the equilibrium dissociation constant for PLP ($K_{D(PLP)}$) with respect to AGT-Ma (Table 1). This explains why in CHO-GO cells expressing the S81L-Ma/S81L-Ma construct grown in a medium containing ∼0.3 μM pyridoxine [a concentration even higher than that present in human plasma (23)], the variant was almost completely present in the apo-form. Unexpectedly, the G170R-Mi/S81L-Ma heterodimer showed a $K_{D(PLP)}$ value ∼7-fold lower than that predicted by averaging the $K_{D(PLP)}$ values of the two parental enzymes (Table 1). In trying to explain this result, we modelled the

putative dimeric structure of AGT through a crystallographic symmetry operation on the available monomeric structure [pdb file 1H0C (5)]. It revealed that the phosphate group of PLP in each active site interacts with the Oβ of Ser81 and with the peptidic N(s) of Gly82 and His83 of the one's own subunit, and with the Oδ of Tyr260 and the peptidic N of Thr263 of the neighbouring subunit (Supplementary Material, Fig. S1A). The Ser81 → Leu substitution (i) eliminates the H bonds that stabilizes the PLP binding, (ii) introduces a bulky hydrophobic side chain in a polar region of the interface between the AGT monomers and (iii) generates a significant steric hindrance that could compromise the H-bond between His262 and Ser79 of the neighbouring subunit (Supplementary Material, Fig. S1B). This in turn can destabilize the loop 251–266 and the stretch 79–83 of the neighbouring subunit, both involved in PLP binding. On these bases, the S81L mutation is predicted to directly affect the cofactor binding to one subunit but also to indirectly perturb the PLP binding pocket of the adjacent subunit. The lack of this indirect effect in the heterodimer G170R-Mi/S81L-Ma may explain its PLP binding affinity higher than that expected.

The steady-state kinetic parameters reported in Table 1 show that the S81L-Ma homodimer was characterized by a 3-fold decrease in the k_{cat} value with respect to AGT-Ma, whereas the G170R-Mi/S81L-Ma heterodimer displayed a k_{cat} value slightly lower than that predicted by averaging the k_{cat} values of the homodimeric counterparts. There were no significant differences in the K_M values of the examined variants.

Taken together, these data provide evidence that (i) the S81L mutation mainly induces a functional defect, by reducing the AGT catalytic activity and strongly decreasing the coenzyme binding affinity and (ii) the heterodimer G170R-Mi/S81L-Ma shows k_{cat} and $K_{D(PLP)}$ values similar to or lower than, respectively, those derived by the average of the corresponding ones of the homodimeric counterparts.

S81L and G170R mutations in homozygous and heterozygous conditions: effect on the AGT subcellular localization

The G170R mutation on the minor allele is known to cause the mitochondrial mistargeting of AGT, owing to the synergism between the polymorphic P11L mutation, generating a putative mitochondrial targeting sequence (MTS), and the G170R mutation, which induces a folding defect on apodimeric AGT that results in the formation of monomeric folding intermediates prone to interact with molecular chaperons and compatible with the mitochondrial import (12,15,17,18,24,25). In fact, while peroxisomes can import fully folded oligomeric proteins, mitochondria import species bearing an MTS in partly folded monomeric conformation. Up to date, no information is available on the effect of the S81L mutation in homozygosis and heterozygosis with G170R on the AGT subcellular localization. Thus, the stable clones of CHO-GO cells expressing the various constructs were analysed by immunofluorescence microscopy (IFM) both qualitatively, by evaluating the RGB profile of the images, and quantitatively, by determining the Pearson coefficient of each sample (Figs 4, 5, Supplementary Material, Figs S2, S3 and 6). The Pearson coefficient can be used to estimate the colocalization between two signals evaluating the correlation between the signal

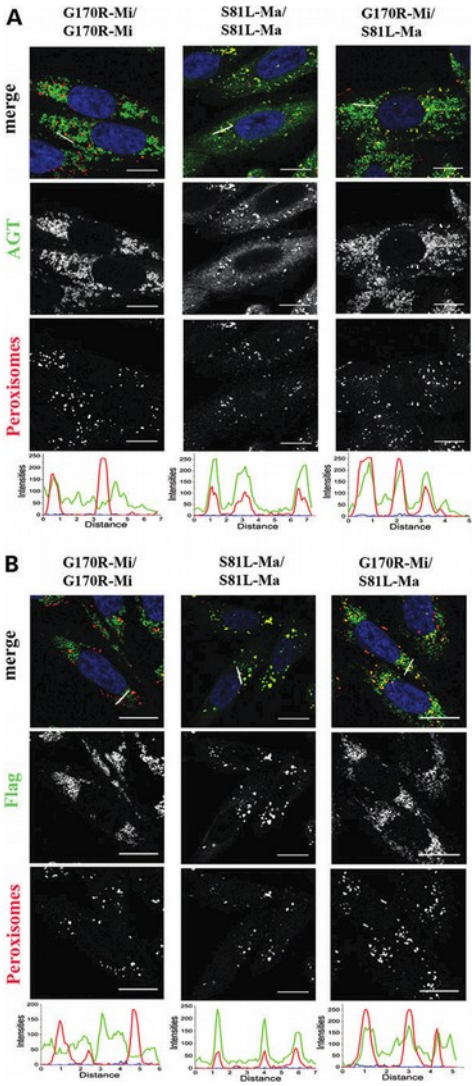

Figure 4. Subcellular distribution of G170R-Mi/G170R-Mi, S81L-Ma/S81L-Ma and G170R-Mi/S81L-Ma variants in stably transformed CHO-GO cells. Twenty-four hours after seeding cells were fixed and coloured as follows: (**A**) anti-AGT (green) and anti-peroxisomal proteins (red) and (**B**) anti-FLAG (green) and anti-peroxisomal proteins (red). Nuclei were stained with Dapi (blue). Below is shown the RGB profile plotted along the line drawn in the merged image. Merge and single-channel images come from a single z-plane. Scale bar: 10 μm.

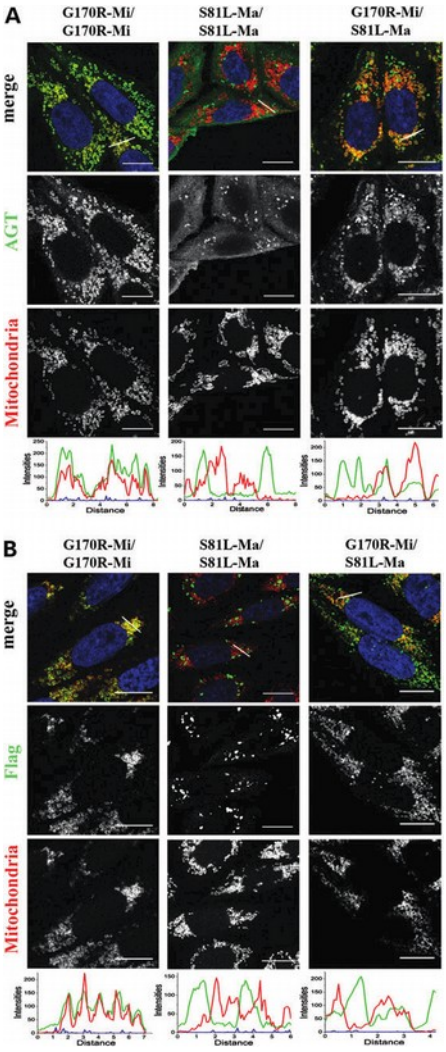

Figure 5. Subcellular distribution of G170R-Mi/G170R-Mi, S81L-Ma/S81L-Ma and G170R-Mi/S81L-Ma variants in stably transformed CHO-GO cells. Twenty-four hours after seeding cells were fixed and coloured as follows: (**A**) anti-AGT (green) and MitoTracker (red) and (**B**) anti-FLAG (green) and MitoTracker (red). Nuclei were stained with Dapi (blue). Below is shown the RGB profile plotted along the line drawn in the merged image. Merge and single-channel images come from a single z-plane. Scale bar: 10 μm.

intensity of the same pixel in two channels. It is generally accepted that only values of >0.5 are indicative of a meaningful colocalization (26). As expected, the localization of AGT-Ma/AGT-Ma,

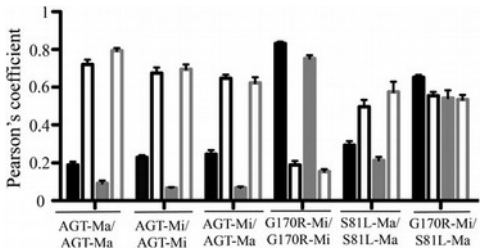

Figure 6. Pearson's coefficients for the subcellular distribution of the analysed AGT variants in stably transformed CHO-GO cells. The histogram bars represent the Pearson's coefficient calculated for the colocalization of: AGT and mitochondria (solid black), AGT and peroxisomes (outlined black), AGT-FLAG and mitochondria (solid grey), AGT-FLAG and peroxisomes (outlined grey). The results are given as means (± S.E.M.) of several images; at least 30 individual cells were analysed for each coefficient.

AGT-Mi/AGT-Mi and AGT-Mi/AGT-Ma was entirely peroxisomal (Supplementary Material, Figs S2 and S3), whereas that of G170R-Mi/G170R-Mi was entirely mitochondrial (Figs 5 and 6). Although S81L-Ma showed a significant peroxisomal localization, a portion of the protein is present in the cell cytosol (Fig. 6). Cross-linking and size-exclusion chromatography experiments of lysates of cells expressing this variant allowed us to exclude that the cytosolic localization was due to the formation of cytosolic aggregates not compatible with the peroxisomal import. Nevertheless, considering that the S81L-Ma homodimer is present in the cell mainly as apoenzyme, it is possible to hypothesize that the S81L mutation induces a conformational change on apoAGT causing the slowing down of the peroxisomal import. Indeed, the purified S81L-Ma apovariant showed with respect to apoAGT-Ma: (i) a slightly lower intrinsic fluorescence emission intensity, (ii) a near-UV CD spectrum characterized by an identical signal at 291 nm and by a completely lost signal at 284 nm and (iii) a 3-fold increased ANS emission fluorescence intensity (Supplementary Material, Fig. S4). We tried to favour the peroxisomal import by culturing the cells expressing the S81L-Ma/S81L-Ma in the presence of 100 μM pyridoxine to favour holoenzyme formation. However, only a partial reduction of the cytosolic amount of the protein could be observed.

Interestingly, the quantitative analysis of the distribution of AGT in cells expressing the G170R-Mi/S81L-Ma construct showed a significant peroxisomal localization as highlighted by staining with both the anti-AGT and the anti-FLAG antibody (Pearson coefficient of 0.55 and 0.53 for the colocalization of the peroxisomal marker with anti-AGT or anti-FLAG, respectively) (Fig. 6). It is worth to point out that cells expressing the G170R-Mi/S81L-Ma construct will produce S81L-Ma/S81L-Ma homodimers, G170R-Mi/G170R-Mi homodimers and G170R-Mi/S81L-Ma heterodimers. However, since the FLAG epitope in the construct is associated with the G170R-Mi moiety, the staining with the anti-FLAG antibody will only label G170R-Mi/G170R-Mi homodimers (known to be targeted to mitochondria) and G170R-Mi/S81L-Ma heterodimers. Therefore, the detection of the FLAG signal in the peroxisomes not only confirms the formation in an appreciable amount of the G170R-Mi/S81L-Ma heterodimers but also indicates their localization into peroxisomes.

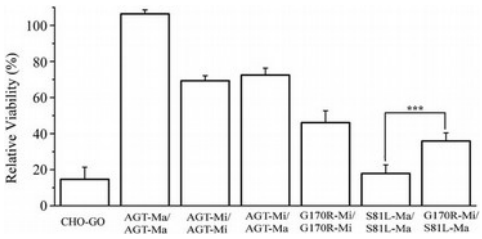

Figure 7. Glycolate cytotoxicity assay of CHO-GO cells expressing the AGT variants. The histogram is representative of the cell viability after 24 h of treatment with 250 μM glycolate expressed as percentage of untreated control. Bar graphs represent the mean ± SD. $^{***}P < 0.001$.

S81L and G170R mutations in homozygous and heterozygous conditions: effect on the AGT functionality

A glycolate-toxicity assay was used to assess the AGT functionality in the analysed clones. CHO-GO cells were treated with 250 μM glycolate for 24 h to allow the formation of peroxisomal glyoxylate by GO. Since glyoxylate induces cell death, the ability to survive in the presence of glycolate is a direct measure of the ability to detoxify peroxisomal glyoxylate. As shown in Figure 7, the protection against glycolate toxicity of AGT-Ma/AGT-Ma, AGT-Mi/AGT-Mi and AGT-Mi/AGT-Ma was 70–100% that of untreated cells. The G170R mutation reduced by about 50% the protection afforded by AGT-Mi/AGT-Mi, whereas the S81L mutation reduced by about 80% the protection afforded by AGT-Ma/AGT-Ma. Finally, the cell line expressing both variants has a viability equal within experimental error to that of cells expressing only the G170R-Mi variant. Thus, the amount of AGT able to detoxify glyoxylate in cells expressing both S81L-Ma and G170R-Mi is equal to that of cells expressing only G170R-Mi and higher than that of cells expressing S81L-Ma. This finding along with the coenzyme binding affinity and the subcellular localization of the heterodimer is relevant for its functionality.

DISCUSSION

In rare recessive diseases, many patients are compound heterozygous. Thus, their enzymatic phenotype results from the combination of the effect(s) of each single mutation when heteromeric protein forms are generated. Positive, negative or no IC effects are usually observed at the clinical level. These effects have been also investigated at the molecular level for few diseases such as phenylketonuria, argininosuccinate lyase deficiency and hypogonadotropic hypogonadism (27–29). These studies have been performed by looking to either the '*in vitro*' catalytic properties of the heteromeric proteins or the specific activity upon coexpression of the two variants in yeast and bacterial systems. Up to now, the correlation between the enzymatic and the clinical phenotype of PH1 compound heterozygous patients has never been investigated, although they represent a large percentage of PH1 patients.

In this work, for the first time, clinical, biochemical and cell biology data have been collected with the aim of understanding the effects of the S81L mutation on the major allele and the G170R on the minor allele under homozygous and heterozygous conditions. Our investigation was not limited to the AGT activity of the purified recombinant forms of S81L-Ma, G170R-Mi and S81L-Ma/G170R-Mi and to the specific activity upon expression or coexpression of the S81L and G170R variants in CHO cells but was extended to their expression level, coenzyme binding affinity, subcellular localization and functionality. This broad spectrum analysis resulted to be useful considering the multiplicity of the molecular mechanisms accounting for AGT deficiency. The results gave new significant insights into the PH1 mechanism, as we report the molecular basis of the pathogenicity of the S81L mutation on the major allele and the interplay between this mutation and the G170R mutation on the minor allele. When expressed individually, the S81L-Ma and G170R-Mi variants show distinct features. As already reported and confirmed in this study, when only G170R-Mi allele is expressed, AGT retains catalytic activity and PLP binding affinity, but it is almost completely redirected to mitochondria (12,15,17,18,24,25). On the other hand, we here report that when the S81L-Ma allele (lacking the putative MTS) is expressed, AGT shows a decreased catalytic activity, is almost completely present in the apo-form and is mainly peroxisomal. Thus, while the pathogenicity of the G170R mutation is related to AGT mistargeting, that of the S81L mutation is linked to the reduction of PLP binding affinity. What happens upon coexpression of both alleles? A pool of monomeric species with either the G170R mutation on the minor allele or the S81L mutation on the major allele should be virtually present in the cytosol. In theory, three different dimeric species, homodimers G170R-Mi, homodimers S81L-Ma and heterodimers G170R-Mi/S81L-Ma would be generated from the combination of the two coexpressed monomers. We were able to recreate the naturally occurring mutations S81L and G170R in AGT identified in compound heterozygous patient and to isolate and characterize the heterodimer in the purified recombinant form. This allowed us to establish that it exhibits a considerable k_{cat} value and, in agreement with its $K_{D(PLP)}$ value, it is present in the cell mainly in the holo-form. More importantly, it shows a peroxisomal localization. The peroxisome-to-mitochondrion mistargeting of AGT in PH1 is due to the combined effects of (i) the generation of an MTS resulting from the P11L polymorphism and (ii) the presence of monomeric AGT. Since in the heterodimer G170R-Mi/S81L-Ma, only the first condition occurs, even if limited to one subunit, its peroxisomal localization is not surprising. It can be also speculated that in cells expressing both S81L-Ma and G170R-Mi, the rate of the formation of the heterodimer is faster than that of the import of G170R-Mi monomers in mitochondria. Moreover, although cells expressing both G170R-Mi and S81L-Ma show a reduced expression level, they display an ability to detoxify glyoxylate greater than that of cells expressing homodimer S81L-Ma and equal to those expressing homodimers G170R-Mi. In the first case, this could be interpreted on the basis of the significant amount of protein present in the holo-form. In the second case, the detoxification ability could be ascribed to the proper subcellular localization of the heterodimer.

Altogether, these results indicate that the resulting hybrid protein has catalytic parameters not significantly different from those obtained theoretically by averaging the homodimeric counterparts, and a reduced expression level in the cellular system. However, the PLP binding affinity, the subcellular localization and the ability to detoxify glyoxylate make the

heterodimer more functional than the homodimeric counterparts. This implies that the association between the G170R and S81L monomers leads to a global positive IC between these mutations. On these bases, it can be speculated that compound heterozygous patients for the S81L mutation on the major allele and the G170R mutation on the minor allele could have a milder phenotype than homozygous S81L patients. In this regard, although it is not possible to drawn definitive conclusions only from two patients, the clinical data reported in this study appear to agree with our biochemical data. Indeed, the G170R/S81L compound heterozygous patient had a late onset of ESRD compared with the one functionally hemizygous for the S81L mutation who only expresses the S81L-Ma variant.

This work constitutes a valid example of an approach useful to understand the enzymatic phenotype in compound heterozygous PH1 patients, with potential applications to the study of the interaction between other pathogenic mutations. In fact, it should be emphasized that the use of different experimental approaches has been crucial to shed light on the molecular mechanisms of IC effects between the S81L and the G170R mutation. Besides providing a better knowledge of the disease pathogenesis, the results obtained could allow to develop proper therapeutic treatments, as the hybrid protein could have defects different from those of the parental enzymes.

MATERIALS AND METHODS

Materials

PLP, L-alanine, glyoxylate, pyruvate, rabbit muscle L-lactic dehydrogenase and mouse anti-FLAG antibody were purchased from Sigma-Aldrich. All other chemicals were of the highest purity available. CHO-GO cells, the rabbit polyclonal anti-AGT human, the anti-AGT-Ma and the guinea-pig anti-peroxisomal protein antibodies were kindly provided by Prof C.J. Danpure of the University College London, UK. Oligonucleotides were purchased from MWG Biotech.

Plasmids construction

To obtain the cDNA encoding the AGT-glutathione S-transferase (AGT-GST) fusion protein, the human AGT cDNA cloned in a pTrcHis2A prokaryotic expression vector (pAGThis) (6) was provided of a 5′ NcoI restriction site, a 3′ thrombin cleavage site and a 3′ BamHI restriction site by three sequential PCR amplifications using the forward primer AGTGSTfwd and the reverse primers AGTGSTrevA, AGTGSTrevB and AGTGSTrevC (Supplementary Material, Table S1). The GST cDNA was amplified from the pGEX-4T vector and provided of a 5′ BamHI restriction site and a 3′ EcoRI restriction site using the forward primer GSTfwd and the reverse primer GSTrev (Supplementary Material, Table S1). The two amplification products were digested with the BamHI restriction enzyme and ligated to obtain the AGT-GST fusion protein cDNA. To construct the plasmid pAGT-GST, the AGT-GST cDNA was subcloned into the pET28a vector using the NcoI and EcoRI restriction sites.

To generate the pAGTFLAG-Ma plasmid, the sequence (GAT TACAAGGATGACGATGACAAG) encoding for the FLAG tag (DYKDDDDK) was introduced at position 762 into the AGT coding sequence by means of site-directed insertion mutagenesis using the pAGThis construct as template, the insertion primers AGTFLAG1 and AGTFLAG2 (Supplementary Material, Table S1) and their complements.

Each construct was confirmed to be free of mutations by DNA sequencing.

Site-directed mutagenesis

Site-directed mutagenesis was performed by the QuikChange II site-directed mutagenesis kit (Stratagene) using the pAGThis-Ma (9) the pAGTFLAG-Ma and the pAGT-GST constructs as templates and the primers reported in Supplementary Material, Table S1.

Construction of pIRES vectors

The pIRES vector is a bicistronic plasmid that allows to express two genes in one transcript in mammalian cells. pIRES vectors encoding two copies of the AGT cDNA in different combinations (Fig. 1) were constructed by two subcloning phases. In the first phase, the cDNA encoding for AGTFLAG-Ma, AGTFLAG-Mi, AGTFLAG-S81L-Ma and AGTFLAG-G170RMa, obtained by site-directed mutagenesis from the pAGTFLAG-Ma construct, were subcloned in the first polycloning site using the primers pIres1fw and pIres1rev (Supplementary Material, Table S1) and the restriction enzymes NheI and EcoRI. In the second phase, the cDNA encoding for AGT-Ma, AGT-Mi, AGT-S81L-Ma and AGT-G170R-Mi were subcloned in the second polycloning site using the primers pIres2fw and pIres2rev (Supplementary Material, Table S1) and the restriction enzymes XbaI and NotI. Each construct was confirmed to be free of mutations by DNA sequencing.

Cell culture, selection and lysis

CHO-GO cells were cultured at 37°C under O_2/CO_2 (19 : 1) in Ham's F12 Glutamax medium (Invitrogen) supplemented with foetal bovine serum (10% v/v), penicillin (100 units/ml), streptomycin (100 μg/ml) and zeocin (0.4 mg/ml). Cells were transfected using the Turbofect^{TM} Transfection Reagent (Thermo-Fisher). After 24 h, G-418 (1.25 mg/ml) was added as selective agent and cells were left to grow for 48 h. To generate monoclonal stably transformed cell lines, the antibiotic-resistant transformants were seeded at limiting dilution of 10 cells/ml in 96-well plates in selective medium and incubated until single colonies started growing. Clones (at least 12 for each construct) were then growth and screened for exogenous gene expression by IFM and western blot. Cells were trypsinized, harvested and lysed in phosphate-buffered saline (PBS), pH 7.2, plus protease inhibitors (Complete Mini, Roche), by five freeze/thaw cycles followed by addition of DNAse (100 units/ml) at room temperature for 45 min. The whole cell extract was then centrifuged at 29,200*g* for 10 min at 4°C to obtain the soluble cellular lysate. Protein concentration was determined using the Bradford protein assay.

Enzymatic activity assays

For the measurement of AGT activity, 90 μg of cell lysate were incubated with 0.5 M L-alanine and 10 mM glyoxylate at 25°C in 100 mM potassium phosphate buffer (KP), pH 7.4, in the

presence or in the absence of 200 μM PLP. In order to obtain a detectable signal of pyruvate in the linear phase of the kinetics, the reaction time was set to 20 min for cells expressing the AGT-Ma/AGT-Ma, AGT-Mi/AGT-Ma and AGT-Mi/AGT-Mi constructs, and to 60 min for cells expressing the other constructs. The reactions were stopped by adding TCA 10% (v/v), and pyruvate production was measured using a spectrophotometric assay coupled with lactate dehydrogenase as previously described (6).

The kinetic parameters for the overall transamination of the pair L-alanine/glyoxylate of normal and mutant AGT were determined by incubating the purified protein (0.1 μM) in the presence of 200 μM PLP in 100 mM KP at 25°C and by varying the substrate concentration at a fixed saturating cosubstrate concentration. Pyruvate formation was measured by the spectrophotometric assay already reported (6). Data were fitted to the Michaelis–Menten equation.

GO enzymatic activity was measured by incubating 10 μg of cell lysate with 4 mM glycolate in 100 mM KP, pH 7.4, previously oxygenated and equilibrated at 25°C. The reaction was stopped after 15 min by addition of 10% TCA, and the glyoxylate produced was detected by HPLC after 2,4 dinitro-phenilhydrazine derivatization (30).

Transcript analysis

The total mRNA content was extracted from cells using the RNAeasy kit (Qiagen) and analysed by real-time PCR as described in Supplementary Material.

Western blot and immunoprecipitation

Four micrograms of cell lysate were loaded per lane on a Mini-Protean TGX™ pre-cast gel (Biorad) along with the Precision plus protein Kaleidoscope™ (BioRad) molecular mass markers. Proteins were transferred on a nitrocellulose membrane by the iBlot device (Invitrogen), and the membrane was blocked in 5% milk solution in TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h at RT. For AGT detection, the membrane was incubated with polyclonal rabbit anti-AGT serum (dilution 1 : 6000), washed three times in TBST and then incubated with peroxidase-conjugated anti-rabbit IgG (dilution 1 : 10 000). Blotted proteins were detected with ECL™ (Millipore), using the ChemiDocXRS Imaging System (BioRad, Hercules, CA, USA). Densitometry analysis was performed using the software ImageJ. For immunoprecipitation, 300 μg of cell lysates were incubated overnight with 300 μl of PBS buffer supplemented with 3 μg of mouse anti-FLAG IgG. The solutions were then incubated with Protein-A-Sepharose for 1 h, and the protein–antibody complexes were recovered by centrifugation. Samples were washed three times with PBS and analysed by western blot using a rabbit anti-AGT-Ma antiserum (1 : 1000).

Glycolate-toxicity assay

The glycolate-toxicity assay was performed as previously reported (15). CHO-GO cells were seeded at 7.000 cells/well in a 96-well plate and incubated 24 h before inducing glyoxylate production by adding Hepes buffered glycolate, pH 7.0, at a final concentration of 250 μM. Cell viability was evaluated after further 24-h incubation using the crystal violet staining (Sigma–Aldrich). Briefly, cells were rinsed with PBS, incubated at room temperature for 5 min with 4% formaldehyde + 0.5% crystal violet solution to perform fixing and staining. Cells were then extensively washed with distilled water to remove the excess of dye and lysed with 1% SDS in PBS to allow crystal violet solubilization and quantification. The absorbance at 595 nm, which is proportional to the number of viable cells, was measured with a TECAN plate reader. Six replicates were performed for each assay condition. Statistical analysis was performed with GraphPad Prism Version 5.0 (GraphPad software, San Diego, CA, USA).

IFM

A total of 3×10^5 cells were grown for 24 h into a 13-mm glass coverslip on a 24-well plate. For the mitochondrial labelling, cells were vitally stained for 30 min with MitoTracker Red (Molecular Probes). Cells were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBS and then blocked in 3% bovine serum albumin. For the immunolabelling, rabbit polyclonal anti-human AGT, anti-peroxisomal protein form guinea-pig and mouse monoclonal anti-FLAG were used as primary antibodies, and Alexa Fluor-conjugated antibodies (AF488 and AF555, Life technologies) were used as secondary antibodies. Nuclei were stained with DAPI (Molecular Probes), and the coverslips were mounted over slides in AF1 medium (Dako). The images were captured using a confocal laser-scanning fluorescence microscope Leica SP5 (Molecular Probes, Leica Microsystem) at 63× magnification. Pearson's coefficients were calculated using the ImageJ (http://rsb.info.nih.gov/ij/) JACoP plugin, and images were processed using Adobe Photoshop.

Expression and purification

The his-tagged AGT-Ma, AGT-Mi, G170R-Mi and S81L-Ma variants were expressed in *E. coli* and purified as already described (6). The S81L-Ma/G170R-Mi heterodimers were obtained by co-transforming *E. coli* BL21 (DE3) cells with vectors encoding the GST-tagged G170R-Mi variant and the his-tagged S81L-Ma variant and purified by a Ni-affinity chromatography followed by a GST-affinity chromatography. Details are given in Supplementary Material. The apo-form of each variant was prepared as previously described (6). The protein concentration in the AGT samples was determined using an extinction coefficient of 9.54 × 104/M/cm at 280 nm (6).

Determination of $K_{D(PLP)}$

The $K_{D(PLP)}$ values of heterodimeric S81L-Ma/G170R-Mi and those of homodimeric S81L-Ma were determined by measuring the quenching of the intrinsic fluorescence of the apoenzyme and the change of the CD signal at 430 nm, respectively, in the presence of PLP at varying concentrations, as described in Supplementary Material.

Spectroscopic measurements

Absorption, fluorescence and CD spectra were registered as described in Supplementary Material.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Appendix 2

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NDT
Nephrology Dialysis Transplantation

NDT Perspectives

Primary hyperoxaluria Type 1: indications for screening and guidance for diagnosis and treatment

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Abstract

Primary hyperoxaluria Type 1 is a rare autosomal recessive inborn error of glyoxylate metabolism, caused by a deficiency of the liver-specific enzyme alanine:glyoxylate aminotransferase. The disorder results in overproduction and excessive urinary excretion of oxalate, causing recurrent urolithiasis and nephrocalcinosis. As glomerular filtration rate declines due to progressive renal involvement, oxalate accumulates leading to systemic oxalosis. The diagnosis is based on clinical and sonographic findings, urine oxalate assessment, enzymology and/or DNA analysis. Early initiation of conservative treatment (high fluid intake, pyridoxine, inhibitors of calcium oxalate crystallization) aims at maintaining renal function. In chronic kidney disease Stages 4 and 5, the best outcomes to date were achieved with combined liver–kidney transplantation.

Keywords: combined liver–kidney transplantation; nephrocalcinosis; oxalosis; primary hyperoxaluria type 1; urolithiasis

Introduction

The term 'primary hyperoxaluria' (PH) encompasses an indeterminate number of rare autosomal recessive calcium oxalate kidney stone diseases, of which three have been described at the molecular level. PH1 is caused by mutations in the AGXT gene, which lead to dysfunction of the vitamin B6-dependent liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT) [1, 2]. PH2 arises from mutations in the GRHPR gene and subsequent dysfunction of the enzyme glyoxylate/hydroxypyruvate reductase (GRHPR) [3–5]. PH3 is caused by mutations in the HOGA1 gene, which is thought to encode the mitochondrial enzyme 4-hydroxy-2-oxoglutarate aldolase [6].

This paper focuses on PH Type 1 (PH1, OMIM 259900), the commonest form of PH. The clinical presentation ranges from asymptomatic through to isolated or recurrent renal stones, nephrocalcinosis and renal impairment. The heterogeneous presentation leads to a diagnostic challenge and therefore, specific biochemical and genetic assessment is required to confirm a diagnosis of PH1 and to institute appropriate treatment.

Materials and methods

PH1 is a very rare inherited disease with a limited access to recommendations for diagnosis and management, due to the lack of randomized clinical trials and meta-analyses. An expert group (OxalEurope) has therefore been established to provide diagnostic and therapeutic recommendations for patients with PH. Experts were selected on both their individual commitment and their peer-reviewed publication activity in this field. Number of PubMed papers were (MeSH: hyperoxaluria, October 2011):

Acquaviva 3, Cochat 34, Danpure 64, Daudon 25, De Marchi 3, Fargue 8, Groothof 6, Harambat 7, Hoppe 43, Hulton 7, Jamieson 10, Kemper 20, Mandrile 3, Marangella 45, Picca 1, Rumsby 41, Salido 15, Straub 2 and van Woerden 6. Due to the rarity of the disease and the lack of evidence coming from randomized clinical trials, recommendations are based on ungraded statements.

Demography

PH1 has an estimated prevalence ranging from one to three per million and an estimated incidence rate of ~1:100 000 live births per year in Europe [7–9]. Higher rates are reported in isolated populations [10]. PH accounts for ~1% of paediatric end-stage renal disease (ESRD) in registries from Europe, USA and Japan [11–13]. In contrast, PH is more prevalent in countries where consanguineous marriages are common: ~10% of Kuwaiti and ~13% of Tunisian children with ESRD have been reported to have PH [14, 15].

Presentation

PH1 may present at any age. The presentation varies from infantile nephrocalcinosis and failure to thrive as a result of renal impairment to recurrent or only occasional stone formation [16]. Although patients with presentation in adulthood often have a history of only sporadic stone disease, over 50% of these patients present with ESRD at the time of diagnosis [9, 17]. Some patients may be identified as presymptomatic subjects with a family history of PH1 [16]. As a result of kidney injury, glomerular filtration rate (GFR) always declines leading to chronic kidney disease (CKD) and ultimately to ESRD and further systemic involvement (named 'oxalosis').

Clinical and radiological assessment

(1) We recommend considering a diagnosis of PH in any child with a first kidney stone and in adults with recurrent stone disease.
(2) We recommend considering a diagnosis of PH in any subject with nephrocalcinosis particularly when associated with decreased GFR.
(3) We recommend searching for PH in the presence of oxalate crystals (calcium oxalate monohydrate) in any biological fluid or tissue.
(4) We recommend screening relatives of index cases.
(5) We do not recommend screening in the general population.

Patients should undergo metabolic screening for PH1 at presentation of a first kidney stone (in a child) or recurrent or familial stone disease (at any age) or if nephrocalcinosis is detected. Stone analysis may reveal characteristic morphology and contain >95% calcium oxalate (CaOx) monohydrate (whewellite) often presenting with a particular morphology [18]. PH1 should be considered in any patient with renal failure of unknown cause, particularly in the presence of nephrocalcinosis or severe stone burden.

Preliminary PH1 diagnostic workup should include 24-h urine collection for oxalate, creatinine and glycolate; plasma oxalate (POx) when GFR is <60 mL/min/1.73m^2.

More specific investigations are covered in the biochemical and genetic sections below.

Ultrasonography (US) of the kidneys may elucidate stones and/or medullary or diffuse nephrocalcinosis. Patients with ESRD from PH may have diffuse cortical nephrocalcinosis which can be hard to distinguish from the common picture of an 'end-stage kidney' by US (Table 1).

Table 1. Organ involvement in PH patients with renal failure[a]

Organ	Symptoms	Diagnosis
Kidney[b]	Stones, medullary or diffuse nephrocalcinosis, cortical nephrocalcinosis	US CT (cortical nephrocalcinosis may be missed on US)
Bone[c]	Fractures, bone pain, growth retardation	X-ray: dense or lucent metaphyseal bands at the growth cartilage plate, vertebral condensations, osteopenia, epiphyseal nuclei (target-like) knee epiphyses
Eye[c]	Disturbed vision, specific brown coloured retinal deposits	Fundoscopy
Arteries[d]	Media calcifications	US, CT
Myocardium[d]	Cardiac failure, arrhythmia, heart block, left ventricular hypertrophy, systolic and diastolic dysfunction	ECG, echocardiography CT: calcifications
Thyroid[d]	Hypothyroidism	US Thyroid function tests
Skin[e]	(Painful) skin nodules, skin necrosis, gangrene, calciphylaxis-like skin lesions, pruritus	Skin biopsy
Nerves[e]	Ischaemic neuropathy	Clinical assessment
Muscle[e]	Myopathy by CaOx deposition	Biopsy, CT
Bowel[e]	Prolonged oxalosis: depositions of CaOx in the intestinal wall	CT
Joints[e]	Arthritis (late sign)	X-ray, CT

[a]CT, computed tomography.
[b]Always involved.
[c]Frequently involved.
[d]Often involved.
[e]Less often involved.

PH patients with a GFR <60 mL/min/1.73m^2 should undergo regular measurement of POx and monitoring of eye involvement by fundoscopy (Table 1). In addition, those with a GFR <30 mL/min/1.73m^2 should undergo assessment of cardiac involvement by ECG—echocardiography, and bone by X-ray as appropriate.

US and computed tomography scan of heart and visceral organs can assist in evaluating the level of calcification in patients with systemic oxalosis.

Biochemical and enzymological assessment

(1) We recommend measuring 24-h urine oxalate, creatinine and glycolate in any patient with a possible diagnosis of PH1 and preserved renal function as a first-line evaluation.

(2) We recommend measuring plasma oxalate in CKD patients.

(3) We recommend measurement of AGT enzyme activity if genetic testing is inconclusive.

The usual biochemical indicator of PH1 is a persistently and markedly elevated urine oxalate (UOx) excretion >0.5 mmol/1.73m^2 per day in the absence of secondary causes of hyperoxaluria. There is no clear cut-off for primary disease versus secondary hyperoxaluria: an excretion >0.7 mmol/1.73m^2 per day is more likely to have a metabolic cause but some secondary cases due to Crohn's disease, other chronic intestinal disease, short bowel syndrome and pancreatic insufficiency secondary to cystic fibrosis [19, 20] can have grossly elevated (>1 mmol/1.73m^2 per day) oxalate excretion. Values which fall between 0.5 and 0.7 cannot exclude PH and a strong clinical suspicion e.g. recurrent stones, young age, heavy crystalluria (>200 calcium oxalate monohydrate crystals/mm^3) should lead to more specific investigations. In children, oxalate to creatinine ratio can be determined on random urine specimens, but ratios fall rapidly in early life and are influenced by prematurity and nutrition; thus, interpretation requires an age-related reference range [21, 22]. Molar creatinine ratios from spot urine may give conflicting results so that confirmation of hyperoxaluria from a 24-h urine collection related to body surface area is recommended at all ages. A raised urine glycolate excretion is suggestive of PH1 but has a low diagnostic sensitivity and specificity; indeed, it is elevated in only two-thirds of patients and can rise as a result of dietary intake.

POx is unhelpful for diagnosis if renal function is normal. POx will increase as GFR falls and there is no clear cut-off to distinguish patients with PH from those with renal failure from any other cause, although values >100 μmol/L are more likely to be due to PH. Plasma glycolate may also be helpful in some patients [23].

The presence of oxalate crystals in a renal biopsy brings further support for a metabolic disturbance as the underlying cause.

The gold standard diagnostic test is the measurement of AGT catalytic and immunoreactivity in a liver biopsy specimen [24], which has a sensitivity >95%; false negatives are possible, albeit rarely, in patients with the p.Gly170Arg mutation [25] in which catalytic activity is preserved but the enzyme is non-functional *in vivo* due to intracellular mislocation. The wider availability of genetic testing has increased use of DNA analysis for diagnosis although there are still some cases in whom no mutation is found in the *AGXT* gene; AGT catalytic activity is therefore needed in these patients in order to completely exclude PH1. Additional genetic analysis of the *GRHPR* and *HOGA1* genes can be performed to confirm/exclude PH2 and PH3, which may have a similar presentation.

Whenever the causative mutation(s) are known, DNA analysis is the method of choice for prenatal testing and diagnosis in other family members.

Genetic tests for mutations in the AGXT *gene*

(1) We recommend genetic testing in subjects with phenotypic characteristics of PH1.

(2) We recommend extending mutation analysis to siblings and parents.

(3) We recommend offering prenatal diagnosis using mutation analysis to parents of an affected child.

Over 150 different mutations have been found so far in the *AGXT* gene [26]. Although most mutations are restricted to individual families, some are found at high frequency e.g. p.Gly170Arg (also known as G170R) found in ~30% of mutant PH1 alleles. Many PH1 mutations, including p.Gly170Arg, segregate and functionally interact with the common intragenic polymorphism Pro11Leu [27], which appears to increase their pathogenicity.

Genomic DNA isolated from ethylenediaminetetraacetic acid blood is the sample of choice. Salivary DNA and chorionic villus (for prenatal) are also suitable for diagnosis and carrier testing. All samples should be accompanied by detailed clinical and laboratory data to support the appropriateness of the request as well as documented consent for the analysis.

Targeted sequence analysis of Exons 1, 4 and 7 of *AGXT* has been proposed for first-line testing, with test sensitivity of 70% for a single mutation in a biopsy-proven population [28]. However, additional sequence of the entire coding region and flanking intronic regions will be required in up to 50% of patients and is strongly recommended. While it is not advised to include polymorphic variants in reports, the presence of the p.Pro11Leu (major/minor) variant is of significance and we recommend that this change is included in the report. It is recommended to always extend the analysis to both parents to confirm the segregation of any identified mutation(s). The results should be reported following recognized guidelines for nomenclature (www.hgvs.org) and content (www.ssgm.ch). For novel mutations, the report should include some indication of the likely pathogenicity.

For affected individuals and families in whom no mutation or only a single disease-causing mutation is identified, additional analyses may be indicated. Multiplex ligation-dependent probe amplification analysis can be used to search for gene deletions or duplications. No recommendation can yet be given on its performance with *AGXT* due to

little experience with the available kit. If no diagnosis is achieved, testing for PH2 and PH3 should be considered, even if evidence is limited to few patients studied. In cases with a strong suspicion but no mutation found in the three genes, a liver biopsy should be considered.

Linkage analysis with intragenic (e.g. the variable number tandem repeat in intron 4) and extragenic markers can be helpful particularly for prenatal diagnosis and family studies [29–31]. The accuracy can be as high as 99% but depends on a correct clinical diagnosis of PH1 in the affected relative(s) and on the informativeness of genetic markers in the family.

Any report of genetic test should be given to the patients by a genetic counsellor or specialist/consultant with a good knowledge of PH. However, prediction on the clinical course of the disease cannot be made on the basis of the genetic findings and the clinical follow-up of the index case, hence, prenatal counselling is extremely difficult.

Conservative treatment

(1) We recommend starting conservative therapy as soon as a diagnosis of PH1 has been suggested.

a) We recommend a high fluid intake, at least 3 L/m^2 per 24 h.

b) We recommend using a nasogastric tube or gastrostomy feeding tube to guarantee adequate hydration, mainly in infants.

c) We recommend administering vitamin B6 (pyridoxine) in any patients with proven PH1, starting at a dose of 5 mg/kg per day and not exceeding 20 mg/kg per day, aiming to decrease urine oxalate excretion by <30%.

d) We recommend calcium oxalate crystallization inhibition by use of alkalization with oral potassium citrate at a dose of 0.10–0.15 g/kg body weight per day (0.3–0.5 mmol/kg) as long as GFR is preserved.

(2) We do not recommend special dietary interventions other than for other concurrent diseases in the absence of CKD.

Conservative measures should be initiated, as soon as investigations are completed and while renal function is maintained. Once ESRD is established, pyridoxine is the only specific treatment that should be pursued.

The following measures apply to all types of PH with the exception of pyridoxine, which is specific to PH1.

High fluid intake. High fluid intake in stone formers has been proven to be effective in epidemiological and prospective intervention studies [32]. In PH, the recommended fluid intake is >3 L/m^2 per day, distributed throughout 24 h. In infants and small children, a feeding or gastrostomy tube is often required. Special care should be taken in situations of fluid losses (diarrhoea, vomiting and fever) or limited oral hydration (surgery) and intravenous (i.v.) fluid intake instituted if necessary.

Vitamin B6. Pyridoxal phosphate, one of the vitamin B6 vitaminers, is a cofactor for AGT. Administration of pyridoxine hydrochloride has been shown to be associated with a decrease in UOx in ~30% of patients with PH [33, 34], but the metabolic basis of pyridoxine responsiveness is not clear. All PH1 patients should be tested for pyridoxine responsiveness, and if responsive, treated until liver transplantation is performed, even if undergoing haemodialysis (HD). The recommended starting dose is 5 mg/kg per day, increasing by 5 mg/kg steps to a maximum of 20 mg/kg per day [35]. Responsiveness has been noted at doses inferior to 5 mg/kg per day. Responsiveness is currently defined by a >30% decrease in UOx excretion after a test period of a minimum of 3 months at maximum dose [36, 37]. Absorption of pyridoxine may vary between patients and assessing plasma levels may be useful, although therapeutic levels are not clearly defined. Side effects are rarely seen and sensory neurotoxicity is unusual. A subset of patients carrying one or two copies of p.Gly170Arg or p.Phe152Ile mutation are more likely to respond to pharmacological doses of pyridoxine, with other mutations possibly similarly responsive [38, 39].

Alkalization of the urine. Alkalization of the urine with alkali citrate can reduce urinary CaOx saturation by forming complexes with calcium thus decreasing stone growth or nephrocalcinosis [40]. Potassium citrate at a dose of 0.10–0.15 g/kg body weight per day (0.3–0.5 mmol/kg) is recommended. It may be replaced by sodium citrate appropriate to GFR and plasma potassium.

Other inhibitors of calcium oxalate crystallization. Other inhibitors of calcium oxalate crystallization such as pyrophosphate ions may decrease CaOx crystallization although orthophosphate has never been evaluated independently of other treatments [41]. Moderate doses of phosphate 20–30 mg/kg per day may be administered. There is no evidence that magnesium monotherapy can prevent stone formation [42, 43]. Despite the ability of *Oxalobacter formigenes* to metabolize oxalate, there is as yet no evidence that probiotics can significantly decrease UOx excretion in PH patients [44, 45].

Diet. A restriction in oxalate intake is of limited use as the main source of oxalate is endogenous and intestinal oxalate absorption is lower in PH patients compared to normal subjects [46]; however, relying on precautionary principle, some experts recommend avoiding oxalate-rich foods in the diet. Calcium intake should remain normal as oral calcium binds intestinal oxalate and dietary calcium restriction results in higher oxalate intestinal absorption [47, 48]. Excessive intake of vitamin C and D is to be avoided. Careful vitamin D supplementation in children is recommended. Ascorbic acid i.v. supplementation should be used with caution in dialysis PH patients [49].

Monitoring. The monitoring of urinary pH, volume and oxalate excretion may be useful. When advanced CKD has been reached, oxalosis bone involvement may be responsible for impaired responsiveness to erythropoiesis-stimulating agents and to growth hormone treatment [50–52].

Surgical management of urolithiasis

(1) We do not recommend any kind of surgical intervention in PH1 patients with uncomplicated urinary stone disease, except when there is obstruction, infection or multiple urolithiasis.

(2) We recommend endoscopic procedure as preferential strategy to manage urolithiasis in patients who require intervention.

The management of intraluminal stones by the urological surgeon is complicated by the potential of concomitant presence of nephrocalcinosis. The successful removal of intraluminal stones can only be assessed by endoscopy, as even successfully treated completely stone-free kidneys will reveal 'residual stones' on imaging when nephrocalcinosis is present. Non-endoscopic treatment (i.e. lithotripsy) holds the risk of misinterpretation, which means that shock waves are applied on nephrocalcinosis spots instead of stones [53]. Different minimally invasive methods like extracorporeal shock wave lithotripsy (ESWL), semi-rigid ureterorenoscopy (URS), flexible ureterorenoscopy (RIRS), percutaneous nephrolithotomy (PCNL) and laparoscopic approaches are currently established for interventional stone treatment. Open surgery has become exceptional in this field.

ESWL has been successfully applied to most kidney and ureter stones and has emerged worldwide as a standard tool [54]. It is the preferred treatment for intrarenal calculi <20 mm diameter. Success rates after ESWL, meaning stone-free kidneys, range from 55 to 90%. However, ESWL means *in situ* fragmentation of the stone, leaving the gravel behind for elimination by natural route. As a consequence, stone clearance of the urinary tract can be incomplete. Up to 60% of patients with small residual stone fragments (<3 mm) after ESWL will have increasing accumulation and formation of new stone masses on these residues [55]. PH1 stone formers experience a high risk of prompt stone re-growth on such residues because of ongoing hyperoxaluria.

While guidelines still recommend ESWL [56], in daily practice, this is completely replaced by endoscopy in PH1 patients when multiple stones are present [57]. URS, RIRS and PCNL allow fragmentation and achieve removal of stone material under direct visual control. Stone size and localization determine whether a retrograde, flexible or percutaneous access is the most appropriate technique. By the aid of pneumatic, electrohydraulic, ultrasound or holmium laser probes, stones are crushed into small removable fragments or even dust which can be flushed out. The patient can expect excellent stone-free rates, reaching 80–100% for all techniques [URS, RIRS (kidney stones < 20 mm) and PCNL]. Endoscopy allows a complete clearance of the urinary tract at the end of the procedure, which is different from ESWL.

Dialysis procedures

(1) We recommend avoiding any form of dialysis unless absolutely necessary and to consider pre-emptive transplantation in PH1 patients with progressive CKD.

(2) We recommend using high efficacy dialysis, such as daily HD, nocturnal dialysis, combination of HD and peritoneal dialysis (PD), in patients where pre-emptive transplantation is not an option.

(3) We do not recommend dialysis in the early postoperative transplantation period other than indications described in the transplant guidelines.

(4) We do recommend haemodialysis/filtration for clearance of oxalate during and after organ transplantation in patients with systemic involvement and/or insufficient urine outflow in the early post-transplant period.

Although the molecular mass of oxalic acid is small (90 Da), conventional dialysis is unable to remove sufficient quantities of oxalate proportionate to the continuous daily production. Oxalate is generated at a rate of 4–7 mmol/1.73m^2 per day in contrast to removal via conventional dialysis at a rate of 1–2 mmol/1.73m^2 per day in adults and 3–4 mmol/1.73m^2 per day in children, resulting in an uncontrolled tissue accretion rate [23, 58, 59].

Consequently, conventional dialysis is not considered ideal for patients with systemic oxalosis who have reached ESRD [16, 60]. However, long-term dialysis may be needed while awaiting organ transplantation and achieving adequate body size. PD and HD have been used either alone or in combination in order to maximize oxalate removal [59, 61]. The peculiar distribution of oxalate mass in the body explains the limitations of dialysis treatment even when optimized. In systemic oxalosis, tissue oxalate is in equilibrium with oxalate in body fluids. When plasma CaOx supersaturation (β_{CaOx}) is reached, oxalate precipitation occurs; the threshold of CaOx supersaturation ($\beta_{CaOx} > 1$) ranges between 30 and 45 μmol/L, also depending on serum calcium concentration [62, 63]. Thus, the goal should be to lower POx enough with dialysis to keep it below β_{CaOx} for as long as possible during the interdialytic period.

Oxalate clearance on HD is greater than on PD (~120 mL/min on HD compared to ~7 mL/min on PD) [64]. Standard HD programmes will result in a weekly clearance of oxalate of 6–9 mmol/1.73m^2 per week equivalent to 2–3 days of endogenous production of oxalate [65]. PD is insufficient to clear adequate quantities of oxalate but in some patients, a combination of overnight PD using continuous cycling PD/nocturnal intermittent PD and intermittent daily HD can enhance the overall clearance of oxalate and attempt to reduce the rebound which occurs after HD. The use of combination therapy, high-flux dialysers or long episodes of haemofiltration has been advocated to improve oxalate removal [60, 66].

However, while HD can reduce POx by ~60% following a dialysis session, this will return to 80% of the pre-dialysis levels within 24 h as HD removes only a small fraction of total body oxalate, followed by a rebound from bone turnover [67].

HD and/or continuous renal replacement therapy may be required following isolated liver transplantation where sequential hepatorenal transplantation is being undertaken or following combined transplantation where there has been a delay in improvement of renal graft function. In

these circumstances, the benefit of intra-operative and post-transplantation dialysis is debated. It can be considered in patients with significant systemic involvement where the urine excretion is limited. Dialysis in these circumstances will produce a rapid fall in POx thus protecting the transplanted kidney from tubulotoxic effects and oxalate deposition. In any case, the risk of CaOx supersaturation should be avoided with accurate fluid management [60, 68].

Transplantation strategy

(1) We recommend planning pre-emptive organ transplantation at CKD Stage 3b to avoid the complications of systemic oxalosis.
(2) We do not recommend isolated kidney transplantation, unless there is no other option.
(3) We recommend combined liver–kidney transplantation in most patients, either simultaneously or sequentially according to patient's condition and to local facilities.
(4) We do not recommend pre-emptive isolated liver transplantation unless in very well-defined and selected patients.

Organ transplantation should be planned prior to systemic oxalosis, i.e. before CKD Stage 4.

Kidney transplantation. There is no scientific rationale for isolated kidney transplantation, and it should be considered only for selected adult patients with confirmed evidence of B6 responsiveness [38].

Liver transplantation. As the liver is the only organ responsible for glyoxylate detoxification by AGT, the excessive production of oxalate will continue as long as the native liver is left in place.

The strategy of liver–kidney transplantation is influenced by the stage of the disease (Table 2) [69]. Simultaneous liver–kidney transplantation is logical in patients with CKD Stage 4 because, at this level, oxalate retention increases rapidly. It has been used successfully with excellent outcome even in small infants [70, 71]. A sequential procedure (first liver transplantation, followed by dialysis attempting to reduce oxalate load from the body, with subsequent kidney transplantation) may be proposed in individual ESRD patients. Pre-emptive isolated liver transplantation may be an option in selected patients supervised by a PH specialist [72–74]. Such a strategy has a strong rationale but raises ethical controversies since the transplant procedure of choice needs to be individualized as conservative management has improved long-term outcome in many patients with PH1.

Most publications currently report on the use of deceased donors but a living related donor for split liver or renal donation may be considered under appropriate conditions.

Post-transplantation reversal of renal and extrarenal involvement. After combined liver–kidney transplantation, UOx can remain elevated for many years due to slow resolubilization of systemic CaOx. Therefore, recurrent nephrocalcinosis or renal calculi is still a risk and may jeopardize kidney graft function. The kidney must therefore be protected by forced fluid intake supported by the use of crystallization inhibitors, and calcineurin inhibitors should be used with caution in order to minimize additional nephrotoxicity. The benefit of post-transplantation HD is still debated and should be limited to patients with significant systemic involvement and those with acute tubular necrosis or delayed graft function.

Conclusions

Hyperoxaluria should be considered in any patient with a history of urolithiasis and/or nephrocalcinosis. Such patients should be referred to reference centres with access to appropriate biochemical and genotyping facilities. An early and accurate diagnosis leading to aggressive supportive treatment is a major factor in short- and long-term outcomes. No method of dialysis is ideal; however, intensive extended daily dialysis should be recommended. Early pre-emptive transplantation should be considered in those with impaired renal function at an early stage (CKD Stage 3b); most experience in PH1 is available with combined liver–kidney transplantation.

Table 2. Suggested transplantation options in pyridoxine-resistant PH1 patients according to residual GFR, systemic involvement and local facilities[a]

Tx options	*Simultaneous liver + kidney*	*Sequential liver–kidney*	*Isolated kidney*	*Isolated liver*
HD strategy	Peroperative ± postoperative according to POx and GFR	Standard HD following liver Tx aiming at POx < 20 µmol/L	Preoperative and peroperative	Sometimes peroperative
CKD Stage 3 (30 < GFR < 59)	No	No	No	Option in carefully selected patients
CKD Stage 4 (15 < GFR < 29)	Yes	Option	Option if B6 response but no evidence	No
CKD Stage 5 (GFR < 15)	Yes	Yes	Option if B6 response but no evidence	No
Infantile form (ESRD < 2 years)	Yes	Yes	No	No

[a]POx, plasma oxalate; Tx, transplantation.

New insights into potential therapies, including restoration of defective enzyme activity through chemical chaperones, hepatocyte cell transplantation or recombinant gene therapy for enzyme replacement, provide some hope for a curative approach of PH1 in the future.

Access to information

Several national and international societies provide information on the PHs. The Oxalosis and Hyperoxaluria Foundation (www.ohf.org) has an extended website with open access and provides regular updates for physicians, patients and scientists. It offers an overview of presentation, diagnosis and treatment of the disease. The European Hyperoxaluria Consortium OxalEurope (www.oxaleurope.org) brings together clinicians and scientists throughout Europe; it hosts a website that directs visitors to country and language-specific websites. UpToDate, Inc. (www.uptodate.com) and Medscape (www.medscape.com) provide useful information. Genetic information can be obtained on www.orpha.net and www.genereviews.org.

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