Primary hyperoxaluria type 1: clinical, genetic and biochemical studies

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CHAPTER 01 GENERAL INTRODUCTION

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This thesis focuses on a single metabolic disorder of the liver, called **primary hyperoxaluria type 1** (PH1; MIM 259900). It is characterised by an overproduction of oxalate because of a deficiency of the peroxisomal enzyme alanine:glyoxylate aminotransferase. The clinical expression and the molecular-biochemical characteristics of PH1 are extremely heterogeneous. Patients may be asymptomatic, suffer from stone disease, or develop end-stage renal disease and systemic oxalosis with multi-organ disease. Presentation occurs at any age. Until recently, several important questions about PH1 were still unanswered:

- Which patients are at risk for a severe course of the disease?
- What is the optimal therapeutic strategy in any particular patient?
- What is the exact source of glyoxylate, the direct precursor of oxalate?

Aiming to find answers to these questions, we investigated the epidemiology of PH1 in The Netherlands. We also looked for associations between clinical characteristics and genetic and biochemical features in patients, in order to develop parameters that may facilitate the diagnostic procedures and decision-making in the treatment of patients with PH1. In addition, we investigated the incidence of hyperoxaluria in the Zellweger spectrum disorders, which comprise a group of patients with a generalized loss of peroxisomal functions. Finally, we aimed to measure the rate of appearance of oxalate in plasma in humans, and to unravel the exact sources of glyoxylate in order to find clues for new therapeutic approaches.

This chapter presents an overview of primary hyperoxaluria type 1:

- 01.01 the history of research in the twentieth century
- 01.02 its classification
- 01.03, 01.04 and 01.05 its metabolic and molecular basis
- 01.06 the pathophysiology and mechanisms leading to renal involvement and symptoms
- 01.07 a description of the clinical characteristics and outcome
- 01.08 and 01.09 an overview of diagnosis and treatment
- 01.10 an introduction to the Zellweger spectrum disorders
- 01.11 a short description of secondary hyperoxaluria
- 01.12 an outline of the research questions that we address in this project.

The colour illustrations of Chapter 01 can be found on the cover.
01.01 HISTORY OF PH1
Renal calculi are one of the oldest reported medical problems in humans, as demonstrated by archaeological studies and descriptions by Hippocrates, Celsus, Galen and Morgagni (68). Oxalate was recognized as a natural product in the eighteenth century. Already in the early 19th century, it had become possible to analyse urine crystals, and calcium oxalate was recognised as a frequent element of kidney stones, as reviewed by Hockaday and associates in 1839 (73). A four-and-a-half year old boy with “infiltration of renal parenchyma with crystalline depositions” was described in 1925 by Lepoutre (108), later marked as the first published case with primary hyperoxaluria. Urinary oxalate could not be measured at that time, but Lepoutre stated that this was a human presentation of renal oxalate calcification. He compared his findings with the animal studies of Ebstein and Nicolaier in 1897 (53). These investigators were able to induce the formation of calcium oxalate kidney stones in rabbits and dogs, following the administration of oxalate and oxamid in drinking water. Until the 2nd half of the 20th century, sporadic cases of the same type of kidney crystal deposition were published. One of the striking clinical stories tells about a boy, 11 years of age in 1942, who received a physical examination and a urine examination at the request of his mother, because of nocturia and polydypsia. It revealed extensive calcification of the renal parenchyma of both kidneys, called nephrocalcinosis, which appeared to involve the medullary pyramids predominantly, with some ureteral dilatation (212). In the early 1950s, two detailed reports appeared of children who died at 7 and 12 years of age of renal insufficiency with systemic oxalate depositions in multiple tissues and organs. This was the first clinical and pathological report of the most severe clinical form of primary hyperoxaluria, called oxalosis (26,50). Adult patients with hyperoxaluria and kidney stones were described as well. By 1960, Antoine and colleagues retrieved 36 cases in whom kidney parenchymal oxalate depositions had been found (4,30). They reviewed all cases, and found that presentation could be as early as 3 months of age up to as late as 57 years of age. An inherited defect was suspected, on the basis of the reports of affected relatives and identical twins (5). The combination of elevated levels of urinary oxalate with this clinical picture of nephrocalcinosis was identified in 1953 and in 1957 (6). Following urine analyses of patients, parents and controls using isotope-dilution techniques, elevated glycolate levels were demonstrated for the first time by Frederick et al. in 1963 (56). A second inherited form of hyperoxaluria was recognized in 1968, with the coincidence of elevated urinary oxalate and L-glycerate instead of glycolate (208). Subsequent studies led to the identification of the enzyme defect in PH2 at the level of D-glycerate dehydrogenase, which also displays glyoxylate reductase activity. Speculations about the metabolism leading to the generation of oxalate were already going on since the early 1950's. Glyoxylate
became recognized as the direct precursor of oxalate and experiments in the early 1960s pointed towards a defect in the glyoxylate to glycine interconversion in primary hyperoxaluria type 1 (56). However, it would take more than 20 years until Danpure and co-workers were the first to identify a deficient activity of the liver specific enzyme AGT (alanine:glyoxylate aminotransferase) in PH1 patients (47). Since then, a number of case reports and some comprehensive epidemiologic studies have been published (27, 72, 112). Insight into the origins of PH1 was further advanced when the gene mutated in PH1 was identified in 1991 which made DNA diagnosis possible (161). The biochemical and genetic causes of PH2 were unravelled later (39, 174) and patients were described, although at a much lower frequency compared to PH1 (95, 207).

01.02

GENERAL CONCEPT AND CLASSIFICATION OF PRIMARY HYPEROXALURIA

Hyperoxaluria can be the result of a genetic metabolic defect, as in primary hyperoxaluria type 1 or type 2, leading to an excessive endogenous oxalate production or can be caused by increased intestinal oxalate absorption, or decreased excretion, called secondary hyperoxaluria (FIGURE 01.1). Urinary oxalate levels are generally higher in primary hyperoxaluria, but the secondary form can lead to severe morbidity as well. Because of the clinical impact of hyperoxaluria in patients with other disorders (e.g. disorders of the gastro-intestinal tract), an overview of secondary hyperoxaluria is given at the end of this chapter.

FIGURE 01.1 Classification of hyperoxaluria. Two genetic metabolic types of primary hyperoxaluria are known, and three ways of developing secondary hyperoxaluria are distinguished

01.02.01 PH1

Primary hyperoxaluria type 1 (PH1) is an autosomal recessive inherited disorder of glyoxylate metabolism due to deficiency of a peroxisomal enzyme, named alanine:glyoxylate aminotransferase, AGT (47). Due to
this deficiency, peroxisomal glyoxylate cannot be transaminated into glycine, but is oxidized into oxalate and reduced into glycolate. A single mutated gene, called the AGXT gene, mapped at chromosome 2q37.3, constitutes the molecular basis of the AGT enzyme deficiency (161).

In humans, oxalate is an end product of glyoxylate metabolism. Any excess of oxalate, either produced endogenously or by ingestion of large amounts of oxalate, is transported via the blood stream to the kidneys and subsequently excreted into the urine. Due to the very low solubility of the calcium salt of oxalate, the concentration process of the urine may lead to crystal formation in the urinary tract as well as in the renal parenchyma (FIGURE 01.2 and 01.3). Crystals are demonstrated by light microscopy in a kidney biopsy specimen, and can be made visible especially using polarized light microscopy (FIGURE 01.4) or after von Kossa staining (FIGURE 01.5). Calcium oxalate crystallization may become visible as nephrocalcinosis or renal stones on ultrasound or abdominal X-ray (FIGURES 01.6 and 01.7). The striking feature of nephrocalcinosis is a diffusely increased echogenicity of the renal parenchyma at normal to low gain settings (116, 210). This abnormal pattern is evident when comparing the kidneys with adjacent liver tissue, which usually contains more internal echoes than the renal parenchyma. More invasive diagnostic procedures may reveal calcium oxalate crystals attached to tubular epithelium of nephrons (FIGURE 01.5). Calcifications can result in interstitial nephritis and fibrosis, eventually leading to renal insufficiency and end-stage renal disease (ESRD). When renal insufficiency develops, calcium oxalate depositions not only accumulate in renal parenchyma but also in other tissues. These extrarenal sequelae are called oxalosis or systemic oxalosis (21).

**PH2**

Primary hyperoxaluria type 2 (PH2) is a disorder of glyoxylate but also of hydroxypyruvate metabolism (208). Mutations in the glyoxylate reductase/ hydroxypyruvate reductase gene, the GRHPR gene, result in a deficiency of the bifunctional enzyme glyoxylate reductase/ hydroxypruvate reductase, GR/HPR, with development of hyperoxaluria and hyper-L-glyceric aciduria (174) (FIGURE 01.8 presents the metabolic scheme). Excess oxalate gives rise to the same phenotype as found in PH1 albeit less severe, and with much less patients recognized so far. A further description of PH2 is beyond the scope of this thesis.

**Unclassified PH**

In six reports, a total number of 13 patients, some of them being sib pairs, with atypical PH leading to renal involvement (urolithiasis and nephrocalcinosis, declining renal function) have been described (38, 98, 132, 134, 140, 193). Common to all patients, AGT and GR/HPR activities were normal, AGT was normally localized in peroxisomes and
DNA analysis of AGXT or GRHPR genes was unremarkable. Urine samples showed hyperoxaluria (six patients, of which one sib pair), hyperglycolic aciduria (three patients of which one sib pair), or a combination of the two (four patients). One report described a decline of urinary glycolate levels upon pyridoxine administration (98). It is suggested that glycolate oxidase (GO) may be involved in this disorder (see below) (134), but the exact nature of this type of primary hyperoxaluria has not been found yet.

### PRIMARY HYPEROXALURIA: THE ORIGIN OF OXALATE

#### Main precursors

Oxalate, excreted in urine, is derived from both dietary sources and from endogenous synthesis. The dietary contribution used to be estimated at 10% of total urinary oxalate (7), but recent investigations have shown that oxalate may be derived in approximately equal parts from the diet and endogenous liver metabolism. The dietary oxalate contribution may vary since oxalate absorption depends on calcium intake (76). Precursors of endogenous oxalate synthesis are sugars and amino acids (171) of which glycine is the most closely related to oxalate (41). The only studies which investigated the biochemical pathways leading to oxalate production report a 40% contribution of glycine to oxalate synthesis (11, 41). Glyoxylate plays a pivotal role in the endogenous oxalate generation (169). Ascorbate is probably the only non-enzymic contributor to the oxalate production, but its role seems insignificant in PH1 patients (11, 12, 14, 42), as studies that suggested an important contribution of ascorbate to urinary oxalate production may have been biased by the use of imprecise, analytical procedures for oxalate detection. Moreover, ascorbic acid may be converted to oxalate after voiding of urine, thus overestimating endogenous oxalate synthesis.

#### Glyoxylate: its transamination, oxidation and reduction

Glyoxylate can be transaminated into glycine (FIGURE 01.8). This conversion detoxifies the cell from the reactive aldehyde glyoxylate. It is only this single conversion, which is catalyzed by the AGT enzyme, which deficiency leads to the appearance of primary hyperoxaluria type 1. Apart from AGT, however, the more widely dispersed glutamate:glyoxylate aminotransferase (GGT) has the same function, although localized in the cytosol. However, for human glyoxylate metabolism, which is mainly localized in liver peroxisomes, AGT is the most important enzyme (145). As common to many aminotransferases, pyridoxal phosphate is the cofactor of this enzyme (60, 188). Pyridoxal phosphate is synthesized from pyridoxine, vitamin B6, and depletion of pyridoxine has been associated with a rise in urinary oxalate excretion in animal experiments (61). A human patient with hyperoxaluria due to pyridoxine deficiency has never been observed, though. Apart from transamination, glyoxylate can also be oxidized to oxalate, or reduced...
to glycolate. Glycolate oxidase (GO) and lactate dehydrogenase (LDH) both catalyze glyoxyxlate oxidation (164). Of these enzymes, glycolate oxidase is exclusively expressed in peroxisomes, catalyzing the conversion of glyoxyxlate into oxalate. However, when abundant glyoxyxlate is present, LDH may catalyze the same reaction in the cytosol, once excess glyoxyxlate has passed into the cytosol (8). The cytosolic enzymes glyoxyxlate reductase (GR) (180, 208) and lactate dehydrogenase (LDH) reduce glyoxyxlate to glycolate (FIGURE 01.8) (180). In PH1, the sum of biochemical pathways involving glyoxyxlate metabolism yield oxalate and glycolate, that are excreted in urine.

**FIGURE 01.8.** Biochemical pathways in primary hyperoxaluria type 1 and type 2. AGT; alanine:glyoxyxlate aminotransferase, GO; glycolate oxidase, DAO; D-amino acid oxidase, LDH; lactate dehydrogenase, GR/HPR; glyoxyxlate reductase/ hydroxyxpyruvate reductase

**01.03.03 Glycine and Glycolate, precursors of Glyoxyxlate in peroxisomes**

Two direct precursors of glyoxyxlate are glycine and glycolate. In liver peroxisomes, D-amino acid oxidase (DAO) catalyzes the conversion of glycine to glyoxyxlate (FIGURE 01.8). Glycolate is oxidized by glycolate oxidase (GO) (219). Both DAO and GO are peroxisomal enzymes. A by-product in the oxidation reaction is H$_2$O$_2$, which is degraded in peroxisomes by catalase.

In contrast to the metabolism of glycine, which is well established, the metabolic origin of glycolate is much more obscure. At least under experimental conditions, the following substrates have been proposed as precursors of glycolate: glycolaldehyde and ethylene glycol (32),
xylitol (58, 92), hydroxypyruvate, ethanolamine (170) and fructose (91, 142, 171). Their exact contribution to glycolate and oxalate synthesis in humans is unknown. However, $[^{14}\text{C}]$hydroxypyruvate and $[^{14}\text{C}]$ethanolamine were studied and appear not to be effective precursors of $[^{14}\text{C}]$oxalate (59). For hydroxypyruvate, reduction to D-glycerate by glyoxylate/ hydroxypyruvate reductase, GR/HPR, is observed, hence preventing glycolate generation in the cytosol (FIGURE 01.8). The importance of this reaction is illustrated by PH2, in which GR/HPR deficiency results in the conversion of hydroxypyruvate into L-glycerate and oxalate (FIGURE 01.8). Clinical studies gave evidence for the role of ethylene glycol, xylitol and fructose as oxalate precursors because patients who were intoxicated with these compounds developed calcium oxalate kidney stones as well as renal insufficiency (66, 90, 109, 126, 142, 222). Another pathway was proposed, in which glycolate dehydrogenase converts glycolate directly to oxalate. This reaction was thought to bypass glyoxylate (FIGURE 01.9). However, no further evidence for such a route was found after analysis of rat liver fractions and human urine samples of LDH subunit deficient patients (217).

**FIGURE 01.9** Xylitol, fructose, glycolate and glyoxylate metabolism, adapted from James et al (172), 3; aldehyde dehydrogenase, 4; glycolate oxidase, 5; glycolate oxidase and lactate dehydrogenase, 6; glycolate dehydrogenase, 7; hydroxypyruvate decarboxylase, 8; alanine:glyoxylate aminotransferase (synonym: serine:pyruvate aminotransferase).

Note: Hypothetical conversion, is not widely believed to occur.

**01.03.04 Hydroxyproline: a precursor of Glyoxylate in mitochondria**

Hydroxyproline, a break down product of collagen (153), is another source for glyoxylate formation. Its importance has been suggested recently (99, 100). Hydroxyproline is metabolized mainly in mitochon-
dria of hepatocytes and renal proximal tubule cells. Its metabolic conversion results in the formation of equal amounts of pyruvate and glyoxylate (99). Daily collagen turnover in humans, which is estimated at 2-3g/day (153), leads to the generation of 240-420mg of hydroxyproline and of 140-240mg of glyoxylate. Another source of hydroxyproline is dietary consumption, especially by ingestion of collagen-containing meat products and gelatine-containing foods. Human experiments demonstrating a rise in oxalate and glycolate excretion upon gelatine consumption, support this notion (100).

ALANINE:GLYOXYLATE AMINOTRANSFERASE (AGT)

AGT deficiency in human PH1

The enzyme alanine:glyoxylate aminotransferase (AGT) is a liver specific, peroxisomal enzyme which is deficient in PH1 patients, as first demonstrated by Danpure and colleagues (47). The immunocytochemical localization was studied by Cooper et al. using protein A-gold immunocytochemistry (33). AGT consists of 392 amino acid residues and is encoded by a single gene, the AGXT gene. In PH1 patients AGT is deficient in its activity, although in many patients a significant residual AGT activity, up to more than 50% of normal value, can be detected (33, 47). Different levels of immunoreactivity of the AGT protein were found as well. No correlation between levels of residual activity on the one hand, and the severity of clinical signs and symptoms on the other hand, could be detected (43, 48).

After its synthesis on free polyribosomes, AGT normally dimerizes and is imported into liver peroxisomes in humans. Dimerization is not a prerequisite per se for peroxisomal routing, but it prevents mitochondrial import (137, 221). The peroxisomal import is facilitated by a peroxisomal targeting sequence Lys-Lys-Leu (KKL), localized at the C-terminus of the polypeptide (137). The KKL-sequence differs from the prototypical, well conserved PTS1 sequence Ser-Lys-Leu (SKL) which is usually found in peroxisomal enzymes. A specific region of human AGT, called PTS1A, probably contains additional peroxisomal targeting information (86). Although the exact mechanism leading to a stable peroxisomal targeting signal is unclear, the PTS1A and PTS1 region interact closely in the three-dimensional crystal structure of AGT. Normally, human AGT is found exclusively in peroxisomes, but surprisingly, based on genetic evidence, 4% of healthy humans is predicted to have a combination of 5% of mitochondrion targeted AGT and 95% peroxisome targeted AGT in liver cells (162). This results from the creation of a mitochondrial targeting signal at the N-terminus that overrules the peroxisomal targeting information.

These peculiarities in combination with their genetic background are described below in the paragraph THE AGXT GENE.
AGT crystal structure

The crystal structure of AGT has been unravelled recently, facilitating predictions regarding genotype and enzymatic phenotype (221). AGT is a homodimer, built from two identical subunits. Each monomer consists of a large N-terminal domain and a smaller C-terminal domain. The core of the dimerization interface is formed by two helices and five connecting loops. This results in a surface, which is in close contact with the same area of the other monomer. The pyridoxal phosphate cofactor binds in a pocket at the dimer interface, and facilitates the catalytic and enzymatic function of AGT.

Species differences in AGT intracellular localization

In most humans, the localization of AGT is restricted to the peroxisomes. Yet, in other vertebrates, like cats and dogs, AGT is predominantly localized in mitochondria and only in a small proportion in peroxisomes. In other mammals, AGT is evenly distributed between both peroxisomes and mitochondria, like in rats or marmosets. The subcellular localization of AGT is thought to have changed during evolution, depending on the dietary contribution of oxalate in different species (45). For example, a vegetable diet, containing relatively high amounts of glycolate or oxalate, requires adequate break down of oxalate precursors to obtain a minimal endogenous production of oxalate. For this reason it is thought that the AGT enzyme is localized in peroxisomes in herbivores such as sheep (45, 75). On the other hand, carnivores have a high intake of collagen, which is also a precursor of oxalate via conversion into hydroxyproline and glyoxylate. Therefore, they require an adequate breakdown of collagen components. Since hydroxyproline metabolism has been localized in mitochondria (99), the AGT localized in mitochondria is likely to detoxify glyoxylate in this compartment. For humans, the expression of so called polymorphic mitochondrion targeted AGT (paragraph THE AGXT GENE) occurs more frequently in areas where meat is an important dietary component in contrast to areas where vegetables are the most common dietary component.

THE AGXT GENE

Cloning and mapping

The AGXT gene encoding the AGT enzyme (161), consists of 11 exons, ranging from 64 to 407 base pairs, and spans about 10 kb at the telomeric end of the long arm of chromosome 2, 2q37.3. It is composed of 11 exons. The open reading frame encodes a polypeptide of 392 amino acids. Soon after its cloning and mapping, a number of mutations and polymorphisms related to PH1 have been sequenced. As of the beginning of the 21st century, the number of sequence variants reported in the Human Gene Mutation Database (www.hgmd.cf.ac.uk) reaches over 50 (34, 35, 57, 218).
Polymorphic variants

Two different haplotypes or polymorphic variants are recognized with a high frequency in European and North American populations: the 'major' allele (frequency ~80%) and the 'minor' allele (frequency ~20%) (162). The frequency of the minor allele is ~3% in South Africans and ~2% in the Japanese population (37, 46). The minor allele is characterized by a 32C>T point mutation resulting in a substitution of the amino acids proline11➔leucine (Pro11Leu). This minor allele is frequently associated with a 74bp-duplication in the first intron, a 1020A>G point mutation leading to an isoleucine340➔methionine (Ile340Met) substitution (159), and an Epstein-Barr-virus-like VNTR in the fourth intron (46). In the (South) African population, another polymorphic variant with a c.976G>A point mutation, yielding a Val326Ile polymorphism is found in ~12% (37).

Genetic basis of mitochondrial mistargeting of AGT

The minor allele alone does not cause PH1, but some interesting biochemical consequences have been observed in homozygous individuals. The most intriguing is the so called mistargeting of a small proportion (5%) of the AGT enzyme to mitochondria instead of peroxisomes (162). In other words, a proportion of AGT, which is normally localized in the peroxisomal matrix, is imported into mitochondria. The mechanism behind this biochemical phenotype is considered to be a change in AGT protein folding. Normal AGT dimerizes after its synthesis. In this state, it can be imported in peroxisomes, but not in mitochondria (106). In vitro studies have shown that the Pro11Leu polymorphism creates a weak mitochondrial targeting sequence at the N-terminus of AGT (115, 158), which could create an alfa-helix at this site of AGT. This in its turn prevents normal dimerization and 5% of AGT is imported in mitochondria in vivo (106, 137). Affinity for mitochondrial import is increased by the additional occurrence of the c.508G>A mutation yielding a Gly170Arg substitution, which is the most common mutation in PH1 found so far (106, 114, 115, 137, 158, 162). Mistargeting of AGT to liver mitochondria is then enhanced, resulting in about 90% of AGT routed to the mitochondria and only 10% to peroxisomes (162). The Pro11Leu polymorphism induces two other changes in the AGT enzyme: it decreases the catalytic activity by 70% and, at elevated temperatures, it decreases the efficiency of dimerization of AGT translated in vitro (114). Furthermore, it sensitizes AGT to the deleterious effects of other mutations that probably would have been innocuous if they had occurred alone (137).

Co-segregation of the minor allele with other AGXT mutations

Apart from the specific enzymic mistargeting phenotype, the minor allele also predisposes AGXT to the pathogenic effects of the three other commonest sequence changes Ile244Thr, Phe152Ile, and Gly41Arg
resulting in aggregation of the AGT protein, as reviewed in the APPENDIX 02. For example, the Ile244Thr mutation, which is the second most common mutation in patient groups studied so far, also segregates with Pro11Leu. Its frequency is 9% in European and North American populations (201). AGT activity is low, and the phenomenon of mitochondrial mistargeting is not seen in this genotype. Santana and associates demonstrated that Ile244Thr AGT forms aggregates in vitro (178). Proteolytic disposal in vivo could explain low AGT levels found in liver biopsies of these patients. The Gly82Glu occurs on the background of the major allele and results in loss of AGT catalytic activity, possibly due to failure of binding the co-factor (114, 160). For the Gly41Arg mutation, segregation with the Pro11Leu polymorphism leads to aggregation into core-like structures in the peroxisomal matrix (49). Without Pro11Leu (155), the specific AGT activity is only reduced in patients (114). Ser205Pro is only observed in Japanese PH1 patients on the major allele. It renders AGT highly unstable and AGT is degraded subsequently (144). A small insertion at codon 11 was found by an Italian group, c.33_34insC (155). A heterogenic picture was seen in these patients but no expression studies were carried out (3, 154). Apart from these specific AGXT gene mutations, a large number of other AGXT mutations have been reported (2, 16, 34, 36, 37, 46, 49, 57, 113, 131, 135, 143, 160, 165, 199, 200, 201, 218). An overview can be found at the website of the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac).

In summary, after the characterization of the AGXT gene and the first PH1-related mutations in 1990 by Purdue et al. (161) a few cross-sectional studies had been reported on the prevalence of AGXT gene mutations (46, 77, 113, 199, 200, 201). Some genetic AGXT mutations had been linked to specific defects of the AGT enzyme, such as intraperoxisomal aggregation, loss of the pyridoxal phosphate binding site and, most fascinatingly, a conformational change of the enzyme leading to a predominantly mitochondrial instead of a solely peroxisomal localized AGT enzyme (44, 105, 106, 158, 160, 162). However, these investigations could not reveal the relationship between genetic characteristics on the one hand and clinical characteristics and outcome on the other hand.

01.06 PATHOPHYSIOLOGY OF RENAL DISEASE IN PRIMARY HYPEROXALURIA

The concentration process of urine in the kidneys results in increasing concentrations of both oxalate and calcium. Since the solubility coefficient of calcium and oxalate is low, they easily precipitate as calcium oxalate crystals. Urine is easily supersaturated with stone forming ions (166) but under normal conditions crystallization is prevented by the presence of crystallization inhibitors such as citrate and magnesium. However, the high output of urinary oxalate in PH1 increases the pre-
cipitation risk. This has already been investigated in animal models in 1926 by Dunn and colleagues and in 1960 by Largiader, as reviewed by Hockaday et al. (1964) (73). In the patient, these calcium oxalate crystals are considered to be the basis for stone formation. Although crystallization of oxalate with calcium was generally presumed to occur in the renal parenchyma or medullary interstitium, the exact nature and extent of crystal deposition and its consequences for renal damage are not known. Massive crystalluria in patients with a severe form of hyperoxaluria could lead to early development of urolithiasis, nephrocalcinosis, and renal insufficiency due to insoluble and obstructive properties of calcium oxalate. Contrary to previous hypotheses, oxalate itself is only toxic at extremely high concentrations (181) and is predicted not to lead to changes in tubular epithelial functioning or damage in vivo. However, the presence of crystalluria has been demonstrated in recurrent renal-stone formers, and appeared to be increased after oral sodium oxalate loading (167). In recent years, another mechanism has become the focus of stone research, namely binding of crystals to the tubular epithelium, which may be mediated by the expression of the receptor CD44 and its ligands hyaluronan (HA) and osteopontin (OPN) (9, 101, 195-197). Asselman et al. have shown that the expression of HA precedes crystal retention in experimental models and, together with OPN, it is also associated with nephrocalcinosis in kidneys of preterm infants and renal transplants (9, 195). Observations in monolayer cell cultures show that HA expression can be induced after creation of an artificial wound in the monolayer. In these circumstances, CD44, which is normally present basolaterally, is expressed at the apical cell surface, as well as HA. They are visible until wound healing is completed. In human renal epithelial cells, the expression of CD44, HA and OPN could well follow damage due to passage of calcium oxalate crystals. Consequently, crystals may attach to these expressed molecules, causing nephrocalcinosis. Hyaluronan has been shown to bind crystals in cell culture experiments of Verkoelen et al., in which it may serve as a stone binding glue (197). The presence of these molecules for PH1 and their implications for clinical management are currently unknown and warrant further investigation.

**CLINICAL PRESENTATION AND CLINICAL STUDIES IN PRIMARY HYPEROXALURIA TYPE 1**

After the description of some case reports in the second half of the previous century, the first large patient cohort was described in Sweden, comprising 17 patients of Scandinavian origin (72). At that time, the enzyme deficiency had not yet been discovered and a complete diagnostic work up was therefore not possible for all patients. Upon the discovery of AGT deficiency as the cause of PH1, many patients with suspected PH1 were analyzed and published. In 2000, at the start of the project leading to this thesis, a French cohort had been published.
(Cochat et al., 1995) (27), a Swiss cohort focused on the infantile presentation (Leumann et al., 1987) (112) and an overview of 330 cases (Latta et al., 1990) (103). One phenomenon was recognized in all of these studies: PH1 is a very heterogeneous disease with respect to age at presentation, outcome of renal function, and biochemical characteristics. The first symptoms in patients with primary hyperoxaluria were usually related to renal stone disease, such as colic pain, hematuria and/or recurrent urinary tract infection (103). However, nephrocalcinosis may also develop without any of these signs and symptoms. Some patients only become clinically symptomatic at the time end-stage renal disease has already developed. Systemic deposition of calcium oxalate may occur throughout the body when plasma oxalate concentration rises above 20 μM and glomerular filtration rate falls below 60ml/min/1.73m² (136). The chemical composition of these systemic depositions was also calcium oxalate (26, 70). The risk for systemic oxalosis increases sharply when the GFR comes down to 20 ml/min/1.73m² (136). When dialysis is started (GFR<10ml/min/1.73m²), further development of oxalosis cannot be prevented. Despite intensive dialysis schedules, patients develop the most severe complications of primary hyperoxaluria due to systemic oxalate depositions, which can occur in many organs and tissues, like bones, muscle fibres and connective tissues (18, 21, 194), and the nervous system, particularly the interstitium of peripheral nerves (69). Other tissues are involved, such as the vascular system, the heart (21, 26, 194), myocardial muscle fibers (21, 194), tracheal cartilage (21), lungs (69), prostate (21), brains, meninges and in walls of congested meningeal vessels (70), choroid plexus, pineal gland, ovaries, fallopian tubes, uterus, thymus, salivary glands, pancreas, bladder (52) and in joints (125). Consequences of oxalosis range from frequent bone fractures and loss of visual acuity, to advanced neural damage, cardiac conductivity blocks, and arterial vessel obstruction with necrosis and gangrene, eventually leading to early mortality (65, 69, 70, 119). Although the liver is the primary site for oxalate production, liver oxalate depositions have only been observed infrequently (1, 69). The most striking features of systemic oxalosis are presented in APPENDIX 01.

The clinical pattern emerging from the initially published patient cohorts showed that infantile patients often presented with end-stage renal disease, failure to thrive, metabolic acidosis, vomiting, anorexia or anemia, generally preceding rapid progression of renal insufficiency. Although most patients presented within the first decade of life with renal involvement, presentation in adulthood was also frequently observed. These observations resulted in a clinical classification of primary hyperoxaluria type 1 into three groups (29):

1. a severe, neonatal or infantile form, with urolithiasis, nephrocalcinosis, or end-stage renal disease before the first birthday.
Outcome, regarding preservation of kidney function or patient survival, was assumed to be poor in this group;  
2. a mild form, with onset of symptoms in late adulthood, and a favourable prognosis of kidney function;  
3. an intermediate group, comprising patients presenting in childhood, with a slow decline of renal function over years and development of ESRD in more that half of them.

Not all these observations were based on comprehensive epidemiological studies. Several case reports of patients with adult onset of symptoms, who presented in end-stage renal disease (102) and reports of patients with neonatal PH1 with a relatively mild course argumented against this classification (129, 203).

**01.08**  
**DIAGNOSTIC APPROACH TO PRIMARY HYPOXALURIA TYPE 1**

Diagnosis of primary hyperoxaluria type 1 is based on clinical, metabolic, genetic and enzymatic studies. The gold standard of diagnosis is the demonstration of absence, or decrease in peroxisomal AGT enzyme activity. As human AGT is almost exclusively expressed in liver, this requires an invasive liver biopsy. Therefore, other less invasive methods are currently regarded as the first choice in the diagnostic work up. In this overview, the approach to diagnosis of primary hyperoxaluria type 1 will be discussed. The appendix provides a table with reference values for relevant diagnostic tests.

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**Clinical diagnosis**

The clinical presentation of PH1 is extremely heterogeneous. Patients may present with symptoms between 0 and at least 60 years of age. Symptoms vary from single urinary stones, recurrent urinary tract infection and hematuria to all stigmata of end-stage renal disease. In children, non-specific symptoms as failure to thrive, feeding problems and vomiting may be the first findings. Weight gain and edema, loss of appetite and fatigue may indicate renal insufficiency at any age. Since calcium oxalate is also deposited at other sites in the body apart from the kidneys, the longer the period of renal insufficiency exists, the more calcium oxalate depositions can be found elsewhere in the body. Typical bone lesions on X-ray and brown retinal maculae on fundoscopy (FIGURE 01.10) characterize systemic oxalosis in patients with severe renal failure. Renal stones, nephrocalcinosis, and in the case of severe renal failure a highly echo dense appearance of the kidney with loss of corticomedullary differentiation are the most common abnormalities found by ultrasound (FIGURE 01.7). Renal biopsy may reveal typical fan-shaped oxalate crystals (FIGURE 01.4) and signs of interstitial nephritis (27, 79, 80, 103, 112, 130). In summary, symptoms of renal calcification, but also signs of renal failure and of bone disease and visual impairment may be the first clues to initiate a diagnostic work up for PH1. In gene-
ral, oxalate crystals can originate either from endogenous oxalate production in patients with primary hyperoxaluria, or excessive oxalate intake due to high dietary oxalate loads, or enhanced oxalate absorption in patients with malabsorption syndromes or inflammatory bowel disorders, or decreased oxalate excretion in patients with end-stage renal disease on dialysis. However, none of the clinical signs and symptoms is pathognomonic for a diagnosis of PH1. Therefore, any patient presenting with these clinical signs and symptoms should be further investigated for PH1.

**Metabolic studies**

Assessment of patients for a diagnosis of primary hyperoxaluria can start with metabolic analysis for elevated levels of oxalate in urine, plasma or peritoneal fluid.

**Urine**

The use of urinary metabolites in the diagnosis of PH1 is restricted to patients with a preserved renal function, since false negative results may be obtained when renal function declines. In case of renal insufficiency, one should proceed to analysis of plasma metabolites. Two mechanisms interfere with reliable determination of oxalate: crystallization and ascorbate interference. For these confounders, urine is collected in acidified containers. If urine is kept at a pH < 2, oxalate remains solved and does not crystallize with calcium ions. Meanwhile, the enzymatic conversion of ascorbate to oxalate in vitro is reduced, though it will take place during storage with time, even at low storage temperatures (211). This may result in false positive values.

Assessment of urinary levels of oxalate and glycolate has always been the first line in the diagnostic assessment of PH1. Due to potential circadian variation in urinary oxalate excretion (148), which parallels variations in creatinine excretion, it is generally advised to study 24-hour samples (128, 152). However, by calculation of the ratio of urinary oxalate to creatinine [mmol/mol] and glycolate to creatinine in spontaneously voided urine samples potential misinterpretation because of sampling errors is avoided. Therefore, analysis of spot-urines produces comparable results to 24-hour urinary collections (88) making urinalysis in young children easier. For children, creatinine and oxalate excretion is higher at younger age. Therefore, age-related reference values have been obtained in a number of studies (15, 62, 111, 163, 198). Results of urinary oxalate excretion between adults and children may also be compared if expressed as mmol per 1.73m² body surface area per day (62, 128). For PH1, levels usually exceed 1 mmol per 1.73m² body surface area. An elegant technique assessing urinary oxalate and glycolate from dried urine filter spots in a pediatric population was developed by Blau et al. (20).
Values of urinary glycolate concentration in control subjects have been reported in 13 studies, and range from 1 to 99.6 mg/24 hr, which is equal to 1 to 135 mmol/mol creatinine/24 hr (206). Urinary glycolate as assessed by gas chromatography produces valid and accurate results (111). Using tetrahydrofuran, the extraction procedure for analysis with gas chromatography was improved by Dietzen et al. (51). The range of normal glycolate values reflects a considerable daily variability of urinary glycolate levels (206). Most reliable results are obtained by expressing the results as the glycolate/creatinine ratio. Whether glycolate levels in PH1 patients also vary to a great extent has not been investigated. If so, it may contribute to the observation of normal urinary glycolate levels in 25% of PH1 patients, which is a major pitfall in the diagnosis of PH1 based on urinalysis.

Plasma

In patients with PH1, plasma levels of oxalate and glycolate not only rise in renal insufficiency, but may also be elevated in patients with a preserved renal function. Therefore, assessment of these metabolites in plasma is of value for diagnosis in all patients with suspected PH1. However, in patients with preserved renal function, normal levels do not exclude PH1.

Elevated plasma oxalate concentration is a common observation in patients with renal insufficiency not due to PH1. However, oxalate levels in non-PH patients with renal insufficiency rarely exceed 50-60 μmol/l (10 times upper range of reference values), in contrast to PH1 patients in whom levels may rise to 200-300 μmol/l. Nevertheless, the isolated determination of plasma oxalate cannot always discriminate between primary hyperoxaluria or high levels of plasma oxalate due to renal insufficiency. Wolthers et al suggested that a plasma oxalate:creatinine ratio exceeding 0.1 and a calculated total quantity of oxalate removed by dialysis exceeding 2 mmol to be supportive of a diagnosis of hyperoxaluria (214). Other causes of secondary hyperoxaluria, resulting in elevated plasma oxalate levels, may include intake of high amounts of vitamin C, and hyperoxaluria caused by inflammatory bowel disease or short bowel syndrome (31, 136, 150). The issue of secondary hyperoxaluria due to high amounts of vitamin C intake remains controversial. Some clinical reports found a significant rise of urinary oxalate excretion upon oral ascorbate loading (17, 71, 191). Two case reports found the occurrence of acute renal failure in otherwise healthy persons due to vitamin C supplementation of 2.5 g – 4 g per day (121, 139). The effect of ascorbic acid supplementation was studied in some controlled studies, using healthy volunteers and stone formers (up to 10 grams per day) (25, 123, 182). Although the conversion rate of ascorbic acid into oxalate was 1-2%, it may become a clinically significant increase in patients with primary hyperoxaluria since
the solubility of calcium oxalate in urine is very limited. However, others could not demonstrate this relationship (23, 192, 205, 211). A study assessing the fate of oral suppletion of isotopic ascorbic acid feedings found that ascorbic acid is only a minor contributor to oxalate excretion in primary hyperoxaluria (10). No other studies assessed the in vivo ascorbate conversion in patients with biochemically or genetically determined PH1.

Determination of glycolate concentration in plasma in addition to plasma oxalate has been suggested to allow discrimination of PH1 patients from patients with other causes of renal insufficiency (87, 117, 118). However, since little studies in patients with PH1 having end-stage renal disease have been performed, the sensitivity of plasma glycolate testing is not known. On the other hand, the specificity seems to be high since studies in patients who have received a curative liver transplantation show a decline of glycolate up to normal values within days (87).

Collection procedure and preparation
As vitamin C in plasma and other potential sources of glyoxylate may cause in vitro oxalate synthesis after collection of a blood sample, resulting in false positively elevated levels of plasma oxalate, plasma should be deproteinized quickly after it has been drawn in pre-cooled heparine vacutainers and stored at -20 ºC and storage time should be as short as possible (149, 151, 213). Interference of ascorbate with the measurement of plasma oxalate is prevented by acidification, prior to analysis, since alkaline pH could lead to generation of oxalate from ascorbate (141, 151).

Analytical methods
Plasma oxalate analysis by means of in vivo dilution, a radiochemical technique using labeled [14C]oxalate, has by far provided the most accurate measurement of plasma oxalate concentration. The reference value has been determined by this method as 1.39 (range 1.04 to 1.78) (31, 74, 157). As this assay is impracticable as it uses a radioactive label, other assays have been developed. Enzymatic measurements using oxalate oxidase reactions are most prone to false results by ascorbate interconversion (40, 96, 107). For ion chromatography (HPLC), the main problem is in-column conversion of endogenous ascorbate to oxalate, which can be avoided either by ascorbate oxidation with iron(III)ions (97) or by means of boric acid dilution (216). Gas chromatography-mass spectrometry (GC-MS) is probably the most accurate technique though more laborious than ion chromatography. Its reliability is very high because of the use of stable isotope labeled internal standards (87).

Plasma oxalate levels in healthy persons have been measured with these different techniques. Levels range from 0.4-6.0 μM, depending on
the method of analysis (152). For PH1 patients with preserved renal function, plasma oxalate levels may be within the normal range. Levels gradually exceed 10 μM when renal function declines from 80 to 40 ml/min/1.73m², however, plasma oxalate levels rise progressively above 30 μM when the glomerular filtration rate falls below 40 ml/min/1.73m² (136).

Plasma glycolate measurements by HPLC and GC-MS methods have revealed glycolate levels of 7.5 ± 2.4 μM in healthy persons (87). Another sensitive HPLC procedure was developed by Hagen et al. (67). Plasma glycolate levels of PH1 patients with preserved renal function can exceed 20 times the upper limit of the normal reference range and are therefore highly discriminative in the diagnosis of PH1 (118). In PH1 patients on hemodialysis or peritoneal dialysis, plasma glycolate levels may even rise up to 150 times the normal range (117), whereas glycolate remains normal in primary hyperoxaluria-unrelated end-stage renal disease.

**Peritoneal fluid**

**Oxalate**

The use of dialysate in the diagnosis of PH1 in patients on peritoneal dialysis (PD) has been investigated in one case-control study: four PH1 patients on PD and four controls. This study revealed an oxalate/creatinine ratio of 512 (range 265-638) mg/g in four PH1 patients on PD as compared to 130 (range 64-191) mg/g in PD patients with other underlying diseases (124).

**Glycolate**

One study has been published on glycolate levels in the dialysate of a PH1 patient in comparison with five non-PH PD patients, which showed significantly elevated levels of glycolate in the PH patient: 48.3 μmol/day in the PH1 patient versus 19.6 (range 15.1-27.5) μmol/day in the non-PH PD patients (215). The analysis was performed by means of standard organic acid analysis using gas chromatography without an isotopic internal standard as control.

Though analysis of oxalate and glycolate in dialysate fluid is not widely practiced, it seems of additional diagnostic value.

**Genetic diagnosis**

After characterization of the AGXT gene as described above, expression studies showed the pathogenicity of the most common mutations, including the amino acid substitutions Gly170Arg, Gly82Glu and Gly41Arg (114).
Enzymatic diagnosis

For patients in whom the diagnosis remains uncertain after urinary and genetic analyses, assessment of the AGT enzyme activity in a liver biopsy specimen may be the ultimate diagnostic procedure. The analysis of the AGT enzyme was first performed in 1972 (138, 172). The analysis was later performed using a Cobas Fara centrifugal analyzer (83, 84), or analogous procedures and can be performed in a semi-automated way (175). The AGT enzyme has a considerable range in residual \textit{in vitro} activity for different genotypes in PH1. Three main forms of AGT deficiency are recognized (48):

1. absence of both immunoreactive and catalytically active AGT (CRM-/ENZ-); the AGT activity may be below the detection level;
2. presence of immunoreactive AGT but absence of catalytically active AGT (CRM+/ENZ-);
3. presence of both immunoreactive and catalytically active AGT (CRM+/ENZ+): the residual \textit{in vitro} activity ranges from 15\% up to 50\% of normal activity. The inconsistency of the apparent \textit{in vitro} activity and lack of \textit{in vivo} activity of the enzyme in the latter situation is due to mitochondrial mistargeting of AGT. In all these 3 situations, the diagnosis of PH1 is confirmed, though heterozygote carriers may also be detected with 50\% residual enzyme activity. However, no reports have demonstrated hyperoxaluria in carriers. Therefore, it is probably without clinical significance. In case of doubt when high residual activity of AGT is found, AGT mistargeting to mitochondria can be assessed by immunocytochemical localization studies of the enzyme (33), though the mitochondrial mistargeting phenotype should have been traced by genetic sequencing at this stage, revealing the Gly170Arg – or possibly Phe152Ile – genotype (158).

Prenatal diagnosis

Molecular genetics is the method of choice for prenatal diagnosis, using mutation analysis (173). It can be used in the first trimester of pregnancy.

THERAPEUTIC APPROACH TO PRIMARY HYPEROXALURIA TYPE 1

Treatment of patients with PH1 is mainly centered on influencing the metabolism of oxalate and glyoxylate, prevention of renal sequelae, treatment of urolithiasis and, in case of end-stage renal disease, institution of renal replacement therapy.

Conservative treatment

As with renal stone disease in general, conservative management consists of increasing fluid intake to dilute urine if renal function permits, dietary modifications, and the administration of pharmacologic agents that minimize the risk of supersaturation by increasing the urinary
concentration of inhibitory substances (81). Maintaining a good hydration is especially important in patients at risk for dehydration, for example in case of diarrhoea. In children, who are at risk for rapid dehydration, early institution of naso-gastric tube hydration or intravenous fluid administration is mandatory. Continuous naso-gastric tube hydration during nighttime should be considered in small children or patients with already severe nephrocalcinosis and renal damage. For dietary measures, a sufficient calcium intake is advised in order to bind any dietary oxalate in the digestive tract before it can be absorbed and excreted by the kidneys. Although excessive ingestion of dietary oxalate should be avoided, absolute elimination of oxalate containing food is probably less important since the ingested amount of oxalate is relatively small compared to endogenously produced oxalate. Citrate, 0.15 g/kg, is considered to be a potent chelator of calcium in urine but under normal conditions it is not sufficient to chelate more than a fraction of the calcium present (85). However, its use in humans strongly inhibits calcium oxalate nucleation and aggregation (89, 110, 179, 186). Potassium citrate is preferred to sodium citrate since it prevents high sodium intake and secondary hypercalciuria due to high sodium intake. In addition, citrate acts as an alkalinizing agent, further reducing the risk of crystallization (168, 220).

Magnesium salts are sometimes used as modifiers of nucleating agents but magnesium may only be added to potassium citrate therapy to further inhibit calcium oxalate crystallization (122, 220). An adverse effect of magnesium administration is the potential occurrence of diarrhoea which may lead to dehydration, greatly enhancing the risk for crystallization of calcium oxalate in the kidney.

The best evidence for successful pharmacological intervention in patients with PH1 comes from studies assessing the use of pyridoxine and orthophosphate. Animal studies had already studied the effects of pyridoxine on oxalate metabolism (60). Pyridoxine was first described for treatment of patients with PH1 in 1967 (185) but the effects were conflicting (63). Orthophosphate alone was known for its inhibitory effect on crystal formation, though the exact mechanism of this phenomenon was not clear (85). A long-term follow-up study in PH1 patients evaluated the use of pyridoxine and orthophosphate and found a decreased urinary calcium oxalate crystallization and preservation of renal function (129). An intake of more than 1000mg per day may lead to reversible, peripheral neuropathy (190). For pyridoxine, an initial dose of 5 mg/kg is usually prescribed. The potential decline of urinary oxalate should be awaited for 2-3 months after which the dosage is optimized under control of urinary oxalate excretion. Usually, either citrate or orthophosphate is added to the treatment regimen. For orthophosphate no further studies are available as to assess its effectiveness.
Transplantation:

Kidney transplantation, Liver transplantation or Combined liver-kidney transplantation

Currently, a definitive cure of the metabolic enzyme deficiency in PH1 is only feasible through liver transplantation. This was initially performed at the time end-stage renal disease had ensued under carefully optimized conditions (104), but later on, pre-emptive liver transplantations were introduced to prevent development of end-stage renal disease. Combined liver-kidney transplantations were also performed to correct both the enzymatic defect and the renal insufficiency.

For patients with PH1, four transplantation procedures are known: (1) sole kidney transplantation; (2) pre-emptive liver transplantation, (3) combined liver-kidney transplantation, or (4) sequential liver-kidney transplantation, in which the kidney transplantation is planned after reduction of plasma oxalate levels, in order to reduce oxalate burden for the kidney transplant. For each of these options, systematic clinical evidence is collected in different cohorts, and adverse effects are considerable: option (1) was thought to be prone for early graft failure due to recurrence of oxalate depositions; option (2) would be reserved to patients who do not respond to conservative treatment, but no patient characteristics could determine this; option (3) needed careful organ selection and operative care; and for option (4), immunization problems were expected since organs from different donors were going to be transplanted. A European cohort study that collected data by questionnaires, found that the outcome of kidney function was inversely related to the time between onset of dialysis treatment and transplantation. They found excellent results with combined liver-kidney transplantations (28, 93, 94) which was confirmed by a single center experience (133). However, incompleteness of data might have resulted in bias towards positive outcome. In a United States follow-up survey, renal allograft survival was equal in patients who had undergone a solitary kidney, as compared to those who had received a combined liver-kidney transplantation; at the same time, the patient survival was better for kidney transplanted recipients (176). A high morbidity and mortality rate was observed in infants and young children undergoing combined liver-kidney transplantation which suggested a pre-emptive liver transplantation or a combined liver-kidney transplantation before reaching end-stage renal disease (54). Few reports have reported the successful use of pre-emptive liver transplantation in order to prevent dialysis-induced oxalosis (146). A better prediction of outcome of patients was needed for appropriate choice of transplantation.
ZELLEWER SPECTRUM DISORDERS

The Zellweger spectrum disorders (ZSDs) belong to the group of peroxisome biogenesis disorders (PBDs), that are characterized by a defect in peroxisomal assembly (202). The failure to assemble peroxisomes in the cells of these patients, results in a failure to import peroxisomal enzymes into the peroxisomal matrix. Subsequently, most of these enzymes, after their synthesis in the cytosol, are rapidly degraded, and multiple peroxisomal functions are lost. However, the AGT enzyme was reported to be stable in the cytosol just like a few other peroxisomal enzymes including catalase (204). We studied the clinical picture of patients with ZSDs, to observe potential effects of cytosolic localized AGT on glyoxylate metabolism. The prototype of PBDs are Zellweger syndrome patients, with an early lethal course, but other patients have milder phenotypes. Still, many have a severe physical and mental retardation, although to different extents, and mild cases survive into adulthood, and are socially and intellectually educable (156). Clinical features include neurodevelopmental impairment, retinopathy, perceptive deafness, and hepatic dysfunction (202). Renal involvement with urolithiasis, nephrocalcinosis and hyperoxaluria was observed in three patients, despite normal AGT activity. The incidence of hyperoxaluria in ZSD patients and its clinical implications was, however, unknown.

SECONDARY HYPEROXALURIA

As previously stated, dietary absorption of oxalate is estimated to account for approximately 10-50% of the total daily oxalate excretion (76, 209). Secondary hyperoxaluria has four pathophysiological origins: 1. an increased ingestion of oxalate from dietary sources such as rhubarb, spinach, cocoa, tea, parsley, pepper, peanuts, beets; 2. an increased absorption due to bowel disorders (187) such as cystic fibrosis (64, 78, 82, 183), inflammatory bowel disease, ileal resection; 3. a decreased clearance of oxalate due to renal insufficiency (214); or 4. as a consequence of long term parenteral nutrition in preterm infants (22). Even without specific disorders of the gastrointestinal tract, there may be a higher oxalate uptake from the diet by recurrent renal-stone formers (167). All these mechanisms can lead to elevated levels of urinary oxalate which may become as high as in primary hyperoxaluria, although levels are generally lower (82, 184). Plasma oxalate may also be increased (64, 78, 82, 183, 187). Contrary to what is observed in primary hyperoxaluria, levels of glycolate and L-glycerate are normal in these patients. Secondary hyperoxaluria may lead to severe morbidity with urolithiasis or nephrocalcinosis as observed especially in patients with malabsorptive syndromes, and eventually renal insufficiency (150, 184) or even ESRD (19). However, large cohorts have never been described. In dialysis-related secondary hyperoxaluria, extra renal depositions of calcium oxalate are rare and do not follow the relentless pattern of PH1 (119).
The mechanism leading to hyperoxaluria in these disorders is not always clear. However, for malabsorptive diseases, several hypotheses exist by which hyperoxaluria is generated. Three mechanisms are mainly suggested to give rise to absorptive hyperoxaluria: first, calcium binds to fatty acids, in steatorrhoea, which is present in patients with malabsorption. Because of this, less calcium is available to bind oxalate and more oxalate is available for uptake in the colon. A higher fat intake per se, however, does not necessarily contribute to higher excretion of urinary oxalate (13, 120). Secondly, as a consequence of elevated fatty acid concentration in the colon, the permeability of the colon for oxalate absorption increases, resulting in more oxalate uptake. Thirdly, a depletion of the oxalate degrading bacteria is seen in some patients with cystic fibrosis, possibly due to frequent use of antibiotics. This also leads to higher oxalate concentrations in the gut, with elevated oxalate uptake (183). Dietary restrictions of oxalate containing products can sometimes prevent or decrease the severity of secondary hyperoxaluria. However, it can be a persistent clinical problem in malabsorptive diseases. For patients on dialysis, hyperoxaluria and hyperoxalemia are caused by the inability of dialysis techniques to remove oxalate sufficiently, and oxalate accumulation occurs. Plasma levels can rise up to 116 μmol/l and systemic oxalosis with organ involvement has been reported frequently (19, 55, 127, 147, 189).

**AIM OF THE STUDY**

Research has elucidated parts of the complicated genetic and metabolic changes that constitute the basis of primary hyperoxaluria type 1. However, a number of questions remain to be answered.

One of the most difficult issues in primary hyperoxaluria type 1 (PH1) is the heterogeneity of the disease, at the genetic, biochemical and clinical level. This heterogeneity prevents a clear prediction of disease severity or of outcome of patients based on patient characteristics. The main aim of this study is to unravel this heterogeneity. For this, a nationwide cohort will be recruited, and patient characteristics will be described, at the genetic, biochemical and clinical level. This facilitates the calculation of the prevalence of PH1 in The Netherlands, and may lead to a correlation between genetic and biochemical studies on the one hand, and clinical outcome of patients on the other hand. Different options for treatment will also be assessed in the patient cohort, including conservative treatment with pyridoxine, and transplantations. Concerning the issue of transplantation, either a (1) kidney, (2) preemptive liver, (3) combined liver-kidney, or (4) sequential liver-kidney transplantation is available. For each of these options, systematic clinical evidence is lacking and adverse effects are considerable. At the time of onset of this study, data on the outcomes of the different transplantation policies were too conflicting to draw definite conclusions.
for therapeutic strategies. To address this issue, we decided to analyze all transplanted patients in our cohort and we designed a strategy for type and timing of transplantations, based on the clinical, biochemical and genetic experience in our national cohort.

Diagnostic studies include the use of mutation analysis of the AGXT gene. The applicability of DNA diagnosis for patients with primary hyperoxaluria was not known in The Netherlands. Furthermore, the relationship between AGXT gene mutations and the clinical phenotype of patients with PH1 was unclear. We aim to assess the spectrum of AGXT gene mutations in Dutch patients with PH1 and evaluate the use of DNA diagnosis in PH1, as a rapid, non-invasive tool in diagnostic screening. Using the clinical information of the Dutch patient cohort, we will try to relate the clinical phenotype to AXGT gene mutations.

Another issue concerning screening for PH1 is the collection of urine for oxalate and glycolate measurements. This is usually performed in acidified containers, which exposes patients to the hazards of hydrochloric acid. No systematic studies have addressed its use in PH1 and we investigate whether hydrochloric acid should still be applied, or it could be added to the urine collection after arrival of the urine specimen at the laboratory.

Regarding the occurrence of hyperoxaluria and renal involvement in some patients with Zellweger spectrum disorders, our aim is to study a cohort of these patients to assess the incidence of hyperoxaluria in them, and the potential implications for renal involvement. Biochemically, patients with Zellweger spectrum disorders lack peroxisomes and therefore, the in vivo results of cytosolically localized AGT enzyme becomes apparent. The correlation between the clinical severity of this neurological disease and the incidence of renal involvement and hyperoxaluria will be studied.

Finally, we initiate research to evaluate potential new treatment in PH1 and to investigate a biochemical model that facilitates studies of the biochemical origin of oxalate. For patients with a preserved renal function, urinary excretion of oxalate is regarded to reflect endogenous oxalate production, unless renal insufficiency occurs. New treatment could be targeted to try to reduce endogenous oxalate synthesis. Evaluation of its efficacy is only possible if a careful estimation of endogenous oxalate synthesis is available. Since endogenous oxalate is produced by the liver, the rate of appearance of oxalate in plasma would accommodate this need. We use an in vivo isotope dilution method to calculate the rate of appearance of oxalate in plasma in patients and control subjects.
The *Agxt* /-- mouse, an animal model for primary hyperoxaluria type 1, was recently developed by Salido *et al.* (177). The *Agxt* /-- mouse is deficient for the AGT enzyme, and displays high levels of urinary oxalate. Development of rational therapies that interfere with endogenous oxalate synthesis is hampered by the lack of knowledge on the exact sources of oxalate, apart from its direct precursor glyoxylate. Therefore, we will isolate hepatocytes from the *Agxt* /-- mouse to study the contribution of potential precursors of oxalate synthesis.

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