Primary hyperoxaluria type 1: clinical, genetic and biochemical studies
van Woerden, C.S.

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CHAPTER 09  General discussion

The work presented in this thesis was carried out to explore a number of clinical and biochemical concepts regarding primary hyperoxaluria type 1 (PH1). The studied concepts comprised the following:

1. Epidemiology of hyperoxaluria:
   Description of the clinical phenotype of patients with PH1 in The Netherlands including the age at presentation, symptoms and clinical outcome of patients.
   The incidence of PH1 in a cohort of routinely screened patients with urolithiasis.
   The occurrence of hyperoxaluria in patients with generalized peroxisomal disorders, i.e. Zellweger spectrum disorders.
2. The association between genetic, biochemical and clinical parameters with respect to outcome of kidney function in PH1.
3. Additional biochemical studies in PH including in vitro and in vivo studies concerning oxalate synthesis and studies on the appropriate collection of urine for diagnostic investigations.

09.01  EPIDEMIOLOGY OF HYPEROXALURIA

Most important conclusions:
1. The prevalence and incidence of PH1 found in the Dutch cohort study is three times higher than expected on the basis of previous data.
2. PH1 is often undetected, especially in adults. This is an important factor in the development of renal failure in PH1.
3. Over 80% of patients with Zellweger spectrum disorders have hyperoxaluria. Clinically important hyperoxaluria occurs predominantly in patients with severe neurological impairment.

At onset of the study, reliable epidemiological data on PH1 were lacking. We therefore decided to study its prevalence and clinical spectrum in a nationwide survey. We recruited the Dutch cohort of PH1 patients by interviewing all nephrology centers in The Netherlands. Due to outstanding contacts of the Dutch Society of Nephrology with her members, we could approach nephrologists, both for pediatric as well as for adult medicine. This resulted in a relatively high number of PH1 patients retrieved in The Netherlands, compared to studies in other countries: 2.9 per million, which is three times higher than previously observed (1). This high number is most probably a reflection of our thorough search strategy. However, we believe that the observed prevalence is still an underestimation of the true prevalence for the following reasons:

1. Our methods of patient retrieval were limited to nephrologists. It is likely that other specialists are also involved in the diagnosis and treat-
ment of these patients, such as general practitioners, internists, or urologists and may be the primary physicians involved in their follow-up. Approaching these specialists might further reduce this under-ascertainment. In a first step to test this hypothesis, we investigated to what extent PH1 had remained undetected using the current routine diagnostic procedures of our own center. Therefore, we performed an automated search on all urinary oxalate measurements that had ever been performed at the general hospital laboratory. Those patients that were found to be hyperoxaluric were reinvestigated by offering analysis of urinary oxalate, glycolate, L-glycerate and if necessary AGXT gene analysis. Even though the search comprised a selected patient group, we found one new PH1 patient. This demonstrates that our estimated prevalence (2.9 per million) is indeed an underestimation of the true prevalence. We conclude that this strategy is an easy and quick way to discover PH1 patients. Neuhaus and co-workers have studied the occurrence of hyperoxaluria in a cohort of pediatric patients with urolithiasis or nephrocalcinosis and found hyperoxaluria in more than 8% of the patients (2).

2. As compared to children, a much higher proportion of adult patients was diagnosed with already end-stage renal disease, despite the presence of renal symptoms in many of them before the onset of decline of renal function. In some patients, the diagnosis of PH1 was suspected only after histological investigation of a kidney biopsy, after early failure of a kidney transplant. These findings illustrate that the diagnosis is often missed or delayed, especially in adults. This is supported by numerous case-reports that describe the diagnosis of PH1 after ‘unexplained’ decline of renal function in patients (3-12).

3. The age at time of development of end-stage renal disease in our cohort was comparable to that determined by Cochat et al. in their cohort study (29 vs. 25 years) (1). In both studies, data were derived from patients in nephrology units. Therefore, the clinical spectrum of these cohorts may be biased towards severe presentation, since nephrologists are often consulted for the first time when renal insufficiency develops. Moreover, data from the Dutch registry RENINE have been used. Input of patient data in this registry is a prerequisite for reimbursement of the dialysis costs. Our search strategy ascertains that we have traced all PH1 patients in end-stage renal disease, covering the severe end of the spectrum. Because of the different methods we used, our cohort is likely to represent the real spectrum of PH1 more accurately than other studies. However, it is very likely that, we may have missed PH1 patients, especially on the mild end of the spectrum of the disease.

In conclusion, we found evidence for an important underdetection of PH1. Timely institution for adequate conservative therapy may prevent renal damage. We therefore advise extensive screening in all patients with urolithiasis, nephrocalcinosis or renal insufficiency.
(ZSDs), we found a high incidence of hyperoxaluria (83%). Although three ZSD patients with hyperoxaluria had been described before, the finding of hyperoxaluria at such a large scale was new. We were able to investigate these patients thoroughly due to the long period of follow-up. Pyridoxine was tried but turned out to be non-efficacious in patients with ZSDs. Since we found marked hyperoxaluria especially in those patients with severe psychomotor developmental delay who were not able to express pain in case of urolithiasis, it is important to screen the kidneys by means of ultrasound at an early age.

A considerable difference in urinary oxalate levels was observed between ZSD patients, with the highest levels found in patients with the severest clinical form of Zellweger spectrum disorders. The variability of the peroxisomal defect may result in varying clinical and biochemical phenotypes, as reflected by varying levels of neurological impairment and varying levels of urinary oxalate. These variations may arise from ghost peroxisomes which have some residual peroxisomal function, or from peroxisome mosaics, in which livers display cells with normal peroxisomes adjacent to cells that resemble Zellweger hepatocytes, i.e. with AGT in the cytoplasm (13). This may result in a milder phenotype. This may also explain how AGT can function to a variable degree in patients with Zellweger spectrum disorders. To prove this hypothesis, we studied a liver biopsy specimen of a patient with mild neurodevelopmental impairment and slightly raised levels of urinary oxalate (60 mmol/mol creatinine, upper level of normal reference range 54 mmol/mol creatinine) (courtesy Prof. M. Espeel). After careful electron-microscopic analysis of the biopsy specimen, peroxisomes were found to be present with a normal appearance in 10% of the investigated liver cells. Since patients with Zellweger spectrum disorders normally synthesize the AGT enzyme, a difference in urinary oxalate excretion cannot be explained by differences in levels of residual AGT activity. A difference in percentage of normal AGT compartmentalization, however, may well explain the variation in urinary oxalate excretion and renal involvement. The finding of peroxisomal mosaics in a mildly affected patient is in line with this thought. Other patients should be studied to further explore this idea.

09.02 CLINICAL, BIOCHEMICAL AND GENETIC PREDICTORS OF OUTCOME IN PH1

Most important conclusions:
1. Age at time of onset is not a good prognostic factor.
2. Adults with PH1 predominantly present with renal insufficiency as first symptom. This implies that the so-called late onset PH1 is not a benign variant of the disease, as previously was suggested.
3. There are strong indications that early conservative treatment
indeed may prevent the development of renal failure.
4. Neonatal PH1 has generally but not always a worse prognosis.
5. Nephrocalcinosis as detected by ultrasound is a bad predictor for end-stage renal disease.
6. Pyridoxine responsive patients have potentially a good prognosis, provided that adequate conservative treatment is established before the onset of renal failure.
7. The level of residual AGT-(in)activity does not predict the outcome.
8. Gly170Arg is the most predominant substitution found in Dutch patients with PH1
9. Patients homozygous for Gly170Arg or Phe152Ile substitutions are responsive to pyridoxine.
10. Patients homozygous for Gly170Arg or Phe152Ile substitutions with renal failure should not be eligible for orthopic liver transplantation.
11. An additional Val336Asp substitution to a Gly170Arg substitution induces pyridoxine unresponsiveness.

In the epidemiologic survey, we investigated the predictive value of five clinical and biochemical outcome parameters and of the various genotypes. The results and clinical implications are discussed below. Since the discovery of the deficiency of the enzyme AGT in PH1 and mutations in the gene AGXT, which codes for AGT, attempts to link clinical prognosis to biochemical levels of AGT enzyme activity, levels of urinary oxalate or AGXT gene mutations had not been very successful. The incompleteness of the cohort studies with respect to the clinical follow-up could have masked the discovery of clear relationships between biochemical and genetic parameters on the one hand and outcome on the other hand. Therefore, in addition to a systematic approach assessing the clinical spectrum of PH1, we evaluated clinically relevant biochemical and genetic parameters in relation to this spectrum.

**Age of first symptoms**

Previously, it was believed that PH1 could be categorized into three groups, namely: (1) a severe form, with infantile presentation before the first year of age and rapid decline of renal function; (2) a mild form with adult onset, few symptoms and preservation of renal function, and (3) an intermediate form with childhood onset and gradual progression to renal insufficiency (14).

We found no prove for this categorization. In strong contrast, in our cohort the percentage of adult patients with end-stage renal disease at the time of diagnosis was significantly larger than of pediatric patients. We also found that patients with first manifestations of the disease in infancy did not always develop early renal insufficiency. Infants and small children who did not present with end-stage renal disease but with urolithiasis or hematuria and were treated timely could preserve their renal function over time. Therefore, a prompt and adequate dia-
gnostic work up seems to predict the outcome and severity of renal insufficiency rather than the age of presentation itself. Other, more recently performed epidemiologic studies in various countries are in line with these findings (15-17).

In conclusion, our study implies that PH1 is an inherited disorder that can become symptomatic at any age. We suggest that all physicians, either dealing with pediatric or with adult patients, should be aware that development of end-stage renal disease is always possible in PH1.

09.02.02

**Pyridoxine responsiveness**

Pyridoxine therapy had been used for more than 20 years in PH1, and Milliner et al. have assessed its benefits in an observational study (18). They demonstrated a favorable effect of pyridoxine by inhibition of urinary crystallization. No cohort studies had addressed the efficacy of pyridoxine since then. In our cohort, we could clearly demonstrate a correlation between pyridoxine responsiveness and outcome. Renal insufficiency occurred more often if urinary oxalate did not decline in response to pyridoxine administration. The efficacy of pyridoxine in patients may have been underestimated in our epidemiologic study since we censored data at renal insufficiency and we did not measure any potential effect of pyridoxine in patients diagnosed in end-stage renal disease. As we demonstrated in our genetic study, efficacy of pyridoxine should also be assessed after renal transplantation since good efficacy of pyridoxine resulted in prolonged preservation of the kidney transplant. Alternatively, pyridoxine efficacy may be assessed using plasma oxalate and plasma glycolate (19,20), as was also demonstrated in one of our patients (21).

Therefore, the addition of data on patients treated with pyridoxine in end-stage renal disease demonstrated that responsiveness to pyridoxine treatment resulted in a clinically relevant decline of urinary oxalate and preservation of renal function.

09.02.03

**Nephrocalcinosis**

As expected, we found an association between the appearance of nephrocalcinosis on ultrasound and the development of renal insufficiency. However, this association was surprisingly weak. On the one hand, we found that more patients with nephrocalcinosis than without nephrocalcinosis at the time diagnosis had an adverse outcome. On the other hand, however, not all patients with nephrocalcinosis developed renal failure over time. We also found patients with end-stage renal disease due to PH1 without apparent (cortical) nephrocalcinosis on abdominal ultrasound, though oxalate crystals were demonstrated in a kidney biopsy specimen. This phenomenon was recently confirmed by Kim et al. (22). Accordingly, it appears that the exact relationship between the results of renal ultrasound and pathological observations is not clear.
in humans, despite the good correlations found in animal studies between the extent of calcium oxalate depositions in kidney biopsies and nephrocalcinosis as detected by ultrasound (23). Apparently, calcifications in a kidney biopsy specimen, as detected by polarized light microscopy or so called von Kossa staining, may be too small to be visible by renal ultrasound. Therefore, renal ultrasound is not always a good predictor of severity of renal involvement and the absence of nephrocalcinosis does not exclude renal involvement in PH1. Moreover, nephrocalcinosis is not an independent patient characteristic, since its presence depends on the moment at which the ultrasound is performed. Since the available renal ultrasounds, as used in the epidemiologic study were made at different time points during the course of the disease in patients, they may have led to incomplete observations. In addition, the follow-up of nephrocalcinosis, as detected by renal ultrasound, may be biased by the growth of the kidneys at more advanced ages of patients. Although they appear less dense on the ultrasound picture, renal calcifications may not have vanished. Instead, the growth of the kidneys may have diluted the density of the kidney lesions. This may lead to an apparent decrease of the degree of nephrocalcinosis. No studies have addressed this problem, so its clinical implication remains to be elucidated. More studies are necessary to assess the relationship between nephrocalcinosis as detected by ultrasound, and the severity of renal pathology, to make renal ultrasound a better predictor of renal involvement.

Levels of AGT activity

We found that levels of AGT activity do not predict outcome of renal function. This heterogeneity had been observed by others as well (24). However, AGT levels show a relationship with the presenting age of patients. Patients with the highest levels of AGT activity (>15%) have the first symptoms at a more advanced age as compared with patients with a lower level of residual AGT activity. On the other hand, as demonstrated by the epidemiologic data of Chapter 3, all patients with renal involvement at neonatal age have very low levels of AGT residual activity. This is partly presented in TABLE 04.3, of Chapter 4, and we herewith provide additional, unpublished data depicted in FIGURE 09.1. In clinical practice, this finding can be of use in patients who have been found by family screening: residual AGT activity of ≥ 15% can be expected to result in later development of symptoms in general. A higher residual AGT activity could result in a better peroxisomal glyoxylate-to-glycine conversion compared with patients with a lower or undetectable activity. This can lead to lower rates of oxalate production, and a lower oxalate burden for the kidneys, with later renal involvement. This hypothesis could not be tested in our cohort, since many patients were diagnosed in end-stage renal disease and no urine was available for oxalate analysis.
Even though we found this relationship between levels of AGT catalytic activity and age of first symptoms, from studies in families, it emerges that the outcome of the disease is quite heterogeneous in patients from the same family (25, 26). It may be difficult to compare the clinical follow-up of the index patient with the patient who has been found by screening, since screening will lead to earlier treatment, and this is likely to influence the clinical outcome. Therefore, despite the association between age at first symptoms and AGT levels, the final outcome also seems to be determined by environmental factors and proper treatment.

**Levels of urinary oxalate**

We did not detect a relationship between levels of urinary oxalate on the one hand, and development of renal insufficiency on the other hand. Until the start of our project, no other studies had addressed this relationship. The failure to find such a relationship could be explained in two ways: first, oxalate levels have been determined at different laboratories, and the use of different analysis techniques could mask a clear relationship. Secondly, many patients, especially those found at adult age, were diagnosed in end-stage renal disease. Since we could not measure urinary oxalate in these patients, data were incomplete. Nevertheless, urinary oxalate concentration may become a predictor of disease severity, if we acquire more prospective data in the future. Recently, the International Registry for primary hyperoxaluria revealed that levels of urinary oxalate above the median level, as found in PH1 patients, predispose to significantly shorter renal survival (27).

**Genotype**

Our genetic studies have clearly shown that a high number of Dutch PH1 patients had the Gly170Arg genotype, as compared to other stu-
dies: 44% versus ~30% (16, 28-30). The German and the Dutch PH1 patient cohorts were different in this respect since the homozygous Gly170Arg genotype was not found in the German cohort. The close geographic relationship between The Netherlands and Germany would predict the same genotypes for PH1. This genotypic difference is probably explained by a difference in patient retrieval. Our systematic search strategy included all nephrologists, both for children and adults, but Hoppe *et al.* only included patients diagnosed by pediatric nephrologists (16). We showed that the Gly170Arg mutation generally resulted in symptoms at adult age, so these patients may not have been found by Hoppe *et al.* in their study. Other studies reported on AGXT data of patients whose DNA had been sent in for diagnostic DNA analysis from different countries. These results are also biased since they were not obtained via a systematic search. Surprisingly, the Ile244Thr genotype was not identified in our cohort. Instead, we observed a high allele frequency of Phe152Ile, which was detected in a homozygous form for the first time. It may therefore be a Dutch founder mutation.

**Gly170Arg and Phe152Ile substitutions**

We found that patients with Gly170Arg and Phe152Ile genotypes had relatively high levels of AGT activity and a relatively late onset of symptoms. The latter finding has also been described by others (30-34). We also found they were all pyridoxine responsive and that early pyridoxine treatment can prevent development of ESRD in these patients. In retrospect, the favorable outcome of patients with this mutation was also found in other patient reports, but because of the limited number of patients in these studies, this relationship was less clear (25,30). In addition to our findings, Monico *et al.* demonstrated a dose dependent relationship of the Gly170Arg genotype in relation with pyridoxine efficacy (31). Pyridoxine responsiveness was already predicted in an earlier report which suggested mitochondrial mistargeting for the Phe152Ile mutation (32) and was demonstrated in another patient in 2005 (35). We were able to demonstrate this relationship convincingly, because we also included patients who already received a kidney transplantation, showing pyridoxine responsiveness thereafter. Yet, some of these patients developed end-stage renal disease despite the presence of the pyridoxine-responsive genotype. This occurred before the diagnosis of PH1 was made, and pyridoxine therapy was initiated. Hence, no Gly170Arg and Phe152Ile patients with end-stage renal disease had been on adequate treatment before the onset of end-stage renal disease. Even after transplantation, oxalate excretion declined and kidney function was preserved as a result of pyridoxine treatment. Based on these observations, we believe that delayed diagnosis and treatment must have caused end-stage renal disease in these patients. Two patients with end-stage renal disease despite having the Gly170Arg genotype received a second renal graft for reasons of increasing syste-
mic oxalosis and renal insufficiency. However, these patients had not been treated with pyridoxine previously. Probably, late diagnosis and late initiation of pyridoxine treatment had caused widespread systemic oxalate depositions, compromising the renal graft. Alternatively, a second, undiscovered AGXT mutation may be present in these patients, since other patients in our cohort and that of others also carried two mutations on the same allele (16). If the clinical response to pyridoxine is different contrary to what is expected based on the AGXT gene profile, such additional mutations should be suspected.

The mechanism of action of pyridoxine is unknown. Our studies did not address this issue, and we can only speculate on the action of pyridoxine. First, a chaperone role for pyridoxine, thereby inhibiting mitochondrial import of AGT, could be envisaged, but there is no true evidence for this postulate (36). Secondly, we know that 10% of AGT can still be routed to the peroxisomes, in the presence of the Gly170Arg, and possibly the Phe152Ile mutation (34). There, it may be functional, and stimulated by the addition of its cofactor pyridoxal-5-phosphate. Since pyridoxine affects both oxalate and glycolate excretion, it is likely that it acts on AGT activity, and not on other enzymes, such as GRHPR, that would selectively reduce oxalate production, by diverting glyoxylate to glycolate.

Although we found this relationship between these mutations and pyridoxine responsiveness, other, yet unknown pyridoxine responsive biochemical phenotypes of AGT and its AGXT mutations may exist. Therefore, we advise that a trial with pyridoxine therapy should always be performed and its efficacy be measured by means of plasma oxalate or glycolate levels.

Val336Asp substitution to the Gly170Arg and Phe152Ile substitution
Interestingly, we observed a previously unknown AGXT mutation Val336Asp, homozygous in one patient (patient 15), and heterozygous in another (patient 22). The mutation was inherited on the same allele as the most common substitution Gly170Arg. Whether this results in elevated urinary oxalate in the parents was not investigated. Although these patients were also homozygous for the favorable Gly170Arg substitution, the outcome was poor. The homozygous Gly170Arg/Val336Asp patient did not respond to pyridoxine and rapidly developed end-stage renal disease. The patient who was compound heterozygous for Phe152Ile on one allele and Gly170Arg, Val336Asp on the other allele, was responsive to pyridoxine. However, levels of urinary oxalate remained higher, than found in patients with only the Gly170Arg or the Phe152Ile genotype (levels about two times the upper limit of reference range). This patient developed nephrocalcinosis and kidney stones, and showed development of a mild renal insufficiency (glomerular
filtration rate 70 ml/min/1.73 m²). Recently, Huber et al. found that a specific region of human AGT, called PTS1A, probably contains additional peroxisomal targeting information (37). This genetic information is most likely located within the sequence coding from the amino acid region between Val-324 and Ile-345 in the AGT protein. The Val336Asp amino acid substitution in these two Dutch patients probably interacts with this region and might prevent peroxisomal import. This could abolish pyridoxine responsiveness and result in lower AGT activity. This implies that screening for the most common mutations can confirm the diagnosis of PH1, but cannot predict the outcome, unless the entire AGXT gene is investigated. In this respect, it is also interesting to look at the Met340Ile mutation that is present on the minor allele in some of the patients. Since this polymorphism is also located in the region where the PTS1A signal is proposed to exist, it may interact with the additional peroxisomal targeting signal. Whether this is clinically relevant, should be evaluated in larger patient groups.

**Gly82Arg substitution, c.33_34insC insertion**
The Gly82Arg substitution and c.33_34insC insertion were all associated with an adverse outcome in our cohort. The c.33_34insC mutation resulted in an absent catalytic AGT activity, absent pyridoxine responsiveness and early development of end-stage renal disease in our cohort. These findings are in line with those found by Milosevic et al (38). For the c.33_34insC mutation, the lack of residual AGT protein is not surprising since a frame shift occurs in the AGXT gene which leads to a truncated protein. However, in an Italian cohort of PH1 patients, the homozygous c.33_34insC mutation was found to be associated with a less severe clinical picture. The favorable outcome may have been the result of earlier diagnostics, but no information on this point was given. In vitro studies for a Gly82Glu substitution show a failure to bind to the co-factor pyridoxal-5-phosphate, which may also occur in Gly82Arg. Nevertheless, one patient found by screening because of an affected brother with the Gly82Arg mutation, preserved her renal function into adulthood, and had an uneventful pregnancy. This demonstrates that early adequate treatment may ameliorate clinical outcome.

**Single heterozygous mutations**
In contrast, if only one heterozygous mutation is found in patients, AGT activity is not necessarily predicted to be normal, as we described in Chapter 4. Mutations that exist outside the regions covered by our primer sets could influence gene transcription or translation, for example if there are sequence variations in the promoter region of the gene. A transcription defect was found in three patients who were homozygous for the minor allele by Purdue et al (34). However, comparable studies could not be undertaken in our patients, because liver samples were not available.
Most important conclusions:
1. 24 hours storage of 12 hours urine collection without acidification does not influence the oxalate measurement.
2. Endogenous oxalate production measurement by stable isotopes is reliable in PH patients but not in healthy controls.

Three biochemical studies were carried out concerning diagnostic procedures in PH1, which will be discussed below.

Procedure for the collection of urine for diagnostic screening in hyperoxaluria
For diagnostic screening of PH1, acidification of urine immediately upon voiding has usually been performed to prevent oxalate precipitation, which could result in false-negative results. As we showed in Chapter 2 acidification can be performed in the laboratory and can be delayed for at least 24 hours. Collection at home is safe and reliable in dry bottles, which are stored in the fridge to prevent bacterial growth. This obviates the use of hydrochloric acid and its dangers at home.

Measurement of endogenous oxalate production by stable isotopes
Plasma oxalate concentration and urinary oxalate excretion have been shown inaccurate parameters to monitor endogenous oxalate production (EOP) in PH1 patients with renal insufficiency. A reliable parameter is needed in these patients to evaluate the efficacy of any therapeutic intervention. Therefore, we devised a stable isotope oxalate tracer method to quantify the rate of appearance of oxalate ($R_a$ oxalate). Urinary oxalate excretion was well reflected by $R_a$ oxalate in all three patients. In healthy control subjects, however, $R_a$ oxalate was higher than expected as it exceeded urinary oxalate excretion several fold. Our results suggest that $R_a$ oxalate as determined by our method results in an overestimation. Furthermore, although a large variation has been reported in plasma oxalate concentration in healthy subjects, as summarized in a recent study describing a stable isotope dilution method to quantify plasma oxalate and glycolate concentration in vitro (19), plasma oxalate concentration as determined in our control subjects was in the higher range of the reported control values. The same dilemma was encountered in the study of France et al who described a method of measuring $R_a$ oxalate after a single injection [1,2-$^{13}$C]sodium-oxalate. They found a daily oxalate production rate of 1.89 mmol in a healthy subject (39). These authors contributed this high production rate of oxalate to in vitro oxalogenesis from ascorbate despite acidification of the plasma samples after collection. We cannot rule out that some in vitro oxalogenesis from ascorbate occurred before analysis of the samples as
this has been shown to occur already in acidified frozen urine during prolonged storage (40). If so, this will have caused an overestimation of plasma oxalate concentration and of $R_a$ oxalate in control subjects. Alternatively, urinary oxalate excretion could have been underestimated in control subjects due to precipitation of oxalate with calcium in vitro. This seems less likely, however, as urinary super saturation is low in control subjects (41) and urinary acidification has been shown effective in preventing calcium-oxalate precipitation (40). Since both $R_a$ oxalate and plasma oxalate concentration are higher in patients with PH1, the relative contribution of in vitro oxalogenesis to the total oxalate concentration in the plasma samples and $R_a$ oxalate is smaller in patients than in control subjects and is probably negligible in patients with renal insufficiency exhibiting a much higher plasma oxalate concentration. To limit the influence of possible in vitro oxalogenesis on the determination of $R_a$ oxalate and plasma oxalate concentration, plasma samples should be immediately acidified and analyzed as soon as possible after their collection. In conclusion, we believe that this method is a reliable tool to establish the effect of oxalate reducing therapy in PH patients with renal insufficiency.

**09.03.03 Oxalate production in $Agxt^{-/-}$ mice hepatocytes**

Little is known about the origin of glyoxylate, the direct precursor of oxalate, in human metabolism. A clear understanding of involved metabolic pathways is a prerequisite for the design of alternative therapies in PH1. Studies providing insight into the biochemistry of oxalate metabolism are limited especially because oxalate metabolism only occurs in hepatocytes, and immortalized hepatocyte cell lines of patients with primary hyperoxaluria are not available for research. We performed the first metabolic studies in AGT deficient mice, which act as a model for primary hyperoxaluria type 1. The experiments indicate a remarkably small flux through the glyoxylate pathway and none of the tested precursors was significantly enhancing oxalate and glycolate production, except for glyoxylate and glycolate. Since the peroxisome is not known as a metabolically active organelle in terms of high throughput of high amounts of metabolic products, this may reflect the true in vivo situation. However, more experiments in separate compartments should be done to observe glyoxylate fluxes through different organelles, such as the peroxisomes or the mitochondria. At this moment, no other precursor is known to influence the oxalate production, which prevents progress in research aiming at dietetic modifications, apart from modification of glyoxylate fluxes. In view of sustained glycine synthesis upon alanine addition and moderate oxalate excretion, our studies support a potential role for a second, potentially mitochondrial, AGT mediated glyoxylate metabolism, at least in mice. The development of mice with different knocked out enzymes, such as glycolate oxidase (GO) or glyoxylate reductase (GR) may facilitate research in this respect.
CLINICAL IMPLICATIONS OF OUR STUDY WITH RESPECT TO DIAGNOSTIC AND THERAPEUTIC STRATEGY IN PH1

FIGURE 09.2  Diagnostic algorithm for primary hyperoxaluria type 1.

FIGURE 09.3  Therapeutic algorithm for primary hyperoxaluria type 1
Update of diagnostic strategy

Patients at any age with signs unexplained urolithiasis, nephrocalcinosis or renal insufficiency should be screened for PH1. The DNA diagnostic approach in most patients eliminates the need for enzyme analysis by taking a liver biopsy. The absence of nephrocalcinosis on ultrasound is not a reliable prognostic determinant with respect to the development of renal damage. Family screening may detect patients at an early or even asymptomatic stage and should always be performed. FIGURE 09.2 provides an algorithm for the recommended diagnostic strategy.

Update of treatment in PH1

Conservative therapy

The patients who were found by family screening, since all of them were able to preserve renal function provide the best illustration of the protective effects of both a timely diagnosis and initiation of treatment. This is remarkable, since the index patients in these families developed renal insufficiency. Conservative treatment consisted of hyperhydration, pyridoxine and potassium citrate in most cases, and magnesium oxide in some. Potassium citrate was frequently prescribed as an anti-precipitating agent by pediatricians but not by adult nephrologists, who more often used magnesium oxide. The lack of citrate therapy was seen in patients who presented with severe secondary hyperoxaluria, as described in Chapter 5. Our observational study does not allow comparison between the efficacy of both drugs and we did not collect data on urinary citrate levels or urinary pH to determine if dosage was adequate, or if patients were compliant. All participating physicians prescribed hyperhydration. Since all patients, who were found by screening, preserved renal function, even though the index patients had end-stage renal disease, and pyridoxine did not reduce urinary oxalate excretion, hyperhydration has proven to be very effective in patients, regardless of the genotype. For pyridoxine, a clear relationship between efficacy and outcome was shown, as discussed in more detail above.

Transplantation

Most patients who received a solitary kidney graft had an excellent survival, contrary to what was usually expected (22,42-47). This is most likely due to the fact that all of these patients had a significant decline of urinary oxalate excretion under pyridoxine therapy. Most of these patients had developed end-stage renal disease before the diagnosis of PH1 was established. Institution of pyridoxine therapy at the time of diagnosis therefore prevented oxalate induced damage in the transplanted kidney. Mutation analysis in our cohort demonstrated pyridoxine responsiveness in patients with the most common Gly170Arg genotype and the Phe152Ile genotype.
Therefore, all PH1 patients who need to have kidney transplantation should be studied by \textit{AGXT} gene analysis to predict pyridoxine responsiveness. Patients with the Gly170Arg or Phe152Ile genotype will benefit from treatment with pyridoxine, regardless of their kidney function: preserved, in renal insufficiency or after kidney transplantation. If results of \textit{AGXT} gene analysis remain inconclusive regarding pyridoxine responsiveness, a trial with pyridoxine should be initiated. Response should be monitored by measuring urine and plasma levels of oxalate and glycolate at baseline and after one to three months of treatment with pyridoxine. Patient compliance can be ascertained by measuring plasma levels of pyridoxine.

\textbf{Choice of transplant}

As a liver or kidney transplantation may become necessary in patients in end-stage renal disease, timing of the procedure is important.

We suggest the following options for transplantation in PH1:

A. \textit{Kidney transplantation alone}

Although the metabolic defect is not corrected by kidney transplantation alone, our studies show that this is a reasonable option for patients in whom pyridoxine responsiveness is established based on either results of genetic studies, or after a trial of pyridoxine with assessment of efficacy by means of studies in plasma oxalate or glycolate.

B. \textit{Pre-emptive liver transplantation}

Although this is the only option to correct the enzyme defect, transplantation related morbidity and mortality prevents its use in all PH1 patients. Since we and others demonstrated a favorable follow-up in many patients with conservative treatment only, especially in those with a pyridoxine-responsive genotype, liver transplantation should not be performed in these patients. Instead, it should be reserved for patients who show no evidence of pyridoxine response, i.e. in patients with other mutations, and without any decline of plasma oxalate or glycolate levels or decline of the rate of appearance of oxalate upon treatment with pyridoxine. Since we also observed a favorable outcome with survival into adulthood of native kidneys even in patients without evidence of pyridoxine responsiveness, we suggest that a liver transplantation should not be performed until renal insufficiency becomes inevitable and the glomerular filtration rate declines below 60 ml/min/1.73m$^2$. This level is generally regarded as the cut-off for renal insufficiency and no morbidity from systemic oxalate deposition is expected at this stage. Preparations for a liver transplantation may be taken at this stage. In patients in whom renal function declines below 20-30 ml/min/1.73m$^2$, an urgent transplantation becomes important in order to prevent systemic oxalate depositions, as investigated previously (48).

C. \textit{Combined liver-kidney transplantation}

In patients with end-stage renal disease and no evidence for responsive-
ness to pyridoxine therapy as discussed under B, combined liver-kidney transplantation should be performed. Unfortunately, these patients are at risk for acute kidney graft failure due to wash out of oxalosis from body stores even after a successful combined transplantation. Sequential liver kidney transplantation is therefore proposed as a good alternative. In this procedure, renal transplantation follows liver transplantation when pre-dialysis plasma oxalate levels have declined up to 25 μM during intensive hemodialysis sessions. A plasma oxalate concentration of 25-50 μM is considered to be safe for kidney transplantation, without early graft failure due to oxalosis in the kidney. However, it usually takes a year or more to achieve such low oxalate levels under these conditions (personal communication with JJ Homan van der Heide) (49,50). Dialysis may need to be continued for some time after the kidney transplantation. Immunological difficulties enhancing transplant rejection may arise as the grafts stem from different donors. However, based on a small group of patients who have undergone this procedure in The Netherlands (n = 7), the outcome may be favorable and no increased incidence of graft rejection was observed in this small group.

**Therapeutic approach to the PH1 patient: an update**

Based on the current evidence we designed an algorithm that may be followed in the choice for optimal treatment:

A. Patients with normal renal function (GFR>80 ml/min)
   1. conservative treatment:
      hyperhydration 3L/m²
      potassium citrate (0.15 g/kg/day, once daily)
      pyridoxine (5-15 mg/kg/day, once daily)
   2. assess urinary oxalate or glycolate before and after treatment
   3. assess genotype to predict pyridoxine responsiveness

B. Patients with decreased renal function on conservative treatment
   GFR 20-80: conservative treatment as under A and:
   GFR 60-80: preparation for liver transplantation in pyridoxine unresponsive patients
   GFR 30-60: consider liver transplantation in pyridoxine unresponsive patients
   GFR<30: absolute indication for (sequential) liver (kidney) transplantation in pyridoxine unresponsive patients; indication for start hemodialysis

C. Patients with end-stage renal disease:
   1. assess genotype for potential pyridoxine responsiveness
   2. medication: start pyridoxine and evaluate responsiveness by plasma glycolate measurements or isotope dilution
   3. intensive (nocturnal) hemodialysis
   4. consider transplantation procedure:
isolated kidney if pyridoxine responsiveness is expected, after intensive dialysis sessions have declined plasma oxalate up to 25-50 μM (pre-dialysis values)
liver-kidney (preferably sequential, with intensive dialysis sessions declining plasma oxalate up to 25-50 μM, i.e. approximately 1 year of dialysis) if pyridoxine responsiveness is not expected.

**LIMITATIONS OF THE EPIDEMIOLOGICAL AND GENETIC STUDY IN PH1 PATIENTS**

In general, the strength of a cohort study depends on the completeness of patient retrieval, the availability of data, the number of patients involved and the length of the follow-up period. The results of our genotype-phenotype study were potentially biased by a limited patient participation and follow-up. First, a considerable number of patients from the epidemiological study have died, before their contribution to the genetic study could be ascertained, which may have created a potential loss at the severe end of the disease spectrum. Secondly, differences in the follow-up period between recruited patients may cause potential bias to observed outcome. The conclusions of our studies can only sustain if a careful prospective follow-up of patient data is carried out. An extended, international registry for careful patient follow-up is therefore warranted.

Another source of potential bias is the difference in treatment strategies followed in our patients. We noticed a difference in conservative treatment as prescribed by pediatric vs. adult nephrologists, as observed in the cohorts of Chapter 3 and Chapter 4. Citrate was widely prescribed by pediatric nephrologists, but scarcely used by nephrologists for adults. Citrate has shown to be efficacious in the reduction of urolithiasis and may therefore have induced a milder phenotype in general in our cohort. The use of intravenous fluids for the prevention of dehydration was strictly used at one of the adult centers which also may have contributed to a milder phenotype. These factors may contribute to differences within a certain genotypic sub group.

**IMPLICATIONS FOR FUTURE RESEARCH**

From the work presented in this thesis, new foci for research emerge.

1. The possibility that patients may remain undetected urges a thorough search strategy among patients with one of the mentioned urinary tract symptoms that may be the initial presentation of primary hyperoxaluria type 1. Family screening is not yet applied systematically and could further increase the prevalence of PH1 provided it is used more widely. Diagnostic studies in patients with unexplained recurrent uroli-
thiasis or other renal involvement should comprise metabolic investigations. Before we have implemented this, research should actively contribute to patient finding. Reinvestigation of patients in whom hyperoxaluria has been detected without any further metabolic studies may also lead to the discovery of new patients.

2. Though heterogeneity regarding genotype and phenotype has been confirmed by our studies, we demonstrated for the first time a relationship between a specific genotype and phenotype. Furthermore, we stated that prognosis was strongly influenced towards favorable outcome by early diagnosis and treatment. Since our studies were censored at a certain time point, follow-up studies should be undertaken to further substantiate the conclusions we extracted. These include (1) the favourable outcome of the Gly170Arg and Phe152Ile genotypes and their obligatory responsiveness to pyridoxine therapy, (2) the devastating clinical outcome of the c.33_34insC insertion and the Gly82Arg and Val336Asp substitutions.

3. An unresolved genetic issue is the detection of PH1 patients with apparently only one AGXT gene mutation. The biochemical profile, including highly elevated levels of urinary oxalate and glycolate, as well as absent catalytical AGT activity suggests that both alleles contain mutations. This needs to be resolved for diagnostic purposes, since heterogeneity of AGXT gene mutations cannot exclude PH1.

If our cohort of patients is too small to reach these goals, a multi-national database should be created to combine data for more power in research.

4. The predictive value of urinary oxalate needs further consideration. Since elevated oxalate excretion is the key stone for renal damage, and since decline of oxalate excretion upon treatment with pyridoxine is related to decrease of symptoms and damage, urinary oxalate levels seem to have a direct correlation with disease severity and prognosis. We were not able to demonstrate this, probably by the fact that many of the more severe patients were diagnosed with anuria and no urine was available for investigation. Collection of data from newly diagnosed PH1 patients may demonstrate such a relationship between biochemical and clinical phenotype. A relationship between genotype and levels of urinary oxalate may also appear.

5. Our studies in patients with Zellweger spectrum disorders demonstrate the importance of compartmentalization for AGT catalytic activity. The levels of hyperoxaluria as observed in these patients varies widely, but renal involvement was only observed in patients with high levels of oxalate and this was only present in patients with a clinically severe form of the Zellweger spectrum disorders. Follow-up should be performed to observe potential renal involvement in the other patients. A relationship with peroxisomal mosaics may be found if liver biopsies are studied in more patients.

6. The use of the stable isotope dilution technique in the evaluation of new potential therapies, that aim to reduce endogenous oxalate pro-
duction, such as scavenger molecules which bind toxic glyoxylate.

7. Metabolic studies in hepatocytes are warranted to identify precursors of endogenous oxalate synthesis. Our first studies in Agxt-/- mice were not conclusive, probably by interference of catalytically active mitochondrial AGT. The clinical significance of alanine supplementation may be investigated in vivo, since the in vitro studies showed restored glycine synthesis in Agxt-/- hepatocytes.

REFERENCES


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