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"Lysinuric Protein Intolerance: Molecular and Cellular Bases of an Inherited Multi-Sistem Disorders"

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A mio marito,
con tanto affetto ed amore

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Chapter 1

AMINO ACID TRANSPORTERS

1.1 Heteromeric Amino Acid Transporters

In the last decade, many amino acid transporters were identified by molecular cloning and assigned to the classically characterized amino acid transport systems.

Amino acid transporters permit the uptake of amino acids from food in the small intestine. They release the amino acids themselves into blood, and then perform either uptake of amino acids from the blood into tissues such as liver or skeletal muscle or they allow amino acids from the urine along the kidney nephron to be reabsorpted (Wagner et al., 2001). Six families of amino acid transporters for the cell plasma membrane have been described in mammals, one of which has a heteromeric structure (Franca et al., 2005; Palacin et al., 2005).

Heteromeric amino acid transporters (HATs) are composed of a heavy subunit and a light subunit (Table 1.1) (Palacin et al., 2005). Two homologous heavy subunits (HSHATs) from the SLC3 family have been cloned and are called rBAT and 4F2hc (also named CD98). Ten light subunits (LSHATs; SLC7 family members from SLC7A5 to SLC7A13) have been identified. Six of them are partners of 4F2hc (LAT1, LAT2, y^+LAT1, y^+LAT2, asc-1, and xCT); one forms a heterodimer with rBAT ($b^{0,+}$AT); and two (asc-2 and AGT-1) seem to interact with as yet unknown heavy subunits (Verrey et al., 2004). Members SLC7A1–SLC7A4 of family SLC7 correspond to system y^+ isoforms (cationic amino acid transporters; CATs) and related proteins, which on average show <25% amino acid identity to the light subunits of HATs (Palacin et al., 2005). The general features of HATs are as follows:

- The heavy subunits (molecular mass of ~90 and ~80 kDa for rBAT and 4F2hc, respectively) are type II membrane N-glycoproteins with a single transmembrane domain, an intracellular NH2 terminus, and

an extracellular COOH terminus significantly homologous to insect and bacterial glucosidases (Fig. 1.1). Recently, X-ray diffraction of the extracellular domain of human 4F2hc revealed a three-dimensional structure similar to that of bacterial glucosidases (Palacin et al., 2005).

- The light subunits (~50 kDa) are highly hydrophobic and not glycosylated. This results in anomalously high mobility in SDS/PAGE (35–40 kDa). Recent cysteine-scanning mutagenesis studies support a 12-transmembrane-domain topology, with the NH2 and COOH terminals located inside the cell and with a reentrant-like structure in the intracellular loop IL2-3 for xCT, as a model for the light subunits of HATs (Fig. 1.1) (Gasol et al., 2004). The light chain and the corresponding heavy chain subunit are linked by a disulfide bridge. For this reason, HATs are also named glycoprotein-associated amino acid transporters. The light subunit cannot reach the plasma membrane unless it interacts with the heavy subunit.

1.2 Functional Proprieties of Heteromeric Amino Acid Transporters

The light subunit confers specific amino acid transport activity to the heteromeric complex. LAT1 and LAT2 correspond to system L isoforms, y^+LAT1 and y^+LAT2 for system y^+L isoforms, asc-1 and asc-2 for system asc isoforms, xCT for system x^-_c isoforms, $b^{0,+}$AT for system $b^{0,+}$ isoforms, and AGT-1 for a system serving aspartate and glutamate transport (Table 1.1). Moreover, reconstitution in liposomes shows that the light subunit $b^{0,+}$AT is fully functional in the absence of the heavy subunit rBAT (Fernandez et al., 2002; Reig et al., 2002). HAT transport activities are, with the exception of system asc isoforms, tightly couplet amino acid antiporters, leading to exchange of amino acids (Palacin et al., 2005).

The general features of amino acid transport activity are as follows (Wagner et al., 2001; Chillaron et al., 2001):

- System L is a sodium-dependent transport of large branched and aromatic neutral amino acids in almost all type of cells;
- System y^+L is a sodium-independent transport of cationic amino acids and sodium-dependent transport for neutral amino acids (see section below);

- System asc is a sodium-independent transport for neutral amino acids of a small side chain, such as alanine, glycine, cystine and serine;
- System x^-_c is a sodium-independent transport for cysteine and anionic amino acids;
- System $b^{0,+}$ is a sodium-independent transport for dibasic amino acids and neutral amino acids, including cystine;
- System AGT-1 serves aspartate and glutamate transport.

1.3 Transport activity of system y^+L

System y^+L was first functionally described in erythrocytes (Raul et al., 1998; Kanai et al., 2000). In polarized epithelia, system y^+L is located at the basolateral membrane and allows for the efflux of cationic amino acids in exchange for preferably large neutral amino acids and sodium (Palacin et al., 2004; Palacin et al., 2005). This pattern of transport distinguishes system y^+L from cationic amino acid transport y^+ (CAT family) (Deves et al., 1998). On a molecular basis, two isoforms of system y^+L have recently been identified, y^+LAT1 and y^+LAT2 (Torrents et al., 1998). Both subunits interact with 4F2hc to form the system y^+L.

Human y^+LAT1 is a protein consisting of 511 amino acids and its mRNA was found mainly in the kidney and small intestine (see below, section lysinuric protein intolerance, cap. 3). Functionally, y^+LAT1 transports cationic amino acids in absence of sodium and neutral amino acids in presence of sodium (Torrents et al., 1998). It seems that y^+LAT1 preferentially mediates the efflux of arginine, which may important in the kidney, where arginine is produced from citrulline and released into the

blood to supply the rest of the body with this amino acid (Palacin et al., 2005).

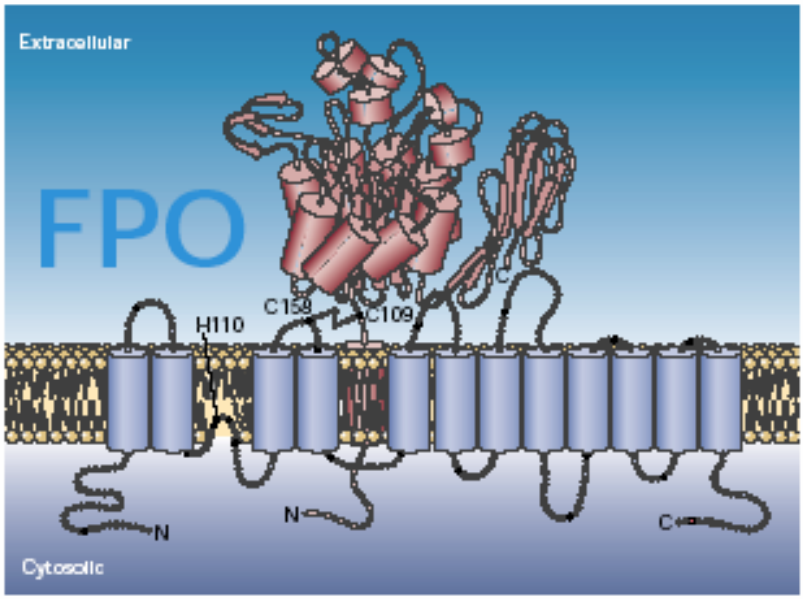
The second isoform, y^+LAT2, constitutes a protein of 515 amino acids. Expression of its mRNA was found in brain, testis, parotis and was found to be weaker in a small intestine, kidney, lung, liver and heart. y^+LAT2 displays the same mechanism as y^+LAT1 but tissues with lower transport capacity probably use y^+LAT2 for transport of arginine (Broer et al., 2000).

Table 1.1. Heteromeric amino acid transporters

Heavy Chain (HSHAT)	Light Chain (LSHAT)	HUGO Nomenclature	Amino Acid Transport	Human Chromosome	Inherited Aminoaciduria
4F2hc		SLC3A2		11q13	
	y^+LAT1	SLC7A7	y^+L	14q11.2	LPI
	y^+LAT2	SLC7A6	y^+L	16q22.1	
	LAT1	SLC7A5	L	16q24.3	
	LAT2	SLC7A8	L	14q11.2	
	asc-1	SLC7A10	asc	19q12-13	
	xCT	SLC7A11	x^-_c	4q28-q32	
rBAT		SLC3A1		2p16.3	cystinuria
	$b^{0,+}$AT	SLC7A9	$b^{0,+}$	19q12-13	cystinuria
?					
	AGT1	SLC7A13	new	8q21.3	
	Asc-2	SLC7A12	asc	not present	

Heteromeric amino acid transporters (HATs) are composed of a heavy and light nchain. Heavy subunits belong to solute carrier family SLC3, and light chain subunits belong to family SLC7. AGT and asc-2 heterodimerize with unknown heavy subunits. A functional asc-2 gene is not present in the human genome (from Palacin et al., 2005).

Figure 1.1: A heteromeric amino acid transporter. The heavy subunit (pink) and the light subunit (blue) are linked by a disulfide bridge with conserved cysteine residues (cysteine 158 for the human xCT and cysteine 109 for human 4F2hc). (From Palacin et al., 2005).



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Chapter 2

PRIMARY INHERITED AMINOACIDURIAS

2.1 Disease with Defective Amino Acid Transport

Primary inherited aminoacidurias (PIAs) are caused by defective amino acid transport activities, which affect renal reabsorption of amino acids and may also affect intestinal absorption of amino acids and transport function in other organs (Palacin et al., 2005). Several PIA have been described (Table 2.1):

1. Reabsorption of dibasic or cationic amino acids is defective in:

- *lysinuric protein intolerance* [LPI, also named hyperdibasic aminoaciduria type 2 or familial protein intolerance (MIM 222700) and first described by Perheentupa et al. (1965)] (Simell, 2001). This disorder will be the subject of the work presented in this thesis and it will be discussed in detail in the next chapter.

- *autosomal dominant hyperdibasic aminoaciduria* type I (MIM 222690) (Whelan and Scriver, 1968).
- *isolated lysinuria* (Omura et al., 1976).

2. Reabsorption of cystine and dibasic amino acids is defective in:

- *cystinuria* (MIM 220100) (Palacin et al., 2001).
- *isolated cystinuria* (MIM 238200) (Brodehl et al., 1967).

3. Reabsorption of zwitterionic amino acids (i.e., neutral amino acids at physiological pH) is defective in:

A. *Hartnup disorder* (MIM 234500) (Babu et al., 2003).

B. *Iminoglycinuria* (MIM 242600) (Rosemberg et al., 1968).

4. Reabsorption of glutamate and aspartate is defective in:

- *Dicarboxylic aminoaciduria* (MIM 222730) (Melacon et al., 1977; Teijema et al., 1974).

2.2 Renal reabsorption and intestinal absorption of amino acids transport

The renal reabsorption of amino acids occurs in the proximal

convoluted tubule (Silbernagl et al., 1983), and the absorption of amino acids occurs in the small intestine (Mariotti et al., 2000). Most of the transporters responsible for these functions are the same in kidney and intestine (Fig 2.1). The transporters with a role in reabsorption of amino acids are highly expressed in the corresponding apical or basolateral plasma membrane of the epithelial cells of the proximal of the proximal convoluted tubules (S1 and S2 segments) in kidney and of the enterocytes of the small intestine (Dave et al., 2004; Fernandez et al., 2002, Furriols et al., 1993).

The molecular basis of cystinuria and LPI instructs us on the molecular bases of cystine and dibasic amino acid reabsorption. Mutations of the apical exchanger $b^{0,+}$ (heterodimer rBAT-$b^{0,+}$AT) lead to hyperexcretion of cystine and dibasic amino acids, as shown in human (Calonge et al., 1994; Feliubadalo et al., 1999), mouse (Feliubadalo et al., 2003; Peters et al., 2003), and canine (Henthorn et al., 2000) cystinuria. Mutations of the basolateral exchanger y^+L (heterodimer 4F2hc-y^+LAT1) produce hyperexcretion of dibasic amino acids (Borsani et al., 1999; Torrents et al., 1999).

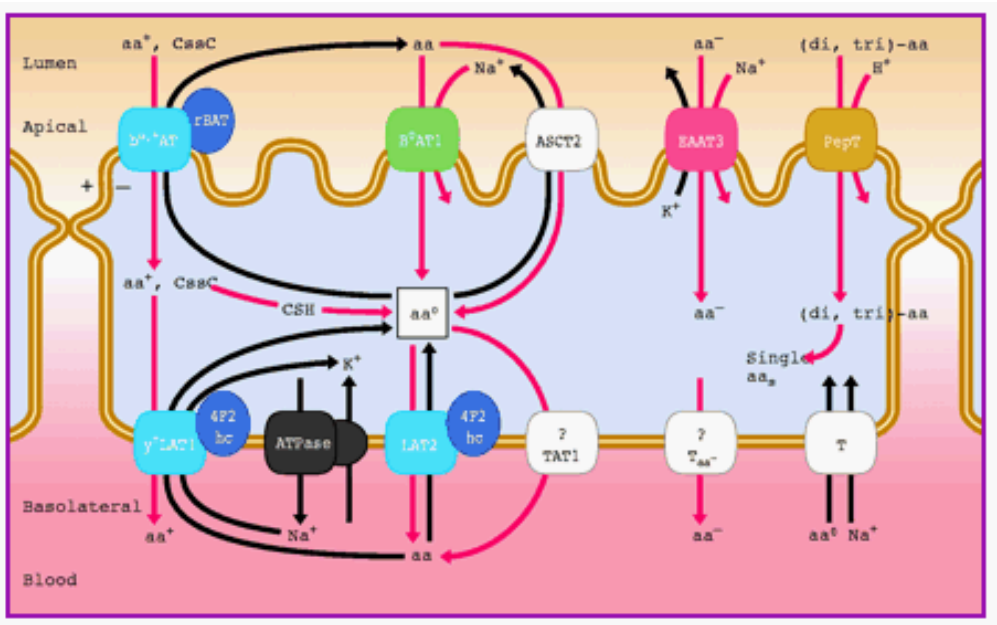
Similarly, there is intestinal malabsorption of cystine and dibasic amino acids in cystinuria and of dibasic amino acids in LPI (Simell 2001). Thus the sequential transport activities of systems $b^{0,+}$ (apical) and $y^{+}L$ (basolateral) play a major role in renal and intestinal reabsorption of amino acids. Besides these two players, not much is known about other transporters with a role in dibasic amino acid reabsorption (Palacin et al., 2004; Palacin et al., 2005).

Table 2.1. Primary Inherited Aminoacidurias

	Prevalence	Type of Inheritance	Trasport System
LPI	> 100 cases	AR	y^+L
Hyperdibasic Aminoaciduria Type 1	very rare	AD	?
Isolated lysinuria	very rare	AR?	?
Cystinuria Type 1 Type 2	1/7.000	 AR ADIP	 $b^{o,+}$
Isolated cystinuria	very rare	AR?	?
Hartnup disorder	1/30.000	AR	B^0
Iminoglycinuria	1/15.000	AR	?
Dicarboxylic aminoaciduria	very rare	AR?	x^-_{AG}

AR, autosomal recessive; ADIP, autosomal dominant with incomplete penetrance; AD, autosomal dominant; AR?, familiar cases in the few cases described for these disease suggest an autosomal recessive mode of inheritance (from Palacin et al., 2005).

Figure 2.1: Transporters involved in the renal and intestinal reabsorption of amino acids (from Palacin et al., 2005).



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Chapter 3

LYSINURIC PROTEIN INTOLERANCE

3.1 Clinical aspects and biochemical investigations

Lysinuric protein intolerance (LPI; [MIM 222700]) represents a disease-model where mutations of the SLC7A7 gene, an amino acid transporter, give rise to variable and mostly unexplainable multiorgan involvement. LPI is a rare disorder relatively common in Finland, in Italy where more than 20 families have already been identified (Sperandeo et al., 2000) and in Japan. LPI is an autosomic recessive dibasicaminoaciduria caused by defective cationic amino acid (CAA; L-arginine; L-lysine; L-ornithine) transport at the basolateral membrane of epithelial cells in intestine and kidney (Simell, 2001; Palacin et al., 2001).

The experiments of the Finnish group (Rajantie et al., 1980) suggest

that system y^+L is the main basolateral absorption system for dibasic amino acids in the small intestine: an oral load with the dipeptide lysyl-glycine increased glycine plasma concentrations, but plasma lysine remained almost unchanged in patients with LPI, whereas both amino acids increased in plasma of control subjects and in patients with cystinuria. This demonstrated the basolateral defect in LPI. Indeed, as a consequence of the renal and intestinal defects in LPI, plasma levels of dibasic amino acids are 1/2 to 1/3 of normal levels (Simell, 2001). These results suggest that system y^+L has a higher impact than system $b^{0,+}$ on renal and intestinal reabsorption of dibasic amino acids.

Arginine and ornithine are intermediates of the urea cycle that provide the carbon skeleton to the cycle. Their reduced availability results in a functional deficiency of the urea cycle. Protein malnutrition and deficiency of lysine, an essential amino acid, contribute to the patient's failure to thrive. LPI patients are usually asymptomatic while breast-feeding; after weaning, the clinical findings include: vomiting, diarrhea, hepatosplenomegaly, bone marrow abnormalities, osteoporosis, episodes of

coma, mental retardation, lung involvement (mainly as alveolar proteinosis), altered immune response and chronic renal disease (Simell, 2001; Palacin et al., 2001).

Metabolic derangement in LPI includes: reduced intestinal absorption of CAA, increased renal excretion of CAA and dysfunction of the urea cycle leading to hyperammonemia and orotic aciduria. The hyperammonemia occurs in inherited defect of urea cycle enzymes but may also results from lack of sufficient amounts of a urea cycle substrate, such as ornithine, in presence of normal enzyme activity. A lack of ornithine results in reduced synthesis of citrulline. Such a mechanism explains the hyperammonemia of LPI. The transport of citrulline in LPI is not affected. Thus the rationale for therapy with cytrulline in LPI is to provide the hepatocyte with limiting substrate for the urea cycle, allowing the cycle to function and also providing for large amounts of intracellular ornithine synthesis. Low-protein diet and citrulline supplementation are used to correct the functional deficiency of urea cycle, but this treatment, however, is insufficient to prevent severe complications such as renal and pulmonary

involvements. Recently, Simell's group reported correction of plasma lysine by oral supplementation with the amino acid (Lukkarinen et al., 2003).

3.2 LPI complications

Renal involvement and pulmonary alveolar proteinosis (PAP) represent life-threatening complications of LPI and almost nothing is known about their pathogenesis; Neither are new strategies for treatment of LPI patients available. Interestingly, after a heart-lung transplant for severe PAP-associated respiratory insufficiency, an Italian LPI patient relapsed and died of respiratory failure (Santamaria et al., 2004). This unfortunate outcome suggests a pathogenetic role for alveolar macrophage in LPI-associated PAP. It also suggests that the lung itself is not affected by the defective y^+L activity but it is likely the target of circulating cells (macrophages?). Severe morphological alterations, associated with a marked decrease in their number, have been described in pulmonary macrophages of LPI patients (Parto et al., 1993; Parto et al., 1994). The role of nitric oxide production from arginine in LPI macrophages seems to be a promising aspect of the pathogenesis of PAP as well as of other uncommon complications of the

disease such as recurrent pancreatitis and renal involvement (Furusu et al., 1998; Al-Mufti et al., 1998). The arginine is also the precursor of important metabolic pathways such as nitric oxide (NO), polyamines, and creatine. Mutations of the SLC7A7 gene can give rise to a paradox: on one hand, low plasma levels of arginine cause vascular dysfunction through reduced NO production (Kamada et al., 2001); on the other hand, arginine could accumulate in polarized cells, for example in renal tubular cells, because of its defective efflux. This accumulation could cause an excessive production of NO that can bring to tubular damage as seen in experimental models of nephritis (Peter et al., 2003).

3.3 Genetics

Putative cationic amino acid transporters were excluded as candidate genes for LPI (Incerti et al., 1994; Lauteala et al., 1997a; Sperandeo et al., 1998). The LPI gene was mapped to chromosome 14q11.2 by linkage analysis near to the T-cell receptor α/δ chains locus in Finnish families (Lauteala et al., 1997b). By same approach, a genetic homogeneity of LPI was demonstrated between Finnish and non-Finnish patients (Lauteala et

al., 1999). Recently, the LPI gene, SLC7A7 (solute carrier family 7A, member 7), located on chromosome 14q11.2 near the TCRA locus, has been identified and mutations were reported in LPI patients (Borsani et al., 1999; Torrents et al., 1999). The human SLC7A7 gene contains 11 exons and 10 introns, harbouring ~20 kb of genomic DNA. The codon for the translation-initiator methionine is located in exon 3, whereas the termination codon is located in exon 11 (Sperandeo et al., 2000). An alternative RNA splicing in the 5' untranslated region (caused by skipping of exon 2) has been found in SLC7A7 mRNA obtained from cultured lymphoblasts, fibroblasts and peripheral blood leukocytes (Noguchi et al., 2000). SLC7A7 is expressed, among other tissues, in all tissues affected in LPI, as kidney, lung, small intestine and white blood cells (Borsani et al., 1999; Torrents et al., 1999).

SLC7A7 gene encodes the y^+LAT1 protein, which belongs to the family of heteromeric amino acid transporters (HATs). The amino acid transporter is composed of a heavy chain subunit, 4F2hc, encoded by SLC3A2 gene, and of a light chain subunit, y^+LAT1, linked to 4F2hc by a

disulfide bond (Chillaron et al., 2001). Co-expression of 4F2hc and y^+LAT1 induces sodium-independent and high affinity transport of CAA, known as y^+L activity. When sodium is present, y^+L is able to exchange CAA for neutral amino acids using an antiport mechanism. The y^+LAT1 protein contains 12 putative transmembrane domains (TM) with the amino (N-) and carboxyl (C-) termini located inside the cell. 4F2hc is a type II membrane glycoprotein consisting of a single transmembrane domain with an intracellular N- terminus and a large extracellular C- terminus. In oocytes, SLC7A7 protein forms a ~135 kDa disulphide bond-dependent heterodimer with 4F2hc, which, after reduction with ß-mercaptoethanol, results in two peptides of ~85 kDa (4F2hc) and ~40 kDa (SLC7A7). SLC7A7 protein does not induce transport of CAA in *Xenopus l*. oocytes when injected alone, but y^+L activity is detected when it is co-injected with 4F2hc (Torrents et al., 1998). There is evidence that the major function of 4F2hc is to traffic the complex to the plasma membrane and membrane topology of the heterodimer, whereas the transport itself is the function of the light chain (Chillaron et al., 2001). In absence of 4F2hc, the expression of the light chain is restricted to the Golgi area (Torrents et al., 1998).

The y^+L activity is also exerted by another heterodimeric complex composed of 4F2hc and y^+LAT2, the latter encoded by the SLC7A6 gene (Torrents et al., 1998). y^+LAT1 and y^+LAT2 share a high identity at both nucleotide and amino acid levels. y^+LAT2 protein is more ubiquitously expressed than y^+LAT1, including tissues such as small intestine and kidney where y^+LAT1 is also highly expressed. Tissues and cell types with lower transport capacity probably use y^+LAT2 to release arginine (Broer et al., 2000), explaining why y^+L activity is not altered in fibroblasts and erythrocytes from LPI patients (Boyd et al., 2000; Dall'Asta et al., 2000).

3.4 Mutations found in the SLC7A7 gene

A total of 31 SLC7A7 mutations have been described in 113 LPI patients originating from different countries (Palacin et al., 2005; Sperandeo et al., 2000; Sperandeo et al., 2005a). Identified SLC7A7 mutations include missense, nonsense, and splicing mutations, insertions, deletions and large genomic rearrangements. No LPI- associated mutations have been found in SLC3A2 gene (Sperandeo et al., 2005b). This strongly suggests that the SLC7A7 is the only gene involved as the primary cause of LPI. The most of

the LPI patients are homozygous for a specific mutation. In three patients, SLC7A7 mutation was detected in one allele only (Palacin et al., 2001; Sperandeo et al., 2005a). The LPI mutations are spread along the entire SLC7A7 gene (Table 3.1). The effect of some SLC7A7 mutations on y^+L activity has been tested by expression in Xenopus oocytes. All mutant proteins fail to co-induce y^+L transport when expressed with 4F2hc (Sperandeo et al., 2005a). Two mutations (G54V and L334R) have been reported as transport-inactivating mutations that are properly targeted to the plasma membrane (Mykkanen et al., 2000). By expression in *Xenopus laevis* oocytes and mammalian cells, the E36del mutant displayed a partial dominant-negative effect. The results reported by Sperandeo et al. (2005b) provide further insight into the molecular pathogenesis of LPI: a putative multiheteromeric structure of both [4F2hc/y^+LAT1] and [4F2hc/y^+LAT2], and the interference between y^+LAT1 and y^+LAT2 proteins (see section chapter 5). This interference can explain why the compensatory mechanism, i.e. an increased expression of SLC7A6 as seen in lymphoblasts from LPI patients, may not be sufficient to restore the y^+L system activity.

Except for the Finnish mutation (IVS6-2A→T), only four others (1670insATCA, 1471delTTCT, W242X and R410X) were found in more LPI-independent families (Palacin et al., 2001; Sperandeo et al., 2000; Shoij et al., 2002). Surprisingly, in contrast to the single mutation found in Finnish LPI alleles, 10 different mutations were identified in LPI patients from Southern Italy, which suggests a higher mutational rate in this region without any reasonable explanation (Sperandeo et al., 2000; Sperandeo et al., 2005a). Data from Italian LPI patients suggest that the apparent worldwide rarity of this disease may be the result of under-diagnosis (Sperandeo et al., 2000).

3.5 Genotype–phenotype correlation

A genotype–phenotype correlation for LPI cannot be established. As seen in Finnish and Italian LPI patients, the same genotype can give rise to extensive clinical variability (Borsani et al., 1999; Sperandeo et al., 2000). Furthermore, intrafamilial phenotypic variability has also been observed in Italian LPI patients all homozygous for the same mutation. Neither age at

onset of the disease nor compliance to treatment explain these differences in the clinical presentation. In addition to mutations of the SLC7A7 gene, other factors might have a role in the pathogenesis and clinical manifestations of LPI.

Table 3.1. List of SLC7A7 Mutations

Nucleotide change in cDNA	Effect	Exon/ intron	Ethnicity	References
Missense				
287A>C	M1L	EX3	Italian	Sperandeo et. 2000
435T>A	M50K	EX3	Italian	Sperandeo et al. 2005a
447G>T	G54V	EX3	Latvian, Estonian	Mykkänen et al. 2000
370T>C	L124P	EX3	Greek	Sperandeo MP, unpublished
740C>T	F152L	EX3	Greek	Sperandeo et al., 2005b
849C>T	T188I	EX4	Greek	Sperandeo et al. 2005a
999C>T	S328F	EX4	Japanese	Shoji et al. 2002
1284G>T	R333M	EX7	Turkish	Sperandeo et al. 2005a
1287T>G	L334R	EX8	Spanish	Torrents et al. 1999, Mykkänen et al. 2000
1299G>A	G338D	EX8	Swedish	Mykkänen et al. 2000
1444C>A	S386R	EX9	Italian	Sperandeo et al. 2000
1751T>C	S489P	EX11	Japanese	Shoji et al. 2002
Nonsense				
1012>A	W242X	EX4	Italian, Maroccan, North African	Sperandeo et al. 2000; Mykkänen et al. 2000
1514C>T	R410X	EX9	Japanese	Noguchi et al. 2000
1657C>A	Y457X	EX10	Greek, French	Sperandeo et al. 2005a
1703C>T	R473X	EX10	Australian	Torrents et al. 2000
Deletion				
242del543	Del of first 168 aa	EX2	Italian	Borsani et al. 1999
381delGGA	E36del	EX3	Greek, Albanese	Sperandeo et al. 2005a
501delCTCT	Frameshift after L71	EX3	Italian	Sperandeo et al. 2000
539delTT	Frameshift after V84	EX3	Turkish	Torrents et al. 2000
786del125	Frameshift after I166	EX4	Pakistan	Sperandeo et al. 2005a
1291delCTTT	Frameshift after L334	EX8	Spanish	Torrents et al. 2000

Table 3.1. List of SLC7A7 Mutations (continued)

Nucleotide change in cDNA	Effect on translation	Exon/ intron	Ethnicity	References
Deletion				
1471delTTCT	Frameshift after L395	EX9	Italian, German, Tunisian, Algerian	Sperandeo et al. 2000; Mykkänen et al. 2000; Sperandeo et al. 2005a
1548delC	Frameshift after F420	EX10	Arabic	Mykkänen et al. 2000
1630delC	Frameshift after A448	EX10	Japanese	Shoji et al. 2002
1673delG	Frameshift after A463	EX10	Japanese, Chinese	Shoji et al. 2002 Sperandeo et al., unpub.
1746delG	Frameshift after L486	EX11	Dutch, Norwegian	Mykkänen et al. 2000
Insertion				
831insT	Frameshift after L182	EX4	Italian	Sperandeo et al. 2000
1438insAACTA	Y384X	EX9	Canadian	Mykkänen et al. 2000
1670insATCA	Frameshift after I461	EX10	Italian	Borsani et al. 1999; Sperandeo et al. 2000
Splicing Mutations				
IVS4+1G>A	Skipping of EX4	Intron 4	Japanese, Turkish, Chinese	Noguchi et al. 2000; Sperandeo et al. 2000 Mykkänen et al. 2000 Sperandeo et al., unpub.
IVS6-2A>T	Frameshift after V298	Intron 6	Finnish	Borsani et al.1999; Torrents et 1999
IVS7+1G>T	No mRNA studies	Intron 7	Japanese	Sperandeo et al. 2000

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Chapter 4

EXPERIMENTAL WORK

4.1 Outline of the Thesis

The overall aim of this Ph.D. thesis was the elucidation of the pathophysiology of LPI. Among all the genetic metabolic disorders, LPI is one of the most puzzling diseases. CAA depletion alone cannot to account for the multiorgan involvement caused by this disorder.

Three different approaches have been designed to achieve this aim:

1. Functional studies of SLC7A7 mutations
2. Creation of mouse models of LPI
3. Creation of a human cellular model of LPI

4.2 Principal results

4.2.1. Functional studies of SLC7A7 mutations

A) The first objective of this part of the thesis was a wider knowledge of the mutational spectrum of the SLC7A7 gene in LPI patients. The work done during the PhD program has led to the identification of eight additional novel mutations of SLC7A7 in six unrelated patients (Sperandeo et al., 2005a; Sperandeo et al., 2005b) (Table 4.1). All these mutations were identified by sequencing the entire coding region and exon-intron boundaries and by Southern blot, when necessary. Any kind of mutation (large genomic rearrangements, missense and nonsense mutations, splicing mutations, insertions and deletions), spread along the entire SLC7A7 gene, was found in LPI mutant alleles (Fig. 4.1). In a noteworthy case, an affected Turkish boy resulted to be homozygous for p.R333M (c.997G>T) mutation and both parents heterozygous for this mutation. The c.997G >T change is located at the last nucleotide of exon 7 and apparently causes a missense mutation of the transcript (Fig. 4.2). In recent years, exonic nucleotidic changes, at specific position, have been shown to alter *cis*-elements that are

important for a correct splicing of the transcript (Carteggi et al., 2002). To test whether the p.R333M mutation affects the splicing mechanism, RT-PCR was performed using total RNA extracted from fibroblasts and renal tubular cells isolated from the patient. In the fibroblast cell line, RT-PCR yielded two different products, the sequencing of which showed a full length transcript, which includes the c.997G >T mutation, and a shorter transcript where exon 7 is entirely skipped. In renal tubular cells, a more appropriate tissue for SLC7A7 expression studies, RT-PCR showed three different transcripts. Sequencing of the most abundant product disclosed the skipping of exon 7. The other two products presented either deletion of exons 7 and 9 or deletion of exons 6, 7 and 9 (Fig. 4.3). Therefore in renal tubular cells, exon 7 is always skipped and premature termination codons are introduced. These results have been reported in enclosed reprint in chapter 5.

Interestingly, this part of the project has also led to the diagnosis of LPI in countries where this disorder was almost unknown (Albania, Algeria, China, Greece, Pakistan, Tunis, Turkey). This suggests that the apparent

rarity of LPI outside Italy, Finland and Japan, is probably due to misdiagnosis of this disease due to the lack of a peculiar clinical presentation and early accessible diagnostic tests (Sperandeo et al., 2000; Sperandeo et al., 2005a; Sperandeo et al., 2005b).

B) The second objective of this part of the thesis was the study of the functional effects of SLC7A7 mutations. Therefore, most of the SLC7A7 mutations have been functionally characterized by expression in *Xenopus l.* oocytes and by transfection in mammalian cell lines. For seven mutations, no arginine transport was observed when mutant cRNAs were co-injected with 4F2hc cRNA in *Xenopus l.* oocytes (Sperandeo et al., 2005a; Sperandeo et al., 2005b) (Fig. 4.4). However, two other mutations, E36del and F152L, found in a LPI compound heterozygote, provided further insight into the molecular pathogenesis of LPI. Expression studies in oocytes demonstrated that the F152L mutation moderately reduces y^+L transport activity when compared to wt SLC7A7, whereas the E36del mutant dramatically interferes with the activity of both wt SLC7A7, F152L mutant and wt SLC7A6, displaying a partial dominant-negative effect. In addition, the deletion of the E36 residue does not alter the binding of the light chain

to 4F2hc and homing to the basolateral membrane as demonstrated by transfection into MDCK cells.

The results of the this study, reported in the enclosed reprint in chapter 6, provided further insight into the molecular pathogenesis of LPI: a putative multiheteromeric structure of both [4F2hc/y^+LAT1] and [4F2hc/y^+LAT2], and the interference between y^+LAT1 and y^+LAT2 proteins. This interference could explain why the compensatory mechanism, (i.e. an increased expression of SLC7A6 as seen in lymphoblasts and renal tubular cells from LPI patients) may not be sufficient to restore the y^+L system activity.

C) The third objective of this part of the thesis was the study of the biological role of other putative CAA transporters, such as SLC7A6 and 4F2hc. By *in silico* analysis, we defined the genomic structure of SLC7A6, which covered a region of 60 kb on chromosome 16q13 and contained 11 exons. No mutations of SLC7A6 were found in LPI patients sequencing all exons. These results reasonably exclude SLC7A6 as a modifier gene in LPI. Finally, 4F2hc cDNA was sequenced in eight independent LPI patients. No mutations were found thus excluding that the co-existence of 4F2hc

mutations might play a role in the phenotypic variability of LPI (Sperandeo et al., 2005b).

Table 4.1: Novel mutations identified in SLC7A7 gene.

Base change[a]	Amino Acid change	Mutation type	Exon
c.106_106delGGA	p.E36del	Deletion	3
c.149T>A	p.M50K	Missense	3
c.370C>T	p.L124P	Missense	3
c.453T>C	p.F152L	Missense	3
c.563C>T	p.T188I	Missense	3
IVS3_IVS4	//	Deletion exon 4	//
c.997G>T	p.R333M	Missense/splicing mutation	7
c.1371C>A	p.Y457X	Nonsense	10

[a]base number according to the international nomenclature and 1 is the adenine of the initiator codon, Met. Nucleotide numbers are derived from SLC7A7 cDNA (GenBank reference sequence AF092032.1).

Figure 4.1: SLC7A7 mutations found in LPI patients.

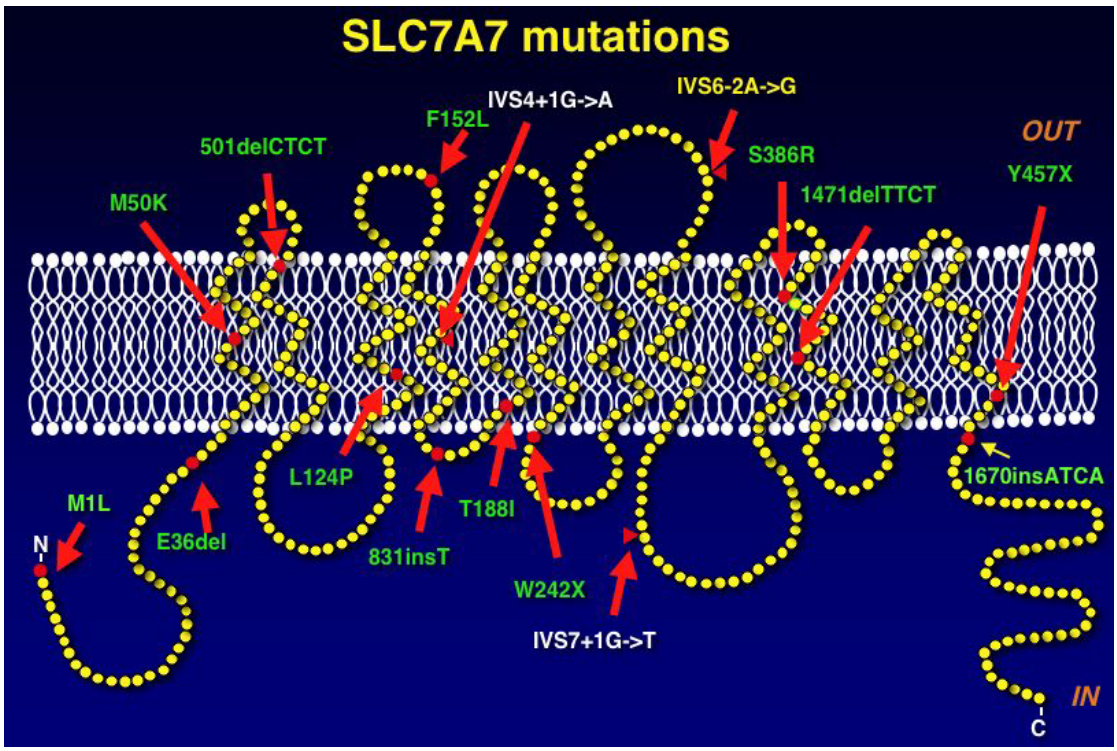


Figure 4.2: c.997G>T (R333M) mutation.

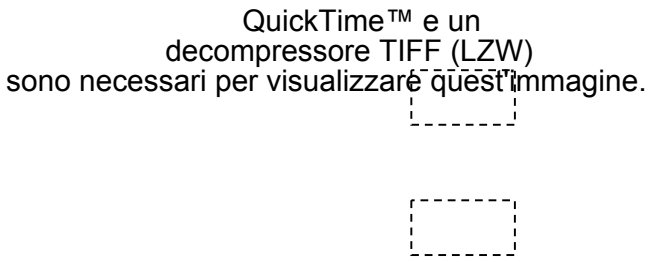


Figure 4.3: *Exon splicing of p.R333M RNA from patient.* **A)** Deletion of exon 7 as found in mRNA from fibroblast and renal tubular cells; **B)** Deletion of exons 7 and 9 as found in mRNA from renal tubular cells; **C)** Deletion of exons 6, 7 and 9 as found in mRNA from renal tubular cells.

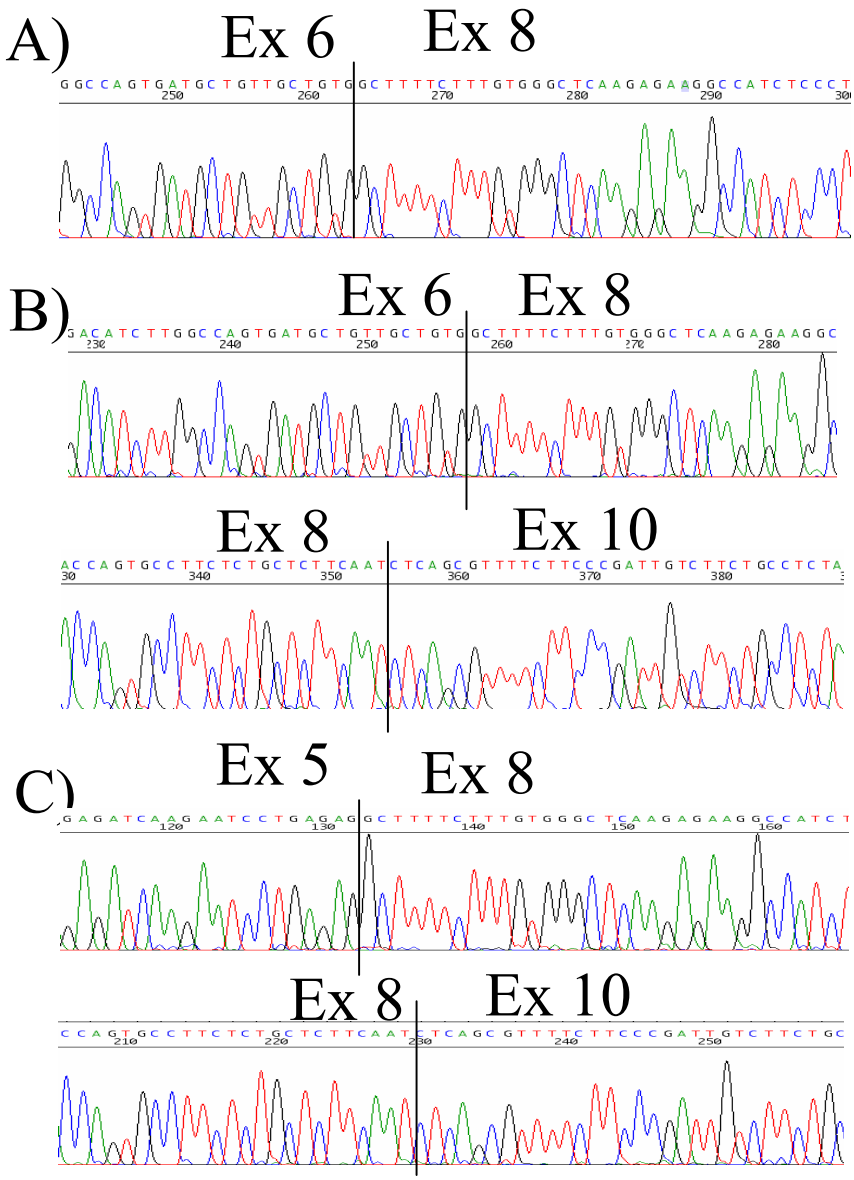
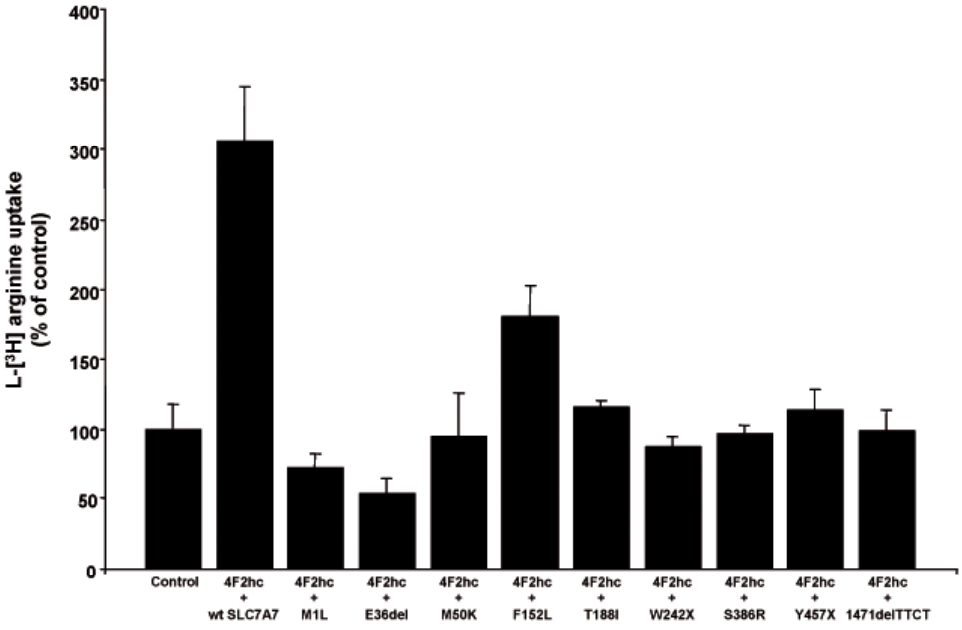


Figure 4.4: *Functional analysis of y^+LAT1 mutants in X. laevis oocytes.* *X. laevis* oocytes were injected with with 4F2hc and either wt SLC7A7 or each mutant cRNA (molar ratio 1:1; 10 ng:10 ng). All SLC7A7 mutations abolished the L-arginine transport except for the F152L mutation, which caused an increase in L-arginine transport activity to 1.6 times that of the activity induced by 4F2hc expressed alone.



4.2.2. Mouse models of LPI

A. Slc7a7 null mouse.

Generation of Slc7a7 null mouse

The objective of this part of the thesis was the creation of a murine model of Slc7a7 knock-out (Sperandeo et al., 2003; Sebastio et al., 2005; manuscript in preparation). As with most inherited disorders, an animal model is necessary to study both pathogenetic mechanisms of the disease and innovative therapeutic approaches.

The Slc7a7 gene maps to chromosome 14 (syntenic region of human chromosome 14). The amino acid sequence of the mouse and human proteins share high homology (90.4% identity; 98.6% similarity). Co-immunoprecipitation experiments demonstrated that human 4F2hc forms disulfide-linked heterodimers with mouse y^+LAT1 upon co-expression in Xenopus oocytes. In addition, there is clear evidence that mouse y^+LAT1 induces a system y^+L activity (Peiffer et al., 1999).

A constitutive Slc7a7 knock-out mouse was generated by screening of the Omnibank library of mouse embryonic stem cell clones using the

Slc7a7 cDNA as a query (Lexicon Genetics Inc, Texas). The ES clones, containing the Slc7a7 gene, which was mutated by insertional mutagenesis using retroviral vectors, were identified and characterized (Fig. 4.5) (Zambrowicz et al., 1998).

Embryo studies and histological examinations

Genotyping analysis of 606 offspring of Slc7a7 heterozygote matings revealed a non-Mendelian distribution of genotypes (28.4% wild type, 68.6% heterozygotes, 3% null homozygotes), being the null allele underscored among the genotypes. Only two null mice survived whereas 16 homozygotes died within 24 hours of birth and had a delayed pattern of growth. The proportion of Slc7a7 (-/-) pups was 8.3-fold lower (3%) than that expected by Mendelian transmission of a recessive trait (25%). This result indicated that the Slc7a7 (-/-) allele resulted in embryonic lethality. Gender did not influence the percentage of heterozygous animals, which also appeared healthy and fertile. They had no overt physiological disadvantage in appearance or somatic growth at maturity (body weight, behaviour, organ size) compared with their wild type littermates. To establish the timing of the observed embryo lethality of the null mouse,

numerous embryos from heterozygous crosses were collected at different embryonic stages (E16.5 and E18.5) and genotyped. We found that the percentages of (-/-), (+/-), and (+/+) embryos perfectly matched with a Mendelian distribution for a recessive trait. This expected Mendelian distribution suggests that most Slc7a7 (-/-) pups died within hours of birth due to maternal cannibalism. As a matter of fact at E 16.5 stage, the Slc7a7 (-/-) embryos were already smaller than +/- or +/+ littermates and a few null mice observed at birth appeared less vital that unaffected pups (Fig. 4.6 a and b). None of the null embryos showed gross morphological abnormalities. Using anti-4F2hc antibodies, histopathological examination revealed differences in renal morphology. The embryos (-/-) showed a decreased number of glomeruli (Fig. 4.7).

Adult Slc7a7 (-/-) mice: analysis of the phenotype

The only two surviving Slc7a7 (-/-) animals showed growth failure compared to their siblings since birth (Fig. 4.8). After weaning, they were fed on a low protein diet and citrulline supplementation. Both Slc7a7 (-/-) animals showed reduced fertility. The older animal, a male, survived until 25 months of age, when the treatment with citrulline and low protein diet

was interrupted and then replaced by a normal diet. After 2 weeks, this animal presented hypotonia, tremors, progressive weight loss and died after 2 weeks later. After an intercross with the Slc7a7 (-/-) male, the second animal, an 11-month-old female, had a pregnancy ended prematurely at E18.5 and cannibalised all the pups. The protein overload, probably, caused its death at 48 hours after delivery. By gross and histological studies both null animals did not display significant differences compared to wild type animals.

Biochemical characterization of Slc7a7 (-/-) adult mice

To assess whether the CAA transport was altered in Slc7a7 (-/-) adult mice, CAA levels and orotic acid were measured in urine. Both Slc7a7 (-/-) mice showed an elevated excretion of arginine and ornithine in their urine when compared to wild type and Slc7a7 heterozygous animals (Fig. 4.9). No increased lysine excretion was found in affected animals when compared with wild type and heterozygous mice. This result might be explained by the extremely low intake of protein after weaning. In addition, this recalls what was reported in LPI patients when put on a restricted protein diet (Simell, 2001).

Expression studies by Real-time PCR

Real-time PCR was used to evaluate the relative abundance of mRNA in a set of selected genes involved in CAA transport (Slc7a1, Slc7a2, Slc7a6, Slc7a7, and Slc3a2), in arginine metabolism (Arg1, Arg2, Nos2) and in embryo growth (Igf1, Igf2, Igf2r). The expression of these genes was tested on mRNA, which was extracted from embryos and adult mouse tissues, and from placenta.

Expression studies in placenta tissue

In placentas isolated from embryos Slc7a7 (-/-) at E18.5 age, the expression of Slc7a1, Slc7a2, Slc7a6 and Slc3a2 genes were identical to those observed in wild type embryos. In this tissue, the expression of Slc7a7 mRNA was 12.5 times lower than the controls, thus confirming the disruption of the Slc7a7 gene function (Fig. 4.10).

Expression studies in Slc7a7 (-/-) embryo tissues

In selected tissues (intestine, kidney, lung, liver) of Slc7a7 (-/-) embryos (E18.5) mRNA Slc7a7 was markedly reduced compared to controls (Fig.4.11 a and b).

As for genes involved in arginine metabolism, the expression of Arg1, Arg2 and Nos2 was tested. Interestingly, in liver tissues the Arg1 expression was 5 times lower than that of wild type animals. In contrast Nos2 expression was up-regolated 8 times compared to wild type animals. The same results were also obtained for Slc3a2 (upregulated 2 times when compared to wild type animals) and for Slc7a6 (upregulated1.6 times when compared to wild type animals) (Fig. 4.11 a and b).

Expression studies in Slc7a7 (-/-) adult tissues

Liver and lung tissues displayed up-regulation of Slc3a2 (2 times higher than wild type animals in both tissues), up-regulation of Slc7a6 (5.7 and 3.3 times higher than wild type animals in liver and lung, respectively). We also found up-regulation of Arg1 in liver (2.5 times higher than wild type animals) and Arg2 in kidney (2 times higher than controls) (Fig. 4.12 a and b).

Igf1, Igf2 and Igf2r assays in null embryos

To elucidate the pathogenesis of the severe intrauterine growth failure we tested the expression of Igf1 and Igf2 and Igf2r, which have a key role

in foetus-placental growth throughout gestation. Igf2 and Igf2r resulted in the down-regulation of Slc7a7 (-/-) placentas (Fig. 4.13). The Igfs and Igf2r were also down-regulated in all selected tissues from Slc7a7 (-/-) embryos (Fig. 4.14 a and b).

In conclusion, the Slc7a7 gene disruption was always associated with a severely delayed foetal growth, indicating the importance of this amino acid transporter's role during embryonic growth (Sperandeo et al, manuscript in preparation). This severe reduced growth was also observed in Slc7a7 (-/-) adult mice. This is also a phenotypic characteristic seen in LPI patients due to protein malnutrition and a deficiency of essential amino acid such as lysine. Igf1, Igf2 and Igf2r were down-regulated in Slc7a7 (-/-) placenta and in target tissues (intestine, kidney, lung, liver) isolated from embryos E18.5. These results appear to be related to the disruption of the Slc7a7 gene function and could explain the severe reduced growth found in embryos and adult Slc7a7 (-/-) mice.

By Real-time PCR we found that the expression of Arg1 in liver tissue from Slc7a7 (-/-) embryos was down-regulated in contrast with the up-regulation of Nos2, whereas in the same tissue from adult null mice, we

found up-regulation of both genes. These results suggest that the accumulation of arginine into the cells, due to the defective efflux, might induce a temporally differentiated regulation of genes involved in the catabolism of the arginine.

Figure 4.5: *Generation of KO mouse. Structure of the gene trap vector integration site in the Slc7a7 locus.* The retroviral construct is inserted in the intron downstream from the ATG-containing Slc7a7 exon. Northern blot analysis of kidney RNA obtained from embryos at stage E18.5.The blot, hybridyzed with the entire Slc7a7 cDNA probe, shows signals in wt and Slc7a7 (+/-) embryos. Transcripts were undetectable in kidney RNA from Slc7a7 (-/-) embryos.

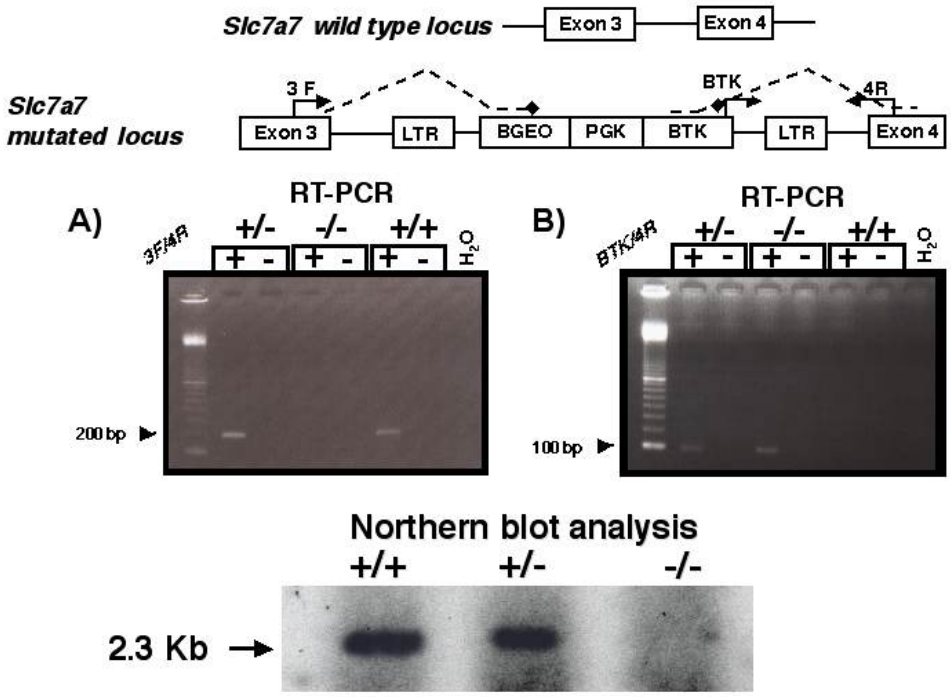
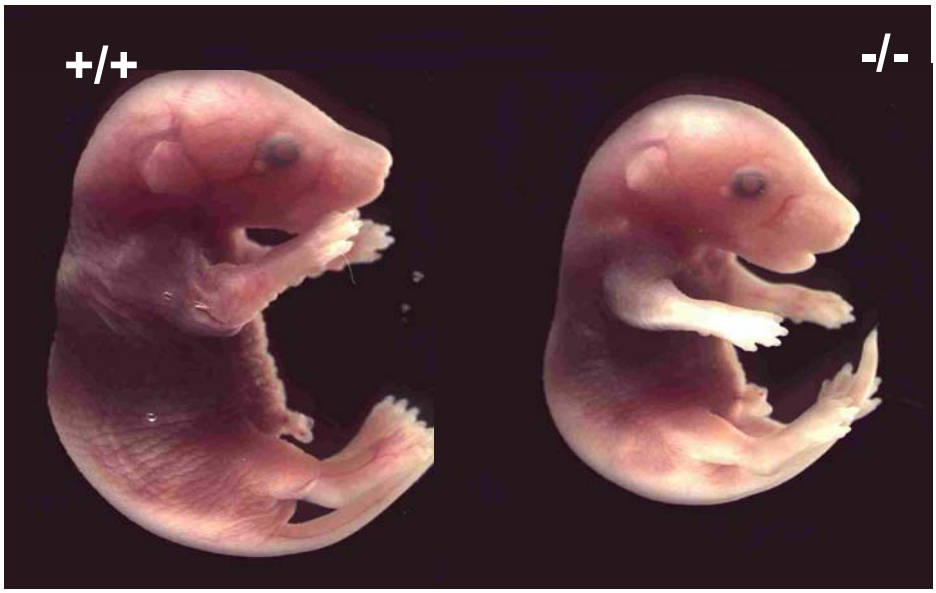


Figure 4.6: *Growth rate and body morphology.* a) wt (+/+) and KO (-/-) embryos at E18.5. b) wt (+/+) and KO (-/-) neonate pups.

a)



b)

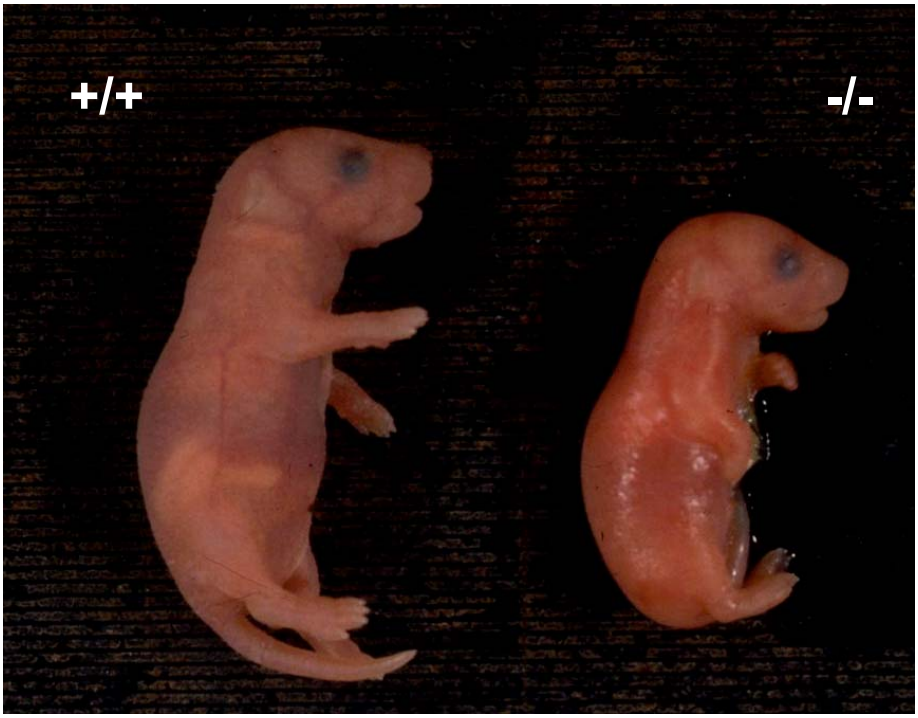
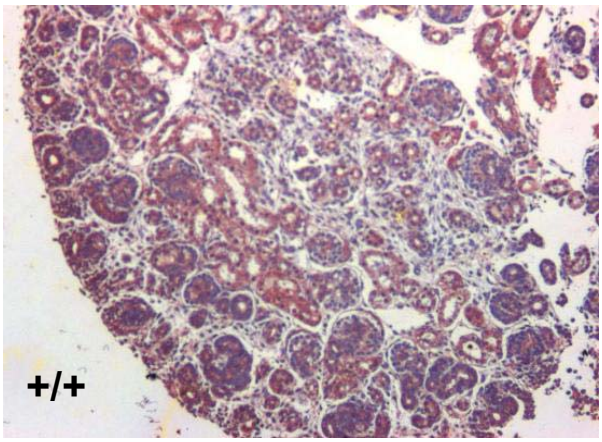


Figure 4.7: *Immunohistochemistry in kidney section.* wt (+/+) and KO (-/-) embryos at E18.5. Slc7a7-/- embryo tissues display very low 4F2hc protein due to marked delay of renal development.



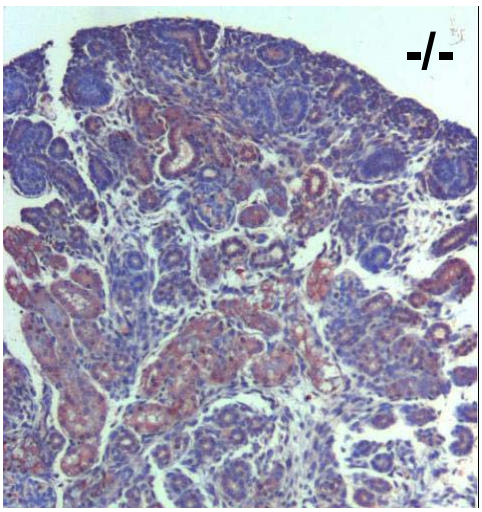


Figure 4.8: *Growth rate and body morphology.* wt (+/+) and KO (-/-) mice.

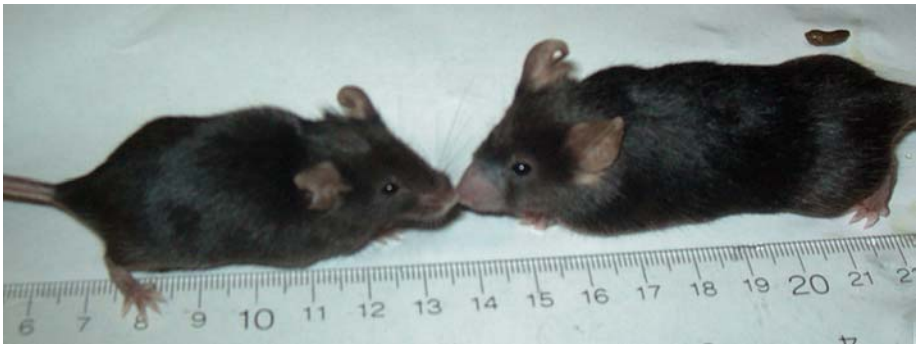
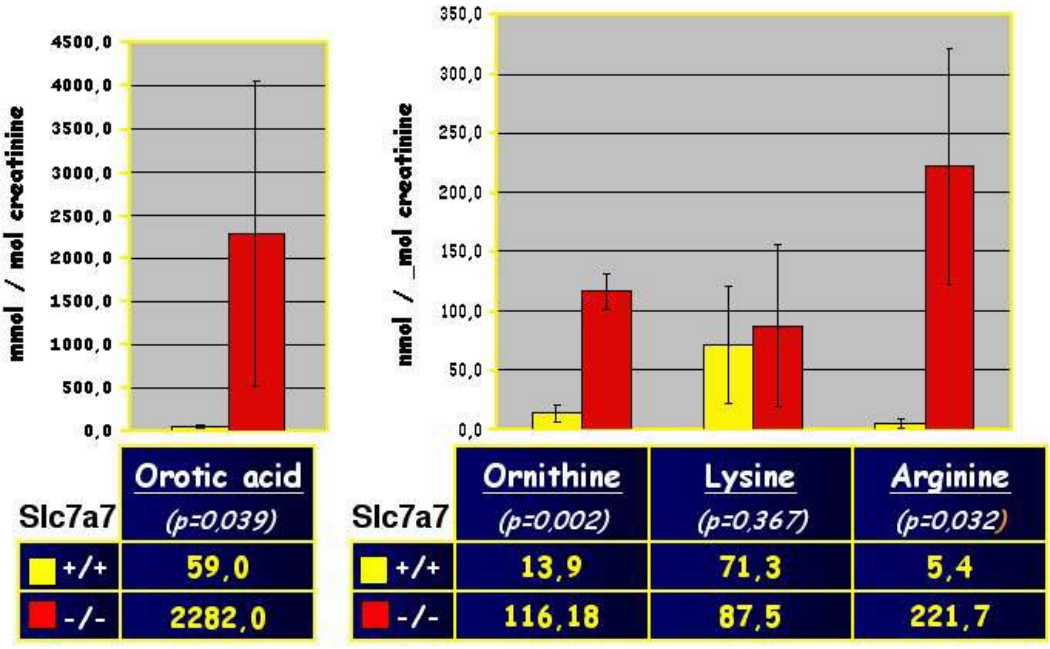


Figure 4.9: *Biochemical characterization of Slc7a7 (-/-) adult mice.* Metabolic derangement found in adult Slc7a7 (-/-) mice (urine from 2 animals).



Slc7a7	Orotic acid (p=0,039)
+/+	59,0
-/-	2282,0

Slc7a7	Ornithine (p=0,002)	Lysine (p=0,367)	Arginine (p=0,032)
+/+	13,9	71,3	5,4
-/-	116,18	87,5	221,7

Figure 4.10: Expression of Slc3a2, Slc7a6 and Slc7a7 in placenta from embryos (-/-) at 18.5 stage *vs* controls.

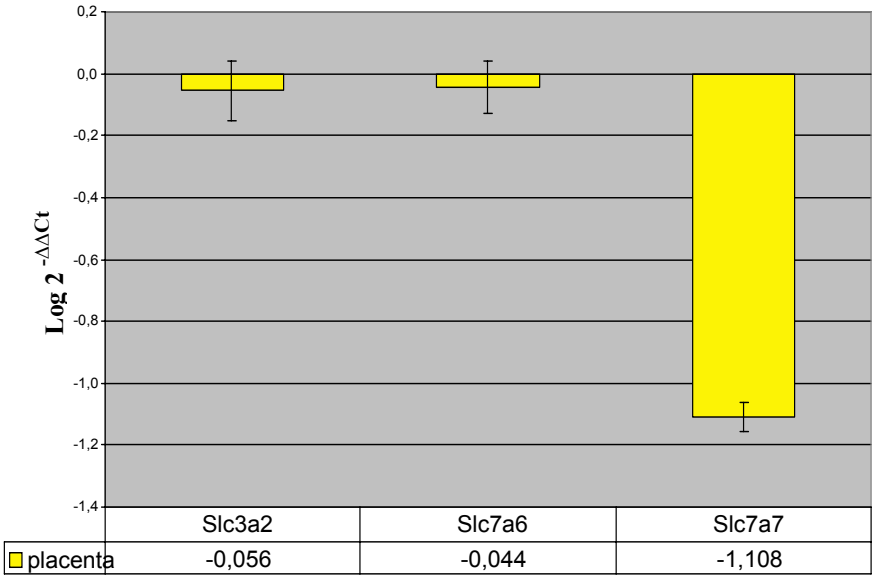
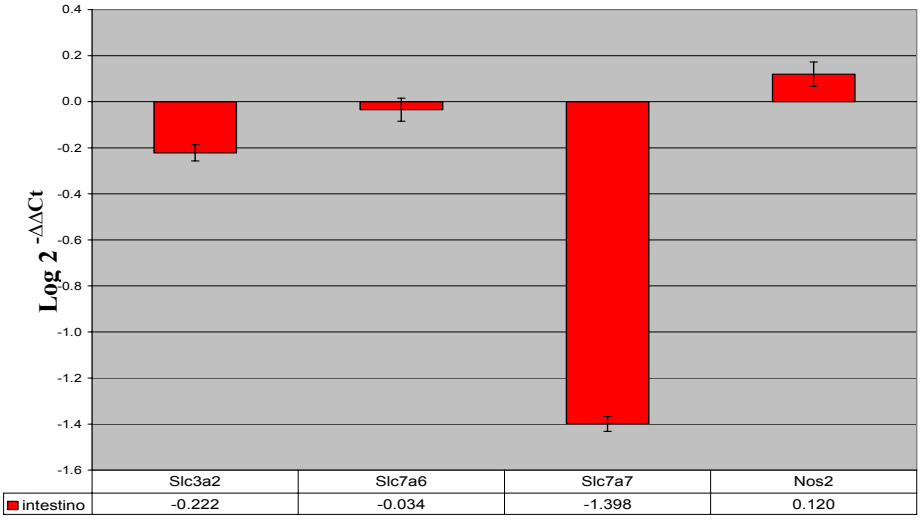


Figure 4.11a: Expression of Slc3a2, Slc7a6, Slc7a7, Arg2 and Nos2 in selected tissues from embryos (-/-) at stage 18.5 *vs* controls.



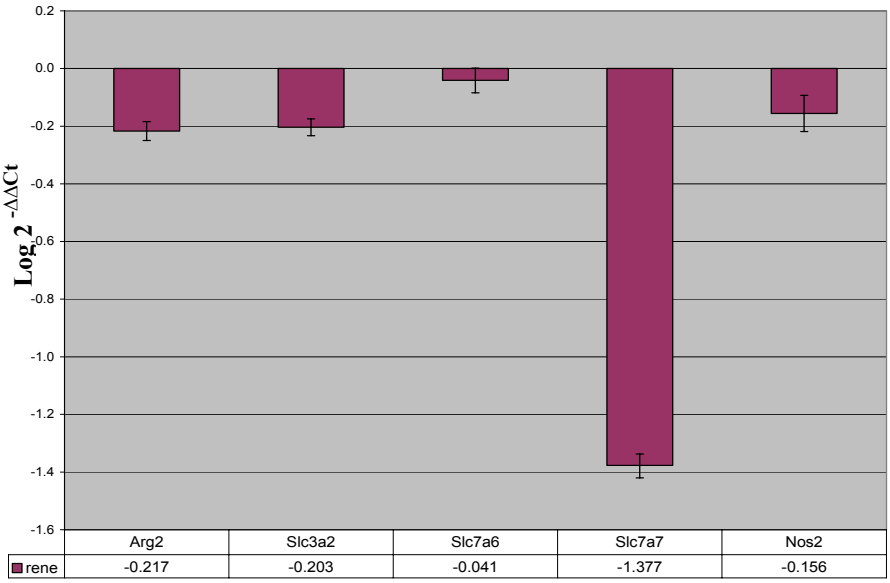
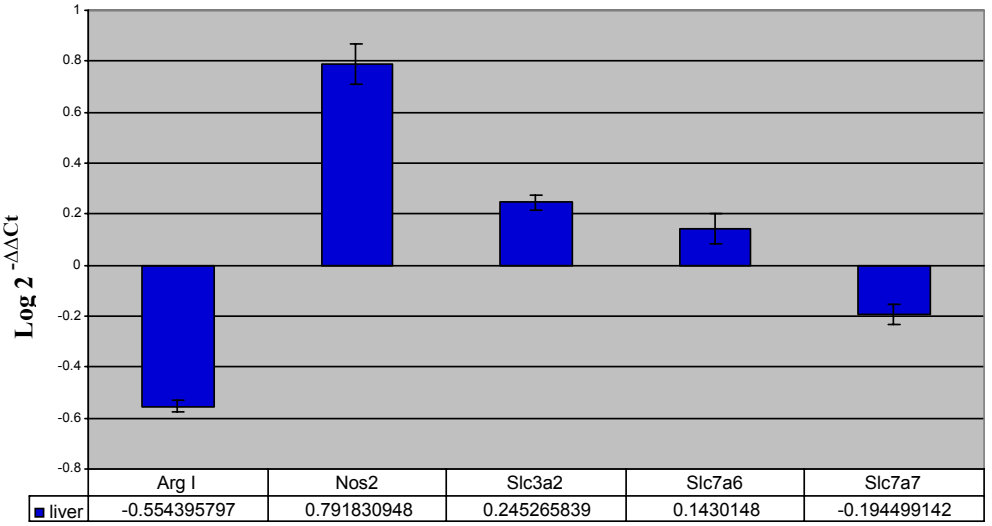


Figure 4.11b: Expression of Slc3a2, Slc7a6, Slc7a7, Arg1 and Nos2 in selected tissues from embryos (-/-) at stage 18.5 *vs* controls.



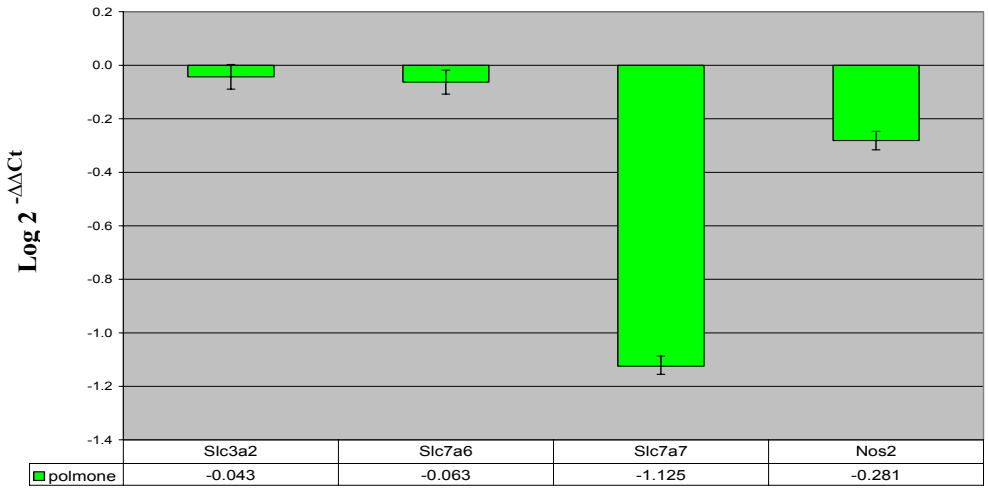
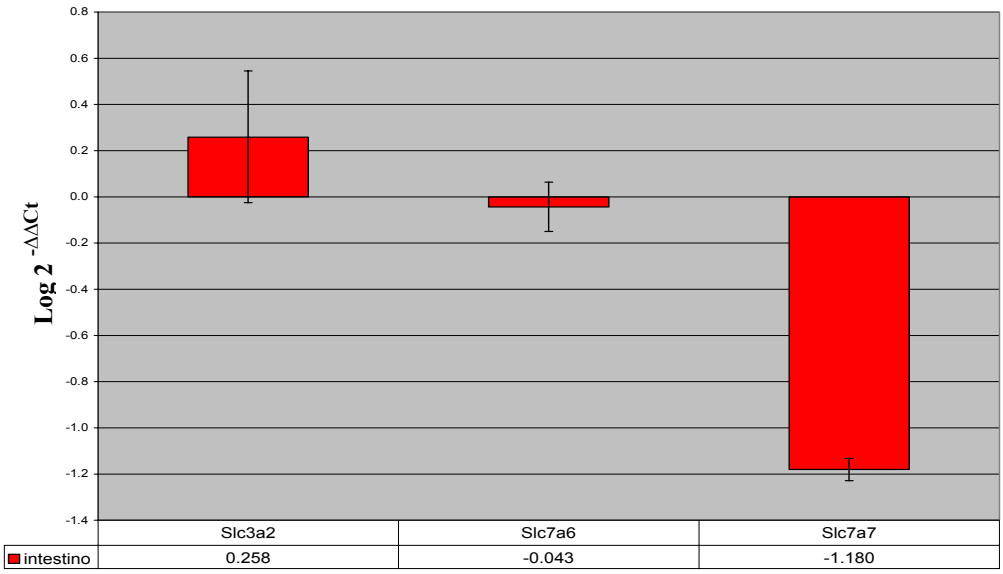


Figure 4.12a: Expression of Slc3a2, Slc7a6 and Slc7a7 in selected tissues from adult mice (-/-) *vs* controls.



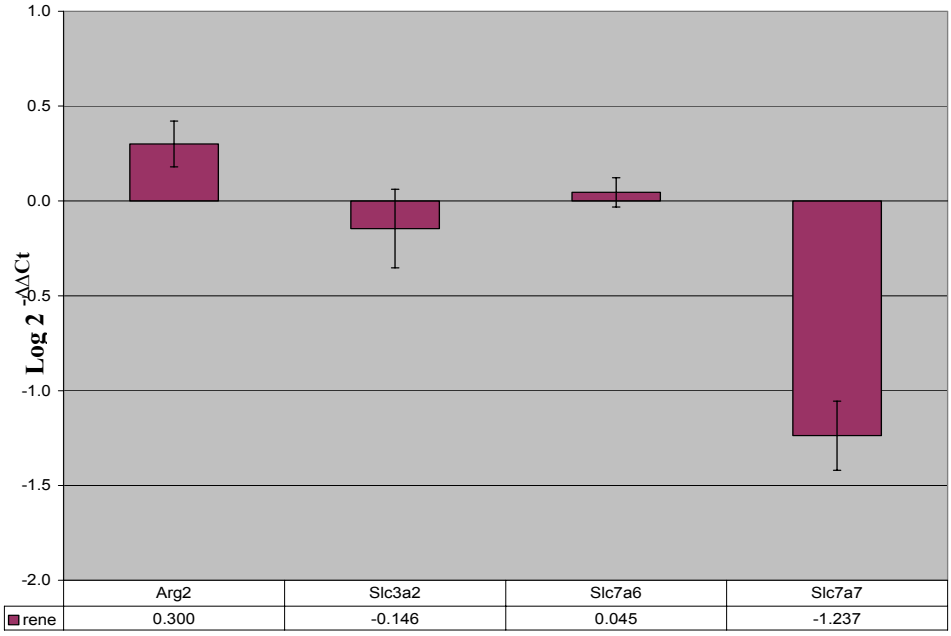
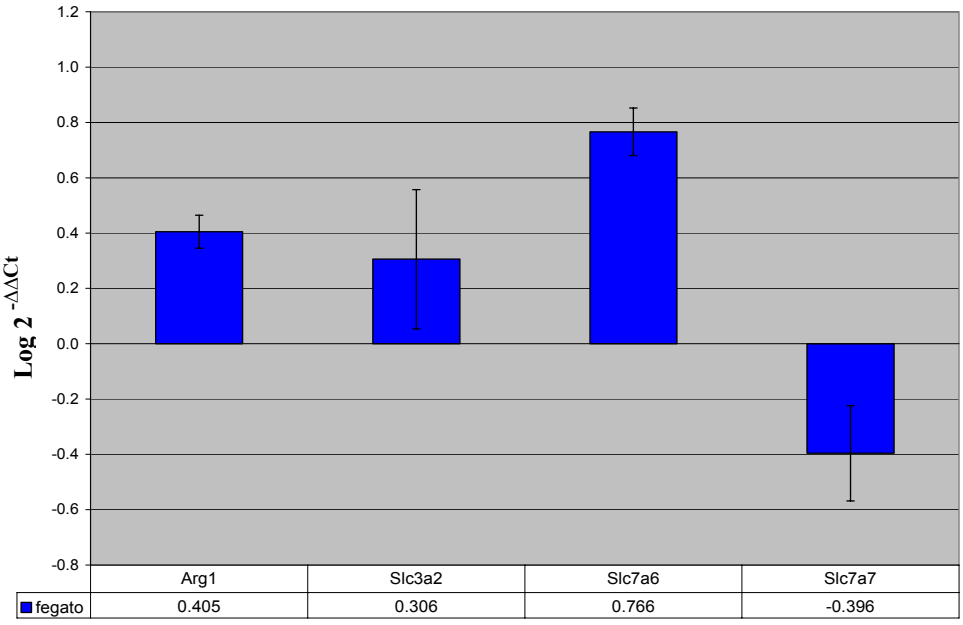


Figure 4.12b: Expression of Arg1, Slc3a2, Slc7a6 and Slc7a7 in selected tissues from adult mice (-/-) *vs* controls.



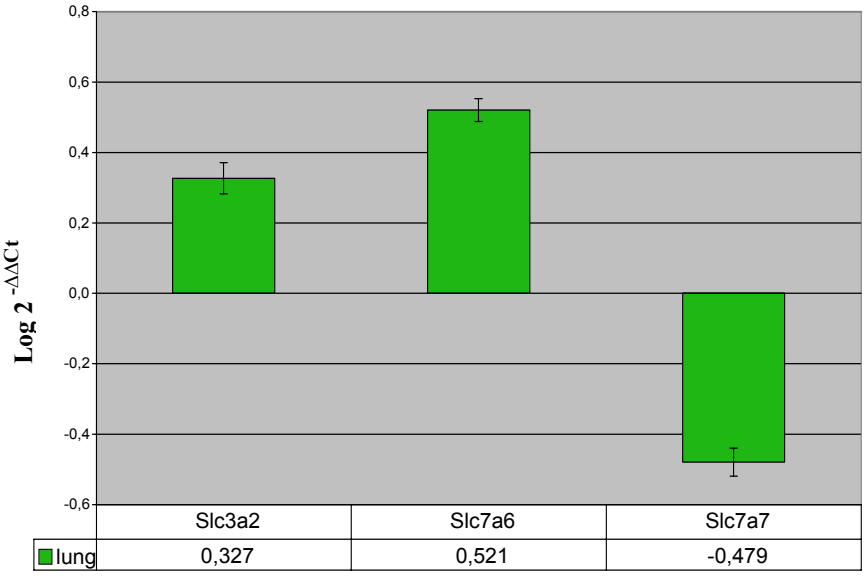


Figure 4.13: Expression of Igf1, Igf2 and Igf2r in Slc7a7 (-/-) placentas *vs* controls.

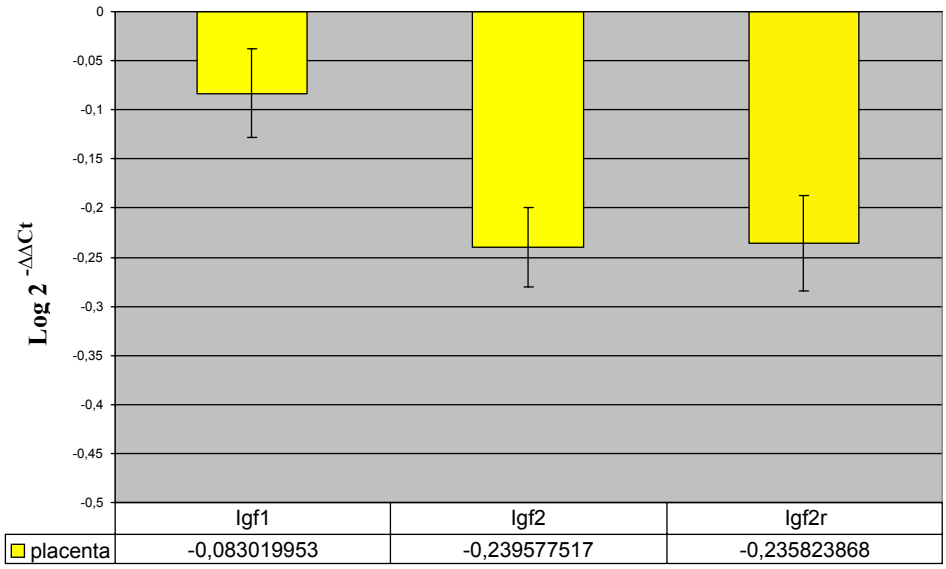
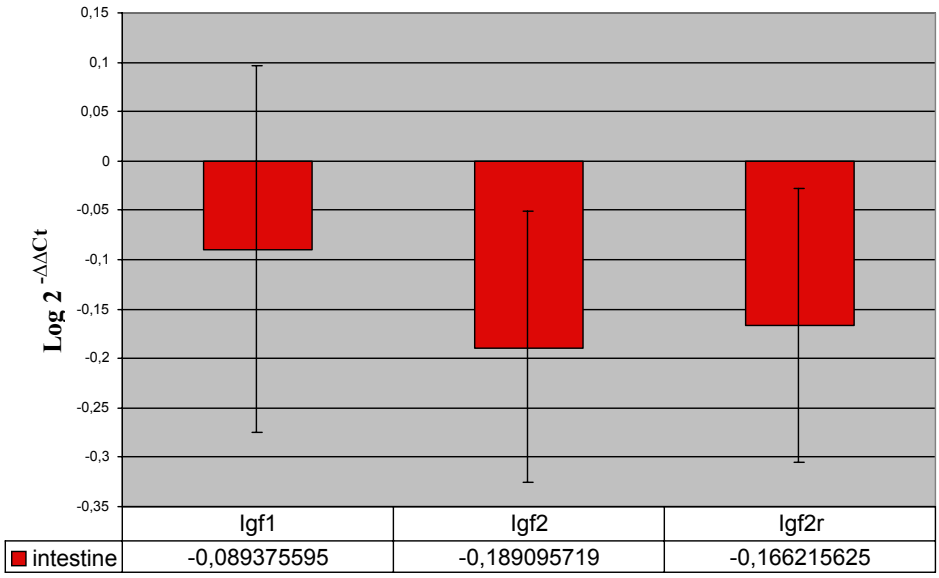
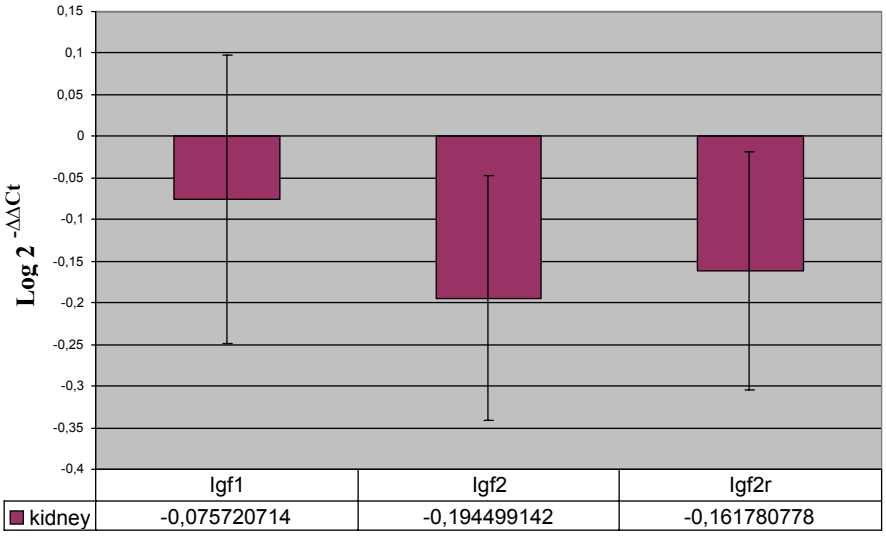


Figure 4.14a: Expression of Igf1, Igf2 and Igf2r in selected tissues of Slc7a7 (-/-) embryos at stage 18.5 *vs* controls.

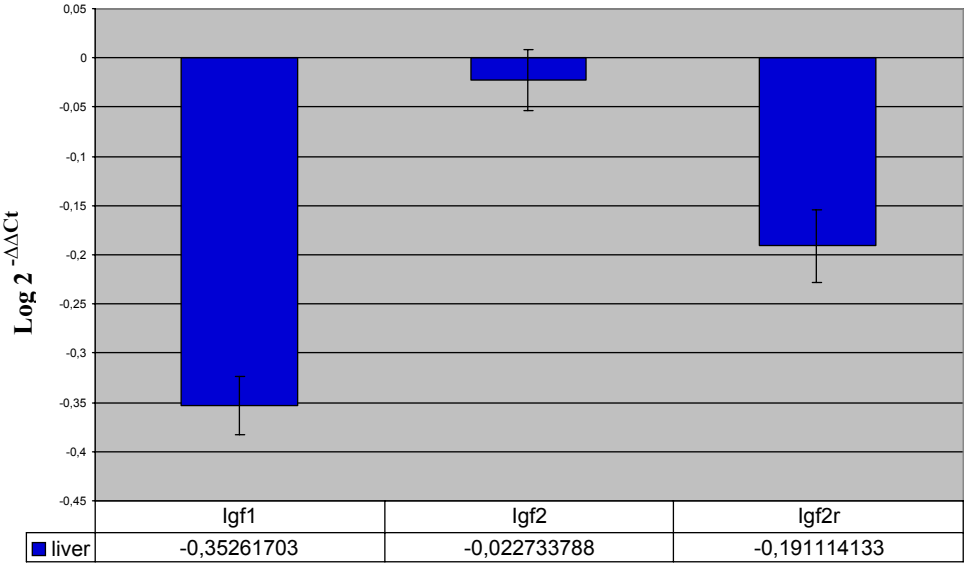


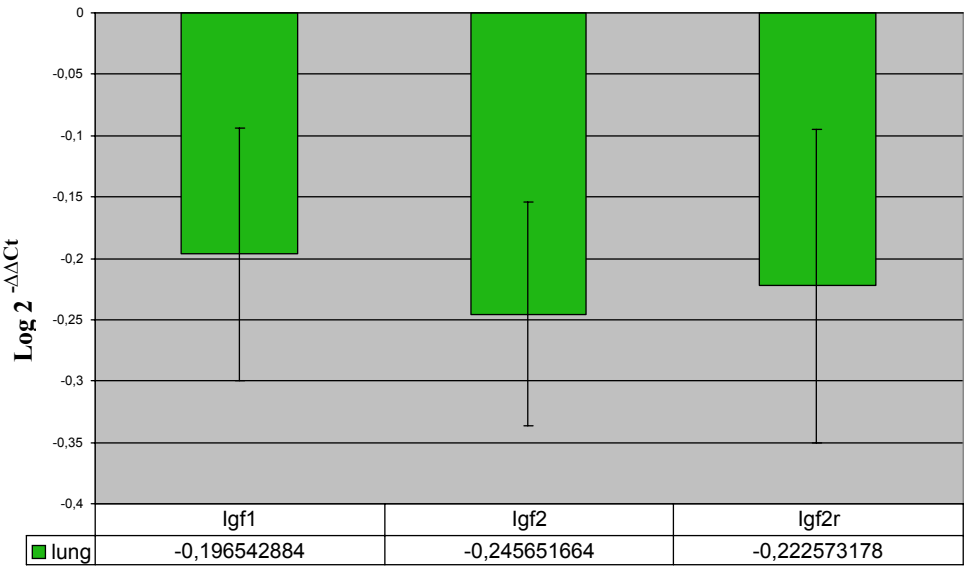
	Igf1	Igf2	Igf2r
intestine	-0,089375595	-0,189095719	-0,166215625



	Igf1	Igf2	Igf2r
kidney	-0,075720714	-0,194499142	-0,161780778

Figure 4.14b: Expression of Igf1, Igf2 and Igf2r in selected tissues of Slc7a7 (-/-) embryos at stage 18.5 *vs* controls.





4.2.2. Mouse models of LPI

B. Slc7a7 conditional mouse model

Generation of a conditional LPI mouse model

With the exception of only 2 animals, our Slc7a7 (-/-) mouse model turned to be a lethal model, which prevented further studies. To circumvent this problem, a conditional Slc7a7 gene mouse model, is now being developed using the Cre/loxP recombination system, This conditional mouse will be generated by floxing exons 3 and 4 of the Slc7a7 gene and breeding with specific Cre-expressing strains (Fig. 4.15). We have already produced the vector with the LoxP sites flanking the ortholog genomic segments necessary for the recombination. The strategy of targeting vector construction required (Fig. 4.16):

a. bioinformatic analysis of the mouse Slc7a7;
b. amplification of the 2 arms of the vector (about 3 kb and 6 kb, respectively);
c. 12 separeted subcloning along with screening by PCR, restriction analysis and sequencing of each subclone

d. for each step of subcloning a further round of sequencing was required (total number of sequencing: 210 and synthesis of 52 different primers).

With this strategy the molecular defect is expressed in a tissue-specific way by specific intercrossing with mouse strains that selectively express the Cre-recombinase. A large number of tissue-specific Cre-expressing mouse strains are already available (http://www.mshri.on.ca/nagy/cre.htm database). With this mouse model, single aspects of the disease can be investigated without the risk of a full-blown clinical picture, which caused the lethality in the constitutive knock-out mouse.

Figure 4.15: *LPI conditional mouse model.* Schematic representation of the Slc7a7 conditional mouse model strategy.

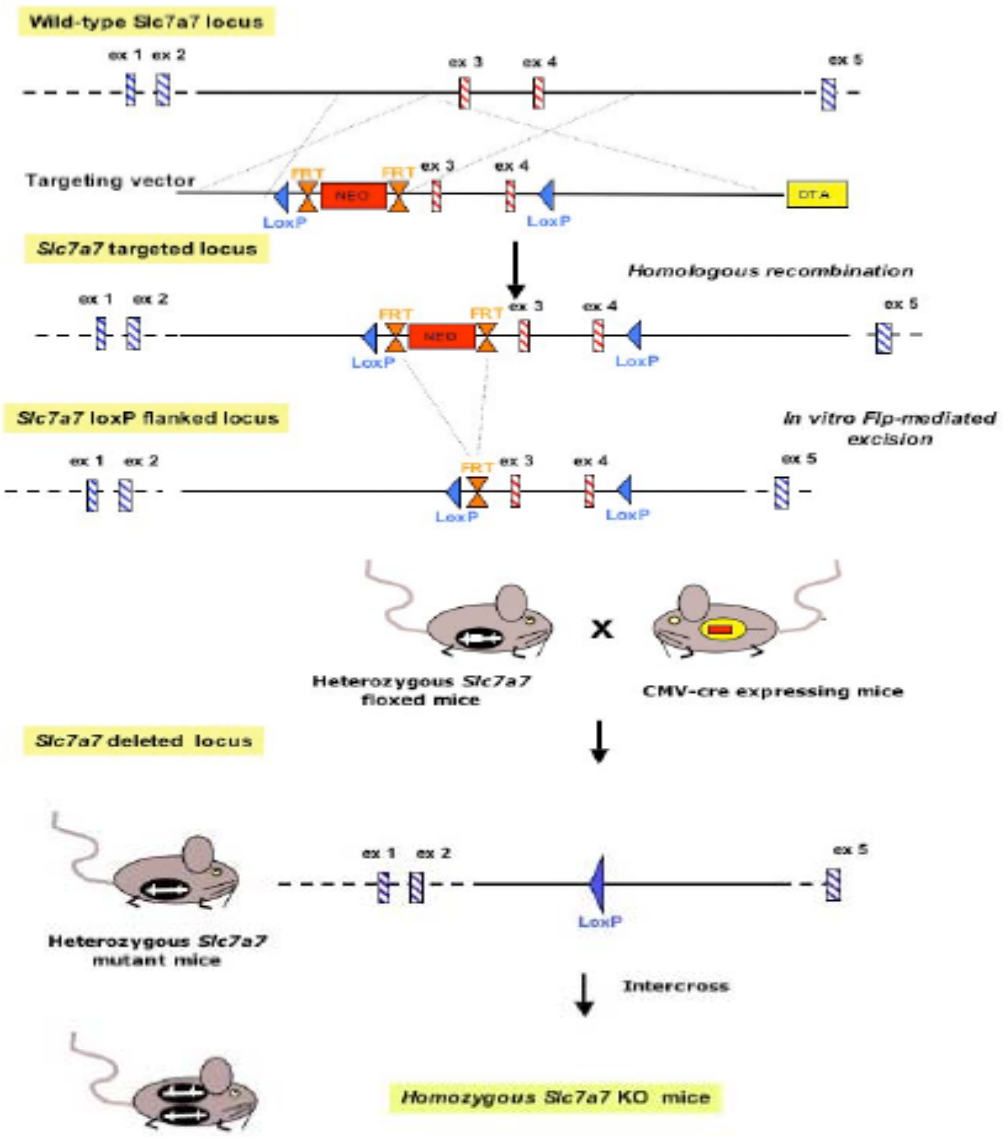
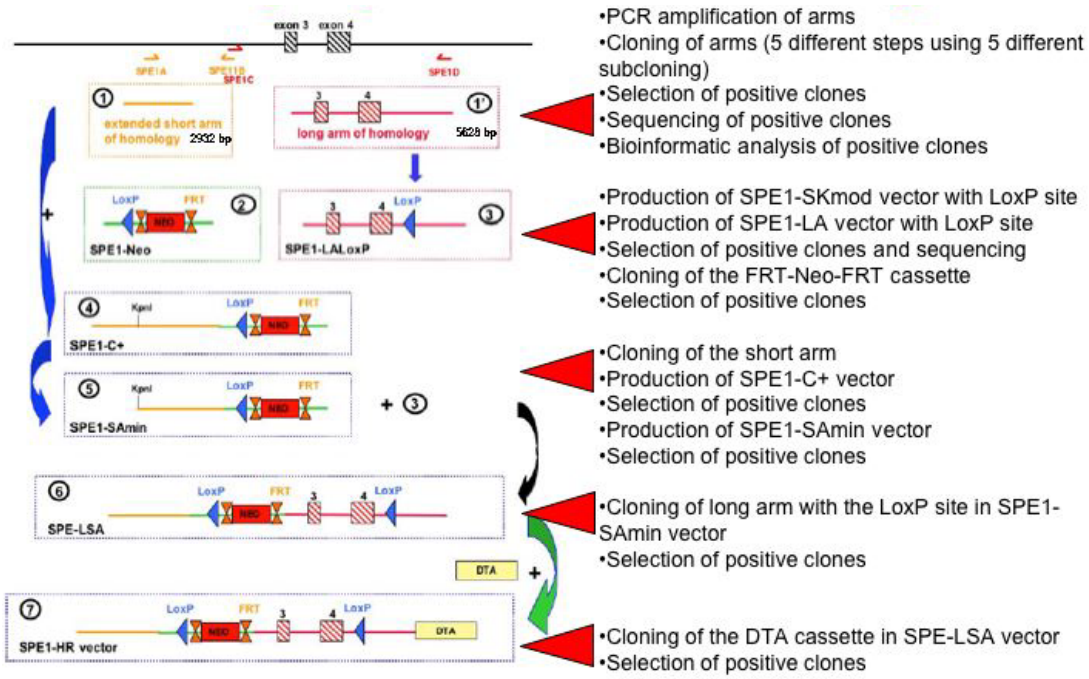


Figure 4.16: *LPI conditional mouse model.* Targeting vector construction strategy.



4.2.3. Creation of a human cellular model of LPI

The objective of this part of the thesis was the creation of a human cellular model of LPI in order to study the role of nitric oxide in the pathogenesis of the complications of the disease. Renal involvement is a life-threatening complication of LPI and almost nothing is known about its pathogenesis. A key-role might be played by unbalanced intracellular homeostasis of arginine. In fact, arginine is the precursor of important metabolic pathways leading to the synthesis of nitric oxide (NO), polyamines and creatine (Fig. 4.17).

Intestine and kidney are the main target tissues of LPI. While intestinal biopsy is an invasive procedure, often not allowed by parents, cultivation of renal tubular can serve as a tool to study SLC7A7 function in the target tissue. Renal tubular cells (RTC) can be isolated and grown from patients with heavy shedding of these cells in urine (Inoue et al., 2000). We have set the methodologies for the isolation and for the cultivation of RTC from the urine of LPI subjects as well as patients with severe tubular nephropathy (Sperandeo et al., 2005c). Urine from these patients were collected and cells were selectively grown in an appropriate medium to

isolate those of renal tubular origin (Fig. 4.18). The origin of RTC was tested by immunohistochemical analysis (Fig. 4.19). In RTC from LPI patients we demonstrated very low level of SLC7A7 transcript as seen by Real-Time PCR. Moreover, CAA transport was tested in these cells and found to be defective as expected in LPI (Fig. 4.20).

Therefore, RTC is the first cellular model where the functional defect of LPI has been demonstrated (sperandeo et al., manuscript in preparation).

The relationship between nitric oxide (NO) production and LPI seems to be an emerging aspect of this disorder. L-arginine is the substrate for the NO synthase-catalyzed reactions and its availability is crucial for the cellular rate of NO synthesis. Deficiency of arginine may lead to a decrease of NO production *in vivo* (Kayanoki et al., 1999). Markedly reduced endothelial-dependent vasodilation, via a decrease of NO production, was demonstrated in LPI patients (Kamada et al., 2001). On the other hand, increased levels of NO, through inducible oxide nitric synthase (NOS2), are fundamental in the pathogenesis of glomerulonephritis and pancreatitis, two clinical presentatios in LPI (Furusu et al., 1998; Al-Mufti et al., 1998).

We investigated NO metabolism in RTC isolated from the urine of LPI patients and controls (Sperandeo et al., 2005c). NO production was investigated in RTC by flow cytometry and DAF-2 diacetate method after stimulation with arginine or citrulline load. Preliminary results indicate an overproduction of NO in LPI cells when compared to controls (Fig. 4.21). In addition, the effect of NO and oxidative stress on cell viability of these cells (apoptosis) was examined in parallel using flow cytometry. Preliminary results suggest that intracellular arginine accumulation induces necrosis in LPI cells (Fig. 4.22).

To investigate the fate of the arginine entrapped intracellularly, the expressions of ARG2 and NOS2, two key-enzymes of the arginine intracellular metabolism, were also studied by Real-Time PCR (Fig. 4.23). The results showed increased expression of both enzymes indicating a constitutional activation of both early and late repair phase of the inflammatory response as seen in experimental glomerulonephritis. Interestingly, also the expression of SLC7A6 mRNA, another gene exerting a y^+L transport activity at the basolateral membrane, was higher than

controls. This might suggest an attempt to restore normal intracellular arginine levels.

Figure 4.17: Metabolic sources and fates of the arginine and citrulline.

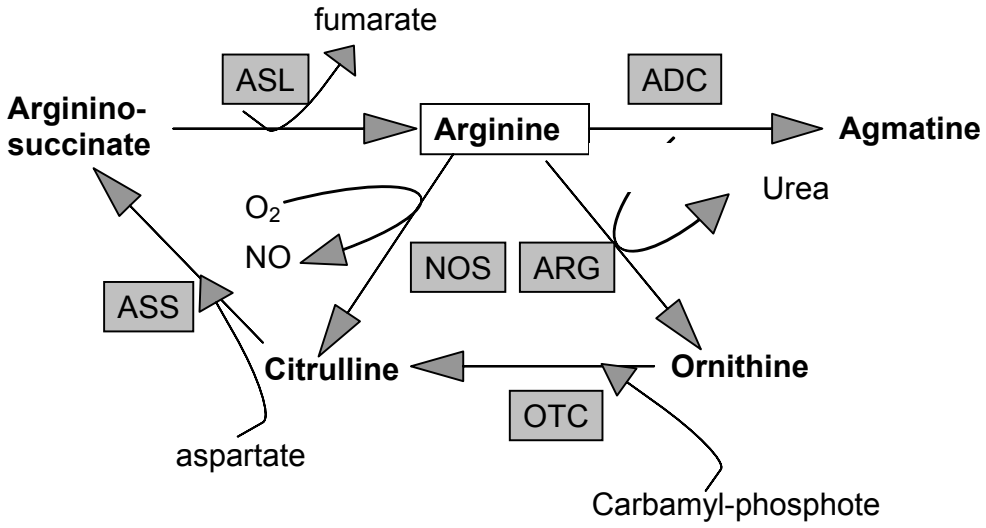


Figure 4.18: Phenotype of renal tubular cells isolated from urine of LPI patients.

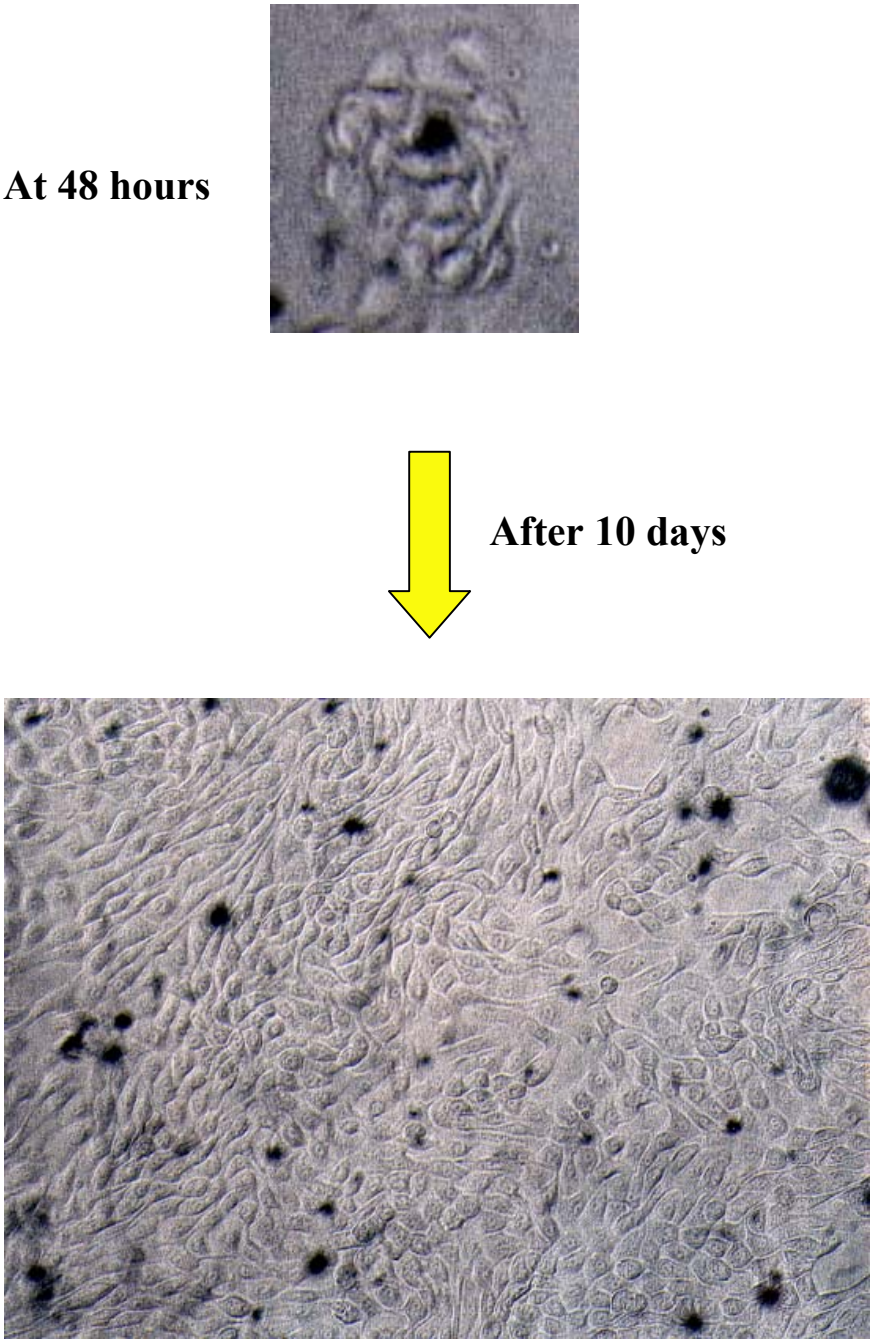


Figure 4.19: Immunohistochemical characterization of renal tubular cells.

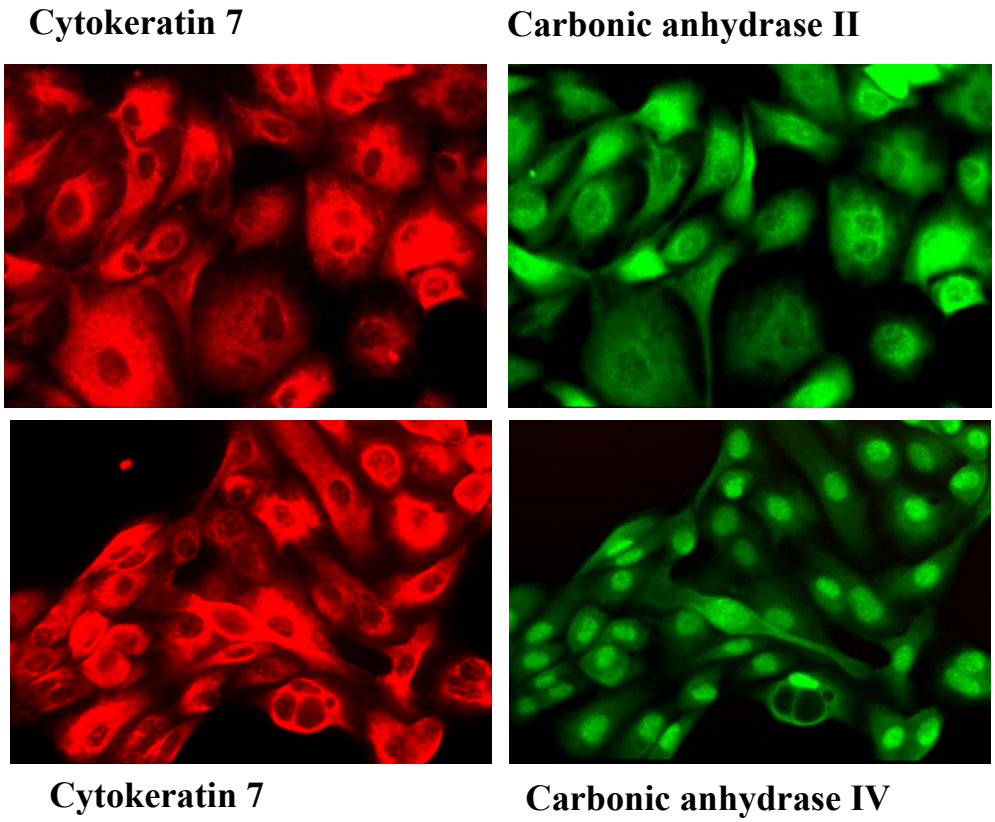


Figure 4.20: Reduced y^+L activity in renal tubular cells isolated from urine of LPI patients.

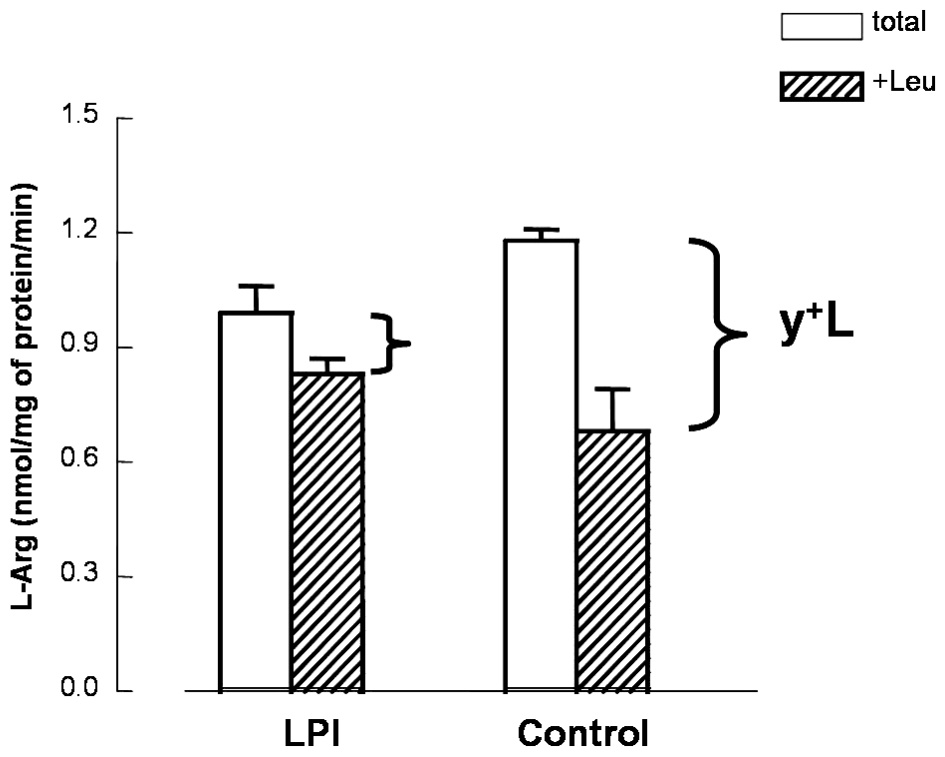


Figure 4.21: Overproduction of nitric oxide in renal tubular cells isolated from urine of LPI patients.

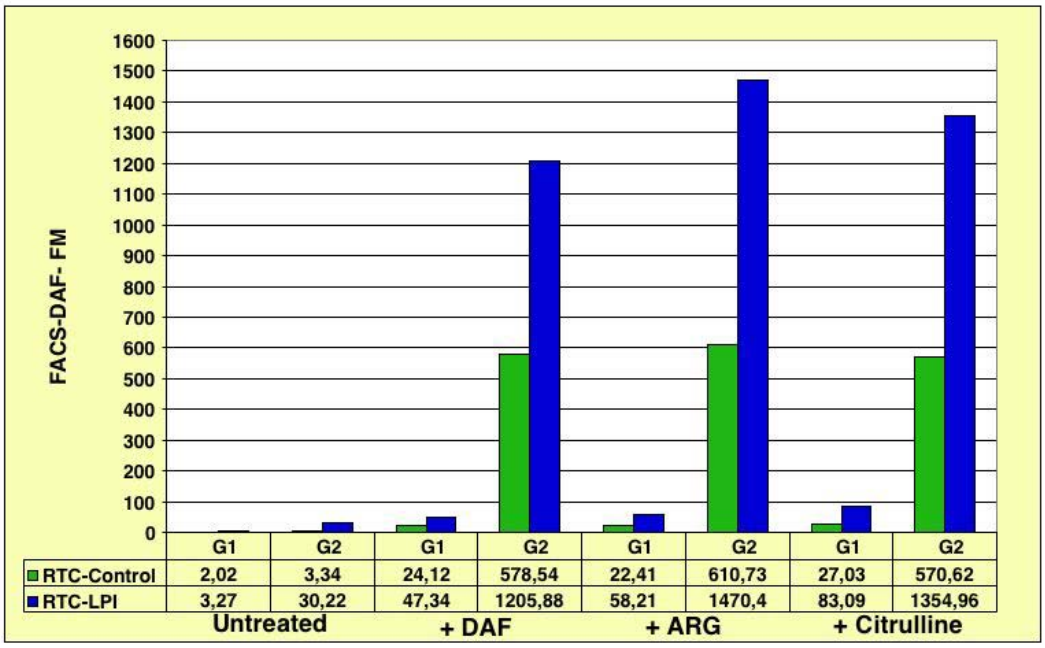


Figure 4.22: Apoptosis analysis in renal tubular cells isolated from urine of LPI patients.

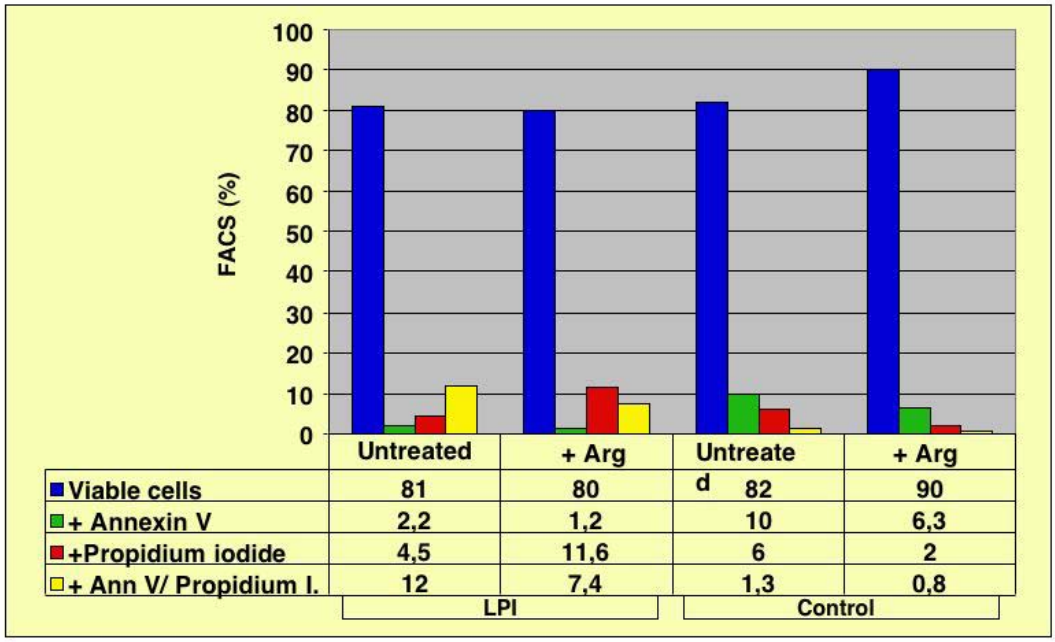
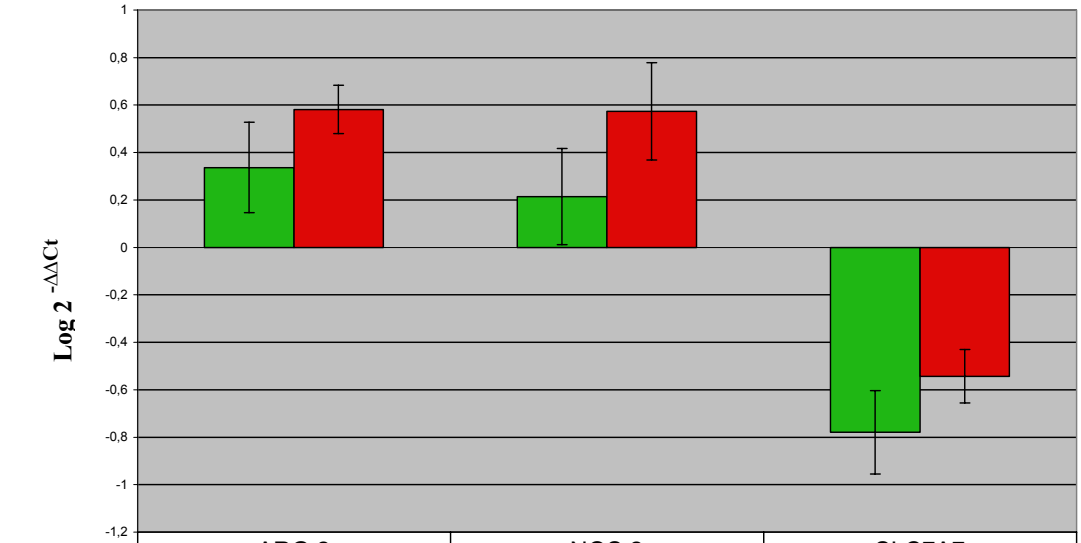


Figure 4.23: Real-time PCR in renal tubular cells isolated from urine of two independent LPI patients *vs* controls.



	ARG 2	NOS 2	SLC7A7
Patient 1	0,336459734	0,214313897	-0,779891912
Patient 2	0,581494542	0,572871602	-0,543633967

4.3 Materials and Methods

4.3.1 Cell cultures

Skin fibroblasts were cultured using standard methods. Renal tubular cells were isolated from urine samples and cultivated according to Inoue et al. (2000).

4.3.2 Mutation analysis of SLC7A7 gene

All exons and intron-exon boundaries of the SLC7A7 gene were amplified from genomic DNA extracted from peripheral blood samples of all family members as previously reported (Sperandeo et al., 2000). Total RNA was extracted from fibroblast and renal tubular cells from patient homozygote for R333M mutation by guanidium/phenol method. First-strand cDNAs were synthesized by RT-PCR by SuperScript Rnase H-Reverse Transcriptase (BRL) and oligo(dt) as primer. Five μl of cDNA was amplified using a forward primer located in exon 5 (5'-TGTTCTCCTACTCAGGCTGGG-3') and a reverse primer located in exon 10 (5'-CAGAGGCAGAAGACAATCGG-3'). PCR products were

cloned into the pBluescript II SK- vector and sequenced by an automated system (ABI 377 DNA, Applied Biosystems, Monza, Italy).

4.3.3 Generation of Mutant Constructs, expression in X. laevis oocytes and L-[^{3}H] arginine uptake

By using the Quick-Change site-directed mutagenesis kit (Stratagene, Amsterdam-Zuidoost, The Netherlands), seven mutant SLC7A7 cDNAs were constructed each containing one of the following mutations: p.M1L, p.E36del, p.M50K, p.F152L, p.T188I, p.W242X, p.S386R, p.Y457X, c.1185_1188delTTCT (previously known as 1471delTTCT). Oocytes (stage V and VI) from *X. laevis* females were isolated by an enzymatic procedure in Sperandeo et al. (1998). Each oocyte was microinjected with either the wt SLC7A7 cRNA or each mutant cRNA together with 4F2hc (molar ratio 1:1; 10 ng:10 ng), using a microinjector device (Drummond Scientific Company, Broomall, PA, USA) as reported in Sperandeo et al.. (2005a). Data are showed as the mean ± SE of L-[^{3}H] arginine uptakes in oocytes from 3 different frogs and expressed as a percentage of the L-[^{3}H] arginine uptake as found in uninjected oocytes (control, 842±85.5 cpm).

4.3.4 Arginine uptake assays in renal tubular cells

Measurements of L-[^{3}H] arginine uptake were obtained according to protocols described elsewhere (Dall'Asta et al., 2000). The uptake tudies were performed in collaboration with Prof. V. dall'Asta (Units of General and Clinical Pathology, Department of Experimental Medicine, Universita degli Studi di Parma, Parma, Italy).

4.3.5 Immunostaining of renal tubular cells

For immunofluorescence studies, renal tubular cells were plated into wells of 8-chamber slides (Nalge Nunc International Corp.,Naperville, USA). After 48-72 hours, cells were fixed with 4% paraformaldehyde for 5 min, permeated with 0.02% Triton X-100 for 15 min, and blocked with 10% bovine serum albumin for 1 hours. Cytokeratin 7, Carbonic Anhydrase II and Carbonic Anydrase IV proteins were detected using specific antibodies. Nuclei were counterstained by DAPI (Roche, Germany; 1□g/□l, dilution 1:4000), and cells were examined by fluorescence microscopy (Zeiss Axioscope 2 equipped with Axiovision camera) with appropriate filters. Digital images were saved and analyzed

with Adobe PhotoShop (Adobe Systems Inc., Mountain View, California, USA).

4.3.6 Mouse care and treatment

Mice were housed at 24°C on a fixed 12-hour light/ 12-hour dark cycle and had free access to water and rodent chow. All Slc7a7 (-/-) mice were fed with a diet containing 8% protein (a special diet produced *ad hoc* by Mucedola, Italy) and citrulline supplementation (100 mg/mL solution; administered at 100 μg/g of body wieght/die) (SIGMA).

4.3.7 Genotyping of Slc7a7 embryos and mice colony

Genomic DNA was extracted by standard protocols from tail biopsies isolated from 3-week old progeny of matings and from E (embryonic day) 16.5 and 18.5 embryos. A PCR method was used with primers that recognize Slc7a7 alleles on mouse chromosome 14. The oligonucleotide primers used were:

A: 5'-GATGAAGTGATCCTAGC CGTAG-3';

B: 5'-GCAGCTCTATGTCACAGGGCG-3'; and

LTR2 5'-AAATGGCGTTACTTAAGCTAGCTTGC-3'.

These primers are located in intron 3 of the Slc7a7 gene, surrounding the gene-trap insertion site. About 100 ng of purified from mice and embryos tail genomic DNA was used as template for PCR in a 25 μl reaction volume. PCR conditions were: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 39 cycles.

4.3.8 Animal experiments

Two groups of mouse embryos were used for this study. One group consisted of three wild type animals that were killed at E16.5 and E18.5. Another group consisted of three Slc7a7 (-/-) killed at same embryonic age.

Two groups of adult mice were used for this study. One group consisted of three wild type adult animals and another group consist of two Slc7a7 (-/-) adult mice.

4.3.9 Gross and immunohistochemical studies

Whole mount embryos and selected tissues such as intestine, liver, lung and kidney, isolated from embryos and adult mice, were fixed by immersion in 4% parafolmaldeyde for 24-48 hours. After fixation,

tissues were routinely processed and embedded in paraffin. In summary, 4 mm sections were deparaffined in xylene and rehydrated and then stained with hematoxylin and eosin for morphological assessment. For immunohistochemical studies, the endogenous peroxidase activity was blocked by incubation in 3 % hydrogen peroxide (SIGMA) in PBS for 10 min. Sections were washed in TBS, then blocked with 10% BSA for 1 hour at room temperature (RT). The primary antibody, goat anti-mouse CD98 (Santa Cruz, Biotechnology, Santa Cruz, CA, USA), was diluted 1:200 in TBS containing 2% BSA for 1 hour at RT. Slides were washed in TBS, then incubated with the biotinylated secondary antibody, rabbit anti goat (Dako) diluted 1:200 at RT for 1 hour. Bound antibodies were detected by an avidin-biotin complex method (Sigma) with 3', 3'-diaminobenzidine as chromogenic substrate following the manufacturer's instructions (SIGMA). Negative controls were included in each experiment by omission of the primary antibody. After counterstaining of nuclei with hematoxylin, sections were mounted with aquamount (BDH). The sections were viewed with a Nikon microscope. Pictures were processed and assembled using Adobe PhotoShop.

To prevent observer bias, all histological specimens were coded and examined without knowledge of animal age and genotype.

4.3.10 Real time PCR

Real time PCR was performed according to the reccomendation supplied by Applied Biosystems (http://europe.appliedbiosystems.com/). For all genes of interest, primers were purchased from Applied Biosystems as 'AssaysOnDemand®. Primers were chosen to result in amplicons of 70-150 bp that span intron-exon boundaries to avoid the effect of potentially contaminating genomic DNA. A 25 μl PCR reaction volume was prepared using 2 μl (about 40 ng) cDNA as template with sense and antisense primers and the labelled probe. Reactions were run in 96-well optical reaction plates using an Applied Biosystems 7300 Real-Time PCR System. Thermal cycles were set at 95°C (10 min) and then 35 cycles at 95°C (15 sec) and 60°C (1 min) with auto ramp time. For analysing the data, the threshold line was set automatically and it was in the linear range of the amplification curves for all mRNA in all experimental runs. All reactions were performed in triplicate. The abundance of the target mRNAs was calculated relative to a reference

mRNA (b_2 microglobulin). Relative expression ratios were calculated as $R=2^{(Ct(b2\ microglobulin)-Ct(test))}$, where Ct is the cycle number at the threshold and the test stands for the tested mRNA. The interval of confidence was fixed at 95%.

4.3.11 Urine collection and analysis

From each experimental animal, urine was collected in a mouse metabolic cage (Momoline, Italy) after a 2-day adaption period. Twenty-four-hour urine was collected and immediately frozen. Amino acid levels and orotic acid content were performed by GC–MS analysis in collaboration with Prof. G. Corso (Federico II University, Naples, Italy and University of Foggia, Foggia, Italy) .

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Chapter 5

Sperandeo MP, Annunziata P, Ammendola V, Fiorito V, Pepe A, Soldovieri MV, Taglialatela M, Andria G, Sebastio G.

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MUTATION IN BRIEF

Lysinuric Protein Intolerance: Identification and Functional Analysis of Mutations of the SLC7A7 Gene

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Lysinuric protein intolerance (LPI) is an inherited hyperdibasic aminoaciduria caused by defective cationic amino acid (CAA) transport at the basolateral membrane of epithelial cells in the intestine and kidney. LPI is relatively common in Finland and a few clusters of patients are known in Italy and Japan. The SLC7A7 gene, mutated in LPI patients, encodes the y+LAT-1 protein which is the light subunit of a heterodimeric CAA transporter. We performed the mutation analysis in seven probands from five unrelated LPI families and identified five novel SLC7A7 mutations (p.M50K, p.T188I, p.R333M, p.Y457X, and c.499+?_629-?). By expression studies in X. laevis oocytes or patient's renal tubular cells, the functional analysis of altogether eight SLC7A7 mutations is here reported. Noteworthy, the p.R333M mutation, caused by a G to T transversion of the last nucleotide at 3' end of exon 7, disrupts a functional splicing motif generating misspliced transcripts. Three of the novel mutations were found in patients originating from Greece and Pakistan thus increasing the list of ethnic backgrounds where LPI mutant alleles are present. This reinforces the view that the rarity of LPI outside Finland might be ascribed to misdiagnosis of this disease. © 2005 Wiley-Liss, Inc.

KEY WORDS: Lysinuric protein intolerance; y+LAT-1; mutation; splicing; *X. laevis* oocytes; SLC7A7

INTRODUCTION

Lysinuric protein intolerance (LPI; MIM# 222700) is an inherited hyperdibasic aminoaciduria caused by defective cationic amino acid (CAA; L-arginine; L-lysine; L-ornithine) transport, normally exerted by y^+L system, at the basolateral membrane of epithelial cells in the intestine and kidney (Palacin *et al.*, 2001).

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Clinical findings in LPI patients include: vomiting, diarrhea, failure to thrive, hepatosplenomegaly, bone marrow abnormalities, osteoporosis, episodes of coma, mental retardation, lung involvement (mainly as alveolar proteinosis), altered immune response and chronic renal disease (Simell, 2001). The diagnosis of LPI is often difficult due to an unspecific clinical presentation. Therefore it is not surprising that LPI is mainly known in Finland, Italy and Japan where clinicians are accustomed to diagnose it (Palacin *et al.*, 2001).

LPI is caused by mutations of the solute carrier family 7A member 7 (SLC7A7) gene (Borsani *et al.*, 1999; Torrents *et al.*, 1999). SLC7A7 (MIM# 603593) gene encodes the y^+LAT-1 protein which is the light chain subunit of a member heterodimeric amino acid transporters (HATs) family. y^+LAT-1 is linked by a disulfide bond to the heavy chain subunit, 4F2hc, encoded by the SLC3A2 gene, (Chillaron *et al.*, 2001).

So far, 26 distinct mutations, spread along the entire SLC7A7 gene, have been identified in 106 LPI patients (Palacin *et al.*, 2001; Shoji *et al.*, 2002). Any kind of mutation (large genomic rearrangements, missense and nonsense mutations, splicing mutations, insertions and deletions) was found in LPI mutant alleles.

Except for the Finnish founder mutation (IVS6-2A>T), only four other mutant alleles (1670insATCA, 1471delTTCT, W242X and R410X) recur in more than one LPI independent family.

Here we report the identification of five novel mutations and the results of functional studies of altogether eight mutations of the SLC7A7 gene.

MATERIALS AND METHODS

Patients

Seven LPI patients from five independent families (family 1, 2, 3, 4,5) were investigated for mutations of the SLC7A7 gene. All patients presented with clinical and biochemical findings compatible with LPI that included: failure to thrive, hyperammonemia after protein-rich meals, low plasma levels and increased urinary excretion of CAA and massive orotic aciduria. The patient from family 4 developed also a severe tubular nephropathy.

Cell cultures

Skin fibroblasts were cultured using standard methods. Renal tubular cells were isolated from urine samples and cultivated according to Inoue *et al.* (2000).

Mutation analysis

All exons and intron-exon boundaries of the SLC7A7 gene were amplified from genomic DNA extracted from peripheral blood samples of all family members as previously reported (Sperandeo *et al.*, 2000). Direct sequencing was performed on two distinct PCR products, and both strands were sequenced by an automated system (ABI 377 DNA, Applied Biosystems, Monza, Italy).

Southern blot analysis was performed by standard methods. Briefly, genomic DNAs of patient from family 3 and of two controls were digested with Taq I restriction enzyme (New England Biolabs, Hitchin, England), according to the manufacturer's recommendations. After electrophoresis in a 1% agarose gel, the digested DNAs were transferred onto a Hybond-N membrane (Amersham Pharmacia Biotech, Milan, Italy). The membrane was hybridised at 65°C in a buffer containing 6X SSC, 1X Denhardt's solution, 0.5% SDS, 0.2 mg/ml sonicated herring sperm DNA, and ^{32}PdCTP-labeled full-length SLC7A7 cDNA as a probe.

Total RNA was extracted from fibroblast and renal tubular cells from patient of family 4 by guanidium/phenol method. First-strand cDNAs were synthesized by RT-PCR by SuperScript Rnase H-Reverse Transcriptase (BRL) and oligo(dt) as primer. Five µl of cDNA was amplified using a forward primer located in exon 5 (5'-TGTTCTCCTACTCAGGCTGGG-3') and a reverse primer located in exon 10 (5'-CAGAGGCAGAAGACAATCGG-3'). PCR products were cloned into the pBluescript II SK- vector and sequenced by an automated system (ABI 377 DNA, Applied Biosystems, Monza, Italy).

Generation of mutant constructs, expression in *X. laevis* oocytes and L-[^{3}H] arginine uptake

By using the Quick-Change site-directed mutagenesis kit (Stratagene, Amsterdam-Zuidoost, The Netherlands), seven mutant SLC7A7 cDNAs were constructed each containing one of the following mutations: p.M1L, p.M50K, p.T188I, p.W242X, p.S386R, p.Y457X, c.1185_1188delTTCT (previously known as 1471delTTCT). The

following mutagenic oligonucleotides (only sense strands are shown, antisense primers have the complementary sequence) were used:

p.M1L mutation: 5'-CTCTCCTCCACCCTGGTTGACAGC-3'
p.M50K mutation: 5'-GATTGTGGGGAACAAGATCGGCTCAGGC-3'
p.T188I mutation: 5'-GGGAACCCTGGTACAAGATATTTTCATCTATGC-3'
p.W242X mutation: 5'-CCTACTCAGGCTGAGACACCCTCAAC-3'
p.S386R mutation: 5'-GCTCATTAACTACTACAGATTCAGCTACTG-3'
p.Y457X mutation: 5'-CCTCTCAGGCCTGCCCTTTTAATTCCTCATC-3'
c.1185_1188delTTCT mutation: 5'-CTGGTTCTTTGTGGGGCT(TTCT)ATTGTGGGTCA-GCTTTATCTG-3'

The mutated bases are underlined. The nucleotides in parentheses were omitted during the synthesis of the corresponding mutant sequences.

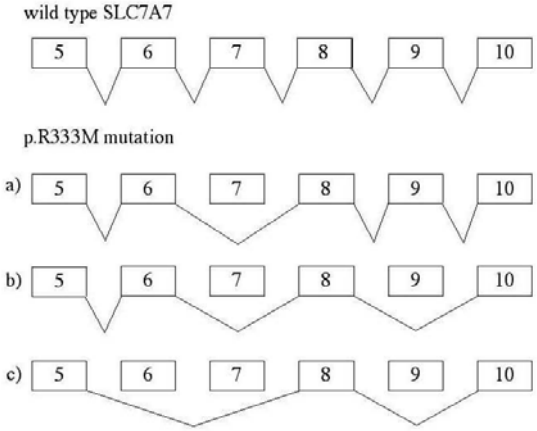

Figure 1. Exon splicing of wild-type SLC7A7 RNA (*top*) and of p.R333M RNA from patient of family 4 (*bottom*). **a.** Deletion of exon 7 as found in mRNA from fibroblast and renal tubular cells; **b.** Deletion of exons 7 and 9 as found in mRNA from renal tubular cells; **c.** Deletion of exons 6, 7, and 9 as found in mRNA from renal tubular cells.

The wt 4F2hc, wt SLC7A7 and all the SLC7A7 mutants cDNAs were *in vitro* transcribed by T3 or T7 RNA polymerase using a cap analogue, according to the manufacturer's instructions of the mCAP RNA capping kit (Stratagene, Amsterdam-Zuidoost, The Netherlands). Oocytes (stage V and VI) from *X. laevis* females were isolated by an enzymatic procedure as described elsewhere (Sperandeo *et al.*, 1998). Each oocyte was microinjected with either the wt SLC7A7 cRNA or each mutant cRNA together with 4F2hc (molar ratio 1:1; 10 ng:10 ng), using a microinjector device (Drummond Scientific Company, Broomall, PA, USA). For each experiment, wt SLC7A7 and 4F2hc cRNAs were co-microinjected as a control for the L-[^{3}H] arginine transport into a group of oocytes. Uptake rates of L-[^{3}H] arginine were measured at day 3 after injection. Each experiment represented the mean value of L-[^{3}H] arginine uptake, obtained from a group of 7-12 oocytes. Each microinjection was performed in triplicate using oocytes from different frogs. Measurements of L-[^{3}H] arginine uptake were obtained according to protocols described elsewhere (Pfeiffer *et al.*, 1999). Uptake experiments were carried out for 5 min. Data in fig. 2 are showed as the mean ± SE of L-[^{3}H] arginine uptakes in oocytes from 3 different frogs and expressed as a percentage of the L-[^{3}H] arginine uptake as found in uninjected oocytes (control, 842±85.5 cpm).

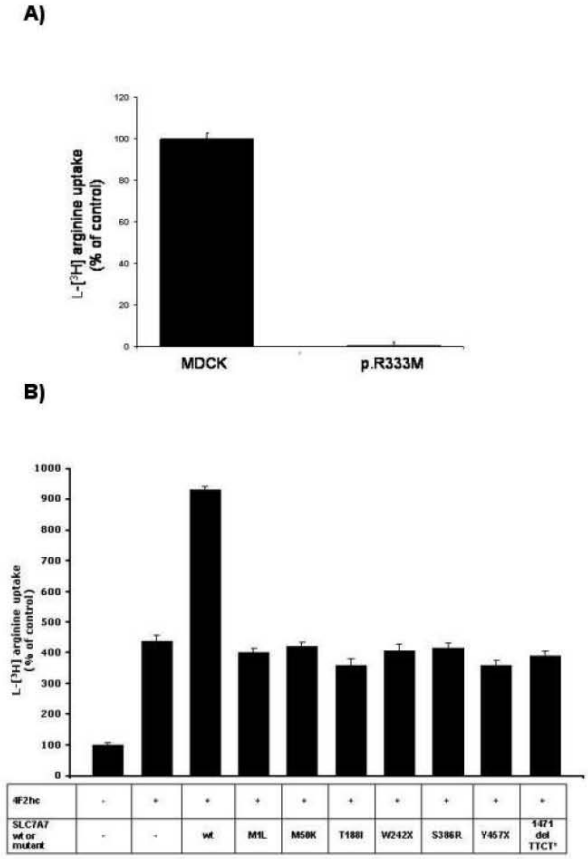

Figure 2. Functional analysis of y^+LAT-1 mutants. **A.** L-[^{3}H] arginine uptake in tubular renal cells. Absent L-[^{3}H] arginine uptake was detected from basolateral surface of patient's renal tubular cells than MDCK cells. **B.** *X. laevis* oocytes were injected with wt SLC7A7 or each mutant cRNA along with 4F2hc cRNA (molar ratio 1:1; 10 ng:10 ng). All SLC7A7 mutations abolished the L-[^{3}H] arginine transport. *The 1471delTTCT mutation corresponds to c.1185_1188delTTCT according to the new nomenclature.

L-[^{3}H] arginine transport assays in renal tubular cells

Renal tubular cells isolated from urine of patient of family 4 and Madin-Darby canine kidney (MDCK) cell line, used as control, were seeded on filters (Corning Costar transwell filter, 0.4 mm pore size, 12 mm diameter). Measurements of L-[^{3}H] arginine uptake were obtained according to protocols described elsewhere (Cariappa *et*

al., 2002). L-[^{3}H] arginine uptake was carried out for 7 min and was measured in triplicate at 37°C after addition of the uptake solution to either the basolateral or apical surface of the filters. L-[^{3}H] arginine uptakes were expressed as a percentage of the L-[^{3}H] arginine uptake as found in MDCK cells (control, 3182±292,7 cpm, corresponding to about 230 pmoli/L).

Mutation nomenclature

All mutation are described according to mutation nomenclature (den Dunnen and Antonorakis, 2000; den Dunnen and Paalman, 2003). Nucleotide number is derived from the human SLC7A7 cDNA sequence (GenBank accession no. AF092032.1).

RESULTS AND DISCUSSION

Here, we report five novel mutations of SLC7A7 gene which strengthen the evidence of a high mutational heterogeneity of LPI observed outside Finland. The novel mutations, reported in table 1, were found in seven LPI patients from five independent families after sequencing of all exons and intron-exon boundaries of the SLC7A7 gene.

A French-Algerian patient (family 1) was found to be a compound heterozygote for the c.1185_1188delTTCT and p.Y457X (c.1371C>A). The c.1185_1188delTTCT mutation had been already reported (Sperandeo *et al.*, 2000; known as 1471delTTCT according to a previous nomenclature). These mutations are located in different transmembrane (TM) domains of the protein, X and XII respectively. The p.Y457X introduces an early termination codon and the predicted mutant protein would lack the last TM domain and the intracellular C-terminus. The c.1185_1188delTTCT mutation was inherited from the father, whereas the p.Y457X mutation from the mother.

In family 2, two affected Greek brothers were compound heterozygotes for mutations p.T188I (c.563C>T) and p.Y457X (c.1371C>A), respectively. The T188 is a highly conserved amino acid residue in the HATs family. The p.T188I mutation was inherited from the father, whereas the p.Y457X mutation from the mother.

In family 3, originating from Pakistan, the patient was homozygous for the c.499+?_629-? mutation, a genomic rearrangement causing the deletion of the entire exon 4 at the cDNA level. Southern blotting analysis showed the absence of a Taq I fragment of about 1.2 Kb (data not shown).

In family 4, an affected Turkish boy was homozygous for p.R333M (c.997G>T). Both parents resulted heterozygous for this mutation. The c.997G >T change is located at the last nucleotide of exon 7 and apparently causes a missense mutation of the transcript. In recent years, exonic nucleotidic changes have been shown to alter *cis*-elements that are important for correct splicing (Cartegni *et al.*, 2002). To test whether the p.R333M mutation affects the splicing mechanism , we performed RT-PCR using total RNA extracted from fibroblasts and renal tubular cells isolated from the patient. In the fibroblast cell line, RT-PCR yielded two different products the sequencing of which showed a full length transcript, which includes the R333M mutation, and a shorter transcript where exon 7 is entirely skipped (Fig. 1). In renal tubular cells, which is a more appropriate tissue for SLC7A7 expression studies, RT-PCR showed three different transcripts. Sequencing of the most abundant product disclosed the skipping of exon 7. The other two products presented either deletion of exons 7 and 9 or deletion of exons 6, 7 and 9 (Fig.1). Therefore in renal tubular cells, at least exon 7 is always skipped and premature termination codons are introduced. We then examined the L-[^{3}H] arginine uptake from both apical and basolateral surface of renal tubular cells of the patient. No L-[^{3}H] arginine uptake was detected from the basolateral surface when compared to controls thus indicating that the p.R333M mutation severely alters the CAA transport (Fig. 2A).

In family 5, only one allele was identified in an LPI patient originating from Italy. This mutation, p.M50K (c.149T>A), is located in the TM domain I and causes a substitution of a highly conserved amino acid. The p.M50K mutation was inherited from the father.

The residual CAA transport activities of three of the novel mutations (p.M50K, p.T188I, p.Y457X) and of four previously described,p.M1L (c.1A>C), p.W242X (c.725G>A), p.S386R (c.1158C>A), c.1185_1188delTTCT, (Sperandeo *et al.*, 2000), were measured by expression of these mutant alleles in *X. laevis* oocytes. Control oocytes were injected with 0.1 M KCl. Their L-[^{3}H] arginine transport rates were identical to those obtained from uninjected oocytes (data not shown). Injection of 4F2hc alone increased L-[^{3}H] arginine transport to about 4 times the value of the uninjected oocytes (control) (Fig. 2B). Co-expression of wt y^+LAT-1 and 4F2hc cRNAs increased

the L-[^{3}H] arginine transport to about 2.5 times the activity obtained by the expression of 4F2hc alone, i.e. about 10-fold higher than controls. When co-injected with 4F2hc, all the SLC7A7 mutant proteins failed to induce L-[^{3}H] arginine thus confirming their disease-causative role (Fig. 2B).

In conclusion, the five novel mutations, here reported, extend to 30 the number of different SLC7A7 mutations found in non-Finnish LPI patients. Surprisingly, this mutational heterogeneity is higher in Italy than elsewhere (ten different mutations found in 16 independent families). With the inclusion of Greece in the list of LPI patients' origins, any country of the Mediterranean seems to be at risk to harbour mutant LPI alleles. In addition, the Pakistani LPI patient, here reported, suggests that LPI mutant alleles may be anywhere and that more attention should be paid for the diagnosis of this disorder.

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Chapter 6

Sperandeo MP, Paladino S, Maiuri L, Maroupulos GD, Zurzolo C, Taglialatela M, Andria G, Sebastio G.

A y^+LAT-1 mutant protein interferes with y^+LAT-2 activity: implications for the molecular pathogenesis of lysinuric protein intolerance.

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ARTICLE

A y^+LAT-1 mutant protein interferes with y^+LAT-2 activity: implications for the molecular pathogenesis of lysinuric protein intolerance

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Lysinuric protein intolerance (LPI) is an inherited aminoaciduria caused by defective cationic amino acid (CAA) transport at the basolateral membrane of epithelial cells in the intestine and kidney. The *SLC7A7* gene, mutated in LPI, encodes the y^+LAT-1 protein, which is the light subunit of the heterodimeric CAA transporter in which 4F2hc is the heavy chain subunit. Co-expression of 4F2hc and y^+LAT-1 induces the y^+L activity. This activity is also exerted by another complex composed of 4F2hc and y^+LAT-2, the latter encoded by the *SLC7A6* gene and more ubiquitously expressed than SLC7A7. On the basis of both the pattern of expression and the transport activity, y^+LAT-2 might compensate for CAA transport when y^+LAT-1 is defective. By expression in *Xenopus laevis* oocytes and mammalian cells, we functionally analysed two SLC7A7 mutants, E36del and F152L, respectively, the former displaying a partial dominant-negative effect. The results of the present study provide further insight into the molecular pathogenesis of LPI: a putative multiheteromeric structure of both [4F2hc/y^+LAT-1] and [4F2hc/y^+LAT-2], and the interference between y^+LAT-1 and y^+LAT-2 proteins. This interference can explain why the compensatory mechanism, that is, an increased expression of SLC7A6 as seen in lymphoblasts from LPI patients, may not be sufficient to restore the y^+L system activity.

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Introduction

Understanding the molecular bases of the pleiotropic effect of mutations of a single gene is a difficult task for most genetic diseases. Lysinuric protein intolerance (LPI [MIM 222700]) represents a disease model where mutations of the *SLC7A7* gene, an amino acid transporter, give rise to variable and mostly unexplainable multiorgan involvement. LPI is an inherited aminoaciduria caused by defective cationic amino acid (CAA; L-arginine; L-lysine; L-ornithine) transport at the basolateral membrane of epithelial cells in the intestine and kidney.

Clinical findings in LPI patients include: vomiting, diarrhoea, failure to thrive, hepatosplenomegaly, bone

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marrow abnormalities, osteoporosis, episodes of coma, mental retardation, lung involvement (mainly as alveolar proteinosis), altered immune response and chronic renal disease.[1]

Metabolic derangements in LPI include: reduced intestinal absorption of CAA, increased renal excretion of CAA and dysfunction of the urea cycle, leading to hyperammonaemia and orotic aciduria. LPI is caused by mutations of the solute carrier family 7A member 7 (*SLC7A7*) gene.[2,3] *SLC7A7* gene encodes the y^+LAT-1 protein, which belongs to the family of heterodimeric amino acid transporters (HATs). The amino acid transporter is composed of a heavy chain subunit, 4F2hc, encoded by *SLC3A2* gene, and a light chain subunit, y^+LAT-1, linked by a disulphide bond.[4] Co-expression of 4F2hc and y^+LAT-1 induces sodium-independent and high-affinity transport of CAA, known as y^+L activity. When sodium is present, y^+L is able to exchange CAA for neutral amino acids using an antiport mechanism. The y^+LAT-1 protein contains 12 putative transmembrane domains (TM) with the amino (N-) and carboxyl (C-) termini located inside the cell. 4F2hc is a type II membrane glycoprotein consisting of a single transmembrane domain with an intracellular N-terminus and a large extracellular C-terminus. There is evidence that the major function of 4F2hc is to traffic the complex to the plasma membrane, where the transport is the function of the light chain.[4] y^+L activity is also exerted by another heterodimeric complex composed of 4F2hc and y^+LAT-2, the latter encoded by the *SLC7A6* gene.[5] y^+LAT-1 and y^+LAT-2 share a high identity at both nucleotide and amino acid levels. The y^+LAT-2 protein is more ubiquitously expressed than y^+LAT-1, including tissues such as small intestine and kidney, where y^+LAT-1 is also highly expressed. Tissues and cell types with lower transport capacity probably use y^+LAT-2 to release arginine,[6] explaining why y^+L activity is not altered in fibroblasts and erythrocytes from LPI patients.[7,8]

Among the genetic defects of membrane transport, LPI is one of the most puzzling diseases. CAA depletion alone is not sufficient to account for multiorgan involvement. At least two aspects of the molecular pathogenesis of LPI need to be elucidated: the extreme variability of the phenotype and the role of y^+LAT-2. The variability of the phenotype cannot be explained by either a straightforward correlation with the genotype or variable compliance to the therapy.[9,10] For example, two of the most severe genotypes reported so far, that is, homozygosity for a large deletion of the gene (patient IV-2, family 1 in Borsani *et al*[2]) or homozygosity for the M1L mutation (patients 1A and 1B in Sperandeo *et al*[9]), both of which result in absent protein, were found in patients with a very mild phenotype under treatment. The ubiquitous expression of SLC7A6 and the overlapping transport activity suggest that y^+LAT-2 may compensate when y^+LAT-1 is defective. In fact, an increased expression of SLC7A6 was found in lymphoblasts from LPI patients.[11]

Here we report the results of functional studies of two mutations of SLC7A7, which provide further insight into the molecular pathogenesis of LPI: a putative multiheteromeric structure of both [4F2hc/y^+LAT-1] and [4F2hc/y^+LAT-2], and the interference between y^+LAT-1 and y^+LAT-2 proteins.

Materials and methods

Subject

A 2-year-old boy, born to nonconsanguineous parents originating from Greece, presented hepatomegaly, recurrent episodes of hyperammonaemia, drowsiness, and acute encephalopathy after chickenpox infection. Biochemical findings were typical of LPI and included: low plasma concentrations of CAA, increased urinary excretion of CAA and orotic acid.

Laboratory procedures

Mutation analysis Oligonucleotide primers, corresponding to exon-flanking intronic sequences, were used to amplify all exons from genomic DNA extracted from peripheral blood samples of all family members by standard methods.[9] Direct sequencing was carried out on PCR products and both strands were sequenced by an automated system (ABI 377 DNA, Applied Biosystems, Monza, Italy).

Generation of mutant constructs Two mutant SLC7A7 cDNAs containing E36del and F152L, respectively, were constructed using the Quick-Change site-directed mutagenesis kit (Stratagene, Amsterdam-Zuidoost, The Netherlands), according to the manufacturer's protocol. The following mutagenic oligonucleotides (only sense strands are shown) were used:

E36del mutation: 5′-GTGAAGCTGAAGAA(GGA)GATCT CACTGCTTAACGG-3′

F152L mutation: 5′-CCCGAGCTGCCTCGCCCCTTATG-3′

The mutated bases are underlined. The nucleotides in parentheses were omitted during the synthesis of the corresponding mutant sequences.

Expression in* Xenopus laevis *oocytes For expression studies, the human wt 4F2hc, SLC7A6, SLC7A7 sequences and all SLC7A7 mutant cDNAs were cloned in pBluescript II SK- vector. For the *in vitro* transcription, plasmid DNAs were linearized by *Xho*I or *Not*I (New England Biolabs, Hitchin, England) and *in vitro* transcribed by T3 or T7 RNA polymerase using a cap analogue, according to the manufacturer's instructions of the mCAP RNA capping kit (Stratagene, Amsterdam-Zuidoost, The Netherlands). Oocytes (stage V and VI) from *X. laevis* females were isolated by an enzymatic procedure as described elsewhere and allowed to recover overnight.[12] In each experiment, wt SLC7A7 and 4F2hc cRNAs were co-microinjected as a control for the L-[^{3}H]arginine transport into a group of

oocytes. Uptake rates of L-[^{3}H]arginine were measured at day 3 after injection.

Arginine uptake Each experiment represented the mean value of uptake obtained from a group of 7–12 oocytes. Each microinjection was performed in triplicate using oocytes from different frogs. Measurements of L-[^{3}H]arginine uptake were obtained according to protocol described elsewhere.[13] Control oocytes were injected with 0.1 M KCl. Their L-[^{3}H]arginine transport rates were identical to those obtained from uninjected oocytes (data not shown). Data are shown as the mean ± SE of L-[^{3}H]arginine uptakes in oocytes from three different frogs and expressed as a percentage of L-[^{3}H]arginine uptake as found in uninjected oocytes (control, 842 ± 85.5 cpm).

Analysis of protein expression by transfection into Madin–Darby canine kidney (MDCK) cells Madin–Darby canine kidney (MDCK) cells were grown in DMEM supplemented with 10% FBS without antibiotics. Wild-type (wt) and mutant SLC7A7 cDNAs, cloned in pBluescript II SK, were amplified using PCR primers (forward primer 5′-CCGCTCGAGCGGATGGTTGACAGCACTGAG-3′ and reverse primer 5′-GCTCTAGAGCTTAGTTAGACTTGGGAT CCC-3′). PCR products were subcloned into pcDNA3My-cEGFP vector (kindly provided by Dr G Meroni, TIGEM, Naples, Italy) with N-terminal GFP tag. wt SLC7A6 cDNA was similarly prepared using different PCR primers (forward primer 5′-CGCGGATCCGCGATGGAAGCCAGGGAGCCTG 3′; reverse primer 5′-GGAATTCCTCAGTCAGTTTTCCTCT CATCC-3′). PCR products were subcloned into pcDNA3My-cEGFP vector. wt and SLC7A7 mutants and wt SLC7A6 were transfected into mammalian MDCK cells using the Lipofectamine 2000 reagent (Invitrogen Life Technologies, Milan, Italy). Stable transfectants were selected by addition of 100 μg/ml G418 (Life Technologies Inc.) into the culture medium, and the cells were cultured for at least 2 weeks before using them for studies. For immunofluorescence, 2.5×10^5 G418-resistant cells were seeded on filters (Corning Costar transwell filter, 0.4 mm pore size, 12 mm diameter) and cultured for 3 days. Cells were then washed twice with PBS containing calcium and magnesium, fixed with 4% paraformaldehyde for 15 min and permeated by 0.02% Triton X-100 for 15 min. wt and mutant recombinant SLC7A7 and wt SLC7A6 proteins were identified by EGFP (green) fluorescence. Cells were examined using a Zeiss laser-scanning confocal microscope (LSM 510). Serial sections (about 28 optical sections) were analysed using LSM Unit software (Carl Zeiss). Digital images were saved and analysed by Adobe PhotoShop (Adobe Systems Inc., Mountain View, CA, USA).

SLC7A6 and 4F2hc genomic sequencing Oligonucleotide primers, corresponding to exon-flanking intronic sequences, were used to amplify all exons of SLC7A6 and 4F2hc from genomic DNA. Primer sequences are available on request. PCR products were direct-sequenced on both strands by an automated system (ABI 377 DNA, Applied Biosystems, Monza, Italy).

Results

Identification of two novel LPI-associated SLC7A7 mutations

The patient was found to be a compound heterozygote for two mutations, E36del and F152L, respectively. The E36del mutation, caused by a 3 bp deletion, nucleotides 104–106 from the first methionine of the SLC7A7 cDNA, deletes a glutamic acid residue at the N-terminus of the protein, leaving an unaltered reading frame downstream. The E36 residue is highly conserved in all members of the HAT light subunits (LSHATs). By contrast, the F152L (from the first methionine, 453T>C) mutation, located in the extracellular loop II, affects a nonconserved amino acid residue.

Functional analysis of y^+LAT-1 mutants

We investigated the capacity of these two novel mutations to mediate CAA transport in *X. laevis* oocytes by co-microinjection of the cRNA encoding wt or mutant y^+LAT-1 proteins along with 4F2hc cRNA. Injection of oocytes with 4F2hc alone increased L-arginine transport to about four times that of the uninjected oocytes (control) (Figure 1). Co-expression of wt y^+LAT-1 and 4F2hc cRNAs increased arginine transport to about 2.5 times that of the activity obtained by the expression of 4F2hc alone, that is, about 10-fold higher than controls. When co-injected with 4F2hc, the F152L mutation induced L-[^{3}H]arginine transport activity 1.6 times that of the single injection of 4F2hc (Figure 1).

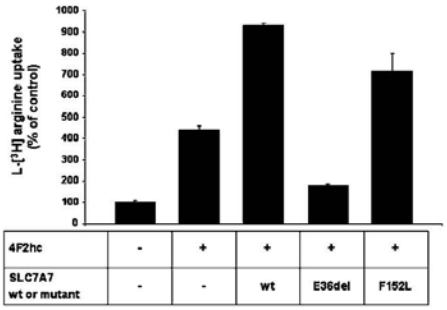


Figure 1 Functional analysis of y^+LAT-1 mutants in *X. laevis* oocytes. *X. laevis* oocytes were injected with wt SLC7A7 or each mutant cRNA together with 4F2hc cRNA (molar ratio 1:1; 10 ng:10 ng). The E36del mutation failed to show transport activity. In contrast, the F152L mutation induced L-[^{3}H]arginine transport activity to 1.6 times the activity obtained with 4F2hc alone.

The E36del mutation failed to show transport activity. Moreover, L-[^{3}H]arginine transport in oocytes injected with E36del mutant cRNA was consistently lower than that found in oocytes injected with 4F2hc alone (Student's *t*-test: $P<0.00002$; Figure 1).

Analysis of protein expression of wild and mutant y^+LAT-1 in MDCK cells

To further elucidate the effects exerted by the E36del and the F152L mutations, respectively, we examined the subcellular fate of these proteins after transfection into MDCK cells. MDCK cells originate from the distal nephron and represent a well-established model for epithelial polarity studies. In addition, MDCK cells constitutively express 4F2hc, as seen by immunolocalization of y^+LAT-1 and L-amino acid transporter-2 (LAT2, encoded by the *SLC7A8* gene, that induces a system L transport activity with 4F2hc) at the basolateral membrane in the absence of exogenous 4F2hc.[14] This allowed us to perform single transfections of GFP-tagged vectors containing either wt or mutant SLC7A7 cDNAs.

As already reported,[14] wt SLC7A7 was mainly localized at the basolateral membrane (Figure 2a). Both F152L and E36del mutants showed definite signals at the basolateral membrane (Figure 2b and c).

Effect of the E36del mutation on y^+L transporter

From the data described above, E36del is a severe mutation despite a correct homing to the membrane, in contrast with F152L, which has a mild effect on CAA transport.

According to the heterodimeric model of [4F2hc/y^+LAT-1] complex, the cumulative effect of E36del and F152L mutations on CAA transport should represent the sum of single effects. To test this model, we then performed a triple injection of 10 ng of 4F2hc cRNA along with 5 ng cRNA of each mutant (F152L and E36del; molar ratio 1:0.5:0.5) into *X. laevis* oocytes. This triple expression was designed to resemble the compound heterozygosity as seen in the patient. As controls, two groups of oocytes were injected with either 4F2hc + F152L or 4F2hc + the E36del mutant (molar ratio 1:0.5), respectively. The E36del mutant completely abolished the L-[^{3}H]arginine transport activity not only when expressed alone (Student's-*t*-test: $P<0.00000001$ compared to the wt SLC7A7) but also when co-injected with the F152L mutant (Student's *t*-test: $P<0.0001$ compared to wt SLC7A7; Figure 3).

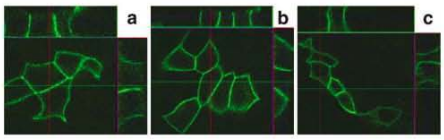


Figure 2 Expression of wt or mutant SLC7A7 in MDCK cells. After transfection, the cells were processed by immunofluorescence using SLC7A7-GFP autofluorescence for detection by confocal microscopy. In each panel, the central part shows the en face image and the top and right parts show the Z-sectioning images. Transfections with (a) wt SLC7A7, (b) F152L mutant, (c) E36del mutant. In wt SLC7A7 as well as in both mutants, immunofluorescence is evident on the surface membrane with negligible amount within the cytoplasm.

To further understand this inhibitory effect, microinjections with different molar ratios of the E36del mutant + wt SLC7A7 and 4F2hc were carried out. This triple injection mimics the heterozygous condition as seen in the patient's father. The L-[^{3}H]arginine transport activity was moderately reduced in oocytes co-injected with E36del mutant by a dose-dependent effect, which disappears only at a 1:10 molar ratio compared to wt SLC7A7 (Student's *t*-test: $P<0.33$; Figure 4).

We examined also the interference of the E36del mutant with the activity of 4F2hc itself. Co-expressions of the 4F2hc with different molar ratios of E36del mutant showed a dose-dependent suppression of the activity induced by 4F2hc alone (data not shown).

Analysis of protein expression of wt y^+LAT-2 into MDCK cells

When MDCK cells were transfected with y^+LAT-2, the pattern of expression was identical to that observed with y^+LAT-1 (data not shown).

Interaction between E36del mutant and y^+LAT-2 in *X. laevis* oocytes

To investigate possible reciprocal interferences between y^+LAT-1 and y^+LAT-2 on the assembly of y^+L transporters, we microinjected *X. laevis* oocytes with different combinations of E36del mutant + wt y^+LAT-2 and 4F2hc. When we co-expressed 4F2hc + wt SLC7A6 + E36del mutant (molar ratio 1:0.5:0.5), a reduction in L-[^{3}H]arginine transport (50% of inhibition compared to 4F2hc + wt SLC7A6; molar ratio 1:0.5; Student's *t*-test: $P<1.3\ 10^{-10}$) was observed (Figure 5).

Sequencing of 4F2hc and SLC7A6 in LPI patients

The crucial role of 4F2hc in the assembly of the y^+L transporter and the possible interference of SLC7A6 led us to search for common or rare mutations of these genes in the present patient as well as in other seven previously characterized at molecular level (data not shown). All exons and intron–exon junctions of 4F2hc and SLC7A6 were sequenced in eight independent LPI patients. No causative mutations or polymorphic changes were found in either of the two genes.

Discussion

Two mutations, E36del and F152L, found in compound heterozygosity in a patient with a full-blown LPI phenotype, turned out to be suitable for further insight into the

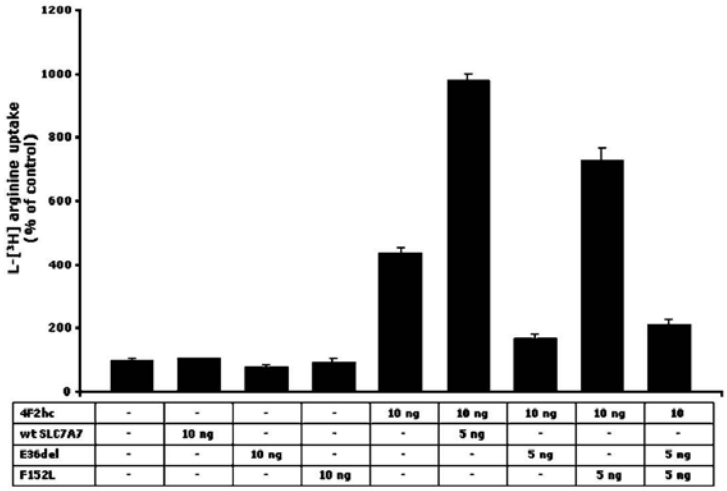
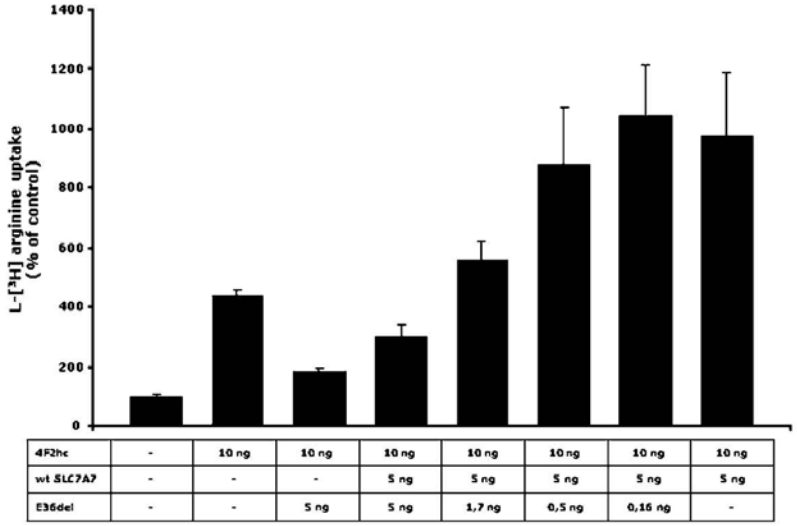


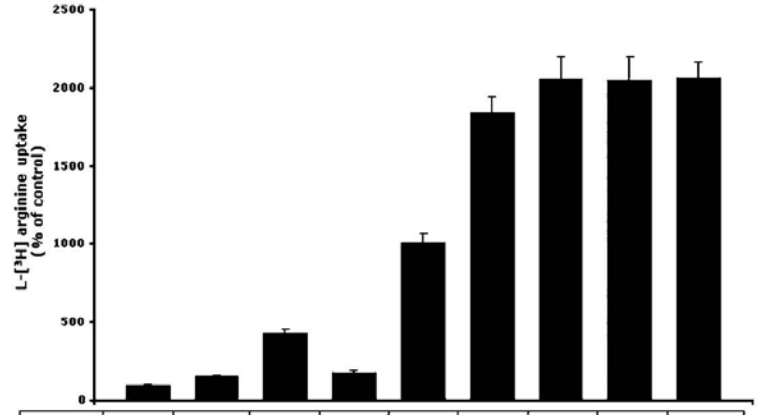
Figure 3 Interaction between different y^+LAT-1 mutants in *X. laevis* oocytes. Arginine uptakes after triple injection (molar ratio 1:0.5:0.5) of 4F2hc + F152L + E36del were compared to those observed in oocytes injected with 4F2hc + F152L and 4F2hc + E36del (molar ratio 1:0.5). The E36del mutant completely abolished the L-[^{3}H]arginine activity when co-injected with the F152L mutant (Student's *t*-test: $P<0.0001$ compared to wt SLC7A7).

molecular pathogenesis of LPI. Expression studies in *X. laevis* oocytes demonstrated that the F152L mutation allows a residual y^+L transport activity moderately reduced when compared to wt SLC7A7, while the E36del mutant chain dramatically interferes with the activity of both wt SLC7A7 and SLC7A7 carrying the F152L mutation. Similarly, E36del protein totally inhibited the L-arginine transport when expressed in the presence of 4F2hc alone. Previously, other SLC7A7 mutant proteins failed to co-induce amino acid transport activity when co-expressed with 4F2hc in *X. laevis*.[3,15] When transfected into MDCK cells, E36del mutant displayed clear signals at the baso-lateral membrane, with a pattern overlapping those observed with both the wt SLC7A7 and the F152L mild mutant. These results suggest that the deletion of the E36 residue does not alter the binding of the light chain to 4F2hc or its transport to the membrane. The F152L and the E36del mutants provided unique opportunity to further investigate the molecular mechanisms underlying the pathogenesis of LPI. We performed triple injections of *X. laevis* oocytes with different combinations of 4F2hc, wt SLC7A7 and/or SLC7A7 mutants to resemble the heterozygous and the compound heterozygous conditions as found in the family. The E36del mutation was able to suppress the residual transport activity allowed by the F152L mutant. In addition, the E36del mutation reduced the L-arginine transport even in the presence of the wt SLC7A7 protein, an effect that might cause some metabolic derangement in the heterozygous carrier. As a matter of fact, the patient's father (carrier of the E36del mutation) showed neither clinical or biochemical abnormalities. This might be explained by mechanisms such as upregulation of the wt SLC7A7 allele's transcription or replacement by other LSHATs, for example, y^+LAT-2. In expression studies carried out in *X. laevis* oocytes, the dose-dependent inhibitory effect of the E36del mutant on y^+L activity, induced by wt SLC7A7 and 4F2hc, is already present at the molar ratio of 1:3 (E36del:wt SLC7A7). This suggests a partial dominant-negative effect of this mutation. We are aware that a dominant-negative effect would contradict with both the autosomal recessive inheritance of the disease and the current view of a heterodimeric structure of the [4F2hc/y^+LAT-1] complex. Indeed, our data would favour a different model for the y^+L transporter: a multiheteromeric structure composed of heterodimers of 4F2hc and y^+LAT-1. Accordingly, a severe y^+LAT-1 mutant, for example, E36del, might interfere with the function of the complex even in the presence of a second mild mutant allele, for example, F152L. A multiheteromeric structure of the y^+L transporter would be also in agreement with a similar model (heterotetrameric structure) recently proposed for the [rBAT/$b^{0,+}$AT] complex,[4,16]



4F2hc	-	10 ng	10 ng	10 ng	10 ng	10 ng	10 ng	10 ng
wt SLC7A7	-	-	-	5 ng	5 ng	5 ng	5 ng	5 ng
E36del	-	-	5 ng	5 ng	1,7 ng	0,5 ng	0,16 ng	-

Figure 4 Interaction between E36del mutant and wt y^+LAT-1 in *X. laevis* oocytes. Arginine uptakes after triple injection with different molar ratios of wt SLC7A7 + E36del together with 4F2hc were compared to those observed in oocytes injected with 4F2hc + wt SLC7A7 and 4F2 + E36del (molar ratio 1:0.5), respectively. The L-[^{3}H]arginine transport activity was significantly suppressed in oocytes co-injected with E36del mutant in a dose-dependent fashion, which disappears only at a 1:10 molar ratio with respect to wt SLC7A7 (Student's *t*-test: $P<0.33$).



4F2hc	-		10 ng	10 ng	10 ng	10 ng	10 ng	10 ng	10 ng
wt SLC7A6	-	10 ng	-	-	5 ng	5 ng	5 ng	5 ng	5 ng
E36del	-	-	-	5 ng	5 ng	1,7 ng	0,5 ng	0,16 ng	-

Figure 5 Interaction between E36del mutant and wt y^+LAT-2. A 50% reduction in the L-[^{3}H]arginine transport was observed in oocytes injected with 4F2hc + wt SLC7A6 + E36del mutant (molar ratio 1:0.5:0.5; Student's *t*-test: $P<1.3\times10^{-10}$).

the transport system defective in cystinuria, a cognate disorder of LPI. Unfortunately, the high identity at both nucleotide and amino acid levels, shared by y^+LAT-1 and y^+LAT-2, prevented the production of specific antibodies in several laboratories. Therefore, further studies at protein level are not feasible at the moment.

Phenotypic variability is an unsolved problem for most genetic disorders. In this respect, LPI is a remarkable example. After the identification of the gene mutated in this disease, efforts aimed at establishing a genotype/phenotype correlation were unsuccessful. To further complicate this point, the molecular physiology of amino acid transport is finely tuned by a large number of specific carriers, which often exhibit similar tissue specificity and biochemical features. The efflux of CAA from cells is under the action of the y^+L system, which is exerted by at least two transporters, [4F2hc/y^+LAT-1] and [4F2hc/y^+LAT-2], respectively. In addition, y^+LAT-2 and 4F2hc are almost ubiquitously expressed in mammalian tissues, including small intestine and kidney, as confirmed by Northern blot (data not shown). As a consequence, one wonders why LPI can turn out to be a life-threatening disease despite the presence of an almost ubiquitous [4F2hc/y^+LAT-2] complex. Our data show that the activity of the [4F2hc/y^+LAT-2] complex can be influenced by the presence of y^+LAT-1 mutants such as E36del. Previously, we demonstrated that the y^+L transport activity is unaffected in the erythrocytes and fibroblasts of LPI patients.[7,8] This can be explained by a predominant or exclusive expression of y^+LAT-2 in those cells. In other tissues, such as the small intestine and kidney, where both SLC7A6 and SLC7A7 are expressed, y^+LAT-1 mutants might interfere with the activity of the [4F2hc/y^+LAT-2] complex. This interference can explain why the compensatory mechanism, that is, an increased expression of SLC7A6 as seen in lymphoblasts from LPI patients,[11] may not be sufficient to restore the y^+L system activity.

It is still unclear why Finnish LPI patients, all affected by the same SLC7A7 genotype, present a wide phenotypic variability. We excluded the possibility that concomitant mutations of the coding region of either SLC7A6 or 4F2hc might be related to the phenotypic variability in eight LPI patients. However, we could not rule out differences of gene expression of either SLC7A6 or 4F2hc.

The present study, although not definitely clarifying the y^+LAT-1 and y^+LAT-2 interference due to lack of specific antibodies, provides a new scenario for understanding the pleiotropic effect of SLC7A7 mutations in LPI.

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Chapter 7

DISCUSSION

The overall aim of the present thesis is to contribute to the elucidation of the pathophysiology of LPI. LPI is a congenital aminoaciduria caused by a defective transport of CAA to the basolateral membrane of the epithelial cells of the intestine and the kidney. In the last 6 years, that is to say after the isolation of the gene, no further progress on the knowledge of the molecular bases of the LPI pathophysiology has been achieved. The therapeutic approach has not changed despite the emerging suspicion that an excess of citrulline might worsen the renal involvement.

Among all the genetic metabolic disorders, LPI is one of the most puzzling diseases, where CAA depletion alone is insufficient to account for the multiorgan involvement.

Three different approaches were taken to study the molecular bases of the LPI pathophysiology:

1. Functional studies of SLC7A7 mutations
2. Creation of mouse models of LPI
3. Creation of a human cellular model of LPI

Our functional data favour a different model for the y^+L transporter: a multiheteromeric structure composed of heterodimers of 4F2hc and y^+LAT1. Accordingly, a severe y^+LAT1 mutant, e.g. E36del, might interfere with the function of the complex even in presence of a second mild mutant allele, e.g. F152L.

A multiheteromeric structure of the y^+L transporter would be also in agreement with a similar model (heterotetrameric structure) recently proposed for [rBAT/$b^{0,+}$AT] complex the transport system defective in cystinuria, a cognate disorder of LPI.

Phenotypic variability is an unresolved problem for most genetic disorders. In this respect, LPI is a remarkable example. After the identification of the mutated gene in this disease, efforts aimed at establishing a genotype/phenotype correlation were unsuccessful. To further

complicate this point, the molecular physiology of amino acid transport is finely tuned by a large number of specific carriers, which often exhibit similar tissue specificity and biochemical features. The efflux of CAA from cells is under the action of y^+L system, which is exerted by at least two transporters, [4F2hc/y^+LAT1] and [4F2hc/y^+LAT2] respectively. In addition, y^+LAT2 and 4F2hc are almost ubiquitously expressed in mammalian tissues, including small intestine and kidney. As a consequence, one wonders why LPI can turn out to be a life-threatening disease despite the presence of an almost ubiquitous [4F2hc/y^+LAT2] complex. Our data show that the activity of the [4F2hc/y^+LAT2] complex can be influenced by the presence of severe y^+LAT1 mutants such as E36del. The y^+L transport activity is unaffected in erythrocytes and fibroblasts of LPI patients. This can be explained by a predominant or exclusive expression of y^+LAT2 in those cells. In other tissues, such as small intestine and kidney, where both SLC7A6 and SLC7A7 are expressed, y^+LAT1 mutants might interfere with the activity of the [4F2hc/y^+LAT2] complex. This interference could explain why the compensatory mechanism (i.e. an increased expression of SLC7A6 as seen in lymphoblasts or in RTC from LPI patients) may not be sufficient to restore the y^+L system activity.

It is still unclear why Finnish LPI patients, all affected by the same SLC7A7 genotype, also present a wide phenotypic variability. We excluded the possibility that concomitant mutations of the coding region of either SLC7A6 or 4F2hc might be related to the phenotypic variability in eight LPI patients. However, we could not rule out differences of gene expression of either SLC7A6 or 4F2hc.

An animal model of LPI is nevertheless essential since the complexity of the pathology and obvious ethical issues prevent some experimental work in humans. The first animal model of LPI, a constitutive knock-out of Slc7a7, was generated to study the pathophysiology of LPI and to explore new therapeutic protocols. Currently, we have succeeded in generating this model but it has turned out to be lethal in the perinatal period. Such lethality limited our studies. To circumvent this problem, we are generating a conditional Slc7a7 gene mouse model using the Cre/loxP recombination system. With this mouse model single aspects of the disease can be investigated without the risk of a full-blown clinical phenotype, which had caused the lethality in the constitutive knock-out mouse.

The production of a human cellular model of the LPI, namely a renal tubular cellular model, is a useful approach to study the role of nitric oxide in the pathogenesis of renal complications of the disease. Renal involvement represents a severe complication of LPI and almost nothing is known about its pathogenesis. The crucial role might be played by an unbalanced intracellular homeostasis of arginine, which is the precursor of important metabolic pathways such as nitric oxide (NO), polyamines and creatine. Mutations of the SLC7A7 gene can give origin to a paradox: on one hand, low plasma levels of arginine cause vascular dysfunction through reduced NO production; on the other hand, arginine could accumulate in polarized cells, for example in renal tubular cells, because of its defective efflux. Furthermore the arginine metabolism studies on RTC could be crucial for gaining knowledge of renal involvement in LPI. Our preliminary results have demonstrated that this accumulation is due to a defective efflux which can cause an excessive production of NO resulting in tubular damage (necrosis) as seen in experimental models of nephritis. In addition, the arginine is also synthetized from citrulline in a pathway known as the arginine-citrulline pathway (see fig. 4.16). Our results demonstrated that overload of citrulline (supplemented daily in agreement with conventional

therapeutic protocols for LPI patients) in RTC might represent a factor that leads to an even greater arginine overload in renal tubuli.

The work described in this thesis has led to the following conclusions:

1. putative new model of y^+L transporter with a putative multiheteromeric structure;
2. ablation of Slc7a7 causes severe prenatal growth retardation with no gross developmental abnormalities but with unbalanced NO metabolism;
3. unbalanced NO metabolism might be the key to understand the pathophysiology of many complications of LPI in man;
4. renal involvement in human LPI has a clear relationship with apoptosis induced by high levels of NO synthesis in renal tubular cells;
5. conventional therapeutic protocols of LPI should be revised immediately in order to avoid excessive citrulline intake and possible iatrogenic complications arising from disease.

Summary

Background: Lysinuric protein intolerance (LPI; MIM 222700) is caused by a defective transport of cationic amino acids (CAA for Cationic Amino Acids) to the basolateral membrane of the epithelial cells of intestine and kidney. Clinical manifestations of LPI include:vomiting, diarrhea, stunted growth, visceromegaly, osteoporosis, episodes of coma, mental delay, severe pulmonary (pulmonary alveolar proteinosis, PAP) and renal involvements. The metabolic derangement of this disease includes: reduced intestinal absorption of CAA, increased CAA renal excretion and impairment of the urea cycle that brings to hyperammonemia and increased orotic aciduria. The molecular basis of LPI is a defective transport of CAA normally exerted by system y^+L. The y^+L activity is induced by a heterodimer composed of:

- the heavy chain 4F2hc, encoded by the SLC3A2 gene;
- a light chain that can be either y^+LAT1 (encoded by the SLC7A7 gene) or y^+LAT2 (encoded by the SLC7A6 gene).

Summary

We found that the SLC7A7 gene is mutated in LPI patients and we also characterized most of the mutations found so far in Italian and non-Italian patients.

***Specific objectives**:* The overall aim of the present thesis is to contribute to the elucidation of the pathophysiology of LPI, using three different approaches:

1. Mutational analysis of LPI patients;
2. Functional studies of SLC7A7 mutants;
3. Creation of animal and cellular models of LPI.

Results: Our functional results revealed a new scenario in the knowledge on LPI disorder. In fact, our results provide further insight into the molecular pathogenesis of LPI: a putative multiheteromeric structure of both [4F2hc/y^+LAT1] and [4F2hc/y^+LAT2], and the interference between y^+LAT1 and y^+LAT2 proteins. This interference can explain why the compensator mechanism (via an increased expression of SLC7A6 seen in lymphoblasts and renal tubular cells from LPI patients) may not be sufficient to restore the y^+L system activity.

The first animal model of LPI, a constitutive knock-out of Slc7a7, has been generated to study the pathophysiology of LPI and explore new therapeutic

protocols. At the moment, we have succeeded in generating this model but it turned out to be lethal in the perinatal period. This lethality limited our studies. To circumvent the problem, we are generating a conditional Slc7a7 gene mouse model, using the Cre/loxP recombination system. With this mouse model, single aspects of the disease can be investigated without the risk of a full-blown clinical phenotype, which caused the lethality in the constitutive knock-out mouse.

The first cellular model of LPI was obtained by isolation of renal tubular cells from urine of LPI patients. Using this cellular model we studied the relation between renal compliance and the nitric oxide metabolism. Our preliminary results confirm a possible relation between production of nitric oxide and renal involvement known in LPI.

Conclusions: The work described in this thesis has led to the following results:

1. putative new model of y^+L transporter with a putative multiheteromeric structure;
2. ablation of Slc7a7 causes severe prenatal growth retardation with no gross developmental abnormalities but with unbalanced NO metabolism;

3. unbalanced NO metabolism might be the key to understand the pathophysiology of many complications of LPI in man;
4. renal involvement in human LPI has a clear relationship with apoptosis induced by high levels of NO synthesis in renal tubular cells;
5. conventional therapeutic protocols of LPI should be revised immediately in order to avoid excessive citrulline intake and possible iatrogenic complications arising from disease.

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Curriculum vitae

Maria Pia Sperandeo was born on October 30, 1965 in Torre del Greco (Naples), Italy. Her education and professional positions are listed below.

Education/Training

- **1990** Federico II University of Naples-Degree in Biology, summa cum laude.
- **1991** Post-graduate fellow at the Cytogenetics Laboratory, Cardarelli Hospital of Naples:
    - Studied the cytogenetic methodologies under the tutelage of Prof. V. Ventruto.
- **1991-94** Research fellow at the Dept. of Pediatrics of Naples, Federico II University:
    - Contributed to the development of molecular methodologies as mutational and linkage analyses for the diagnosis of metabolic disease under the tutelage of Prof. G. Andria and Prof. G. Sebastio.
- **1992-95** Residency in Clinical Pathology at Federico II University of Naples.
- **1995** Research Fellow at the Telethon Institute of Genetics and Medicine –TIGEM of Milan:
    - Studied bioinformatic approaches in medical research under Prof. G. Borsani.

- **1997** Visiting Scientist at the CSS-IRCCS Hospital of San Giovanni Rotondo (FG):
    - Studied the methodologies for linkage analysis studies under Prof. P. Gasparini.
- **1998** Visiting Scientist at the IIGB-CNR of Naples:
    - Studied methodologies for the YAC analysis under Dr. M. D'Urso and Dr. A. Ciccodicola,.
- **2001-05** PhD program in 'Riproduzione, sviluppo e accrescimento dell'uomo', XII ciclo, Federico II University, Naples.

Employment and Experience

- **1993-94** CNR fellowship at the Dept. of Pediatrics, Federico II University:
    - Worked with Prof. G. Andria and Prof. G. Sebastio on two projects: 1) Molecular characterization of patients with Fragile X mental retardation; 2) Molecular analysis of patients affected by homocystinuria due to cystathionine ß-synthase deficiency (CBS).
- **1995-96** CNR fellowship at the Dept. of Pediatrics, Federico II University, Naples
- **1996-2000:** Scientific collaboration (Telethon Grant E.436-E.652 to Prof. G. Sebastio) at the Dept. of Pediatrics, Federico II University:
    - Involved in studies on the isolation and identification of a candidate gene for lysinuric protein intolerance.
- **2000- present** Assistant Telethon Scientist (project TCP 99029).

Full papers

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