

LINKÖPING UNIVERSITY MEDICAL DISSERTATIONS
No. 1034

MOLECULAR GENETIC STUDIES
ON CYSTINURIA

LOTTA HARNEVIK



Linköping University
FACULTY OF HEALTH SCIENCES

LINKÖPING 2007

© 2007 Lotta Harnevik

Original publications have been reprinted with permission from copyright holders:

© 2001 Wiley-Liss, Inc.

© 2003 Mary Ann Liebert, Inc.

© 2003 Springer Verlag

© 2006 Springer Science+Business Media, Inc.

Printed by LiU-Tryck, Linköping, Sweden, 2007

ISBN 978-91-85895-22-9

ISSN 0345-0082

“Du blir aldrig färdig, och det är som det skall.”

TOMAS TRANSTRÖMER
För levande och döda
1989

ABSTRACT

Cystinuria is defined as an inherited disorder characterised by increased urinary excretion of cystine and the dibasic amino acids arginine, lysine and ornithine. The only clinical manifestation of cystinuria is renal cystine stone formation due to the low solubility of cystine in the urine. Cystinuria can be attributed to mutations in the SLC3A1 and SLC7A9 genes in the majority of all cases and it has been a common expectation that molecular genetic studies of cystinuria would aid in understanding of the varying clinical outcome seen in the disease. Besides human, the disease has been most extensively studied in the domestic dog.

The present study was undertaken to investigate the molecular genetic basis of cystinuria in patients from Sweden and to correlate genetic findings with phenotypes produced regarding cystine and dibasic amino acid excretion. Further, attempts were made to elucidate the molecular genetics of cystinuria in the dog.

The entire coding sequences of the SLC3A1 and SLC7A9 genes were analysed by means of SSCA and DNA sequencing in 53 cystinuria patients and genetic findings were related to urinary excretion of cystine and dibasic amino acids in a subset of the patient group. We detected a total number of 22 different mutations in the SLC3A1 and SLC7A9 genes, 18 of which were described for the first time. We have found a probable genetic cause of cystinuria in approximately 74 % of our patients and a possible contribution to the disease in another 19 %. Mutations in the SLC3A1 gene is the major cause of cystinuria in our group, with only a minor contribution of SLC7A9 mutations. The group of patients presenting SLC3A1 mutations in a heterozygous state or lacking mutations in both genes had higher values of total urinary cystine and dibasic amino acids compared to patients homozygous for SLC3A1 mutations. The reason for this discrepancy remains unclear, but the possible impact of medical treatment with sulfhydryl compounds on total cystine values was ruled out.

Sequencing of the full-length canine SLC7A9 cDNA was accomplished using the RACE technology and results from mutation analyses of SLC7A9 and SLC3A1 in cystinuric dogs showed that only two out of 13 dogs have mutations with possible impact on protein function in these genes. DNA sequencing was used for all exons of both genes in the dog, and in human cystinuria patients, all samples lacking mutations or showing heterozygosity after SSCA screening were sequenced in both genes as well. This implies that all point mutations present have been detected, but the possibility of mutations escaping PCR based methods as well as mutations in regulatory parts of the SLC3A1 and SLC7A9 genes remains in cases lacking a full molecular genetic explanation of the disease.

Finally, clinical and genetic data from our study of cystinuria both in man and dog exemplifies that manifestation and clinical severity of cystinuria is not determined by genetic alterations in the SLC3A1 and SLC7A9 alone. Environmental factors, congenital malformations and modulating genetic factors are all possible contributors to the clinical outcome of cystinuria.

LIST OF PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by their Roman numerals, I-IV.

- I** **HARNEVIK, L.**, E. FJELLSTEDT, A. MOLBAEK, H. G. TISELIUS, T. DENNEBERG and P. SÖDERKVIST
Identification of 12 novel mutations in the SLC3A1 gene in Swedish cystinuria patients.
Human Mutation **18**: 516-525, 2001
- II** **HARNEVIK, L.**, E. FJELLSTEDT, A. MOLBAEK, T. DENNEBERG and P. SÖDERKVIST
Mutation analysis of SLC7A9 in cystinuria patients in Sweden.
Genetic Testing **7**: 13-20, 2003
- III** FJELLSTEDT, E., **L. HARNEVIK**, J. O. JEPSSON, H. G. TISELIUS, P. SÖDERKVIST and T. DENNEBERG
Urinary excretion of total cystine and the dibasic amino acids arginine, lysine and ornithine in relation to genetic findings in patients with cystinuria treated with sulphhydryl compounds.
Urological Research **31**: 417-425, 2003
- IV** **HARNEVIK, L.**, A. HOPPE and P. SÖDERKVIST
SLC7A9 cDNA cloning and mutational analysis of SLC3A1 and SLC7A9 in canine cystinuria.
Mammalian Genome **17**: 769-776, 2006

TABLE OF CONTENTS

INTRODUCTION	- 1 -
CYSTINURIA	- 1 -
<i>Historical review</i>	- 1 -
RENAL HANDLING OF CYSTINE AND DIBASIC AMINO ACIDS	- 2 -
<i>Amino acid transport in the renal proximal tubule</i>	- 2 -
<i>Driving forces of renal amino acid reabsorption</i>	- 3 -
<i>Biochemistry of cystine and the dibasic amino acids</i>	- 4 -
<i>Evidence for a transporter system shared by cystine and dibasic amino acids</i>	- 5 -
CLINICAL CHARACTERISTICS OF CYSTINURIA	- 6 -
<i>Cystinuria is inherited in a recessive or incompletely recessive manner</i>	- 6 -
<i>Cystinuria classification</i>	- 6 -
<i>Cystine excretion and stone formation in cystinuria</i>	- 7 -
<i>Diagnosis of cystinuria</i>	- 8 -
<i>Medical and prophylactic treatment of cystinuria</i>	- 8 -
MOLECULAR BIOLOGY OF CYSTINURIA	- 10 -
<i>Cloning of an amino acid transporter subunit exhibiting $b^{0,+}$-like transport</i>	- 10 -
<i>Linkage of cystinuria to chromosome 2 and mutation analysis of SLC3A1</i>	- 10 -
<i>Linkage of cystinuria to chromosome 19 and mutation analysis of SLC7A9</i>	- 12 -
<i>Genotype - phenotype correlations in cystinuria</i>	- 12 -
<i>Functional and expression studies of rBAT and $b^{0,+}$-AT</i>	- 14 -
<i>HSHATs, LSHATs and the model proposed for rBAT-$b^{0,+}$-AT interaction</i>	- 15 -
CANINE CYSTINURIA.....	- 16 -
<i>Characteristics of canine cystinuria</i>	- 16 -
<i>Inheritance and molecular genetics of canine cystinuria</i>	- 17 -

AIMS OF THE THESIS - 19 -

MATERIAL AND METHODS - 21 -

MOLECULAR GENETIC STUDIES ON CYSTINURIA IN HUMANS - 21 -

Cystinuria patients and normal controls - 21 -

Mutation screening of SLC3A1 and SLC7A9 - 21 -

DNA sequencing of SLC3A1 and SLC7A9 exons - 22 -

Southern Blot analysis - 22 -

Amino acid analysis - 22 -

MOLECULAR GENETIC STUDIES ON CANINE CYSTINURIA - 23 -

Cystinuric dogs and amino acid analysis - 23 -

Identification of the canine SLC7A9 cDNA - 23 -

Mutation analysis of the SLC3A1 and SLC7A9 genes - 23 -

RESULTS - 25 -

MOLECULAR GENETIC STUDIES ON CYSTINURIA IN HUMANS - 25 -

SLC3A1 mutations in cystinuria patients and controls - 25 -

SLC7A9 mutations in cystinuria patients and controls - 26 -

Genotype-phenotype correlations in cystinuria patients - 26 -

MOLECULAR GENETIC STUDIES ON CANINE CYSTINURIA - 28 -

Structure of the canine SLC7A9 gene - 28 -

Amino acid excretion in cystinuric dogs - 28 -

Genetic variants detected in SLC3A1 and SLC7A9 in cystinuric dogs - 29 -

DISCUSSION - 31 -

SLC3A1 and SLC7A9 mutations in Swedish cystinuria patients..... - 31 -

Mutational pattern in cystinuria in different populations..... - 32 -

Genotype-phenotype correlations - 33 -

Cause of cystinuria in patients lacking SLC3A1 and SLC7A9 mutations..... - 34 -

Absence of SLC3A1 and SLC7A9 mutations in cystinuric dogs..... - 35 -

Complexity of cystine stone formation..... - 36 -

CONCLUSIONS..... - 39 -

ACKNOWLEDGEMENTS..... - 41 -

REFERENCES..... - 43 -

PAPER I-IV

INTRODUCTION

CYSTINURIA

Cystinuria is defined as an inherited disorder characterised by increased urinary excretion of cystine and the dibasic amino acids arginine, lysine and ornithine. The pathophysiological mechanism behind cystinuria is a defective renal reabsorption of cystine and dibasic amino acids and due to the low solubility of cystine in the urine, the disorder is clinically manifested as recurrent stone formation in affected individuals. The incidence of cystinuria varies among different populations (table I) and although cystinuria is a rare condition the overall prevalence of the disease of 1/7,000 makes it one of the most common inherited disorders (SEGAL and THEIR 1995).

Historical review

The history of cystinuria begins in 1810 when Wollastone discovered a urinary stone that was different from stones previously described.

He named the calculi cystic oxide because of its presumed composition and hypothesised a bladder origin of the stone. Twenty years later Berzelius analysed the same calculi and concluded that the molecule did not consist of any oxygen and renamed it cystine.

The chemical structure of cystine was described nearly a century later and in 1908 Garrod proposed the theory of a defect cystine metabolism as the pathological basis of cystinuria. In the late 40's, lysinuria and argininuria was observed in a cystinuria patient and a few years later Dent and Rose hypothesised that cystinuria is caused by a defect renal transport of amino acids rather than an inborn error of metabolism (DENT and ROSE 1951).

Association of cystinuria within certain families was observed in 1929 and the inheritance pattern was further refined in the 50's when Harris demonstrated a recessive inheritance pattern in cystinuria (HARRIS and WARREN 1953). Further progress in the genetics behind the disease was made in 1966 when Rosenberg suggested genetic heterogeneity and allelism in cystinuria and classified the disease into three different subtypes; I, II and III (ROSENBERG 1966; ROSENBERG *et al.* 1966b).

In parallel with the progress on the genetics of cystinuria, a number of *in vitro* and *in vivo* studies further characterised the intestinal and renal transport of cystine and dibasic amino acids and it was shown that cystine and the dibasic amino acids shared a common resorption mechanism (DENT and ROSE 1951; KATO 1977; SEGAL *et al.* 1977).

Table I. Incidence of cystinuria in different populations

Incidence	Population	Reference
1/2,500	Libyan Jewish	(WEINBERGER <i>et al.</i> 1974)
1/5,600	Czech	(SKOPKOVA <i>et al.</i> 2005)
1/15,000	United States	(LEVY and BARKIN 1971)
1/16,000	Japan	(ITO <i>et al.</i> 1983)
1/100,000	Sweden	(BOSTROEM and HAMBRAEUS 1964)

Development of modern technologies during the 80's and 90's together with an increased understanding of human genetics has led to the molecular characterization of at least one transporter system for cystine and dibasic amino acids and to genetic linkage of cystinuria. Two cystinuria loci are known at present, located to chromosome 2 and 19 (BISCEGLIA *et al.* 1997; PRAS *et al.* 1994; WARTENFELD *et al.* 1997). The disease genes within these loci are the amino acid transporter genes SLC3A1 and SLC7A9, together encoding a functional transporter protein complex. A number of studies have identified mutations in the two known disease genes and experimental studies supports the linkage between SLC3A1 and SLC7A9 mutations and cystinuria (CALONGE *et al.* 1994; FELIUBADALO *et al.* 1999b).

RENAL HANDLING OF CYSTINE AND DIBASIC AMINO ACIDS

Amino acid transport in the renal proximal tubule

Amino acid reabsorption in the kidney is localized to early proximal tubule segments along the nephron (GONSKA *et al.* 2000). The majority of all amino acids are reabsorbed from the tubule to >99% independently of species, *L*-isomers preferred over *D*-isomers since protein and peptide amino acids are always the *L*-amino acid (SILBERNAGL 1988). Hence, most amino acids are protected from being wasted in the urine and normal excretion of amino acids has been estimated to approximately 0.6 % of the intake in humans, if protein uptake is 100 g/day. Normal urinary excretion depends on the filtered load and the concentration of the substance in the filtrate, tubular reabsorption and/or secretion of the substance and metabolic formation or degradation of the substance within the lumen of the tubule. The first two factors differ a lot between different amino acids whereas the third factor applies only to a few amino acids.

The transepithelial transport of amino acids is mediated via functionally and structurally different amino acid transporters at the luminal and basolateral surface of the epithelial cells. The specific mechanisms of amino acid transport are often referred to as "systems", which bind and translocate amino acids of a certain group for which the molecular characteristics are quite similar. The systems show overlapping specificities and criteria to define the systems have been based on functional characteristics; the type of amino acid transported (acidic, zwitterionic or basic) across the membrane and the thermodynamic properties of the transport (PALACIN *et al.* 1998). In the proximal tubule, system x-AG, B⁰ and b⁰⁺ for acidic, neutral and basic amino acids respectively operates at the luminal side of the epithelium, while system L, A and ASC for neutral amino acids and y⁺L for basic amino acids are situated on the basolateral side, mediating entrance to adjacent renal capillaries (figure 1)(KANAI *et al.* 2000).

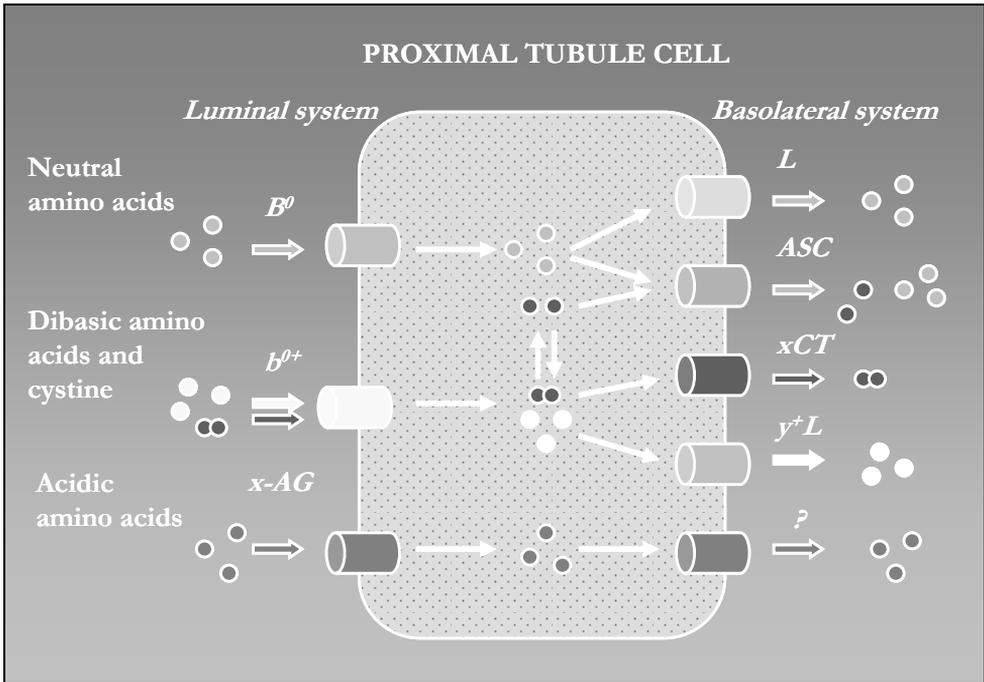


Figure 1. Amino acid transporter systems and the net transport of amino acids in the renal proximal tubule. Different amino acid transporter systems are expressed on the luminal and basolateral side of the proximal tubule cell. Most transporter systems depend on co-transport of Na^+ (indicated by capital letter) and many also function as obligatory exchangers of amino acids. The Na^+ -independent transporter system b^{0+} -AT, transports cystine and dibasic amino acids into the cell in exchange for neutral amino acids that are recycled by system B^0 . Cystine can be transported by the basolateral system xCT but the major part of the cystine is reduced to two cysteines and transported by system ASC together with neutral amino acids. The dibasic amino acids exit the cell via system y^+L . This exemplifies the co-operation between different transporter systems to achieve a net reabsorption of amino acids from urine to the blood.

Driving forces of renal amino acid reabsorption

Urinary concentrations of amino acids can be lower than plasma levels, suggesting that tubular amino acid reabsorption is an active process with a net solute transport against an electrochemical potential gradient (SCHAFER and BARFUSS 1980). The majority of all amino acid transport depend on co-transport of Na^+ and amino acid across the membrane and the driving forces proposed for this type of transport includes pre-existing membrane potential, presence of luminal Na^+ gradient directed into the cell and transmembranal concentration gradient of the amino acid involved (SILBERNAGL 1988).

Active transport of amino acids across the brush border membrane produces a high intracellular concentration gradient, which favours passive amino acid exit from the cell across the basolateral membranes (SILBERNAGL 1988). Hence, the net transepithelial transport is determined by both luminal amino acid uptake and the rate of basolateral movement out of the cell. Basolateral exit of amino acids may regulate transepithelial transport independent of the luminal uptake process;

however the basolateral transport mechanisms are not as well understood as the luminal uptake.

Biochemistry of cystine and the dibasic amino acids

Cystine is a non-essential amino acid synthesised from methionine and although major losses of cystine occur in cystinuria patients, the plasma levels of methionine and cystine are essentially normal (FRIMPTER 1963; LONDON and FOLEY 1965).

Cystine is the condensation product of two cysteines and is highly insoluble in the urine. Cysteine and cystine exist in a chemical equilibrium in the tubule cell and cysteine is the predominant intracellular form since cystine is quickly reduced to cysteine within the tubule cells (CRAWHALL and SEGAL 1966; CRAWHALL and SEGAL 1967). Cysteine presented to the lumen is largely converted into its oxidized form cystine.

The characteristic of cystinuria is elevated urinary cystine levels accompanied by increased excretion of the dibasic amino acids arginine, lysine and ornithine. Arginine and lysine are essential amino acids while ornithine is the only one not used for protein synthesis (SILBERNAGL 1988). The dibasic amino acids possess an additional positive charge in the side chain hence the name dibasic amino acids.

Cystine and the dibasic amino acids share certain structural features as shown in figure 2, since the molecules all have two amine groups separated by 4-6 atoms and at least one of the amine groups is linked to an asymmetric carbon atom (DENT and ROSE 1951).

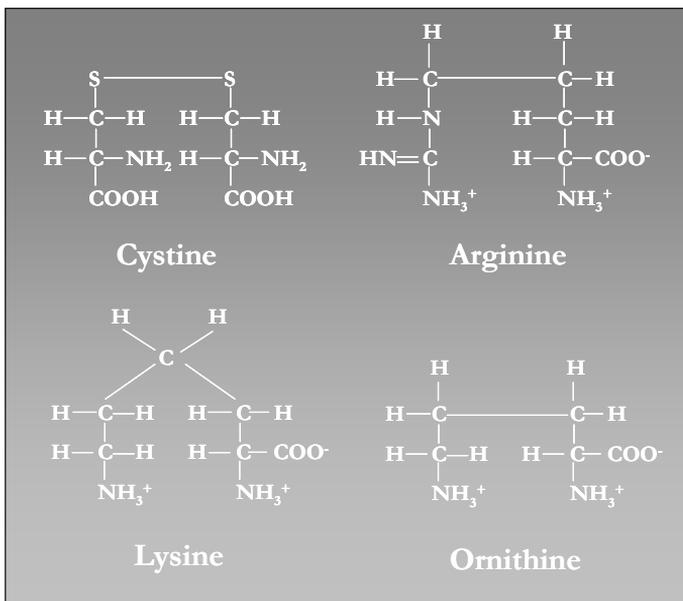


Figure 2. Chemical structure of cystine and the dibasic amino acids, arginine, lysine and ornithine.

Evidence for a transporter system shared by cystine and dibasic amino acids

It was hypothesised very early, based on the clinical findings in cystinuria patients, that cystine and the dibasic amino acids share a common resorptive mechanism in the kidney (DENT and ROSE 1951). The theory was later confirmed in a study of renal clearances of cystine, arginine and ornithine in cystinuria patients and normal subjects after intravenous lysine infusion (ROBSON and ROSE 1956). It was demonstrated that increased filter load of lysine caused a transient increase of cystine, arginine and ornithine in normal individuals but not in cystinuria patients.

Results from *in vitro* studies performed by Thier and co-workers was later presented, supporting a shared transporter system defect in the disease as suggested by Dent and Rose (THIER *et al.* 1964; THIER *et al.* 1965). Amino acid transport in jejunal mucosa was examined and showed cystine and dibasic amino acid accumulation against a concentration gradient in normal subjects whereas the majority of the cystinuria patients were unable to accumulate cystine and dibasic amino acids.

To further characterise the transport system shared by cystine and dibasic amino acids, Kato performed a series of experiments resembling those conducted by Dent and Rose (KATO 1977). Using intravenous infusion of *L*-lysine and *L*-arginine to study the tubular reabsorption of cystine and dibasic amino acids in cystinuria patients and normal controls the existence of two different reabsorption systems shared between cystine and dibasic amino acids was shown. One system appeared to be of low capacity and one seemed to work at higher concentrations since homozygous cystinuria patients could not reabsorb elevated amino acid levels until the filtered load was increased seven- to ten-fold the normal filtered load. Kato suggested a defective low-capacity system and an intact high-capacity system in cystinuria patients.

Kato's findings were supported by *in vitro* experiments on cystine uptake by rat renal brush-border vesicles which also pointed at two distinct transport systems, one of high affinity, inhibited by the dibasic amino acids, and one of low affinity, unaffected by dibasic amino acids (SEGAL *et al.* 1977). Cystine and dibasic amino acid uptake was also examined in isolated renal tubules and the results demonstrated two saturable systems for cystine, a low K_m system that interacts with dibasic amino acids and a high K_m which was affected only by arginine (FOREMAN *et al.* 1980).

CLINICAL CHARACTERISTICS OF CYSTINURIA

Cystinuria is inherited in a recessive or incompletely recessive manner

The first attempts to investigate the inheritance pattern of cystinuria came in the 50's, when Harris and co-workers examined the renal excretion of amino acids in cystinuria patients and their relatives (HARRIS *et al.* 1955; HARRIS and ROBSON 1955; HARRIS and WARREN 1953).

Two different phenotypes were described in the patient group, one that was characterised by highly increased values of urinary cystine and dibasic amino acids with a high frequency of stone formation, and one phenotype, although variable, with moderately increased cystine and lysine excretion and arginine excretion in the range between normal or slightly elevated. Stone formation was seen only occasionally in the latter phenotype and the families studied were divided into two groups with respect to occurrence of this phenotype. In the first group this phenotype was not found and the inheritance pattern was determined as recessive. In the other group, the second phenotype was frequently found and the mode of inheritance was described as incompletely recessive.

Cystinuria classification

On the basis of biochemical findings in cystinuria patients and obligate heterozygous individuals (parents and children), a classification into different subtypes was proposed in the 1960's. Rosenberg and co-workers performed a series of *in vitro* and *in vivo* studies in cystinuria families and normal controls including uptake of cystine, lysine and arginine by intestinal mucosa, oral loading with cystine and examination of urinary excretion of cystine and dibasic amino acids in patients and obligate heterozygous individuals (ROSENBERG 1966; ROSENBERG *et al.* 1966b; ROSENBERG *et al.* 1965). The results from the urinary excretion patterns confirmed the previously observed inheritance pattern that cystinuria occurs in a recessive and an incompletely recessive form (HARRIS *et al.* 1955; HARRIS and ROBSON 1955) and the *in vitro* studies further subdivided the incompletely recessive form into two subtypes.

Rosenberg suggested at least three biochemically distinct subtypes named cystinuria subtypes I, II and III, where homozygous individuals of any subtype show increased urinary excretion of cystine and dibasic amino acids (ROSENBERG *et al.* 1966a). Type I is further characterised by normal amino acid profile in heterozygotes and absence of mediated transport of cystine, lysine and arginine in homozygotes in jejunal mucosa. Type II and III heterozygotes show excessive excretion of cystine and dibasic amino acids in the urine and mediated cystine transport was demonstrated in type III homozygotes whereas type II homozygotes only showed transport of cystine in jejunal mucosa. The oral loading studies further support the *in vitro* studies since only type III show normal plasma concentrations after cystine load. The features of the cystinuria subtypes according to Rosenberg are summarised in table II.

Table II. Characteristics of cystinuria subtypes according to Rosenberg. The classification is based on urinary concentration and intestinal transport of cystine and dibasic amino acids and plasma concentrations of cystine after oral loading experiments (ROSENBERG *et al.* 1966a).

	Biochemical manifestation	Type I	Type II	Type III	Controls
Homozygotes	Urinary excretion of cystine and dibasic amino acids	Elevated	Elevated	Elevated	<1%
	Active intestinal transport of cystine, lysine and arginine	Absent	Cystine present Lysine absent	Present	Present
	Plasma concentration of cystine after oral load	No increase	No increase	Increase	Increase
Heterozygotes	Urinary excretion of cystine and dibasic amino acids	<60 mg/g creatinine	>175 mg/g creatinine	80–150 mg/g creatinine	<60mg/g creatinine

The classification of cystinuria into different subtypes was an important step in understanding the genetic basis of the disease. Based on examination of double heterozygous individuals (type I/III, II/III and I/II) Rosenberg later concluded that the different subtypes seen in cystinuria were due to different alleles on the same locus (ROSENBERG *et al.* 1966b). The results indicated that individuals heterozygous for two different subtypes was clinically indistinguishable from homozygotes of any subtype and conclusion about their double heterozygous state came from examination of obligate carriers.

Cystine excretion and stone formation in cystinuria

The only clinical manifestation of cystinuria is the formation of kidney stones in affected individuals (figure 3), which can occur when urinary excretion of cystine exceeds solubility threshold values. The normal upper limit of urinary excretion of cystine is 8.4 $\mu\text{mol}/\text{mmol}$ creatinine (CRAWHALL *et al.* 1969) which correspond to less than 30 mg/day (JOLY *et al.* 1999). Limit of cystine solubility at pH 4.5-7.0 is approximately 250 mg/l (DENT and SENIOR 1955), a threshold value frequently exceeded in cystinuric patients that generally excrete between 600-1,400 mg/day (JOLY *et al.* 1999). At pH higher than 7, solubility of cystine increases up to 500 mg/l or more above pH 7.5.



Figure 3 Cystine urolith.

Cystinuric patients present a life-long risk of stone formation and although stone formation may occur at any time in life, age of onset peaks during the first three decades of life (JOLY *et al.* 1999; MILLINER 1990). In 80 % of patients, the first stone is presented during this period with an earlier appearance and more severe clinical outcome in males (DELLO STROLOGO *et al.* 2002).

Uroliths formed in cystinuric patients may be composed of pure cystine but stones of mixed composition such as cystine, struvite, calcium oxalate and calcium phosphate are frequently found. There are indications that cystinuria may be a predisposing factor to calcium oxalate stone formation, which is one of the most common types of uroliths (RESNICK *et al.* 1979). Resnick and co-workers concluded that heterozygous cystinuria is a risk factor for calcium oxalate stone formation after screening 126 stone forming patients and 84 normal controls for elevated cystine levels in the urine. This is further supported by *in vitro* studies where the addition of cystine to undiluted human urine resulted in enhancement of calcium oxalate crystal precipitation (MARTINS *et al.* 2002). The result from Martins and co-workers implies that urinary cystine is a risk factor for calcium oxalate calculi and that the mechanism of crystal formation was a salting out effect since cystine was not present in any calcium oxalate crystals.

Diagnosis of cystinuria

Screening methods in the diagnosis of cystinuria include non-quantitative colorimetric methods i.e. Brand's test (Brand 1930) and Urocystin test (Urocystin Kit®; Santeen Pharmaceutical, Osaka Japan)(KALLISTRATOS *et al.* 1983). In Brand's test the reaction between cyanide nitroprusside and cystine produce a purple colour which will detect cystine levels above 75-125 mg cystine/g of creatinine. Homozygous individuals will thus be detected as will some, but not all of the heterozygous individuals and a positive result is not exclusive for cystinuria. In the commercially available Urocystin Kit®, the red complex formed between cystine and nickel ions instead of nitroprusside is detected.

Microscopic examination of urinary sediment provide another diagnostic tool, although the sensitivity is low since the typical cystine crystals are found in only 17-26% of homozygous cystinuria patients (DAHLBERG *et al.* 1977; EVANS *et al.* 1982).

To confirm the diagnosis, patients are further evaluated with regard to quantitative measurement of cystine and dibasic amino acids in the urine. Ion-exchange chromatography may be preferred for this purpose since the mixed disulfide with D-penicillamine and tiopronin can be simultaneously analysed (JEPPSON and KARLSSON 1972).

Medical and prophylactic treatment of cystinuria

Medical treatment of cystinuria aims at decreasing the cystine concentration and increasing the solubility of cystine in the urine (JOLY *et al.* 1999; KNOLL *et al.* 2005). Treatment is thus preventive with regard to formation of new stones but will also help dissolve pre-existing cystine calculi. This is achieved using a combination of hydration, urine alkalinisation and sulfhydryl (SH) compound and cystinuria patients are carefully monitored to continually adjust medical and prophylactic treatment (figure 4). A low-sodium diet has also been proposed to reduce cystine excretion (JOLY *et al.* 1999; LINDELL *et al.* 1995a).

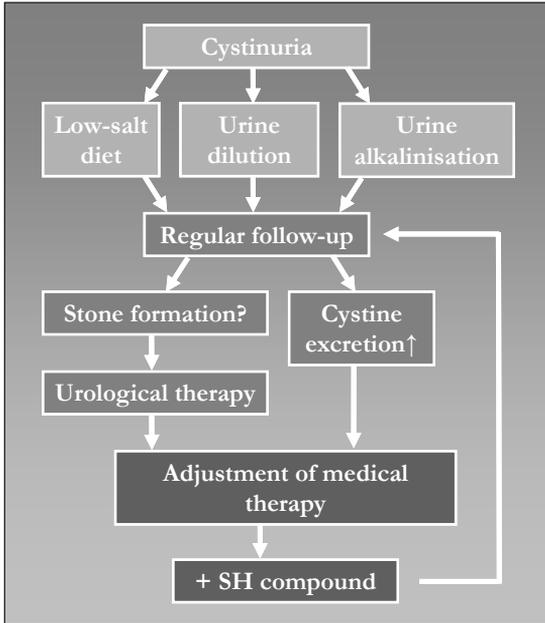


Figure 4. Treatment regimen in cystinuria.

The obvious disadvantage of such a regimen is that alkalinisation favours precipitation of calcium salts and since cystinuria patients sometimes form mixed cystine-calcium stones, and heterozygotes seem to be predisposed to calcium stone formation, alkalinisation should be used together with high water intake.

The combination of high fluid intake and alkalinisation of the urine is insufficient as a treatment in many patients and a sulfhydryl compound such as tiopronin or D-penicillamine is often used in addition to further increase solubility of cystine and lower the concentration of free cystine (DENT *et al.* 1965). The sulfhydryl compound forms a mixed disulfide with cystine that is 50 times more soluble than cystine (figure 5). Adverse effects are common *e. g.* proteinuria, skin rash, fever, nausea, epidermolysis, nephritic syndrome and thrombocytopenia (JOLY *et al.* 1999; KNOLL *et al.* 2005)

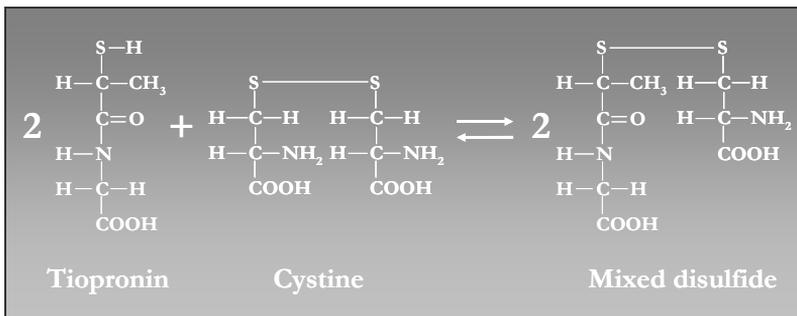


Figure 5. The sulfhydryl compound tiopronin forms a mixed disulfide with cystine in the urine.

MOLECULAR BIOLOGY OF CYSTINURIA

Cloning of an amino acid transporter subunit exhibiting b^{0,+}-like transport

Amino acid transport system b^{0,+} denotes transport of neutral and basic (indicated by ^{0,+}) amino acids in a Na⁺ independent manner (indicated by the lower case letter *b*). In 1992, both rat and rabbit kidney cDNAs exhibiting this transport activity was identified (BERTRAN *et al.* 1992; WELLS and HEDIGER 1992). The cDNAs were referred to as rBAT, related to b-like amino acid transport and induced Na⁺ independent uptake of cystine and dibasic amino acids when expressed in *Xenopus* oocytes. The tissue distribution was mainly localised to kidney and small intestine. A year later Bertran and colleagues isolated the human homologous cDNA exhibiting the same transporter properties using the same expression cloning approach (BERTRAN *et al.* 1993) and the human rBAT gene was mapped to chromosome 2p16 (CALONGE *et al.* 1995a; LEE *et al.* 1993).

A more detailed study of the rBAT expression pattern showed localisation of the protein mainly in the proximal straight tubule in renal rat tissue and to some extent in the proximal convoluted segments (KANAI *et al.* 1992; PICKEL *et al.* 1993). Corresponding analyses of human renal tissue confirmed these results and further defined the expression to the microvilli of the proximal straight tubule, and it was postulated that rBAT was a part of the high affinity system for cystine absorption previously detected in this region (FOREMAN *et al.* 1980; FURRIOLS *et al.* 1993; SEGAL *et al.* 1977).

The role of the new protein in amino acid resorption *in vivo* was suggested as regulatory or as a component of the renal b^{0,+}-like system since the predicted protein structure was not very hydrophobic (BERTRAN *et al.* 1992; WELLS and HEDIGER 1992). Further, rBAT showed high sequence similarity to the 4F2 protein, a subunit of the y⁺L-like amino acid transport system known to heterodimerise to other proteins. Taken together, these findings pointed at the involvement of rBAT in cystine and dibasic amino acid reabsorption with an expression pattern in concordance with the defects seen in cystinuria, and the novel protein was inferred in the pathogenesis of cystinuria.

Linkage of cystinuria to chromosome 2 and mutation analysis of SLC3A1

The search for cystinuria candidate genes started in the early 90's and the previous cloning and chromosomal location of the rBAT cDNA focused the attention to chromosome 2. Linkage between cystinuria and chromosome 2 markers were investigated in affected Middle East families with several consanguineous marriages and the disease locus was located to 2p16 (PRAS *et al.* 1994). Mutations in the gene encoding the rBAT protein, SLC3A1 (figure 6), were demonstrated simultaneously by Calonge and co-workers. By the use of illegitimate transcription technique from lymphoblastoid cell lines derived from cystinuria patients followed by RT-PCR, it was possible to analyse the main part of the coding sequence using SSCA analysis and DNA sequencing (CALONGE *et al.* 1994). Six different cystinuria specific

mutations were identified and distributed in 11 out of 36 unrelated cystinuria patients. M467T was the most frequent mutation and associated with an almost complete loss of *L*-cystine and *L*-arginine transport when expressed in *Xenopus* oocytes.

Several studies later confirmed the association of SLC3A1 mutations with cystinuria (BISCEGLIA *et al.* 1996; CALONGE *et al.* 1995b; ENDSLEY *et al.* 1997; PRAS *et al.* 1995) but mutations were never found in all patients, observations that could only in part be explained by the methodology used (usually SSCA in combination with DNA sequencing). Mutation analysis of cystinuria patients with different subtypes in French Canadians (HORSFORD *et al.* 1996), Italian and Spanish (GASPARINI *et al.* 1995) implied genetic heterogeneity in cystinuria since mutations were detected only in type I cystinuria patients. Further, linkage analysis of chromosome 2 markers in type I and type III cystinuria patients demonstrated linkage only in type I patients (CALONGE *et al.* 1995b).

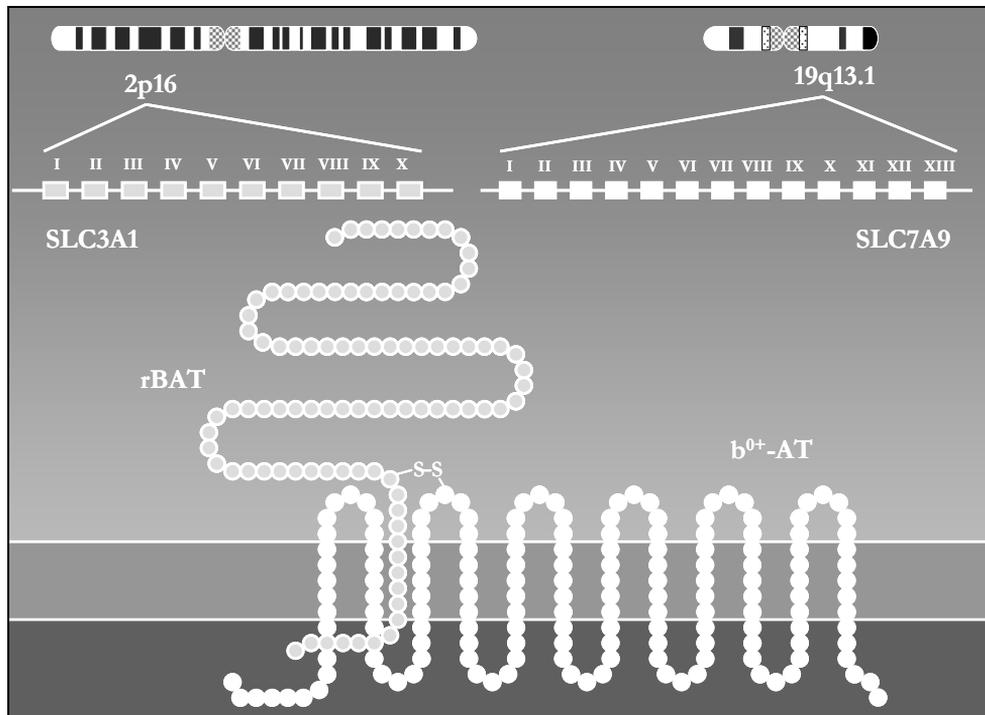


Figure 6. Cystinuria loci and structure of disease genes and the encoded proteins. The SLC3A1 gene is divided into ten exons and spans approximately 45 kb. Most of the 685 amino acid long rBAT protein encoded by SLC3A1 is found extracellularly where a disulfide bond is formed with the b⁰⁺-AT protein. The b⁰⁺-AT protein is encoded by another member of the solute carrier family of genes, SLC7A9 which spans 40 kb divided into 13 exons. The twelve transmembrane segments of the b⁰⁺-AT protein form the channel that translocates amino acids across the membrane.

Linkage of cystinuria to chromosome 19 and mutation analysis of SLC7A9

The absence of SLC3A1 mutations in non-type I cystinuria led to further search for new cystinuria loci and genome wide screens in non-type I patients finally located a new locus on chromosome 19 (BISCEGLIA *et al.* 1997; WARTENFELD *et al.* 1997). Bisceglia and colleagues demonstrated linkage between cystinuria and markers located to 19q13.1 in Italian families including mainly cystinuria type III patients and the same locus was linked to cystinuria in Libyan Jewish cystinuria families by Wartenfeld and co-workers. Linkage of cystinuria type II and III to 19q13.1 was later confirmed and refined by different groups (FELIUBADALO *et al.* 1999a; PRAS *et al.* 1999; STOLLER *et al.* 1999).

In 1999, the International Cystinuria Consortium identified the SLC7A9 gene located to the new cystinuria locus on chromosome 19 (figure 6)(FELIUBADALO *et al.* 1999b). The cDNA sequence was identified by cDNA library screening and translates into the b^{0,+}-protein that forms the actual amino acid transporting channel through the membrane of proximal tubule cells. Three exons of the SLC7A9 gene were screened for mutations in non-type I and unclassified cystinuria patients, revealing seven different cystinuria specific mutations distributed in 41 out of 133 independent cystinuria chromosomes. The most frequent mutation, V170M, a founder mutation in a Libyan Jewish population, abolished L-arginine uptake induced by co-transfection with rBAT in COS cells. The intracellular location of the protein was investigated with and without co-transfection of rBAT and the results showed that rBAT directs the b^{0,+}-protein to the plasma membrane. Northern Blot analysis showed a tissue distribution of the transcript restricted to kidney, liver, small intestine and placenta.

Genotype - phenotype correlations in cystinuria

Progress made on the molecular genetics of cystinuria in the 90's provided new possibilities for studying the relation between genotype and phenotype in cystinuria. Goodyer found that children with a I/I genotype presenting homozygous SLC3A1 mutations excreted significantly greater amounts of cystine than did children with a I/III genotype in a study of 23 cystinuria probands identified by the Quebec neonatal screening program (GOODYER *et al.* 1998). The patients were classified by analysing urinary excretion of cystine and dibasic amino acids in their parents according to Rosenberg (ROSENBERG 1966; ROSENBERG and DOWNING 1965; ROSENBERG *et al.* 1966b). A follow-up of this study indicated an increased risk of stone formation in type I/I probands during the first decade of life (GOODYER *et al.* 1998). Later, the same group detected SLC7A9 mutations in all three subtypes and suggested a new classification for the disorder based on inheritance pattern; a dominant and a recessive form of cystinuria (LECLERC *et al.* 2002).

The non-type I cystinuria patients in a Libyan Jewish population provided a unique opportunity to study the disease since the patients share a specific chromosome 19 haplotype which suggests a common founder (PRAS *et al.* 1998).

Urinary cystine and dibasic amino acids were measured and oral loading tests were performed in cystinuria patients and their relatives, affected status of which were previously determined by haplotype analysis (WARTENFELD *et al.* 1997). The results showed that the sum of urinary cystine and dibasic amino acids could be used to separate between the normal, the heterozygous and the homozygous group (PRAS *et al.* 1998). Urinary cystine alone was also helpful in distinguishing normal from heterozygous but not between heterozygous and homozygous individuals due to considerable overlap. Arginine and ornithine were the best markers for discriminating between heterozygotes and homozygotes since excretion values were always in the low hundred ($\mu\text{mol/g}$ of creatinine) while homozygotes were in the thousands. This phenomenon may be explained by different affinity of the transporter system for cystine and the dibasic amino acids. Thus, a milder defect as seen in heterozygotes results in a relatively large excreted amount of lysine and cystine due to a higher transporter affinity for arginine and ornithine.

In the same study oral lysine load in two homozygous and two normal individuals resulted in elevated plasma concentrations in all cases, however the peak in the homozygous individuals was only half of the normal (PRAS *et al.* 1998).

Despite a genetic homogenous group of cystinuria patients, phenotype expression varied considerably in both heterozygotes and homozygotes. 85 % of the homozygotes and 13 % of the heterozygotes suffered from kidney stones and the mean age of onset was 12 ± 10 years in the homozygotes. These findings emphasises the importance of other environmental and genetic factors for the development of kidney stones in cystinuria patients.

A few years after the identification of SLC7A9, the genomic structure was determined by the International Cystinuria Consortium, facilitating mutation analyses of this gene in cystinuria patients (FONT *et al.* 2001). The open reading frame was analysed in 124 cystinuria patients, 61 of which were classified according to subtype. Samples were screened using SSCA and DNA sequencing and mutations were detected in 79 % of the non-type I alleles and in 31 % of the untyped probands. Functional analysis of the most common mutations, where mutated b^{0,+}-protein were co-expressed with rBAT in HeLa cells, showed a complete or almost complete lack of L-cystine uptake. Genotype–phenotype correlations where mutations were related to amino acid excretion in heterozygotes showed a clear individual variation for a given SLC7A9 mutation, but also a varying degree of severity among different mutations. Correlation was also found between amino acid transport function in transfected HeLa cells and the urinary phenotype in heterozygotes for different mutations.

The largest genotype–phenotype correlation study conducted so far was performed by Dello Strologo and co-workers (DELLO STROLOGO *et al.* 2002). A database over 150 cystinuria families from Italy, Spain and Israel was established including clinical and mutational data. Two different classifications were attempted, the traditional type I-III classification and another based on genetic findings; homozygotes for SLC3A1 mutations were defined as type A, homozygotes for

SLC7A9 mutations type B and a third type AB, with compound mutations in SLC3A1 and SLC7A9. Age of onset, clinical outcome and severity of the disease did not differ between the groups in spite of classification used, although treatment of the patients may have influenced the outcome. Significant gender differences were however observed for age of onset and number of stone events with more severe outcome in males, but no correlation was found between urinary excretion values of the amino acids elevated in cystinuria and stone event frequency. Finally, silent SLC7A9 carriers were observed in 14% of the cases, supporting previous results where SLC7A9 mutations were reported in all subtypes (LECLERC *et al.* 2002). The authors suggested a new classification, the A, B and AB system described, which is based on genetics rather than clinical findings.

Taken together, the correlation studies performed in cystinuria points at the need for a new classification or a revision of the Rosenberg classification. No consensus regarding the basis of the classification has been made and both genotype (A-B system) and phenotype (recessive-dominant) have been put forward as classification criteria.

Functional and expression studies of rBAT and b⁰⁺-AT

After the molecular cloning of the SLC3A1 and SLC7A9 genes and characterisation of the proteins encoded, rBAT and b⁰⁺-AT, it was shown that these proteins heterodimerise to form a functional amino acid transporting complex (CHAIROUNGDUAN *et al.* 1999; PFEIFFER *et al.* 1999; RAJAN *et al.* 1999). Human cDNAs as well as rat and rabbit cDNAs were used in *Xenopus* and COS-7 expression system to demonstrate the b⁰⁺-like amino acid transport defective in cystinuria and a covalent interaction that cause the heterodimerization between rBAT and b⁰⁺-AT was proved. Further, *in situ* hybridisation and immunofluorescence studies showed the distribution of b⁰⁺-AT over the proximal tubule with the highest expression in the proximal convoluted tubule and lowest in the proximal straight tubules. Surprisingly, the expression pattern of rBAT was reversed along the proximal tubule with regard to b⁰⁺-AT.

In order to solve the expression paradox of rBAT and b⁰⁺-AT, Fernandez and co-workers performed Western blot analyses of mouse and human brush border membranes co-precipitated with anti-bodies against rBAT and b⁰⁺-AT (FERNANDEZ *et al.* 2002). The results indicated that the proteins are expressed solely as heterodimers in the renal brush-border membrane, and that most of the b⁰⁺-AT associates exclusively with rBAT. A substantial part of the rBAT proteins were however not co-precipitated with b⁰⁺-AT and may have another binding partner in the proximal straight tubule. It was concluded that the rBAT-b⁰⁺-AT complex is the main apical reabsorption system for cystine in the kidney since both rBAT and b⁰⁺-AT previously had been associated with the system located to the proximal convoluted tubule where most of the cystine is reabsorbed and expression of b⁰⁺-AT is the highest.

HSHATs, LSHATs and the model proposed for rBAT-b^{0,+}-AT interaction

The assumption of the heterodimeric constitution of the cystine and dibasic amino acid transporter system defective in cystinuria came from studies of other amino acid transporters that need association with the 4F2hc protein which is structurally related to rBAT. rBAT is expressed mainly in the renal proximal tubule and the small intestine while 4F2hc is more widely distributed. Although both proteins are localised to the renal reabsorptive epithelia, rBAT is located to the apical side of the membrane whereas 4F2hc is expressed basolaterally.

4F2hc and rBAT constitute a family referred to as HSHATs (heavy subunits of heterodimeric amino acid transporters) that associates with a member of the LSHAT family (light subunits of heterodimeric amino acid transporters) to form a functional transporter (CHILLARON *et al.* 2001; WAGNER *et al.* 2001). The HSHAT proteins share a common structure with an intracellular NH₂-terminus, an extracellular glycosidase-like domain and an extracellular cysteine residue located near the transmembrane domain which makes the intermolecular link with the LSHAT proteins (exemplified in figure 4).

The LSHAT proteins also share some structural features, including conserved amino acid residues in the protein. They are approximately 500 amino acids in length with twelve transmembrane domains and a cysteine residue located in the loop between putative transmembrane domain three and four which forms the disulfide with rBAT and 4F2hc respectively. Several LSHATs are known to heterodimerise with 4F2hc, but so far only b^{0,+}-AT is known to associate with rBAT. Recent studies also suggest the existence of an unknown HSHAT, since a novel LSHAT was identified that does not heterodimerises with either 4F2hc or rBAT (VERREY *et al.* 2004). HSHATs and LSHATs known at present are summarised in table III.

Table III. Heterodimeric amino acid transporters of the HSHAT and LSHAT families. The heavy chains rBAT and 4F2hc are listed together with light chain amino acid transporter subunits known to heterodimerise with the heavy chains. LSHATs that associate with unknown HSHATs are included as well (PALACIN and KANAI 2004; VERREY *et al.* 2004).

<i>HSHAT</i>	<i>Gene</i>	<i>LSHAT</i>	<i>Gene</i>	<i>System</i>	<i>Tissue distribution</i>
rBAT	SLC3A1	b ^{0,+} -AT	SLC7A9	b ^{0,+}	kidney, small intestine(apical)
4F2hc	SLC3A2	LAT1	SLC7A5	L, type 1	ovary, placenta, brain, spleen, testis
		LAT2	SLC7A8	L, type 2	kidney, small intestine
		y ⁺ LAT1	SLC7A7	y ⁺ L, type 1	kidney, small intestine
		y ⁺ LAT2	SLC7A6	y ⁺ L, type 2	non-epithelial tissues
		xCT	SLC7A11	xc ⁻	activated macrophages
		Asc-1	SLC7A10	asc	brain, heart, placenta, skeletal muscle, kidney
Unknown	---	Asc-2	SLC7A---	asc	kidney, placenta, spleen, lung, skeletal muscle
		AGT-1	SLC7A---	---	kidney (basolateral)

Work by Bauch and Verrey shows the requirement of co-expression of subunits from both the HSHAT and the LSHAT families to receive a functional amino acid transporter (BAUCH and VERREY 2002). Their experiments, including

immunofluorescence microscopy and immunoprecipitation of rBAT and b^{0,+}-AT in MDCK cells, clearly demonstrates that the two proteins require each other for proper localisation to the membrane. MDCK cells expressing b^{0,+}-AT alone showed that the protein was stable but accumulated intracellularly and cells expressing rBAT alone showed a decreased protein stability.

A proposed model for folding and post-translational modifications of rBAT and b^{0,+}-AT suggests that b^{0,+}-AT folds into a functional protein structure without interaction with rBAT (REIG *et al.* 2002). b^{0,+}-AT in turn favours tertiary structure formation of rBAT and finally the rBAT-b^{0,+}-AT complex is transported to the plasma membrane. Processing of rBAT from the ER to the Golgi, *i.e.* maturation of the *N*-glycosylation of the rBAT, depends on the presence of the b^{0,+}-AT, which also seems to protect rBAT against cellular degradation. In contrast, the level of expression of b^{0,+}-AT seems to be independent of the expression of rBAT.

CANINE CYSTINURIA

Characteristics of canine cystinuria

Selective breeding of the dog has generated a great number of dog breeds and currently, ~400 breeds are recognized over the world (LINDBLAD-TOH *et al.* 2005). The selective pressure on a rather small number of founding animals of specific breeds has led to a limited genetic diversity in respective breed and consequently the accumulation of recessive disease alleles. Approximately 1000 inherited disorders has been recognized in the dog, among them cystinuria, reported in more than 60 different breeds (CRAAN 1981).

In the early days of canine cystinuria research, Treacher addressed the issue of renal or metabolic origin of the disorder. Cystine concentrations in plasma and urine were analysed in normal and cystinuric dogs of different breeds to investigate the mechanisms of cystinuria (TREACHER 1964). Urinary levels, but not plasma levels, differed between normal and cystinuric dogs indicating a renal origin of the disease. A considerable overlap in excretion values was observed between these two groups, reflecting the importance of other factors than urinary cystine for cystine stone formation in the dog. In following experiments, lysine was fed to normal and cystinuric dogs and the observed results in cystinuric dogs suggested an impaired intestinal absorption of lysine as seen in human cystinuria subtypes (TREACHER 1965), although later work did not support this finding (HOLTZAPPLE *et al.* 1971; TSAN *et al.* 1972).

The amino acid excretion pattern in canine cystinuria tends to vary to a greater extent compared to human cystinuria and show substantial differences between and within breeds. The obligatory dibasic aminoaciduria as seen in man is not always present in the dog and cystinuria with lysinuria but with normal excretion of the other dibasic amino acids is a frequent finding in canine cystinuria as well as the presence of isolated cystinuria which is rarely found in humans (BOVEE *et al.* 1974; HOLTZAPPLE *et al.* 1971). Yet another study showed significant differences in

urinary excretion between normal and cystinuric dogs of different breeds for cystine, threonine and serine (CLARK and CUDDEFORD 1971).

The most extensive study so far, where urinary excretion of twenty amino acids in fifteen normal and twenty-four cystinuric dogs were analysed, was conducted by Hoppe and co-workers (HOPPE *et al.* 1993b). Even though all cystinuric dogs had formed cystine stones, seven of the dogs did not excrete abnormal amounts of cystine. Ten different amino acids were positively correlated to urinary cystine and three amino acid patterns were observed: i) increased excretion and significant correlation with cystine of the three dibasic amino acids, ii) increased excretion but no correlation with cystine for glutamine, citrulline and threonine, and iii) correlation with cystine but no increased excretion of 1-methyl-histidine, phenylalanine, 3-methyl-histidine, leucine or alanine. Cystinuria in combination with dibasic aminoaciduria was found in ten dogs, although only five had increased levels of all three dibasic amino acids as seen in human cystinuria. Of importance is the finding that cystinuric dogs have lower diuresis than normal dogs, resulting in higher concentrations of cystine, thereby increasing the risk of stone formation.

Inheritance and molecular genetics of canine cystinuria

Mode of inheritance in canine cystinuria has been studied by Treacher who observed an incompletely recessive inheritance pattern (TREACHER 1964). Results from Tsan and co-workers indicated on the other hand a sex-linked inheritance extracted from genetic analysis of cystinuric Scottish Terrier and Irish Terrier pedigrees (TSAN *et al.* 1972). Urinary cystine, lysine and arginine were elevated in cystinuric dogs and only males were affected. The anatomical difference between male and female was put forward as an alternative explanation and an autosomal recessive inheritance pattern as seen in humans was not ruled out.

Cystinuria in Newfoundlands has been characterised by Casal and co-workers and show striking similarities to cystinuria in man (CASAL *et al.* 1995). The classic urinary excretion pattern of cystine and dibasic amino acids was demonstrated in both male and female cystine stone forming dogs although males were more severely affected in terms of urinary tract obstructions and stone event frequency. In consistence with an autosomal recessive inheritance, obligate carriers did not present clinical signs of cystinuria. In all, cystinuria in Newfoundlands resembles the type I phenotype in human cystinuria and after cloning and sequencing of the canine SLC3A1 gene, a non-sense mutation was detected in cystinuric Newfoundlands (HENTHORN *et al.* 2000). Compared to other species where the gene has been identified, the canine SLC3A1 gene shows the highest similarity to the human SLC3A1 gene. The mutation analysis performed by Henthorn included other breeds as well but no deleterious sequence alterations were detected, and so far there are no successive studies further describing the molecular genetics behind cystinuria in the dog.

AIMS OF THE THESIS

The present study was undertaken to investigate the molecular genetic basis of cystinuria in patients from Sweden and to correlate genetic findings with phenotypes produced regarding cystine and dibasic amino acid excretion. Further, attempts were made to elucidate the molecular genetics of cystinuria in the dog.

When the project was initiated, the SLC3A1 gene had recently been identified and analysed in a limited number of populations. The results indicated population specific distribution of SLC3A1 mutations, with only a few mutations reported in more than one population. In order to investigate the involvement of SLC3A1 and describe the mutational pattern in a Swedish population, the SLC3A1 gene was analysed for mutations in cystinuria patients from the South and South-East of Sweden.

A few years after the identification of SLC3A1, the second cystinuria disease gene, SLC7A9, was cloned and since a substantial proportion of the patients in our study lacked mutations in the SLC3A1 gene, analysis of SLC7A9 was performed to further characterise the molecular genetics behind cystinuria in our patients.

A common expectation after identification of the two genes involved in cystinuria was that knowledge on the molecular genetic basis of the disease would help explain the wide range in clinical outcome of cystinuria. The results from our mutation analysis of SLC3A1 and SLC7A9 formed the basis for investigating the relation between genetic findings and clinical characteristics in our patients, *i.e.* urinary excretion of cystine and dibasic amino acids.

The similarities seen between cystinuria in humans and a number of dog breeds naturally leads to the hypothesis that mutations in homologous genes in the dog are responsible for cystinuria. Only one of the genes involved in cystinuria in humans, SLC3A1, had been identified in the dog and to enable analysis of SLC7A9 in cystinuric dogs along with SLC3A1, identification of the canine SLC7A9 gene preceded our mutation analysis of the disease genes in cystinuric dogs.

Explicitly, the specific aims of our research on cystinuria were as follows:

- Analyse the amino acid transporter genes SLC3A1 and SLC7A9 in cystinuria patients from South and South-East of Sweden to elucidate the genetic basis of cystinuria in this population.
- Relate genetic findings to urinary excretion of cystine and dibasic amino acids in cystinuria patients.
- Identify the SLC7A9 gene in the dog to enable mutation analysis of this gene in cystinuric dogs
- Perform mutation analysis of SLC3A1 and SLC7A9 in cystinuric dogs.

MATERIAL AND METHODS

MOLECULAR GENETIC STUDIES ON CYSTINURIA IN HUMANS

Cystinuria patients and normal controls

53 cystinuria patients and their relatives representing 43 unrelated families from the South and South-east of Sweden were included in this study. 51 of the patients were of Swedish ancestry, one was of Greek origin and one of Iranian origin. 25 of the patients were women and 28 were men, all but two demonstrating urinary cystine excretion in the homozygous range (LINDELL *et al.* 1995b). These two patients showed excretion levels intermediate between normal and homozygous and could therefore be denoted as heterozygous non-type I and these patients produced calcium rather than cystine stones. No further classification according to Rosenberg was determined since urinary excretion values of obligate carriers were not available. Cystine stones were found in all patients, with the exception of two women newly diagnosed and 38 of the patients were on long-term treatment with sulfhydryl compounds. Patient characteristics and mutation data are summarised in table IV.

A DNA bank comprising randomly chosen individuals from South-east of Sweden selected from the population register was used for investigating frequencies of mutations and polymorphisms detected.

The study design was approved by the local ethics committee at Linköping University and informed consent was given from all patients and relatives before collecting blood and urine samples.

Mutation screening of SLC3A1 and SLC7A9

All 10 exons of the SLC3A1 gene and the 12 coding exons of SLC7A9 were PCR amplified and radioactively labelled for single-stranded conformation analysis, SSCA. PCR primers were found in the literature or designed with a range of PCR product size generally between 150 and 300 bp. Large exons were divided in two fragment to ensure a fragment size suitable for the SSCA analysis. All primers annealed to intronic sequences flanking the exons in order to include coding regions as well as intron/exon boundaries. To further increase the sensitivity of the method, all samples were separated on two different gel matrices, 6 % polyacrylamide gel and MDE™ gel (BMA, Rockland, ME).

SSCA is a mutation screening method based on the assumed different secondary structures formed in normal and mutated single-stranded fragments respectively. PCR products are radioactively labelled and heat denatured before separation onto a non-denaturing polyacrylamide gel. The result is visualised using autoradiography and mutant samples will show a different migration pattern in the gel compared to normal samples caused by the different single-strand conformations.

Mutation and polymorphism frequencies were determined in a normal control population using SSCA or when appropriate restriction enzymes were available, restriction fragment length polymorphism, RFLP.

DNA sequencing of SLC3A1 and SLC7A9 exons

Samples displaying aberrant SSCA migration pattern were selected for DNA sequencing using commercially available kit based on Sanger's dideoxy sequencing technique. In a second round of sequencing, all samples where no mutations or only heterozygous mutations were detected in the SLC3A1 and SLC7A9 genes were selected for sequencing of all exons of both genes. This was done in order to ensure that no mutations were missed because of an imperfect sensitivity of the SSCA analysis. Samples selected for sequencing were analysed using a second PCR product as template for the reaction.

Southern Blot analysis

Southern Blot analysis was used to detect large deletions and chromosomal rearrangement in patients heterozygous for SLC3A1 mutations and for patients that did not carry any mutations in the SLC3A1 gene.

The principal steps in Southern Blot analysis include digestion of genomic DNA, separation on an agarose gel, transfer of the DNA fragments to a membrane and hybridisation of a labelled probe to the fragments.

A full-length cDNA-probe of the SLC3A1 gene was used for hybridisation. Due to experimental difficulties in generating SLC7A9 probes, the SLC7A9 gene was not analysed by means of this method.

Amino acid analysis

A smaller cohort of patients from the original patient material was used for genotype-phenotype correlation studies with regard to genetic findings in the SLC3A1 and SLC7A9 genes and urinary excretion of cystine and dibasic amino acids (table IV). This group consisted of 33 patients families, 8 of which were heterozygous for SLC3A1 mutations and two patients with a lack of mutations in this gene and 23 of which were homozygous for SLC3A1 mutations. None of the 33 patients had mutations in the SLC7A9 gene.

Urinary concentrations of cystine and the mixed disulfides tiopronin-cysteine and D-penicillamin-cysteine were measured using ion-exchange chromatography in four separate 24-h collections and the total urinary cystine was calculated as the sum of free cystine and the amount of cystine corresponding to the cystine in the disulfides. For the dibasic amino acids one single 24-hour collection was used for each patient.

The Mann-Whitney U-test was used as the statistical test for group comparison and a *p*-value of <0.05 was considered statistically significant.

MOLECULAR GENETIC STUDIES ON CANINE CYSTINURIA

Cystinuric dogs and amino acid analysis

Thirteen cystinuric dogs of ten different breeds were included in this study (table V), all being males and diagnosed before the study started by means of infrared spectroscopy of surgically removed cystine uroliths. Before the start of prophylactic treatment with tiopronin, routine urinalysis, CBC, serum biochemistry analysis and cyanide nitroprusside reaction test were performed on each dog. In all except two dogs a quantitative measurement of the urinary excretion of cystine and the dibasic amino acids was performed using ion-exchange chromatography and automatically evaluated as described (HOPPE *et al.* 1993b).

Blood samples were collected from all cystinuric dogs for DNA analysis as well as a kidney biopsy from a dog with no history of cystine stone formation. The protocol was approved by the local ethics committee at Uppsala University.

Identification of the canine SLC7A9 cDNA

The assumption that the SLC7A9 gene is highly conserved between the dog and other species served as a starting-point for the identification of the canine SLC7A9 cDNA and definition of its genomic structure.

Database searches were conducted in order to find sequences in the dog genome that showed similarities to the human SLC7A9 gene. Primers were designed in putative canine exons and used for PCR amplification using cDNA from canine kidney biopsy as a template. This procedure resulted in the sequencing of a PCR product covering almost the entire coding region of SLC7A9. In order to sequence the full-length 5'- and 3'-regions, rapid amplification of cDNA ends, the RACE technique, was used. This method utilizes primer adapter sequences that are ligated to the 5' and 3' end respectively, which later serves as binding sequences for PCR primers. The second primer is complementary to the known cDNA sequence which enables amplification of PCR products even though the end sequences are unknown. In a series of nested PCR reactions where the primer in the known sequence is moved towards the end more specific PCR products are generated that can ultimately be sequenced, possibly following cloning of the PCR products.

Mutation analysis of the SLC3A1 and SLC7A9 genes

To enable mutation analysis of SLC7A9 exons in cystinuric dogs, the genomic organisation of the canine SLC7A9 gene was determined by means of database searches of the canine genome using the full-length cDNA sequence as search query.

All exons of the SLC3A1 and SLC7A9 genes of all cystinuric dogs were PCR amplified using intronic primers covering the coding regions and intron/exon boundaries. The samples were subjected to automatic sequencing.

RESULTS

MOLECULAR GENETIC STUDIES ON CYSTINURIA IN HUMANS

SLC3A1 mutations in cystinuria patients and controls

A total number of 18 different SLC3A1 mutations were identified among the cystinuria patients, 15 of which were not previously reported in other populations (figure 7). 15 of the SLC3A1 mutations were reported in paper I and the additional three, R227W, Y259X and S547L, were detected after sequencing all patients that lacked or showed heterozygosity for mutations in SLC3A1 or SLC7A9 after SSCA screening. 38 of 53 patients were homozygous for SLC3A1 mutations, nine were heterozygous and in six patients no SLC3A1 mutations could be identified. The most common mutation was M467T, detected on 43 out of 106 chromosomes.

The new mutations include 13 missense mutations (Y151C, R227W, N253K, Y259X, R362H, G398R, G481V, E482K, Q510R, S547L, R584T, F599S, and G600E) one deletion (1999-2000delTT) and one splice site mutation (1136+2T>C). M467T was the only mutation detected (4/794) in the normal population with a frequency of 0.5 % for this allele.

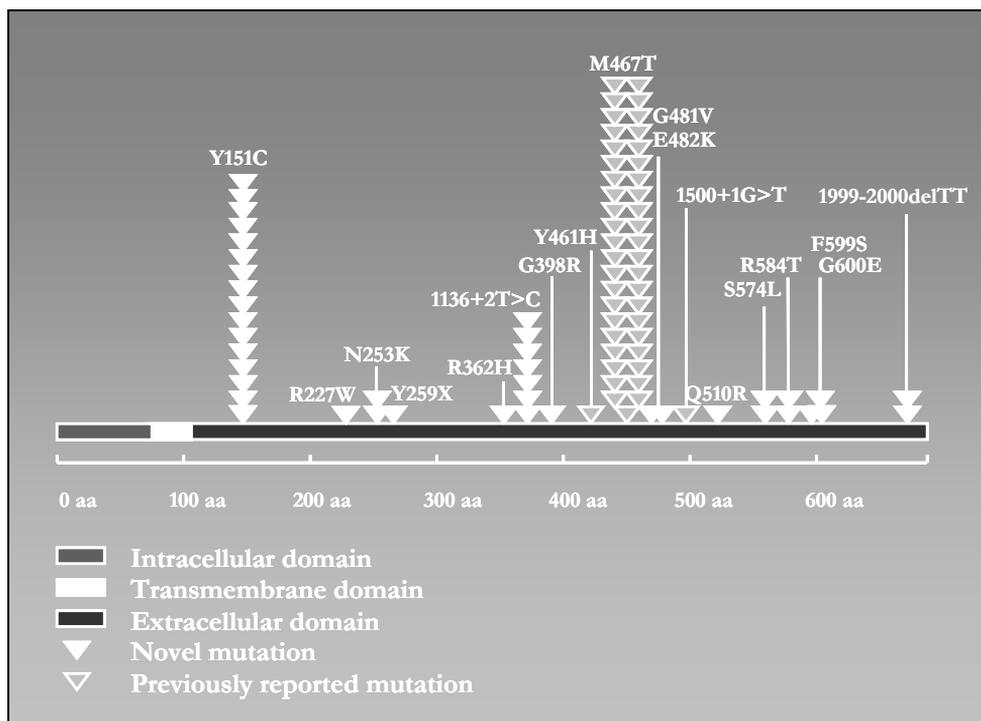


Figure 7. SLC3A1 mutations detected in cystinuria patients. The figure illustrates the distribution and frequency of mutations in the rBAT protein.

Multiple sequence alignment between human, rat, mouse, rabbit and dog amino acid sequences showed that all missense mutations affect conserved positions. Distribution of all mutations in the patient group is shown in table IV.

In addition, a genetic alteration resulting in an amino acid shift (I618M) was detected in several patients but also in high frequency in the normal population (24/100), indicating the polymorphic nature of this alteration.

The Southern Blot analysis showed no aberrant pattern in the SLC3A1 gene in any of the patients analysed indicating that no gross genetic alterations explain the absence of point mutations in non-mutated patients.

SLC7A9 mutations in cystinuria patients and controls

In the SLC7A9 gene, four different mutations were detected in our patient group, two novel missense mutations (V330M and P261L) and one deletion (1009delA) along with A182T, previously reported. V330M and the single nucleotide deletion occurred in the same patient, probably explaining cystinuria in this patient and V330M was also present in the normal population (1/188). P261L was detected in a patient carrying two SLC3A1 mutations and was not detected in the normal population. A182T was present in heterozygous state in one patient and one control (1/96). V330M and P261 both affect conserved amino acid residues and the 1009delA creates a premature stop codon resulting in a protein lacking 127 amino acids. Table IV shows the SLC7A9 mutations identified in our cystinuria patients.

Two genetic alterations probably not contributing to cystinuria detected in the patient group (V142A and L223M) was also present in the normal population at 33/100 and 27/96 chromosomes respectively.

Genotype-phenotype correlations in cystinuria patients

Cystinuria patients were divided in two groups based on genetic findings in SLC3A1 and SLC7A9 genes. Group 1 comprised 10 individuals either lacking SLC3A1 mutations (2 patients, subgroup 1a) or showing heterozygosity for SLC3A1 mutations (8 patients, subgroup 1b). Group 2 included 23 patients all homozygous for SLC3A1 mutations. Total cystine and dibasic amino acids were measured and compared between the two groups and the results show a statistically significant higher excretion of all amino acids in group 1. When excluding patients in subgroup 1a (in order to get a more genetically homogenous group) *p*-value for cystine, lysine and ornithine excretion is still below 0.05 and 0.052 for arginine

The mean value of the administered doses of sulphhydryl substances was higher in group 1 (2,333 mg) compared to group 2 (1,889 mg) and there was no statistical significant difference in urinary volumes.

Table IV. Cystinuria patients and SLC3A1 and SLC7A9 mutation data. All patients have a history of cystine stone formation except 13 and 14 that were newly diagnosed and 36 and 46 that formed calcium stones.

<i>Patient</i>	<i>Sex</i>	<i>Age</i>	<i>Age at diagnosis</i>	<i>SH-treatment</i>	<i>SLC3A1 mutation</i>	<i>SLC7A9 mutation</i>	<i>No in paper</i>	
							<i>II</i>	<i>III</i>
1	F	31	17	no	M467T/M467T	wt/wt	-	-
2 ^{a)}	M	49	25	yes	M467T/M467T	wt/wt	-	21
3	M	71	25	yes	M467T/M467T	wt/wt	-	30
4	M	34	16	no	M467T/M467T	wt/wt	-	-
5 ^{b)}	F	20	19	yes	M467T/M467T	wt/wt	-	15
6 ^{c)}	F	42	18	yes	M467T/M467T	wt/wt	-	17
7	F	37	18	yes	M467T/M467T	wt/wt	-	16
8 ^{a)}	M	15	2	yes	M467T/M467T	wt/wt	-	20
9 ^{d)}	F	85	uncertain	yes	M467T/M467T	wt/wt	-	19
10	F	67	19	yes	M467T/M467T	wt/wt	-	18
11	F	68	uncertain	no	M467T/M467T	wt/wt	-	-
12 ^{c)}	F	50	39	no	M467T/M467T	wt/wt	-	-
13 ^{b)}	F	19	19	no	M467T/M467T	wt/wt	-	14
14 ^{a)}	F	14	14	no	M467T/M467T	wt/wt	-	-
15 ^{e)}	M	68	13	yes	Y151C/Y151C	wt/wt	-	27
16 ^{c)}	M	70	23	yes	Y151C/Y151C	wt/wt	-	28
17 ^{e)}	M	58	18	yes	Y151C/Y151C	wt/wt	-	26
18	M	55	34	no	Y151C/Y151C	wt/wt	-	-
19 ^{e)}	F	79	uncertain	yes	Y151C/Y151C	wt/wt	-	33
20	M	38	6	yes	Y151C/Y151C	wt/wt	-	25
21	F	49	20	yes	wt/wt	A182T/wt	2	-
22 ^{f)}	F	53	19	yes	1136+2T>C/1136+2T>C	wt/wt	-	-
23 ^{f)}	F	50	36	no	1136+2T>C/1136+2T>C	wt/wt	-	-
24	M	75	32	yes	1999-2000delTT/1999-2000delTT	wt/wt	-	11
25	F	18	13	yes	M467T/G481V/E482K	wt/wt	-	-
26	M	43	22	no	M467T/Y151C	wt/wt	-	-
27	M	48	29	yes	M467T/Y151C	wt/wt	-	32
28	M	79	19	yes	M467T/Y151C	wt/wt	-	24
29	M	34	20	yes	M467T/R362H	P261L/wt	3	-
30	F	13	13	yes	M467T/N253K	wt/wt	-	-
31 ^{d)}	M	57	3	yes	M467T/R584T	wt/wt	-	12
32	M	78	44	yes	M467T/Q510R	wt/wt	-	31
33	M	45	34	yes	M467T/F599S	wt/wt	-	13
34	F	55	23	yes	M467T/1136+2T>C	wt/wt	-	29
35	F	38	13	yes	Y151C/1500+1G>T	wt/wt	-	23
36	M	37	5	no	G398R/Y461H	wt/wt	-	-
37	F	44	15	no	1136+2T>C/R584T	wt/wt	-	-
38	F	64	34	yes	M467T/wt	wt/wt	6	3
39	M	78	27	yes	M467T/wt	wt/wt	4	5
40	M	35	20	yes	M467T/wt	wt/wt	5	4
41	M	30	19	yes	M467T/ Y259X^{g)}	wt/wt	-	22
42	F	41	21	no	M467T/wt	wt/wt	7	-
43	F	43	18	yes	N253K/wt	wt/wt	8	6
44	M	47	18	yes	1136+2T>C/wt	wt/wt	12	10
45	M	58	24	yes	wt/wt	1009delA/V330M	1	-
46	F	62	25	no	wt/wt	wt/wt	16	-
47	M	62	14	no	G600E/G600E	wt/wt	-	-
48	F	41	12	no	wt/wt	wt/wt	13	-
49	F	55	28	yes	wt/wt	wt/wt	14	1
50	M	24	11	yes	R227W^{h)}/wt	wt/wt	11	7
51	M	57	28	yes	wt/wt	wt/wt	15	2
52 ^{b)}	M	27	22	yes	S547L^{g)}/wt	wt/wt	10	9
53 ^{b)}	M	21	20	yes	S547L^{g)}/wt	wt/wt	9	8

a) Father, son and daughter

c) Sisters

e) 19 is cousin to brothers 15-17

g) Unpublished results

b) Sisters

d) Aunt/nephew f) Sisters

h) Brothers

MOLECULAR GENETIC STUDIES ON CANINE CYSTINURIA

Structure of the canine SLC7A9 gene

The canine SLC7A9 cDNA identified was 1602 bp with a coding sequence of 1470 bp that translates into a 490-amino acid protein. Multiple sequence alignment between rat, mouse, human, rabbit and canine SLC7A9 amino acid sequences show that highest similarity is found between dog and human sequences (figure 8).

The gene is separated into 13 exons with the translator initiator methionine located to exon 2 and the termination codon in exon 13. Exon sizes range between 45-243 bp and all splice sites conform to the GT-AG donor/acceptor consensus sequence with the exception of the donor sequence GC of intron 2.

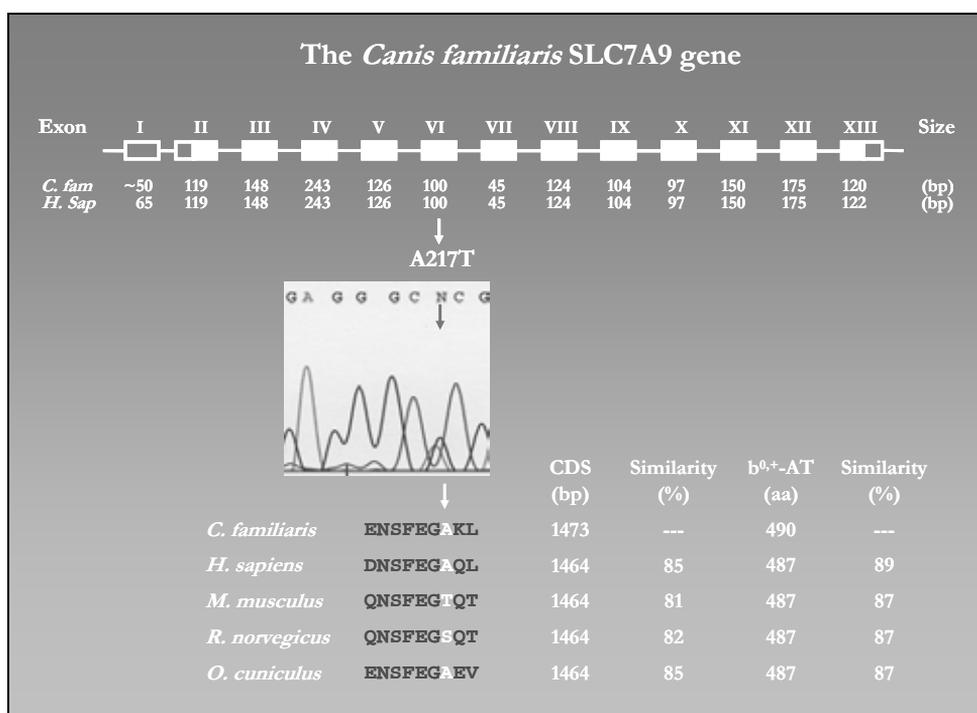


Figure 8. The *Canis familiaris* SLC7A9 gene. The 13 exons of the canine SLC7A9 gene are similar in size to human SLC7A9 exons and exon/intron splice sites occur at the same positions in the two orthologous genes. High sequence similarity is found both on nucleotide and amino acid level between species. At position 217, there is an alanine in the human, canine and rabbit sequences, whereas mouse has a threonine and rat has a serine arguing against a deleterious nature of A217T detected in English Bulldog.

Amino acid excretion in cystinuric dogs

Urinary excretion of cystine and dibasic amino acids was measured in 11 dogs producing pure cystine stones (table V). Cystine excretion values varied widely between 13 and 650 mmol/mol creatinine and the dogs could be divided into three separate groups based on cystine excretion as previously described (HOPPE and

DENNEBERG 2001). Dibasic amino acid excretion was compared to cystine excretion and evaluated using both parametric and non-parametric statistical analyses.

Table V. Urinary excretion of cystine and dibasic amino acids and SLC3A1 and SLC7A9 mutations detected in cystinuric dogs of different breeds.

<i>Patients</i>	<i>Breed</i>	<i>Cystine</i> (mmol/mol creatinine)	<i>Lysine</i> (mmol/mol creatinine)	<i>Ornithine</i> (mmol/mol creatinine)	<i>Arginine</i> (mmol/mol creatinine)	<i>Mutation</i> (SLC3A1/SLC7A9)
1	Irish Terrier	-	-	-	-	-/-
2	Border collie	-	-	-	-	-/-
<i>High excretion</i>						
3	Scottish Terrier	146	274	37	39	-/-
4	Dachshound	650	367	84	63	-/-
5	French Bulldog	146	334	37	42	I192V, S698G/-
<i>Moderate excretion</i>						
6	Tibetanian spaniel	63	233	21	25	-/-
7	Scottish Deerhound	45	88	6	15	-/-
8	Irish Terrier	97	148	18	20	-/-
9	Irish Terrier	78	33	14	5	-/-
10	English Bulldog	72	101	20	32	I192V, S698G/A217T
<i>Low excretion</i>						
11	Labrador Retriever	13	12	6	7	-/-
12	Welsh corgi cardigan	14	25	7	7	-/-
13	Welsh corgi cardigan	15	22	13	5	-/-

Genetic variants detected in SLC3A1 and SLC7A9 in cystinuric dogs

Twelve different SLC3A1 genetic variants were detected in the cystinuric dogs, five of which were located to intronic sequences and the remaining seven to the exons. Only two genetic alterations result in amino acid shift, I192V and S698G, both detected in homozygous form in French and English bulldog (table V). These missense mutations both affect non-conserved amino acid residues.

In SLC7A9, we detected seven genetic variants, five occurring in introns and two located in exon sequences. A217T was the only mutation resulting in a change of amino acid, affecting an amino acid position not conserved between species (table V, figure 8).

DISCUSSION

SLC3A1 and SLC7A9 mutations in Swedish cystinuria patients

The mutational spectrum in cystinuria is continually expanding and more than 100 SLC3A1 and 60 SLC7A9 mutations have been identified (www.hgmd.cf.ac.uk). The present study describes mutational patterns found within the SLC3A1 and SLC7A9 genes in cystinuria patients from Sweden. A total number of 22 mutations were detected, among them 15 SLC3A1 and three SLC7A9 mutations described for the first time. Missense mutations are the most frequent alterations in our study but deletions and splice site mutations were detected as well.

All missense mutations in both SLC3A1 and SLC7A9 occur at conserved amino acid residues indicating an important functional or structural property at these specific positions in the proteins. Where compound heterozygous mutations were detected, the mutations were confirmed to occur on both chromosomes in several cases by analysing relatives being obligate carriers of the mutation. Most of the mutations detected were absent or detected only on a single chromosome in the normal population. Taken together, this information provides argument for the deleterious nature of the missense mutations detected and their respective contribution to the disease in our patients.

The deletions detected in SLC3A1 and SLC7A9 (1999-2000delTT and 1009delA) both cause frame shift mutations resulting in five additional amino acids in the rBAT protein and in a 127 amino acid truncation of the b⁰⁺-AT protein. The novel splice site mutation in SLC3A1 (1136+2T>C) affect the second nucleotide of the donor consensus sequence, thereby assumed to influence splicing. GT-AG introns are by far the most frequent, accounting for approximately 98 % of all introns, followed by GC-AG introns accounting for less than 0.8 % (CHONG *et al.* 2004). The 1136+T>C mutation replaces the GT-AG intron with a GC-AG but although GC-AG are functional donor/acceptor sequences, it may have an impact on splicing in this specific genomic context.

Three genetic alterations detected in our patients, I618M in SLC3A1 and V142A and L223M in SLC7A9, were also present at high frequencies in the normal population, indicating that they are not contributing to impaired protein function. Similar conclusion has been reached by several independent investigations (GASPARINI *et al.* 1995; HORSFORD *et al.* 1996; SHIGETA *et al.* 2006). Further, expression analysis of I618M in *Xenopus* oocytes showed uptake of cystine similar to controls (SAADI *et al.* 1998) and V142A and L223M showed only a slight decrease in cystine uptake in transiently transfected COS-7 cells (SHIGETA *et al.* 2006).

The screening method used for mutation analysis, SSCA, has been reported to show a varying sensitivity (HAYASHI 1991; HAYASHI and YANDELL 1993; ORITA *et al.* 1989; SHEFFIELD *et al.* 1993). In order to obtain high sensitivity at reasonable time and effort, fragments analysed usually did not exceed 300 bp and all samples

were analysed on two different gel matrices. All samples where only heterozygous or no mutations were detected in the SSCA screening were selected for direct sequencing of all SLC3A1 and SLC7A9 exons. These measures were taken to confirm all negative SSCA results and these indeed revealed three additional SLC3A1 mutations (R227W, Y259X and S547L, unpublished results). Consequently, the lack of mutations in a number of patients in our study can not be explained by lack of sensitivity of methods chosen.

Mutational pattern in cystinuria in different populations

Mutations in SLC3A1 and SLC7A9 are present in 88 out of 106 cystinuria chromosomes which give a detection rate of 83 %, explaining cystinuria in 73.6 % of the patients in our study. Another 18.9 % of the patients have a partial genetic explanation of the disorder and in the remaining 7.5 % no contribution to the genetic cause could be detected in our study. Our results are in agreement with results from Font-Llitjós and co-workers that detected disease alleles on 282 out of 325 cystinuria chromosomes accounting for an 86.8 % detection rate (FONT-LITJOS *et al.* 2005). In this study SLC3A1 and SLC7A9 mutations explain 76.8 % of all cystinuria cases and give a partial explanation in 20.1 %. Only 3 % completely lacked a genetic explanation of cystinuria. This is the most extensive mutation analysis performed in cystinuria patients so far and the slightly higher detection rate of cystinuria alleles compared to our study can in part be inferred by the complementation of methods for detection of large deletions and chromosomal rearrangements in addition to mutation screening and direct sequencing.

The mutational pattern in cystinuria in our patients is typical of a European cystinuria population in several respects. The most frequent mutation was M467T which is seen in all European populations studied so far, and a number of mutations are specific for the study population and occur on only one chromosome in one individual or one family (ALBERS *et al.* 1999; BISCEGLIA *et al.* 1996; BISCEGLIA *et al.* 2001; CALONGE *et al.* 1994; GASPARINI *et al.* 1995; LANGEN *et al.* 2000). In addition, detection rates are similar as discussed for the Spanish, Italian and Israelian populations above and also for a Czech population with a complete genetic explanation for 70.8 %, partial for 25 % and no genetic explanation in 4.2 % of the patients. The major difference between the Swedish population and other European populations is the limited contribution of SLC7A9 mutations in cystinuria in Sweden.

Cystinuria has been studied in other populations as well and a recent report describes the genetics of cystinuria in a Japanese population (SHIGETA *et al.* 2006). 41 patients were studied and the novel SLC7A9 mutation P482L was presented in 25 individuals being homozygous for the mutation and in another six being heterozygous. Thereby, P482 is present on 75.6 % of all cystinuria chromosomes (assuming recessive inheritance) probably reflecting genetic homogeneity in this population. The detection rate in this cohort of patients was 84.6 %. In accordance with the results from Shigeta and co-workers, previous investigation of cystinuria in

Japan revealed a low prevalence of SLC3A1 mutation with only five individuals out of 36 carrying mutations in this gene (EGOSHI *et al.* 2000). Similar to cystinuria in the Japanese population is the common founder mutation V170M, detected in all cystinuria cases studied a Libyan Jewish population of 23 individuals (COLOMBO 2000; FELIUBADALO *et al.* 1999b).

Genotype-phenotype correlations

In a subset of our patient group, the genetic findings were found to correlate with urinary excretion of cystine and dibasic amino acids. Patients heterozygous for SLC3A1 mutations (n=8) or being wildtype (n=2), had a significantly higher excretion compared to homozygously mutated individuals (n=23).

Since all patients but one had a history of stone formation, it was not possible to discontinue medical treatment administrated for prophylactic or stone dissolving purposes. The concept of total cystine used in this study allows for the detection of cystine bound to the SH substance as well as for free cystine, which gives a more accurate measurement of cystine excretion compared to free cystine alone (LINDELL *et al.* 1995b). In a number of studies, sulfhydryl substances have shown to result in a decreased total urinary cystine indicating that these compounds affect cystine metabolism through other mechanisms than forming a disulfide complex alone (LINDELL *et al.* 1995b; LOTZ *et al.* 1966). This phenomenon could influence the result of the present study and result in lower total cystine in group 1, which has the highest intake of SH substances, but in contrast our observations show a higher total cystine in group 1 in spite of higher doses of SH complexing substance.

The molecular genetic or biological explanation for a difference between SLC3A1 hetero- and homozygotes seen in our patient group still remains unclear. Assuming that the difference in urinary cystine excretion between the groups truly reflects a more severe tubular defect in group 1 and that the rBAT-b⁰⁺-AT complex is the only transporter of cystine and dibasic amino acids in the proximal tubule, then the mechanisms of the undetected mutations more severely affects the function of the transporter complex.

All patients in our genotype-phenotype correlation study lacked SLC7A9 mutations but could still carry mutations such as large deletions or rearrangement or mutations in regulatory parts of this gene, although in the light of other studies this seems not very likely. Patients displaying one mutation in each of the SLC3A1 and SLC7A9 would be referred to as a type AB according to Dello Strologo and have only occasionally been reported in the literature (DELLO STROLOGO *et al.* 2002). If this represent a significant explanation in our group of patients, the proportion of type AB would require a larger subset of patients displaying type B (homozygous mutations in SLC7A9) but in total, only one patient present this genotype. In addition, according to the literature, cases of type AB show a milder phenotype (DELLO STROLOGO *et al.* 2002), which seems contradictory to the finding of higher total urinary cystine in our subgroup hypothetically being type AB.

Cause of cystinuria in patients lacking SLC3A1 and SLC7A9 mutations

The speculations about the involvement of further, yet unidentified disease genes in cystinuria have been inferred by us as well as others. Recent studies do not exclude this possibility although the evidence against involvement of additional genes is accumulating. First of all, the detection rates of SLC3A1 and SLC7A9 mutations in cystinuria patients as discussed above leave a very small possible contribution for additional genes in the pathogenesis. Second, as shown by Fernandez and co-workers, the rBAT-b⁰⁺-AT complex is the main transporter (if not the only) of cystine and dibasic amino acids in the proximal tubule. The proportion of rBAT that does not bind to b⁰⁺-AT may have an additional binding partner, X, although the complex rBAT-X would probably be affected in carriers of homozygous SLC3A1 mutations and result in a different amino acid excretion profile compared to homozygotes for SLC7A9 mutations. The similar aminoaciduria seen in both SLC3A1 and SLC7A9 homozygotes argues against the involvement of the X protein in cystinuria and implies that the function of the assumed rBAT-X complex is not affected by SLC3A1 mutations. Further, polymorphisms in SLC3A1 or SLC7A9 in combination with mutations in these genes may offer a possible mechanism of pathogenesis as has been suggested (CHATZIKYRIAKIDOU *et al.* 2006; SCHMIDT *et al.* 2003).

No studies so far have focused the mutation analysis to regulatory parts of the SLC3A1 and SLC7A9 genes, and knowledge about regulation of the expression of the genes is limited. An obvious choice in analyzing such regulatory sequences would be to investigate the SLC3A1 and SLC7A9 promoters and the 5' UTR of the SLC7A9 gene including non-coding parts of exon 1 and 2. In order to elucidate the involvement of promoter mutations in cystinuria, we have sequenced parts of the immediate upstream sequence of the transcription start site in all patients in our group lacking a complete genetic explanation for cystinuria. Approximately 800 bp were sequenced in both promoters as well as the non-coding exon 1 in SLC7A9 and parts of SLC7A9 intron 1. Preliminary results show a number of sequence variants in the promoter region not recognized in the SNP database (www.ncbi.nlm.nih.gov/SNP/). In the SLC7A9 promoter, a patient that completely lack SLC3A1 and SLC7A9 mutations shows two different genetic variants that are not reported in the SNP database; an A>G substitution at -411 bp and a G>T substitution at +620 bp relative transcription start site.

In the SLC3A1 promoter a T>C substitution was detected at nucleotide -89 bp relative to transcription start site. The patient was heterozygous for this promoter variation and also carries the R227W mutation in the SLC3A1 gene. Simultaneous sequence alignment and search for conserved transcription factor binding sites in the human and canine promoter regions showed that this variation is located within a predicted aMEF2 (transcription factor expressed in muscle tissue) binding site.

Absence of SLC3A1 and SLC7A9 mutations in cystinuric dogs

With knowledge of the complete genome sequence and the haplotype structure of the domestic dog, it can be speculated that if SLC3A1 and SLC7A9 mutations indeed cause cystinuria, they are probably breed specific, and occur in homozygous form in affected dogs.

The genetic structure of the dog has been formed by the domestication process and the selective breeding and maintenance of breed barriers. It has been shown that different breeds are distinct genetic units and that mutations causing recessive disorders often are identical by descent (MEYERS-WALLEN 2003; PARKER *et al.* 2004). The Boxer genome, that was selected for the canine genome sequencing project, is composed of large regions of alternating high homozygosity and heterozygosity and displays linkage equilibrium extending over distances of several Mb (LINDBLAD-TOH *et al.* 2005). Regions of chromosome 1 and 10, harbouring SLC7A9 and SLC3A1 respectively, show almost complete homozygosity and inasmuch as the Boxer is representative for other breeds in these genomic regions, SLC3A1 and SLC7A9 mutations could be expected to be homozygous. The SLC3A1 nonsense mutation detected only in cystinuric Newfoundlands, provides an excellent example of breed specific, homozygous mutations in a recessive disorder.

Our successful sequencing of the full-length canine SLC7A9 cDNA along with recent progress in canine genome research facilitated our attempts to define the canine SLC7A9 gene structure and perform mutation analysis of this gene in cystinuric dogs. In our panel of cystinuric dogs, three different missense mutations were detected in the SLC3A1 and the SLC7A9 genes. The SLC3A1 mutations S698G and I192V, both detected in homozygous form in French and English bulldog, do not affect conserved residues which also apply for the SLC7A9 A217T mutation found in a heterozygous English bulldog. In addition, the A217T mutation affects a position that is not conserved in the LSHAT family. The influence of these mutations on rBAT-b⁰⁺-AT function is therefore uncertain and unfortunately, we did not have DNA samples from healthy control subjects from these breeds.

If the SLC3A1 mutations (S698G and I192V) are responsible for cystinuria, then the impact of A217T seems even more speculative, since all three mutations were detected in the same dog. It seems unlikely that disease mutations in two different disease genes of a rare disorder would present in the same dog. Homozygous mutations in SLC3A1 alone, as seen in French bulldog, would result in cystinuria and the addition of A217T, as seen in English bulldog, would probably not make a difference to the clinical outcome. If the opposite applies, that the SLC3A1 mutations are only benign polymorphisms, then A217T could possibly contribute to the disease. Even if cystinuria in French and English bulldog could in fact be ascribed S698G, I192V or A217T, detection rate would still be only 15 % (4/26) or 4 % (1/26) respectively, assuming recessive inheritance. Compared to detection

rates seen in human cystinuria, this is a remarkably low frequency of point mutations detected in SLC3A1 and SLC7A9 in canine cystinuria.

The absence of mutations in most of our cystinuric dogs could not be attributed to the method used, direct DNA sequencing. Any point mutations present is detected using this method. Still the possibility of large deletions or chromosomal rearrangements or mutations in regulatory elements of SLC3A1 and SLC7A9 remains as in the case of our human cystinuria patients.

Complexity of cystine stone formation

Characteristics of cystinuria in humans, the dog and in the two mouse models generated, illustrate different aspects of complexity in cystine stone formation. Although an elevated excretion of cystine in cystinuria is genetically determined, the formation of cystine stones seem to be a complex event involving both tubular defect in amino acid reabsorption, the concentration, composition and pH of the urine, and possibly also other modifying genetic factors as well as congenital malformations in the urinary system.

The difference seen in cystine excretion among our patients between hetero- and homozygotes for SLC3A1 mutations do not necessarily indicate an increased risk of stone formation in the heterozygous group, having higher total cystine values in the urine. The study conducted by Dello Strologo reported a lack of correlation between urinary cystine and renal stone event frequency although medical treatment and the cystine bound to SH-compounds were not taken into consideration (DELLO STROLOGO *et al.* 2002). It thus appears that once the solubility threshold of approximately 250 mg/l is exceeded, the risk of stone formation arises regardless of excretion level above this threshold.

Several interesting cases described in the literature reflect the complexity of cystine stone formation. Two sisters 27 and 28 years included in the study by Skopkova and co-workers both carried the SLC3A1 mutations R365P and T216M and excreted similar amounts of cystine and dibasic amino acids (SKOPKOVA *et al.* 2005). Despite medical treatment, one of them suffered from recurrent stone episodes since ten years, whereas the other was free of symptoms without medical or prophylactic treatment for the same time period. A similar situation is also seen in our study, in two sisters 50 and 53 years old, being homozygous for the SLC3A1 splice site mutation 1136+2 T>C (table IV). The older sister (receiving SH-treatment) suffered from stone formation since the age of 19, whereas her younger sister (no SH-treatment) did not present with stones until 36 years of age.

An even more peculiar phenomenon is our and others observations of pure cystine stones formed in dogs excreting very low levels of cystine in the urine. Normal reference value of cystine excretion in the dog is <10 mmol/mol creatinine and among our dogs, two Welsh Corgi Cardigans and one Labrador Retriever excreted between 13-15 mmol/mol creatinine, although this was measured from a single 24-hour urine collection. Cystine stone formation has been reported to generally occur at cystine concentrations greater than 104 mmol/mol creatinine,

but is also seen in dogs with very low cystine excretion (BOVEE 1986; HOPPE *et al.* 1993a; TSAN *et al.* 1972). Threshold value of cystine solubility of cystine in the dog urine therefore seems inconclusive and points at other factors involved in cystine stone formation.

Finally, the two mouse models of cystinuria both show that SLC3A1 and SLC7A9 mutation status is not the only determinant in stone formation and severity of the disease. In the *pebbles* mouse, homozygous for the SLC3A1 D140G mutation, genetic background was shown to modulate the clinical outcome of the mutation (PETERS *et al.* 2003). Homozygously mutated male mice with a mixed genetic background had an earlier onset and faster progression of renal failure than mutants of an inbred strain. Similarly, it has been speculated that the mixed genetic background of the *stones* mouse, lacking expression of the SLC7A9 gene, cause the difference in cystine stone phenotype between different mice (FELIUBADALO *et al.* 2003). All mice in the study produced excessive values of urinary cystine and dibasic amino acid, but only 42 % formed cystine stones.

The complex process of cystine stone formation in cystinuria addresses the question of the use of molecular genetic information in management of cystinuria in man and dog. In the dog, identification of breed specific mutations would facilitate a molecular genetic diagnosis and would have immediate impact on breeding programs. With knowledge on carrier status, carrier to non-carrier mating could be used to prevent that affected dogs are produced and ensure genetic diversity within the breed (MEYERS-WALLEN 2003). The SLC3A1 nonsense mutation detected in New Foundlands provides an excellent example of a breed specific mutation in cystinuria and a time and cost-effective dHPLC method has already been evaluated in this breed (MATOS *et al.* 2006).

A molecular genetic diagnostic approach may seem appealing in genetically homogenous populations where a single or limited number of disease alleles are responsible for the majority of cystinuria cases. In outbred populations the mutations are distributed throughout the SLC3A1 and SLC7A9 genes and to get an acceptable sensitivity, the entire genes have to be analysed and the method of choice for such an analysis has to detect all mutations present. Direct sequencing is the most accurate method though still time consuming despite automatisation and even if this methodology was applied in diagnosis, the detection rate of cystinuria alleles would probably still not exceed 85 %.

The ethical considerations in using molecular genetic diagnosis in cystinuria regards the possibility of administrating prophylactic treatment to and monitoring risk individuals versus the impact on quality of life in individuals taking these precautions. Even though SLC3A1 and SLC7A9 mutations cause the excessive excretion of cystine and that elevated urinary cystine is the most important risk factor for cystine stone formation, manifestation and clinical severity of cystinuria is not determined by genetic alterations in the SLC3A1 and SLC7A9 alone. Additional modifying factors, environmental and genetic as well as congenital malformations are all possible contributors to the clinical outcome of cystinuria.

CONCLUSIONS

The present investigations were undertaken in order to survey the molecular genetic basis of cystinuria in patients from Sweden and to correlate genetic findings with urinary excretion patterns. Further, we have investigated the possible involvement of the SLC3A1 and SLC7A9 genes in cystinuria in the dog.

We have found a probable genetic explanation of cystinuria in approximately 74 % of our patients, and a possible contribution to the disease in another 19 %, corresponding to a detection rate of 83 %. The remainder had no mutations in the coding sequence or intron/exon boundaries of the SLC3A1 and SLC7A9 genes. Mutations in the SLC3A1 are the major cause of cystinuria in our group, with only a minor contribution of SLC7A9 mutations. The mutational pattern of cystinuria seen in our studies is comparable to other European populations, with the exception of the low proportion of SLC7A9 mutations in our group.

The genetic findings in a subset of our patient group were related to urinary excretion of cystine and dibasic amino acids. The group of patients presenting SLC3A1 mutations in a heterozygous state or lacking mutations had higher values of total urinary cystine and dibasic amino acids compared to patients homozygous for SLC3A1 mutations. The reason for this discrepancy remains unclear, but the possible impact of medical treatment with SH-compounds on total cystine values was ruled out.

The similarities between cystinuria in dogs and in humans points at the involvement of canine SLC3A1 and SLC7A9 homologous genes in canine cystinuria. Our results however, show that only two out of 13 dogs have point mutations with possible impact on protein function in these genes. Sequencing of the full-length canine SLC7A9 cDNA showed a high similarity between this gene in the dog and other mammals and the deduced genomic structure of the canine SLC7A9 gene showed conservation of size and position of exons and intron compared to the human homologue.

The lack of mutations in our cystinuria patients, both in man and dog, could not be ascribed the methods used for mutation analysis. In the dog, all exons of both genes were sequenced in all patients, and in human cystinuria patients, all samples lacking mutations or showing heterozygosity after SSCP screening were sequenced in both genes as well. The possibility of large deletions or chromosomal rearrangements as well as mutations in regulatory parts of the SLC3A1 and SLC7A9 genes remains.

Finally, clinical and genetic data from our study of cystinuria both in man and dog exemplifies that manifestation and clinical severity of cystinuria is not determined by genetic alterations in the SLC3A1 and SLC7A9 alone. Environmental factors, congenital malformations and modulating genetic factors are all possible contributors to the clinical outcome of cystinuria.

ACKNOWLEDGEMENTS

Jag vill rikta ett stort tack till alla som på ett eller annat sätt funnits med under tiden för arbetet med denna avhandling.

Till min handledare:

Tack **Peter Söderkvist** för att du är precis så entusiastisk, begåvad, och kaotisk som en professor ska vara och därtill en väldigt snäll och generös person med stor förmåga till medkänsla. Du har visat mig ett förtroende som fått mig att växa och uppmuntrat mig på ett sätt som gör att jag vill satsa på forskning i framtiden. Tack!

Till mina medarbetare:

Mitt varmaste tack till eldsjelen bakom detta forskningsprojekt, **Torsten Denneberg**. Din entusiasm smittade mig redan första gången vi träffades och jag har haft stor glädje av ditt engagemang för projektet och dina stora kunskaper inom området.

Erik Fjellstedt, tack för ett gott samarbete och för diskussioner som fördjupat min förståelse av kliniska aspekter av detta forskningsområde.

Tack till **Astrid Hoppe** för ett gott samarbete med cystinuri hos hund men också för att du är en så varm och omtänksam person.

Tack till ”mina” studenter **Karolin Hansén Hallor**, **Sofia Johansson** och **Ana Vujic**, för att jag fick förtroendet att handleda era projekt- och examensarbeten. Tack för ert engagemang och för att ni har hjälpt mig på vägen både med praktiskt labarbete, entusiasm och goda samtal.

Till mina arbetskamrater:

Jag har haft glädjen att jobba i en grupp med engagerade och duktiga personer där man verkligen bryr sig om och ställer upp för sina arbetskamrater och där alla bidrar till att skapa ett öppet klimat. Ni är guld!

Annette Molbæk, tack för att du tog dig an mig när jag kom som student till gruppen, jag kände mig välkommen och omhändertagen och fick den allra bästa guidningen till molekylärbiologiskt labarbete. Även ett stort tack för ditt deltagande i mitt forskningsprojekt.

Åsa Schippert, du är en klippa i gruppen! Jag uppskattar också att du är så bra på att ta tag i att uppmärksamma alla som fyllt år, gift sig eller fått barn, tack för din omtanke.

Deepti Verma, tack för alla goda pratstunder i fikarummet; **Anneli Karlsson**, tack för alla mer och mindre arbetsrelaterade diskussioner under tiden vi delade rum; **Lena Thunell**, tack för att du tålmodigt bistått mig i undervisningsfrågor och delat med dig av all din erfarenhet på det området.

Jonas Ungerbäck, under den hårda ytan finns ett hjärta av guld! Som tack för att du tagit på dig att bli min PT så ska du få ett ♥ av mig för då vet jag att du blir glad!

Tack till **Nils Elander**, min rumskamrat som jag kan prata med om allt från forskning till dagisångest. Din otippade humor är underbar och du är en förebild för mig då det gäller att få ihop tillvaron med arbete och familj.

Patiyan Andersson, vi har följts åt under många år, tack för att du är en så god arbetskamrat och vän. Jag kommer att sakna dig förfärligt mycket när du börjar ditt nya liv på andra sidan jorden, men jag är ändå så glad för din skull!

Till alla på Cellbiologen plan 9

Tack till alla på **Cellbiologen plan 9** för trevliga fika- och lunchraster och andra trevliga arrangemang. Tack också till **Malin** och **Pia** för alla mer och mindre seriösa pratstunder om karriären, familjen och livet i största allmänhet, likaså till **Pernilla**, **Pia** och **Amanda**.

Till alla från Medicinsk Biologi:

Alla eldsjälar och engagerade lärare bakom Medicinsk Biologi: **Peter**, **Anders**, **Eva**, **Magnus**, **Eva**, **Olle** och alla ni andra: Jag och många med mig är tacksamma för att ni genomförde er vision om Medicinsk Biologi, utbildningen blev den bästa tänkbara språngbrädan in i forskningen för mig.

Tack till gamlingarna från Medicinsk Biologi för vänskap, glada tillställningar och en helt fantastisk sammanhållning under och efter studietiden: **Mimi**, **Karin**, **Toffe**, **Stina**, **Jocke**, och så **Patiyan** igen förstås!

Till min familj:

Jag har alltid varit omgiven av en stor och kärleksfull familj, ni betyder allt!

Mamma och **pappa**: Jag har blivit uppmuntrad i allt jag gjort och har alltid känt att vad jag än väljer så väljer jag rätt. Jag har fått den bästa tänkbara grund att stå på och jag är så tacksam för det. **Mormor** och **morfar**, jag har er att tacka för så mycket och jag är så glad över att ni alltid funnits nära. Tack också till moster **Lena**, min morbror och idol **Stig-Ola** och alldeles underbara **Agneta** för att ni finns! **Janne**, tack för att du alltid ställer upp!

Mina systrar **Sara**, **Malin** och **Kajsa**: Ni känner mig bäst av alla och älskar mig ändå, det är en sådan trygghet att ha er. Ingen kan muntra upp, trösta och stötta som en nära syster och jag är så lyckligt lottad som har tre! Ni är underbara. Tack också till goa, rara och roliga **Marcus**, **Fredrik** och **Jesse**.

Till **Niclas**: Det har funnits stunder då jag inte trodde att jag skulle nå hit men du fanns där under hela resan och hjälpte mig att komma fram. Tack för att du alltid finns vid min sida, både i nöd och i lust. Tack för din kärlek, omtanke och ditt välsignade tålamod!

Till **Molly**: Min älskade dotter, med dig i mitt liv är allt mycket roligare, tack för att du varje dag påminner om vad som verkligen är viktigt.

REFERENCES

- ALBERS, A., S. LAHME, C. WAGNER, P. KAISER, K. ZERRES *et al.*, 1999 Mutations in the SLC3A1 gene in cystinuric patients: frequencies and identification of a novel mutation. *Genet Test* **3**: 227-231.
- BAUCH, C., and F. VERREY, 2002 Apical heterodimeric cystine and cationic amino acid transporter expressed in MDCK cells. *Am J Physiol Renal Physiol* **283**: F181-189.
- BERTRAN, J., A. WERNER, J. CHILLARON, V. NUNES, J. BIBER *et al.*, 1993 Expression cloning of a human renal cDNA that induces high affinity transport of L-cystine shared with dibasic amino acids in *Xenopus* oocytes. *J Biol Chem* **268**: 14842-14849.
- BERTRAN, J., A. WERNER, M. L. MOORE, G. STANGE, D. MARKOVICH *et al.*, 1992 Expression cloning of a cDNA from rabbit kidney cortex that induces a single transport system for cystine and dibasic and neutral amino acids. *Proc Natl Acad Sci U S A* **89**: 5601-5605.
- BISCEGLIA, L., M. J. CALONGE, L. DELLO STROLOGO, G. RIZZONI, L. DE SANCTIS *et al.*, 1996 Molecular analysis of the cystinuria disease gene: identification of four new mutations, one large deletion, and one polymorphism. *Hum Genet* **98**: 447-451.
- BISCEGLIA, L., M. J. CALONGE, A. TOTARO, L. FELIUBADALO, S. MELCHIONDA *et al.*, 1997 Localization, by linkage analysis, of the cystinuria type III gene to chromosome 19q13.1. *Am J Hum Genet* **60**: 611-616.
- BISCEGLIA, L., J. PURROY, M. JIMENEZ-VIDAL, A. P. D'ADAMO, F. ROUSAUD *et al.*, 2001 Cystinuria type I: identification of eight new mutations in SLC3A1. *Kidney Int* **59**: 1250-1256.
- BOSTROEM, H., and L. HAMBRAEUS, 1964 Cystinuria in Sweden. Vii. Clinical, Histopathological, and Medico-Social Aspects of the Disease. *Acta Med Scand* **175**: SUPPL 411:411+.
- BOVEE, K. C., 1986 Canine cystine urolithiasis. *Vet Clin North Am Small Anim Pract* **16**: 211-215.
- BOVEE, K. C., S. O. THIER, C. REA and S. SEGAL, 1974 Renal clearance of amino acids in canine cystinuria. *Metabolism* **23**: 51-58.
- CALONGE, M. J., P. GASPARINI, J. CHILLARON, M. CHILLON, M. GALLUCCI *et al.*, 1994 Cystinuria caused by mutations in rBAT, a gene involved in the transport of cystine. *Nat Genet* **6**: 420-425.
- CALONGE, M. J., M. NADAL, S. CALVANO, X. TESTAR, L. ZELANTE *et al.*, 1995a Assignment of the gene responsible for cystinuria (rBAT) and of markers D2S119 and D2S177 to 2p16 by fluorescence in situ hybridization. *Hum Genet* **95**: 633-636.

- CALONGE, M. J., V. VOLPINI, L. BISCEGLIA, F. ROUSAUD, L. DE SANCTIS *et al.*, 1995b Genetic heterogeneity in cystinuria: the SLC3A1 gene is linked to type I but not to type III cystinuria. *Proc Natl Acad Sci U S A* **92**: 9667-9671.
- CASAL, M. L., U. GIGER, K. C. BOVEE and D. F. PATTERSON, 1995 Inheritance of cystinuria and renal defect in Newfoundlands. *J Am Vet Med Assoc* **207**: 1585-1589.
- CHAIROUNGDUA, A., H. SEGAWA, J. Y. KIM, K. MIYAMOTO, H. HAGA *et al.*, 1999 Identification of an amino acid transporter associated with the cystinuria-related type II membrane glycoprotein. *J Biol Chem* **274**: 28845-28848.
- CHATZIKYRIAKIDOU, A., N. SOFIKITIS, V. KALFAKAKOU, K. SIAMOPOULOS and I. GEORGIU, 2006 Evidence for association of SLC7A9 gene haplotypes with cystinuria manifestation in SLC7A9 mutation carriers. *Urol Res* **34**: 299-303.
- CHILLARON, J., R. ROCA, A. VALENCIA, A. ZORZANO and M. PALACIN, 2001 Heteromeric amino acid transporters: biochemistry, genetics, and physiology. *Am J Physiol Renal Physiol* **281**: F995-1018.
- CHONG, A., G. ZHANG and V. B. BAJIC, 2004 Information for the Coordinates of Exons (ICE): a human splice sites database. *Genomics* **84**: 762-766.
- CLARK, W. T., and D. CUDDEFORD, 1971 A study of the amino-acids in urine from dogs with cystine urolithiasis. *Vet Rec* **88**: 414-417.
- COLOMBO, R., 2000 Dating the origin of the V170M mutation causing non-type I cystinuria in Libyan Jews by linkage disequilibrium and physical mapping of the SLC7A9 gene. *Genomics* **69**: 131-134.
- CRAAN, A. G., 1981 Cystinuria: the disease and its models. *Life Sci* **28**: 5-22.
- CRAWHALL, J. C., P. PURKISS, R. W. WATTS and E. P. YOUNG, 1969 The excretion of amino acids by cystinuric patients and their relatives. *Ann Hum Genet* **33**: 149-169.
- CRAWHALL, J. C., and S. SEGAL, 1966 The intracellular cysteine-cystine ratio in kidney cortex. *Biochem J* **99**: 19C-20C.
- CRAWHALL, J. C., and S. SEGAL, 1967 The intracellular ratio of cysteine and cystine in various tissues. *Biochem J* **105**: 891-896.
- DAHLBERG, P. J., B. VAN DEN, S. B. KURTZ, D. M. WILSON and L. H. SMITH, 1977 Clinical features and management of cystinuria. *Mayo Clin Proc* **52**: 533-542.
- DELLO STROLOGO, L., E. PRAS, C. PONTESILLI, E. BECCIA, V. RICCI-BARBINI *et al.*, 2002 Comparison between SLC3A1 and SLC7A9 cystinuria patients and carriers: a need for a new classification. *J Am Soc Nephrol* **13**: 2547-2553.
- DENT, C. E., M. FRIEDMAN, H. GREEN and L. C. WATSON, 1965 Treatment of Cystinuria. *Br Med J* **1**: 403-408.
- DENT, C. E., and G. A. ROSE, 1951 Aminoacid metabolism in cystinuria. *Q J Med* **20**: 205-219.
- DENT, C. E., and B. SENIOR, 1955 Studies on the treatment of cystinuria. *Br J Urol* **27**: 317-332.

- EGOSHI, K. I., K. AKAKURA, T. KODAMA and H. ITO, 2000 Identification of five novel SLC3A1 (rBAT) gene mutations in Japanese cystinuria. *Kidney Int* **57**: 25-32.
- ENDSLEY, J. K., J. A. PHILLIPS, 3RD, K. A. HRUSKA, T. DENNEBERG, J. CARLSON *et al.*, 1997 Genomic organization of a human cystine transporter gene (SLC3A1) and identification of novel mutations causing cystinuria. *Kidney Int* **51**: 1893-1899.
- EVANS, W. P., M. I. RESNICK and W. H. BOYCE, 1982 Homozygous cystinuria--evaluation of 35 patients. *J Urol* **127**: 707-709.
- FELIUBADALO, L., M. L. ARBONES, S. MANAS, J. CHILLARON, J. VISA *et al.*, 2003 Slc7a9-deficient mice develop cystinuria non-I and cystine urolithiasis. *Hum Mol Genet* **12**: 2097-2108.
- FELIUBADALO, L., L. BISCEGLIA, M. FONT, L. DELLO STROLOGO, E. BECCIA *et al.*, 1999a Recombinant families locate the gene for non-type I cystinuria between markers C13 and D19S587 on chromosome 19q13.1. *Genomics* **60**: 362-365.
- FELIUBADALO, L., M. FONT, J. PURROY, F. ROUSAUD, X. ESTIVILL *et al.*, 1999b Non-type I cystinuria caused by mutations in SLC7A9, encoding a subunit (bo,+AT) of rBAT. *Nat Genet* **23**: 52-57.
- FERNANDEZ, E., M. CARRASCAL, F. ROUSAUD, J. ABIAN, A. ZORZANO *et al.*, 2002 rBAT-b(0,+)-AT heterodimer is the main apical reabsorption system for cystine in the kidney. *Am J Physiol Renal Physiol* **283**: F540-548.
- FONT-LLITJOS, M., M. JIMENEZ-VIDAL, L. BISCEGLIA, M. DI PERNA, L. DE SANCTIS *et al.*, 2005 New insights into cystinuria: 40 new mutations, genotype-phenotype correlation, and digenic inheritance causing partial phenotype. *J Med Genet* **42**: 58-68.
- FONT, M. A., L. FELIUBADALO, X. ESTIVILL, V. NUNES, E. GOLOMB *et al.*, 2001 Functional analysis of mutations in SLC7A9, and genotype-phenotype correlation in non-Type I cystinuria. *Hum Mol Genet* **10**: 305-316.
- FOREMAN, J. W., S. M. HWANG and S. SEGAL, 1980 Transport interactions of cystine and dibasic amino acids in isolated rat renal tubules. *Metabolism* **29**: 53-61.
- FRIMPTER, G. W., 1963 Cystinuria: Metabolism of the Disulfide of Cysteine and Homocysteine. *J Clin Invest* **42**: 1956-1964.
- FURRIOLS, M., J. CHILLARON, C. MORA, A. CASTELLO, J. BERTRAN *et al.*, 1993 rBAT, related to L-cysteine transport, is localized to the microvilli of proximal straight tubules, and its expression is regulated in kidney by development. *J Biol Chem* **268**: 27060-27068.
- GASPARINI, P., M. J. CALONGE, L. BISCEGLIA, J. PURROY, I. DIANZANI *et al.*, 1995 Molecular genetics of cystinuria: identification of four new mutations and seven polymorphisms, and evidence for genetic heterogeneity. *Am J Hum Genet* **57**: 781-788.

- GONSKA, T., J. R. HIRSCH and E. SCHLATTER, 2000 Amino acid transport in the renal proximal tubule. *Amino Acids* **19**: 395-407.
- GOODYER, P., I. SAADI, P. ONG, G. ELKAS and R. ROZEN, 1998 Cystinuria subtype and the risk of nephrolithiasis. *Kidney Int* **54**: 56-61.
- HARRIS, H., U. MITTWOCH, E. B. ROBSON and F. L. WARREN, 1955 Phenotypes and genotypes in cystinuria. *Ann Hum Genet* **20**: 57-91.
- HARRIS, H., and E. B. ROBSON, 1955 Variation in homozygous cystinuria. *Acta Genet Stat Med* **5**: 381-390.
- HARRIS, H., and F. L. WARREN, 1953 Quantitative studies on the urinary cystine in patients with cystine stone formation and in their relatives. *Ann Eugen* **18**: 125-171.
- HAYASHI, K., 1991 PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods Appl* **1**: 34-38.
- HAYASHI, K., and D. W. YANDELL, 1993 How sensitive is PCR-SSCP? *Hum Mutat* **2**: 338-346.
- HENTHORN, P. S., J. LIU, T. GIDALEVICH, J. FANG, M. L. CASAL *et al.*, 2000 Canine cystinuria: polymorphism in the canine SLC3A1 gene and identification of a nonsense mutation in cystinuric Newfoundland dogs. *Hum Genet* **107**: 295-303.
- HOLTZAPPLE, P. G., C. REA, K. BOVEE and S. SEGAL, 1971 Characteristics of cystine and lysine transport in renal jejunal tissue from cystinuric dogs. *Metabolism* **20**: 1016-1022.
- HOPPE, A., and T. DENNEBERG, 2001 Cystinuria in the dog: clinical studies during 14 years of medical treatment. *J Vet Intern Med* **15**: 361-367.
- HOPPE, A., T. DENNEBERG, J. O. JEPSSON and B. KAGEDAL, 1993a Canine cystinuria: an extended study on the effects of 2-mercaptopropionylglycine on cystine urolithiasis and urinary cystine excretion. *Br Vet J* **149**: 235-251.
- HOPPE, A., T. DENNEBERG, J. O. JEPSSON and B. KAGEDAL, 1993b Urinary excretion of amino acids in normal and cystinuric dogs. *Br Vet J* **149**: 253-268.
- HORSFORD, J., I. SAADI, J. RAEISON, P. R. GOODYER and R. ROZEN, 1996 Molecular genetics of cystinuria in French Canadians: identification of four novel mutations in type I patients. *Kidney Int* **49**: 1401-1406.
- ITO, H., M. MURAKAMI, T. MIYAUCHI, I. MORI, K. YAMAGUCHI *et al.*, 1983 The incidence of cystinuria in Japan. *J Urol* **129**: 1012-1014.
- JEPSSON, J. O., and I. M. KARLSSON, 1972 Ion-exchange chromatography of physiological sulphur amino acids on a highly crosslinked resin. *J Chromatogr* **72**: 93-103.
- JOLY, D., P. RIEU, A. MEJEAN, M. F. GAGNADOUX, M. DAUDON *et al.*, 1999 Treatment of cystinuria. *Pediatr Nephrol* **13**: 945-950.
- KALLISTRATOS, G., C. DIMOPOULOS, V. KALFAKAKOU-VADALOUKA, A. EVANGELOU, D. STOCKIDIS *et al.*, 1983 Familial cystinuria in Ioannina District (Greece). Diagnosis and treatment. *Urol Res* **11**: 291-296.

- KANAI, Y., Y. FUKASAWA, S. H. CHA, H. SEGAWA, A. CHAIROUNGDUAN *et al.*, 2000 Transport properties of a system y⁺L neutral and basic amino acid transporter. Insights into the mechanisms of substrate recognition. *J Biol Chem* **275**: 20787-20793.
- KANAI, Y., M. G. STELZNER, W. S. LEE, R. G. WELLS, D. BROWN *et al.*, 1992 Expression of mRNA (D2) encoding a protein involved in amino acid transport in S3 proximal tubule. *Am J Physiol* **263**: F1087-1092.
- KATO, T., 1977 Renal handling of dibasic amino acids and cystine in cystinuria. *Clin Sci Mol Med* **53**: 9-15.
- KNOLL, T., A. ZOLLNER, G. WENDT-NORDAHL, M. S. MICHEL and P. ALKEN, 2005 Cystinuria in childhood and adolescence: recommendations for diagnosis, treatment, and follow-up. *Pediatr Nephrol* **20**: 19-24.
- LANGEN, H., D. VON KIETZELL, D. BYRD, M. ARSLAN-KIRCHNER, U. VESTER *et al.*, 2000 Renal polyamine excretion, tubular amino acid reabsorption and molecular genetics in cystinuria. *Pediatr Nephrol* **14**: 376-384.
- LECLERC, D., M. BOUTROS, D. SUH, Q. WU, M. PALACIN *et al.*, 2002 SLC7A9 mutations in all three cystinuria subtypes. *Kidney Int* **62**: 1550-1559.
- LEE, W. S., R. G. WELLS, R. V. SABBAG, T. K. MOHANDAS and M. A. HEDIGER, 1993 Cloning and chromosomal localization of a human kidney cDNA involved in cystine, dibasic, and neutral amino acid transport. *J Clin Invest* **91**: 1959-1963.
- LEVY, H. L., and E. BARKIN, 1971 Comparison of amino acid concentrations between plasma and erythrocytes. Studies in normal human subjects and those with metabolic disorders. *J Lab Clin Med* **78**: 517-523.
- LINDBLAD-TOH, K., C. M. WADE, T. S. MIKKELSEN, E. K. KARLSSON, D. B. JAFFE *et al.*, 2005 Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* **438**: 803-819.
- LINDELL, A., T. DENNEBERG, E. EDHOLM and J. O. JEPSSON, 1995a The effect of sodium intake on cystinuria with and without tiopronin treatment. *Nephron* **71**: 407-415.
- LINDELL, A., T. DENNEBERG and J. O. JEPSSON, 1995b Urinary excretion of free cystine and the tiopronin-cysteine-mixed disulfide during long term tiopronin treatment of cystinuria. *Nephron* **71**: 328-342.
- LONDON, D. R., and T. H. FOLEY, 1965 Cystine metabolism in cystinuria. *Clin Sci* **29**: 129-141.
- LOTZ, M., J. T. POTTS, JR., J. M. HOLLAND, W. S. KISER and F. C. BARTTER, 1966 D-penicillamine therapy in cystinuria. *J Urol* **95**: 257-263.
- MARTINS, M. C., A. A. MEYERS, N. A. WHALLEY and A. L. RODGERS, 2002 Cystine: a promoter of the growth and aggregation of calcium oxalate crystals in normal undiluted human urine. *J Urol* **167**: 317-321.

- MATOS, A. J., C. MASCARENHAS, P. MAGALHAES and J. P. PINTO, 2006 Efficient screening of the cystinuria-related C663T Slc3a1 nonsense mutation in Newfoundland dogs by denaturing high-performance liquid chromatography. *J Vet Diagn Invest* **18**: 102-105.
- MEYERS-WALLEN, V. N., 2003 Ethics and genetic selection in purebred dogs. *Reprod Domest Anim* **38**: 73-76.
- MILLINER, D. S., 1990 Cystinuria. *Endocrinol Metab Clin North Am* **19**: 889-907.
- ORITA, M., H. IWAHANA, H. KANAZAWA, K. HAYASHI and T. SEKIYA, 1989 Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci U S A* **86**: 2766-2770.
- PALACIN, M., R. ESTEVEZ, J. BERTRAN and A. ZORZANO, 1998 Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* **78**: 969-1054.
- PALACIN, M., and Y. KANAI, 2004 The ancillary proteins of HATs: SLC3 family of amino acid transporters. *Pflugers Arch* **447**: 490-494.
- PARKER, H. G., L. V. KIM, N. B. SUTTER, S. CARLSON, T. D. LORENTZEN *et al.*, 2004 Genetic structure of the purebred domestic dog. *Science* **304**: 1160-1164.
- PETERS, T., C. THAETE, S. WOLF, A. POPP, R. SEDLMEIER *et al.*, 2003 A mouse model for cystinuria type I. *Hum Mol Genet* **12**: 2109-2120.
- PFEIFFER, R., J. LOFFING, G. ROSSIER, C. BAUCH, C. MEIER *et al.*, 1999 Luminal heterodimeric amino acid transporter defective in cystinuria. *Mol Biol Cell* **10**: 4135-4147.
- PICKEL, V. M., M. J. NIRENBERG, J. CHAN, R. MOSCKOVITZ, S. UDENFRIEND *et al.*, 1993 Ultrastructural localization of a neutral and basic amino acid transporter in rat kidney and intestine. *Proc Natl Acad Sci U S A* **90**: 7779-7783.
- PRAS, E., N. ARBER, I. AKSENTIJEVICH, G. KATZ, J. M. SCHAPIRO *et al.*, 1994 Localization of a gene causing cystinuria to chromosome 2p. *Nat Genet* **6**: 415-419.
- PRAS, E., I. KOCHBA, A. LUBETZKY, M. PRAS, Y. SIDI *et al.*, 1998 Biochemical and clinical studies in Libyan Jewish cystinuria patients and their relatives. *Am J Med Genet* **80**: 173-176.
- PRAS, E., Y. KREISS, Y. FRISHBERG, L. PROSEN, I. AKSENTIJEVICH *et al.*, 1999 Refined mapping of the CSNU3 gene to a 1.8-Mb region on chromosome 19q13.1 using historical recombinants in Libyan Jewish cystinuria patients. *Genomics* **60**: 248-250.
- PRAS, E., N. RABEN, E. GOLOMB, N. ARBER, I. AKSENTIJEVICH *et al.*, 1995 Mutations in the SLC3A1 transporter gene in cystinuria. *Am J Hum Genet* **56**: 1297-1303.

- RAJAN, D. P., R. KEKUDA, W. HUANG, H. WANG, L. D. DEVOE *et al.*, 1999 Cloning and expression of a b(0,+)-like amino acid transporter functioning as a heterodimer with 4F2hc instead of rBAT. A new candidate gene for cystinuria. *J Biol Chem* **274**: 29005-29010.
- REIG, N., J. CHILLARON, P. BARTOCCIONI, E. FERNANDEZ, A. BENDAHAN *et al.*, 2002 The light subunit of system b(o,+) is fully functional in the absence of the heavy subunit. *Embo J* **21**: 4906-4914.
- RESNICK, M. I., H. O. GOODMAN and W. H. BOYCE, 1979 Heterozygous cystinuria and calcium oxalate urolithiasis. *J Urol* **122**: 52-54.
- ROSENBERG, L. E., 1966 Cystinuria: genetic heterogeneity and allelism. *Science* **154**: 1341-1343.
- ROSENBERG, L. E., and S. DOWNING, 1965 Transport of Neutral and Dibasic Amino Acids by Human Leukocytes: Absence of Defect in Cystinuria. *J Clin Invest* **44**: 1382-1393.
- ROSENBERG, L. E., S. DOWNING, J. L. DURANT and S. SEGAL, 1966a Cystinuria: biochemical evidence for three genetically distinct diseases. *J Clin Invest* **45**: 365-371.
- ROSENBERG, L. E., J. L. DURANT and I. ALBRECHT, 1966b Genetic heterogeneity in cystinuria: evidence for allelism. *Trans Assoc Am Physicians* **79**: 284-296.
- ROSENBERG, L. E., J. L. DURANT and J. M. HOLLAND, 1965 Intestinal absorption and renal extraction of cystine and cysteine in cystinuria. *N Engl J Med* **273**: 1239-1245.
- SAADI, I., X. Z. CHEN, M. HEDIGER, P. ONG, P. PEREIRA *et al.*, 1998 Molecular genetics of cystinuria: mutation analysis of SLC3A1 and evidence for another gene in type I (silent) phenotype. *Kidney Int* **54**: 48-55.
- SCHAFFER, J. A., and D. W. BARFUSS, 1980 Membrane mechanisms for transepithelial amino acid absorption and secretion. *Am J Physiol* **238**: F335-346.
- SCHMIDT, C., J. TOMIUK, E. BOTZENHART, U. VESTER, M. HALBER *et al.*, 2003 Genetic variations of the SLC7A9 gene: allele distribution of 13 polymorphic sites in German cystinuria patients and controls. *Clin Nephrol* **59**: 353-359.
- SEGAL, S., P. D. MCNAMARA and L. M. PEPE, 1977 Transport interaction of cystine and dibasic amino acids in renal brush border vesicles. *Science* **197**: 169-171.
- SHEFFIELD, V. C., J. S. BECK, A. E. KWITEK, D. W. SANDSTROM and E. M. STONE, 1993 The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* **16**: 325-332.
- SHIGETA, Y., Y. KANAI, A. CHAIROUNGDUAN, N. AHMED, S. SAKAMOTO *et al.*, 2006 A novel missense mutation of SLC7A9 frequent in Japanese cystinuria cases affecting the C-terminus of the transporter. *Kidney Int* **69**: 1198-1206.
- SILBERNAGL, S., 1988 The renal handling of amino acids and oligopeptides. *Physiol Rev* **68**: 911-1007.

- SKOPKOVA, Z., E. HRABINCOVA, S. STASTNA, L. KOZAK and T. ADAM, 2005 Molecular genetic analysis of SLC3A1 and SLC7A9 genes in Czech and Slovak cystinuric patients. *Ann Hum Genet* **69**: 501-507.
- STOLLER, M. L., J. E. BRUCE, C. A. BRUCE, T. FOROUD, S. C. KIRKWOOD *et al.*, 1999 Linkage of type II and type III cystinuria to 19q13.1: codominant inheritance of two cystinuric alleles at 19q13.1 produces an extreme stone-forming phenotype. *Am J Med Genet* **86**: 134-139.
- THIER, S., M. FOX, S. SEGAL and L. E. ROSENBERG, 1964 Cystinuria: in Vitro Demonstration of an Intestinal Transport Defect. *Science* **143**: 482-484.
- THIER, S. O., S. SEGAL, M. FOX, A. BLAIR and L. E. ROSENBERG, 1965 Cystinuria: Defective Intestinal Transport of Dibasic Amino Acids and Cystine. *J Clin Invest* **44**: 442-448.
- TREACHER, R. J., 1964 The aetiology of canine cystinuria. *Biochem J* **90**: 494-498.
- TREACHER, R. J., 1965 Intestinal absorption of lysine in cystinuric dogs. *J Comp Pathol* **75**: 309-322.
- TSAN, M. F., T. C. JONES, G. W. THORNTON, H. L. LEVY, C. GILMORE *et al.*, 1972 Canine cystinuria: its urinary amino acid pattern and genetic analysis. *Am J Vet Res* **33**: 2455-2461.
- WAGNER, C. A., F. LANG and S. BROER, 2001 Function and structure of heterodimeric amino acid transporters. *Am J Physiol Cell Physiol* **281**: C1077-1093.
- WARTENFELD, R., E. GOLOMB, G. KATZ, S. J. BALE, B. GOLDMAN *et al.*, 1997 Molecular analysis of cystinuria in Libyan Jews: exclusion of the SLC3A1 gene and mapping of a new locus on 19q. *Am J Hum Genet* **60**: 617-624.
- WEINBERGER, A., O. SPERLING, M. RABINOVITZ, S. BROSH, A. ADAM *et al.*, 1974 High frequency of cystinuria among Jews of Libyan origin. *Hum Hered* **24**: 568-572.
- WELLS, R. G., and M. A. HEDIGER, 1992 Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. *Proc Natl Acad Sci U S A* **89**: 5596-5600.
- VERREY, F., E. I. CLOSS, C. A. WAGNER, M. PALACIN, H. ENDOU *et al.*, 2004 CATs and HATs: the SLC7 family of amino acid transporters. *Pflugers Arch* **447**: 532-542.