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THE EFFECTS OF MUTATED CATIONIC AMINO ACID TRANSPORTER y^+LAT1 AT THE CELLULAR AND SYSTEMIC LEVEL

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To all my loved ones

ABSTRACT

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The effects of mutated cationic amino acid transporter y$^+$LAT1 at the cellular and systemic level

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y$^+$LAT1 is a transmembrane protein that, together with the 4F2hc cell surface antigen, forms a transporter for cationic amino acids in the basolateral plasma membrane of epithelial cells. It is mainly expressed in the kidney and small intestine, and to a lesser extent in other tissues, such as the placenta and immunoactive cells. Mutations in y$^+$LAT1 lead to a defect of the y$^+$LAT1/4F2hc transporter, which impairs intestinal absorbance and renal reabsorbance of lysine, arginine and ornithine, causing lysinuric protein intolerance (LPI), a rare, recessively inherited aminoaciduria with severe multi-organ complications.

This thesis examines the consequences of the LPI-causing mutations on two levels, the transporter structure and the Finnish patients' gene expression profiles. Using fluorescence resonance energy transfer (FRET) confocal microscopy, optimised for this work, the subunit dimerisation was discovered to be a primary phenomenon occurring regardless of mutations in y$^+$LAT1. In flow cytometric and confocal microscopic FRET analyses, the y$^+$LAT1 molecules exhibit a strong tendency for homodimerisation both in the presence and absence of 4F2hc, suggesting a heterotetramer for the transporter's functional form.

Gene expression analysis of the Finnish patients, clinically variable but homogenic for the LPI-causing mutation in *SLC7A7*, revealed 926 differentially-expressed genes and a disturbance of the amino acid homeostasis affecting several transporters. However, despite the expression changes in individual patients, no overall compensatory effect of y$^+$LAT2, the sister y$^+$L transporter, was detected. The functional annotations of the altered genes included biological processes such as inflammatory response, immune system processes and apoptosis, indicating a strong immunological involvement for LPI.

Key words: Lysinuric protein intolerance (LPI), y$^+$LAT1, *SLC7A7*, amino acid transport, FRET, protein-protein interaction, confocal imaging, flow cytometry, gene expression analysis, transcriptome

TIIVISTELMÄ

Maaria Tringham

Kationisten aminohappojen kuljetinproteiini y⁺LAT1:n mutaatioiden vaikutus systeemisellä ja solutasolla

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y⁺LAT1 on solukalvoproteiini, joka yhdessä solun pinnan yleisen 4F2hc-antigeenin kanssa muodostaa epiteelisolujen basolateraalikalvolle paikantuvan kationisten aminohappojen kuljettimen. Sen pääasialliset ilmenemiskudokset ovat munuainen ja ohutsuoli, mutta se toimii pienemmässä määrin myös joissakin muissa kudoksissa, kuten istukassa ja immuuniaktiivisissa soluissa. y⁺LAT1:n mutaatiot aiheuttavat virheen y⁺LAT1/4F2hc-kuljettimeen heikentäen lysiinin, arginiinin ja ornitiinin imeytymistä ohutsuolesta verenkiertoon sekä niiden takaisinottoa alkuvirtsasta munuaisissa. Tämä johtaa harvinaiseen perinnölliseen aminohappoaineenvaihduntasairauteen, lysinuurisen proteiini-intoleranssiin, johon liittyy vakavia monielinkomplikaatioita.

Tämä väitöskirja tarkastelee LPI-mutaatioiden seurauksia kahdella eri tasolla: toisaalta aminohappokuljettimen rakenteen tasolla ja toisaalta suomalaisten LPI-potilaiden geeniekspressioprofiilien kautta kuljettimen toiminnan tasolla. Tässä työssä optimoitiin fluorescence resonance energy transfer (FRET) –konfokaalimikroskopiamenetelmä, jota käyttäen havaittiin kuljettimen alayksiköiden dimerisoituvan keskenään y⁺LAT1:n mutaatioista huolimatta. Virtaussytometriset ja FRET-konfokaalimikroskopiakokeet osoittivat y⁺LAT1-molekyylien pyrkivän muodostamaan homodimeerejä sekä ilman 4F2hc:ta että sen läsnä ollessa viitaten vahvasti siihen, että kuljettimen toiminnallinen kokonaisuus on heterotetrameeri.

Suomalaisilla LPI-potilailla on kaikilla *SLC7A7*-geenissä sama LPI-mutaatio, mutta heidän kliiniset taudinkuvansa eroavat toisistaan suuresti. Potilaiden geeniekspressioanalyysi paljasti 926 ekspressioltaan muuttunutta geeniä sekä laajan, useita aminohappokuljettimia koskevan aminohappotasapainon häiriön. Yksittäisten potilaiden y⁺LAT2-kuljettimen ekspressiomuutoksista huolimatta tämän kuljettimen ei havaittu yleisesti kompensoivan y⁺LAT1:n vähentynyttä ekspressiota. Muuttuneiden geenien toiminnot liittyivät mm. tulehdusvasteeseen, immuunipuolustustoimintoihin ja apoptoosiin, mikä viittaa siihen, että LPI:llä on vahva yhteys immuunipuolustukseen.

Avainsanat: Lysinuurinen proteiini-intoleranssi (LPI), y⁺LAT1, *SLC7A7*, aminohappokuljetus, FRET, proteiini-proteiini-interaktio, konfokaalimikroskopia, virtaussytometria, geeniekspressioanalyysi, transkriptomi

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ABBREVIATIONS

4F2hc	4F2 cell surface antigen heavy chain
CAA	Cationic amino acid
CAT	Cationic amino acid transporter
CFP	Cyan fluorescent protein
CNS	Central nervous system
ECFP	Enhanced cyan fluorescent protein
EGFP	Enhanced green fluorescent protein
EYFP	Enhanced yellow fluorescent protein
E_F	Fluorescence resonance energy transfer efficiency
ER	Endoplasmic reticulum
ES cells	Embryonic stem cells
EYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence-activated cell sorting
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
HEK293	Human embryonal kidney cell line 293
HLH	Haemophagocytotic lymphohistiocytosis
LPI	Lysinuric protein intolerance
LPI_{FIN}	The Finnish founder mutation for LPI, c.895-2A>T, (IVS6AS, A-T,-2)
MDCK	Madin-Darby canine kidney cells
NO	Nitric oxide
ORF	Open reading frame
PCR	Polymerase chain reaction
PAP	Pulmonary alveolar proteinosis
ROI	Region of interest
SLC7A6	Solute carrier family 7, member 6
SLC7A7	Solute carrier family 7, member 7
YFP	Yellow fluorescent protein
y^+LAT1, 2	System y^+L amino acid transporter 1, 2

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I-III):

I Kleemola, M., Toivonen, M., Mykkänen, J., Simell, O., Huoponen, K. and Heiskanen, K. M. (2007). Heterodimerization of y^+LAT-1 and 4F2hc visualized by acceptor photobleaching FRET microscopy. *Biochimica et Biophysica Acta – Biomembranes* 1768:2345-2354.

II Tringham, M., Kurko, J., Toivonen, M., Simell, O. and Mykkänen, J. The dimerisation of cationic amino acid transporter y^+LAT1/4F2hc – a FRET study. Manuscript.

III Tringham, M., Kurko, J., Tanner, L., Tuikkala, J., Nevalainen, O.S., Niinikoski, H., Näntö-Salonen, K., Hietala, M., Simell, O. and Mykkänen, J. (2012). Exploring the transcriptomic variation caused by the Finnish founder mutation of lysinuric protein intolerance (LPI). *Molecular Genetics and Metabolism.* 105: 408-415.

1. INTRODUCTION

Lysinuric protein intolerance (LPI) is a rare, recessively inherited aminoaciduria with severe, often life-threatening multi-organ complications. It is caused by mutations in the *SLC7A7* gene that encodes y$^+$LAT1, a transmembrane protein that dimerises with 4F2hc, a common cell membrane antigen, to form a transporter for cationic amino acids. The main functions of this transporter are to facilitate the absorbance of cationic amino acids from the small intestine into the blood flow and to catalyse their reabsorbance in the kidney, though it is also expressed to a small extent in some other tissues, including the placenta, monocytes and lymphocytes. The cellular trafficking and the resulting cellular location of the mutated y$^+$LAT1 vary depending upon the mutation type: the molecules with point mutations are located in the plasma membrane in a manner similar to the wild-type proteins, whereas those with frameshift mutations are retained in the cytoplasm. Despite this, all *SLC7A7* mutations, regardless of their type, abolish the transporter's amino acid transport function with no genotype-phenotype correlation to be observed.

This was the starting point for the investigations included in this thesis. The cause of LPI, the mutations in *SLC7A7*, had been discovered a few years earlier and, at the time, heteromeric amino acid transporters and their function was a popular topic of research. The structure of the y$^+$LAT1/4F2hc transporter was less well studied. It was only known that it was formed by these two subunits and that 4F2hc was required for the correct localisation of y$^+$LAT1 in the plasma membrane. The visualisation of the transporter in intact cells was problematic as while there were specific antibodies for 4F2hc (CD98), there were none for y$^+$LAT1. The LPI research group had just begun using fluorescent reporter proteins as visualisation aids in confocal microscopy, and y$^+$LAT1 had been fused into an EGFP vector. The group was intrigued by the different effect of the frameshift and point mutations on the cellular trafficking of y$^+$LAT1 and wished to discover whether this was due to discontinuation of the interaction with 4F2hc caused by frameshift mutations. The aim was to uncover this by means of fluorescence resonance energy transfer (FRET) techniques, using flow cytometry and confocal microscopy as imaging methods. After establishing the FRET techniques, it was decided that they be utilised to further explore the structure and characteristics of the y$^+$LAT1/4F2hc transporter: The dimer of the two subunits is united by a disulphide bond, the significance of which to the dimer formation was still somewhat under debate. Similarly, it was as then unknown whether the transporter complex was formed as a simple dimer of y$^+$LAT1 and 4F2hc or consisted of two or more of these dimers.

Curiously, despite the fact that the Finnish LPI patients all share the same Finnish founder mutation, their clinical symptoms vary considerably, to the extent that even siblings can have a radically different clinical picture, ranging from extremely mild symptoms to fatal complications. In order to explain the reasons for this, a microarray study was performed to explore the patients' transcriptomic variance, thereby uncovering factors contributing to the variance.

This thesis thus consists of three distinct parts: The first consists of introducing and optimising acceptor photobleaching, a variant of the FRET technique, for the confocal microscopic imaging of the y^+LAT1/4F2hc transporter. The second utilises that technique for visualising the effects of different mutations in y^+LAT1 and/or 4F2hc on the protein-protein interactions between the two and explores the heteromeric structure of the transporter by using FRET in flow cytometry. The third part of this thesis moves the research focus to the systemic level and investigates the transcriptomes of the Finnish LPI patients in the quest for the causes of their vast clinical variation.

My thesis work began by establishing fluorescence resonance energy transfer confocal microscopy for imaging the y^+LAT1/4F2hc transporter, a method that forms a central theme of this thesis to the extent that two thirds of the experiments revolve around it. However, the fluorescence resonance energy transfer in itself, although providing a powerful tool for the protein-protein interaction required for uncovering the structures and characteristics investigated here, is not central for the biological theory behind the research questions. For this reason, rather than beginning the literature review by discussing its principles, I shall leave these to the end of the chapter, along with the other method, transcriptomics. Therefore, in the pages that follow, I shall first shed some light on what we know about heteromeric amino acid transporters y^+LAT1 and 4F2hc, their substrates and function, and what happens when things go wrong.

2. LITERATURE REVIEW

2.1 Cationic amino acids

2.1.1 Amino acids classified

Amino acids are the elemental pieces of a living organism as they are the intermediates of metabolic processes and the building blocks of all proteins. As such, they play a vital role because the availability and properties of amino acids also govern and determine the rates and function of metabolic processes and the properties of bioactive proteins, respectively. Altogether, 20 protein-forming amino acids have been identified, of which ten can be synthesised by humans whilst the remainder need to be obtained from outside the body. On this basis, the amino acids can be divided into essential (need to be obtained from food), including histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, and non-essential (can be synthesised), including alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine. Arginine can be included in both categories since it is non-essential for adults but essential for children as the arginine synthesis in the body may be insufficient to meet its increased demand during periods of rapid growth (Ritter 1996).

Amino acids can also be classified based on their structure. They are formed by a carbon backbone with an amino group at one end and a carboxyl group at the other, and with a side chain of varying electrochemical properties branching out from the mid carbon of the backbone, ranging from the single hydrogen in glycine to the large aromatic side chain in tryptophan. On the basis of their polarity, and the electrochemical properties of their side chains, amino acids fall into the following categories: aromatic (phenylalanine, tryptophan and tyrosine), non-polar non-charged (alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline and valine), polar non-charged (asparagine, cysteine, glutamine, histidine, serine, threonine, tyrosine), polar cationic (lysine and arginine), and polar anionic amino acids (aspartate and glutamate)(Ritter 1996).

Given that the focus of this thesis, y^+LAT1, is a transporter for cationic amino acids, I will next provide a short summary on the biological function and importance of its substrates, lysine, arginine and ornithine, and their main metabolic derivatives.

2.1.2 Lysine

Lysine is an essential amino acid that is particularly important for protein synthesis as it is present in virtually all proteins. Therefore, it plays an important part in a vast spectrum of biological processes because its amine side chains serve as sites for post-translational modifications, such as acetylation, methylation, ubiquitination and succinylation. Lysine acetylation, for example, controls gene expression by regulating histone binding to DNA in nucleosomes, lysine methylation in histones regulates chromatin structure and function, and lysine ubiquitination marks proteins for degradation (Liao, Wang & Regmi 2015).

In studies on livestock, lysine has been proven to be vital for muscle growth and bone development: Dietary lysine supplement has been linked to increased muscle protein accretion and whole-body growth in pigs (Liao, Wang & Regmi 2015), and increased total weight gain and changes in body composition (increase in the muscle to abdominal fat ration) in poultry (Leclercq 1998, Tesseraud et al. 1999, Li et al. 2013). Similarly, dietary lysine deficiency has been documented to reduce muscle weight by up to 50% in chicks, depending upon the strain and muscle type (Tesseraud et al. 1996). Lysine residues in different proteins are also important for the maintenance of the tissue structures; for example, the amine side chains mentioned above facilitate the cross-linking of collagen and elastin into fibrous proteins (Eyre, Paz & Gallop 1984, Liao, Wang & Regmi 2015).

In bone development, malnutrition has been shown to lead to reduced bone formation in rats (Shires et al. 1980), rhesus monkeys (Jha, Deo & Ramalingaswami 1968) and humans (Einhorn, Bonnarens & Burstein 1986), presenting as a reduced quantity of compact bone, osteoblasts and osteoclasts. More specifically, dietary lysine deficiency has been discovered to lead to decreased calcium deposition in bones and defective bone growth in lysine-starved rats (Likins, Bavetta & Posner 1957).

Lysine has also been implicated in various other biological functions: For example, it is an important precursor for the *de novo* synthesis of the neurotransmitter glutamate (Papes et al. 2001). It has a stimulating effect on the growth hormone (Knopf et al. 1965) and insulin secretion (Floyd et al. 1966), and a lysine supplement increases the concentration of postprandial IGF1, whereas dietary lysine restriction reduces the plasma IGF1 level (Takenaka, Takahashi & Noguchi 1996, Liao, Wang & Regmi 2015). Lysine is also essential in lipid metabolism as it is the precursor for carnitine (Kakuda & MacLeod 1994), which normalises blood cholesterol and triglyceride levels and transports long-chained fatty acids from the cytoplasm to be betaoxidated in the mitochondria for ATP production in insulin-sensitive tissues (Steiber, Kerner & Hoppel 2004). In addition to its contributions to the lipid and energy metabolism, carnitine has a multitude of other biological functions, such as improving cardiac performance, counteracting oxidative stress and promoting substrate oxidation in brown adipose tissue (Ferrari et al. 2004).

Lysine is known to be required for the proper maintenance of immunological functions as its deficiency has been linked to an increased susceptibility of mammals to infectious diseases (Datta, Bhinge & Chandran 2001, Li et al. 2007, Liao, Wang & Regmi 2015). Chickens with deficient dietary lysine produced a reduced response to vaccinations, and their spleen was reduced in size relative to body weight, contrary to the usual finding that immune-activation frequently causes spleen enlargement (Chen, Sander & Dale 2003). In contrast to the findings in chickens, the LPI patients, who also exhibit immune system related symptoms, do display marked splenomegaly (Lukkarinen et al. 1999).

2.1.3 Arginine and nitric oxide

Arginine is a semi-essential amino acid that has been implicated in various biological processes and functions. Its *de novo* synthesis occurs mainly in the kidney, in which it is

produced by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) from citrulline (which then, in turn, is recreated either in the urea cycle or in cells with NOS activity). Citrulline is mainly synthesised from glutamine in the small intestine, where it is released into the circulation and transported to the kidney for arginine synthesis. This is known as the renal-intestinal axis. During periods of intensive growth, the *de novo* synthesis may not supply the body with sufficient amounts of this amino acid and, therefore, an addition of dietary arginine is needed, hence its semi-essential nature. (Kakuda & MacLeod 1994, Baylis 2006, Wijnands et al. 2015). It has also long been known to stimulate growth hormone secretion (Knopf et al. 1965, Alba-Roth et al. 1988).

Arginine is essential for nitric oxide production by being its precursor. Nitric oxide, NO, is a highly diffusible bioactive gas with a short lifetime generated by nitric oxide synthase from arginine in a 1:1 reaction with L-citrulline. It was originally identified as an endothelium-derived relaxing factor as it is responsible for vasodilation. Due to its relaxing effect on the vascular smooth muscle cells, it has been been widely and successfully employed in medicine to relieve, for example, hypertension, cardio-pulmonary conditions and pre-eclampsia, the leading cause of maternal and perinatal death (Bhatraju et al. 2015, Sasser, Murphy & Granger 2015). In LPI patients, NO deficiency due to reduced arginine availability has been documented to cause vascular endothelial dysfunction and exacerbate intravascular coagulation (Kayanoki et al. 1999, Kamada et al. 2001). NO is involved in several different signalling pathways, its effect most probably depending upon the concentrations of NO: in lower, neuroprotective concentrations it mediates physiological signalling, such as vasodilation and neurotransmission, whereas in higher concentrations, its effect is neurotoxic and contributes to immune reactions, such as tumour immunity and non-specific host defence (Kakuda & MacLeod 1994, Steinert, Chernova & Forsythe 2010).

Arginine-NO reactions have been implicated in various immunological functions, and arginine deficiency has been linked to sepsis. Despite the increased release of arginine as a result of protein degradation, its consumption, coupled with impaired *de novo* synthesis, reduces its bioavailability. As with arginine, the whole-body levels of NO of patients with sepsis have been equally low, potentially due to precursor depletion, leading to endothelial dysfunction (Bronte et al. 2003, Wijnands et al. 2015).

As a neurotransmitter, NO is involved in synaptic plasticity (learning), neuronal plasticity and excitability, and memory. Its synthesis in the brain is stimulated by NMDA receptors activated by glutamate. The diffusing NO activates the production of cGMP which has a role in several cognitive functions (Susswein et al. 2004, Steinert, Chernova & Forsythe 2010).

Prolonged hyperammonaemia has an effect on cognitive development, and the mechanism is mediated by NO. In a hyperammonaemic state, ammonia detoxification in the brain leads to accumulation of glutamine. As glutamine shares a transporter route with the cationic amino acids, its accumulation reverts the y^+L transport, preventing arginine uptake by the NOS-containing cells, thereby inhibiting NO synthesis and reducing the production of cGMP

(Zielinska et al. 2011). Abnormal NO signalling has also been suggested to play a part in neurodegenerative conditions, such as stroke, Alzheimer's disease and Parkinson's disease (Steinert, Chernova & Forsythe 2010).

2.1.4 Ornithine and the urea cycle

Ornithine is a cationic amino acid that can be synthesised in the body from glutamate or arginine. It has strong links to the development and maintenance of tissues as it is a precursor for polyamines, putrescine, spermidine, and spermine. They have a function in cellular division and proliferation as they prevent cell death and promote growth, wound healing, tissue repair and neuronal development (Kakuda & MacLeod 1994, Lange et al. 2004). Low plasma levels of ornithine and deficiencies in its biosynthesis from glutamate have been linked to spastic paraplegia (Coutelier et al. 2015). On the other hand, increased ornithine concentrations have been associated with pathological aspects of vascular disease; vascular hypertrophy, fibrosis and stiffness (Lange et al. 2004), and an increase in polyamine synthesis has been observed in several types of cancer (Nowotarski, Woster & Casero 2013).

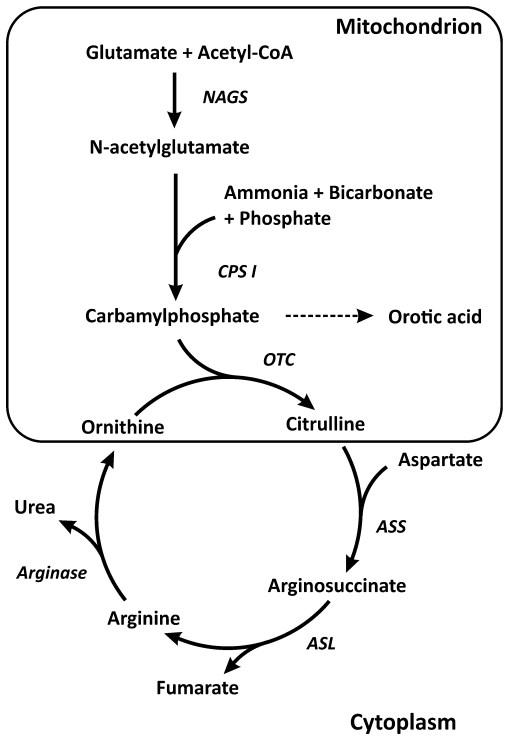

Figure 1. The urea cycle.
The urea cycle converts highly toxic ammonia into urea, which is then expelled from the system in urine. The cycle consists of six enzymatic reactions that take place in hepatocytes, three in the mitochondrial matrix (N-acetylglutamate synthase (NAGS), carbamyl phosphate synthetase 1 (CPS1) and ornithine transcarbamylase (OTC)), and three in the cytosol (argininosuccinate synthase (ASS), argininosuccinate lyase (ASL) and arginase).

Ornithine is also an intermediary in the urea cycle (Figure 1), which converts ammonia into urea and relieves the body of excess nitrogen, thus preventing hyperammonaemia. In the first stages of ammonia detoxification, N-acetylglutamate synthase (NAGS) converts glutamate and acetyl-CoA into N-acetylglutamate, to which carbamyl phosphate synthetase 1 (CPS1) joins ammonia, bicarbonate and phosphate, forming carbamylphosphate, which then enters the urea cycle. The ornithine transcarbamylase (OTC) joins it to ornithine, forming citrulline and releasing orotic acid. Citrulline is modified by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) and turned into arginine, which in turn is processed by arginase, creating ornithine and releasing urea (Caldwell et al. 2015). The urea cycle takes place in the liver; NAGS, CPS1 and OTC function in the mitochondria, whereas the ASS, ASL and arginase activity takes place in the cytosol (Wijburg & Nassogne 2012).

2.1.5 Cationic amino acid transport

Cationic amino acids pass through the plasma membrane assisted by four different amino acid transport systems: y^+, y^+L, $b^{0,+}$ and $B^{0,+}$. Of these, y^+ is selective for cationic amino acids only, whereas the other systems also transport neutral amino acids (Dye et al. 2004). System $B^{0,+}$ transporter $ATB^{0,+}$ even transports selected D-enantiomers (D-serine, D-alanine, D-methionine, D-leucine and D-tryptophan (Hatanaka et al. 2002)). In polarised cells, system $b^{0,+}$ and $B^{0,+}$ transporters are located in the apical plasma membrane of a cell and therefore facilitate the entrance of cationic amino acids into the cell, whereas system y^+L functions in the basolateral plasma membrane, facilitating the exit of cationic amino acids from the cell to the extra-cellular matrix. Together, these apical and basolateral transporters provide a route for cationic amino acid through the epithelial cells and thus have an important role in the absorbance of amino acids in the small intestine, thus introducing them into the system.

System y^+ contains four transporters, CAT1, CAT2A, CAT2B and CAT3 (encoded by *SLC7A1-3*), which are, with the exception of CAT1, mainly expressed in non-epithelial cells, particularly in the liver (CAT2A) and brain (CAT3). CAT1 is expressed almost ubiquitously, with a basolateral localisation in polarised cells (Verrey et al. 2004, Closs et al. 2006). The system y^+ transporters function in facilitating the Na^+- and pH-independent influx of cationic amino acids into these cells (Dye et al. 2004, Verrey et al. 2004, Closs et al. 2006, Bröer 2008, Fotiadis, Kanai & Palacín 2013).

The system $B^{0,+}$ transporter $ATB^{0,+}$ (encoded by *SLC6A14*) is a multi-purpose transporter with a wide range of substrates. It is mainly expressed in the intestine, lung and mammary gland and functions in Na^+- and Cl^--coupled absorption of neutral and cationic amino acids through epithelial cells. In addition to L- and D-amino acids, it can also facilitate the transport of carnitine and γ-aminobutyrate (Munck 1995, Sloan & Mager 1999, Nakanishi et al. 2001, Hatanaka et al. 2002, Sloan, Grubb & Mager 2003, Gupta et al. 2005).

Systems $b^{0,+}$ and y^+L both facilitate the obligatory exchange of cationic amino acids for neutral amino acids in polarised, epithelial cells, system $b^{0,+}$ in the apical and system y^+L in the basolateral membrane. System $b^{0,+}$ was first described in murine blastocysts in

1988. It operates independently of sodium, catalysing the influx of cationic amino acids and cysteine, assisted by their extra-cellular high affinity and the membrane potential. Similarly, it catalyses the efflux of neutral amino acids, apparently due to their intra-cellular accumulation (Van Winkle, Campione & Gorman 1988, Verrey et al. 2004). System y^+L transporters, discovered in 1992 in human erythrocytes, require sodium for the influx of the neutral amino acids (mainly glutamine and leucine) that they mediate in exchange for the efflux of cationic amino acids (lysine, arginine, ornithine) (Devés, Chavez & Boyd 1992). As both systems operate by antiport, no net transport of amino acids is achieved. The system $b^{0,+}$ and y^+L transporters are each formed by two protein subunits and thus belong to the family of heteromeric amino acid transporters, discussed in greater detail in the following chapter.

2.2 Heteromeric amino acid transporters

The heteromeric amino acid transporters (HATs) are a family of plasma membrane amino acid antiporters involved in intercellular amino acid transfer. Their substrate spectrum is broad, consisting of neutral amino acids (amino acid transport systems L and asc), negatively charged amino acids (system x_c^-), and cationic amino acids exchanged with neutral amino acids (systems y^+L and $b^{0,+}$). The heteromeric amino acid transporters facilitate sodium-independent amino acid transport, with the exception of system y^+L, which, as noted in the previous chapter, requires sodium for transporting neutral amino acids exchanged for cationic ones (Fotiadis, Kanai & Palacín 2013).

HATs are formed by two distinct subunits, a heavy chain and a light chain. The heavy chains (the SLC3 family of transporters) are glycoproteins with a single transmembrane unit, a small intracellular amino terminus and a large glycosylated extra-cellular carboxy terminus. To date, two heavy chains, 4F2hc and rBAT, are known to associate with seven light chains, $b^{0,+}$AT (rBAT), and LAT1, LAT2, y^+LAT1, y^+LAT2, xCT and asc1 (4F2hc). The light chains all belong to the LAT family of transporters (the SLC7 family, based on their genes, *SLC7A5-11*), which is a subgroup of the amino acid-polyamine-organocation (APC) superfamily of transport proteins. Based on hydrophobicity studies, they are predicted to have 12 transmembrane domains with cytoplasmic termini, and form a barrel-like pore for amino acids to cross the plasma membrane. It has been demonstrated by Western blotting and immunofluorescence studies that, when expressed alone, the light subunit cannot be inserted into the plasma membrane (Palacín, Borsani & Sebastio 2001, Toivonen et al. 2002); the heavy chains are required for the proper localisation of the complex, apical or basolateral, depending on the heavy chain. In polarised epithelial cells, rBAT guides $b^{0,+}$AT to the apical plasma membrane, whereas the transporter complexes formed by 4F2hc are confined to the basolateral membrane (Chillarón et al. 2001, Wagner, Lang & Bröer 2001, Fotiadis, Kanai & Palacín 2013). The transporter's substrate specificity, however, is defined by the light chain, which is the catalytic subunit of the transporter complex. In some complexes (such as y^+LAT1/4F2hc, y^+LAT2/4F2hc and LAT2/4F2hc), the heavy subunit also facilitates the transport activity (Chubb et al. 2006, Torrents et al. 1998), although some of the light chains ($b^{0,+}$AT and LAT1)

are also capable of residual amino acid transport in the absence of the heavy subunit (Reig et al. 2002) or its extracellular terminus (Bröer et al. 2001, Chubb et al. 2006). All these heteromeric amino acid transporters operate as obligatory amino acid exchangers and thus do not generate net amino acid movement across the plasma membrane (Boyd 2008).

The first of the light subunits to be identified and cloned by Mastroberardino and others, and simultaneously also by Kanai and their group, was LAT1, which catalyses the membrane transport of large neutral amino acids in a sodium-independent manner, its preferred substrates being leucine and tryptophan. It is a 507-amino-acid product of the *SLC7A5* gene with a calculated size of 55 kDa. LAT1 is expressed ubiquitously in non-epithelial cells: for example, in the brain, spleen, thyroid, stomach, testis, liver, placenta and skeletal muscles (Kanai et al. 1998, Mastroberardino et al. 1998, Devés & Boyd 2000, Wagner, Lang & Bröer 2001).

The following year, a second transporter with similar transport characteristics was discovered and named, accordingly, LAT2. Its substrate spectrum is wider than that of LAT1, as apart from the large neutral amino acids it also transports small neutral amino acids, such as glycine and alanine. In addition to amino acids, both LAT1 and LAT2 also transport thyroid hormones (Kinne, Schulein & Krause 2011). LAT2 is encoded by *SLC7A8*, and its amino acid sequence consists of 535 amino acids, of which 50% are identical to that of LAT1. It is strongly expressed in the basolateral plasma membrane of epithelial cells of the proximal kidney tubules, and in decreasing amounts in the placenta, brain, liver, spleen, skeletal muscles, heart, small intestine and lungs (Pineda et al. 1999, Rossier et al. 1999).

The same year, Rajan and others cloned $b^{0,+}$AT, the only light chain dimerising with rBAT (Rajan et al. 1999). True to its name, it transports cationic and neutral amino acids and cysteine independently of sodium, in line with transport system $b^{0,+}$, in the proximal kidney tubules and small intestine, as described in the previous chapter. It is the 54-kDa product of the *SLC7A9* gene and shares up to 65% similarity (40 % identity) with the other light chains (Rajan et al. 1999, Wagner, Lang & Bröer 2001). The heavy chain that forms a $b^{0,+}$ transporter with it, rBAT (encoded by *SLC3A1*), was discovered a few years earlier, in 1993, in rabbit kidney cells (Bertran et al. 1992, Bertran et al. 1993). It is the larger of the two heavy chains, consisting of 685 amino acids and being 78 kDa (unglycosylated) or 85 kDa (glycosylated) in size. It has four extremely conserved sites that share 67-80% identity with similar areas in 4F2hc, the overall identity and likeness of the heavy chains being 30% and 50%, respectively. In contrast to 4F2hc, rBAT locates apically in epithelial cells (Devés & Boyd 2000, Wagner, Lang & Bröer 2001). It appears that both subunits, rBAT and $b^{0,+}$AT, are active in the transport function as the mutations in both have been published as causes for cystinuria, type I and non-type I, respectively (Calonge et al. 1994, Palacín 1994, Feliubadaló et al. 1999).

The only HAT light chain transporting anionic amino acids is xCT, representing transport system x^-_c, first described in human fibroblasts in 1980 (Bannai & Kitamura 1980). The *SLC7A11*-encoded protein is 501 amino acids long and approximately 55 kDa in size. xCT dimerises with 4F2hc to form a sodium-independent cystine-glutamate exchanger, mainly

active in the brain and pancreas. Its function is activated by chemical or physical stress as it supplies cystine for the synthesis of glutathione, an antioxidant protecting cells against oxidative stress (Sato et al. 1999, Sato et al. 2000, Bassi et al. 2001).

The latest-discovered HAT light chain dimerising with 4F2hc is ascAT1, identified and cloned in 2000. It is encoded by *SLC7A10,* and consists of 523 amino acids. ascAT is a sodium-independent transporter of small neutral amino acids that, in addition to L-amino acids, such as serine, alanine, cysteine, glycine and threonine, also transports some D-isoforms, particularly D-serine and D-alanine. Its main mode of function is amino acid exchange, but it can also operate as a channel for facilitated diffusion. Its expression has been documented, for example, in the kidneys, brain, placenta, heart, skeletal muscles, lungs, liver and pancreas (Fukasawa et al. 2000, Wagner, Lang & Bröer 2001).

As 4F2hc and the system y^+L amino acid transporters, y^+LAT2 and, particularly, y^+LAT1 are central to this thesis, I shall concentrate on these in the chapters below and focus less upon the other HATs.

2.2.1 4F2hc

The heavy chain of the human 4F2 cell-surface antigen (4F2hc, FRP-1 or CD98 cell surface antigen, OMIM #158070) was first detected with a 4F2 antibody in monocytes and activated lymphocytes and, in varying amounts, in every tissue culture cell line tested (Haynes et al. 1981). The antigen was a protein complex of approximately 120-130 kDa which was later revealed to be formed by two subunits of approximately 40 kDa and 85 kDa. The antibody recognition site resided in the heavy chain (Hemler & Strominger 1982).

Subsequently, it was discovered that 4F2hc is a type II glycoprotein with a 50-81-amino acid intra-cellular N terminus, a single 23-amino acid transmembrane domain and an extensive extra-cellular C-terminal domain containing two cysteine residues and four potential glycosylation sites. It is slightly smaller in size than the other HAT heavy chain, rBAT, being 72 kDa when unglycosylated but 94kDa when glycosylated (Wagner, Lang & Bröer 2001). It is encoded by the single-copy *SLC3A2* gene, which is an 8-kb gene located in 11q12.3. The full length cDNA is 1854 bp, producing an open reading frame of 1587 bp and resulting in a 529-amino acid-long protein, highly conserved during mammalian evolution. Its amino acid sequence is 30% identical to that of rBAT, with four highly conserved areas sharing 67-80% identity with the sequence with rBAT (Quackenbush et al. 1987, Gottesdiener et al. 1988, Wagner, Lang & Bröer 2001, Fotiadis, Kanai & Palacín 2013).

4F2hc has many roles in various dynamic cell functions: It has been reported to be involved in cell adhesion and cell fusion, for example in fertilisation (Takahashi et al. 2001), implantation of the blastocyst in the endometrium (Domínguez et al. 2010) and formation of the syncytiotrophoblasts of the placenta (Boyd 2008). It is vital for activated vascular smooth muscle cells (Fogelstrand et al. 2009), the formation of osteoclasts (Mori et al. 2001) and skeletal muscles, and the maintenance of the skin structure (Lemaître et al.

2011). The 4F2hc protein is expressed at low levels in quiescent cells but at high levels in all actively proliferating cells. It has been demonstrated to promote rapid proliferation in various cells, most notably in B and T lymphocytes, thus enabling the clonal expansion that is vital for adaptive immunity (Cantor & Ginsberg 2012), and also in invasive and metastatic cancers (Ohkame et al. 2001, Kaira et al. 2008, Kaira et al. 2009, Nguyen et al. 2011, Fei et al. 2014). It mediates these functions by regulating amino acid transport (Devés & Boyd 2000, Verrey et al. 2000, Chillarón et al. 2001, Palacín & Kanai 2004) and by governing integrin signalling (Fenczik et al. 1997, Fenczik et al. 2001, Feral et al. 2005, Cantor & Ginsberg 2012). 4F2hc also promotes anchorage independence, thus facilitating metastasis (Feral et al. 2005). 4F2hc is ubiquitously expressed in all vertebrates, conveying with it the benefits of adaptive immunity specific to vertebrates and, as a trade-off, the caveats of metastatic cancer (Cantor & Ginsberg 2012).

No natural mutations have been detected in 4F2hc, not even in disease states such as LPI (Palacín & Kanai 2004). Experimental disruption of the gene dramatically reduced the growth of ES cells and fibroblasts (Fenczik et al. 2001), prevented clonal expansion of both T and B lymphocytes (Cantor & Ginsberg 2012) and proved embryonically lethal for mice (Tsumura et al. 2003). As its expression is associated with poor prognosis and malignancy in cancers (Kaira et al. 2008, Kaira et al. 2009) and blocking 4F2hc with the specific anti-CD98 antibodies inhibited the growth of tumour cells *in vitro*, 4F2hc has demonstrated some potential as a cancer treatment target (Cantor, Ginsberg & Rose 2008, Hayes et al. 2015).

As mentioned above, part of the stimulatory effect of 4F2hc on actively proliferating cells is achieved through its role as an amino acid transporter with the light chains. In epithelial cells, 4F2hc coupled with the y^+L transporters form a functional entity with $b^{0,+}$/rBAT and LAT2/4F2hc in order to facilitate trans-epithelial amino acid flow: $b^{0,+}$/rBAT is located in the apical plasma membrane and catalyses the sodium-independent influx of cationic amino acids and cysteine in exchange for neutral amino acids. The efflux of cationic amino acids occurs in the basolateral membrane through the y^+L/4F2hc transporter, facilitated by the simultaneous influx of neutral amino acids and sodium (or another positively charged ion). The neutral amino acids are recycled back out through the basolateral membrane by the LAT2/4F2hc, and the ions through the Na^+-K^+-ATPase (Chillarón et al. 2001). Similarly, the transport function of 4F2hc/LAT1 is vital for rapidly proliferating cells as it exchanges glutamine for essential amino acids, for example leucine, supplying the cells with growth-limiting nutrients. Disruption of LAT1 has indeed been proven to arrest tumour cell growth in rat models (Ohkame et al. 2001).

2.2.2 y^+LAT1

The system y^+L amino acid transporter 1 (y^+LAT1 or y^+LAT-1, OMIM #603593) was first identified and described in 1998 (Torrents et al. 1998). It is the *SLC7A7*-encoded glycoprotein-associated trans-membrane protein formed by 511 amino acids with a predicted molecular mass of approximately 56 kDa. Based on its amino acid sequence and hydrophobicity studies, its 3D structure is predicted to be similar to the other HAT light chains: 12 TM

domains, with extra- and intracellular loops between them and intracellular N and C termini (Wagner, Lang & Bröer 2001).

y^+LAT1 dimerises with 4F2hc, which dictates its basolateral localisation in epithelial cells. It is mainly expressed in the epithelial cells of the small intestine (jejunum and ileum, (Dave et al. 2004)) and proximal kidney tubules and, to a lesser extent, also in peripheral lymphocytes, monocytes/macrophages, brain microglia (Rossi et al. 2015), liver, pancreas, epididymis, testis, ovary, placenta, lungs and thyroid. Dave and others performed a detailed assay of assorted mouse tissues that confirmed intense expression of y^+LAT1 in the small intestine and in the proximal kidney tubules, where its expression follows a decreasing axial gradient (Bauch et al. 2003). No expression was detected in the stomach, colon, or brain (Dave et al. 2004).

y^+LAT1 has the largest physiological significance in the small intestine, facilitating absorbance of free cationic amino acids from the gut to the blood circulation, and in the kidney, where it provides a way for cationic amino acids to be reabsorbed from the primary urine. As the kidney also supplies the body with arginine by conversion of plasma citrulline, y^+LAT1 is also responsible for the release of this arginine from the kidney (Bröer et al. 2000). With regard to its substrates, it has the strongest affinity for arginine, lysine and ornithine (of the cationic amino acids), and leucine (of the neutral amino acids) (Devés & Boyd 1998).

In addition to the above, y^+LAT1 is suggested to be involved in immunological functions, although this involvement is yet to be thoroughly researched. Rotoli and others noted that in monocytes, arginine transport was carried out by y^+LAT1, which could be stimulated by INFγ (Rotoli et al. 2004). The group also discovered that in monocytes/macrophages *SLC7A7* expression, regulated by GM-CSF, seems to be required for the appropriate phagocytotic function of these cells, since a defect in *SLC7A7* was observed to weaken their phagocytotic activity (Barilli et al. 2010).

SLC7A7 – the genetic aspect

The y^+LAT1-encoding gene, *SLC7A7* (NM_001126106), is located in 14q11.2, in the minus orientation. It is 46.6 kb in size and contains 11 exons and 10 introns (Figure 2). The coding region starts at the beginning of the third exon (c.349), resulting in an open reading frame of 1536 bp (Torrents et al. 1998).

Three different transcript variants of human *SLC7A7* have been detected, one noncoding and the two others differing from each other at the 5' untranslated region but resulting in the same open reading frame (Noguchi et al. 2000, Puomila et al. 2007). In transcript variant 1, the second exon is skipped, whereas transcript variant 2 only begins at the second exon. Transcript variant 1 has been detected in the brain, peripheral leukocytes, cultured lymphoblasts and fibroblasts, and transcript variant 2 in the kidney, small intestine and also in the brain. The alternative transcription results from utilising the two alternative promoters regulating the gene expression, promoter 1 and 2 in front of exons 1 and 2, respectively. The downstream promoter contains a TATA box motif, whereas the upstream promoter is TATA-less (Puomila et al. 2007).

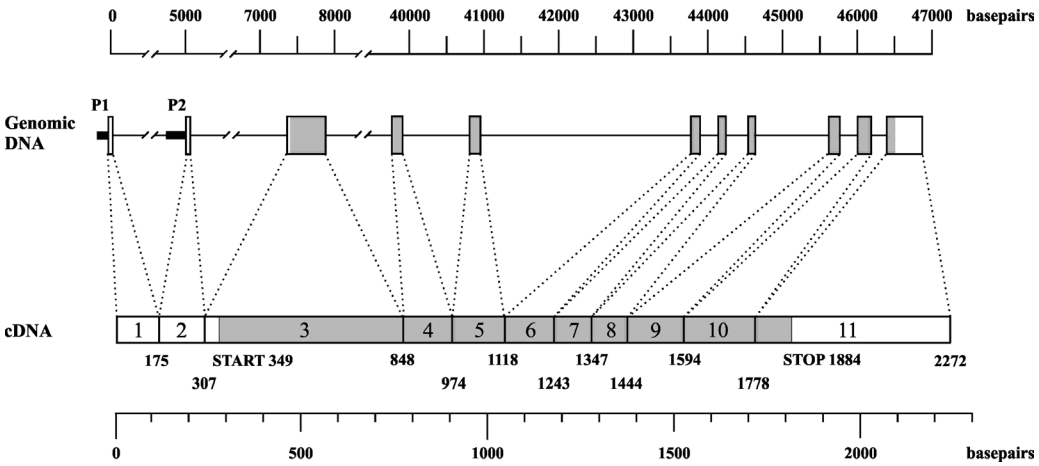

Figure 2. The schematic representation of the *SLC7A7* gene.
The *SLC7A7* genomic DNA contains 11 exons (illustrated by boxes) and 10 introns (lines). The sizes of the discontinued introns 1, 2 and 3 are 4.2, 1.9 and 32.9 kb, respectively. P1 and P2 mark the locations of the alternative promoters in front of exons 1 and 2. The grey shading represents the coding region, starting from the beginning of exon 3 (START 349 in cDNA). The numbers under the cDNA continuum state the first bases of each exon. The image was modified from Mykkänen et al. (2000) with the kind permission of the copyright holder, Oxford University Press (©Oxford University Press).

2.2.3 y^+LAT2

The second system y^+L transporter, y^+LAT2, was identified by Torrents and others based on its similarity to y^+LAT1 (Torrents et al. 1998). It is the 56-kDa product of the *SLC7A6* gene (OMIM #605641) and consists of 515 amino acids that form a sequence 75% identical to that of y^+LAT1. Similarly to y^+LAT1, it has 12 TM domains, and it forms a y^+L transporter with 4F2hc, its main substrates being arginine, lysine, leucine, glutamine, histidine and methionine (Bröer et al. 2000). y^+LAT2 is more ubiquitously expressed than y^+LAT1, but usually in tissues in which y^+LAT1 does not feature, particularly in the brain, but also in the stomach, colon, testis and parotis, and to a lesser extent in the small intestine, kidney, heart, spleen, lung and liver (Bröer et al. 2000, Wagner, Lang & Bröer 2001, Bröer & Wagner 2002, Shoji et al. 2002). y^+LAT2 preferentially exchanges L-arginine with L-glutamine and Na^+, and it has an important role in the brain, releasing arginine from the arginine-producing cells in exchange for glutamine (surplus in the brain) in order to be available for cells expressing nitric oxide synthase as a precursor for nitric oxide (NO) (Zielinska et al. 2011, Zielinska et al. 2012).

To my knowledge, only one mutation in *SLC7A6* has ever been reported, and this was in connection with schizophrenia (Fromer et al. 2014).

2.2.4 Dimer formation of the heteromeric amino acid transporters

True to their name, the heteromeric amino acid transporters are formed as a heteromer of one or more pairs of a heavy chain and a light chain, the dimer formation being essential for the transport function. The interaction of the heavy chains and light chains appears to

be similar to that of Na^+- or H^+K^+-transporter subunits: the β subunits lead the catalytic α subunit to the plasma membrane and stabilise the complex, thus facilitating its function (Møller et al. 1996).

As mentioned before, the catalytic subunit in the heteromeric amino acid transporter complex is the light subunit (LAT1, LAT2, y^+LAT1, y^+LAT2, xCT, asc1 and $b^{0,+}$AT) which can reach the plasma membrane only when dimerised with the heavy subunit (4F2hc or rBAT). This process is assisted by a covalent connection between the chains in the form of a disulphide bridge (Figure 3). However, this bridge is not an essential requirement for the expression of the light chain at the plasma membrane but does seem to stabilise the complex, thus preventing its dissociation (Bröer et al. 2001). The importance of this connection for the transport function seems to vary depending on the light chain; if the connection is abolished, the transport activity of y^+LAT1 and y^+LAT2 is severely hindered, whereas LAT1 is able to continue (Torrents et al. 1998, Chubb et al. 2006). The conserved cysteine residues in the extra-cellular domains of the subunits seem likely anchoring points for the disulphide bridge: C109 in 4F2hc, C151 or C174 in y^+LAT1 and C159 or C182 in y^+LAT2, for example. Upon the discovery of y^+LAT1 in 1998, Torrents and others tested the importance of C109 in 4F2hc for the dimer formation and discovered that mutating the residue led to severing of the dimer bond in an immunoprecipitation experiment. Mutating the C109 residue also abolished the transport capacity of the complex. It remained unclear which was the counterpart of the disulphide bond-forming cysteine residue in the light unit: C151 located in the second predicted extracellular loop or C174 in predicted transmembrane domain IV (Torrents et al. 1998).

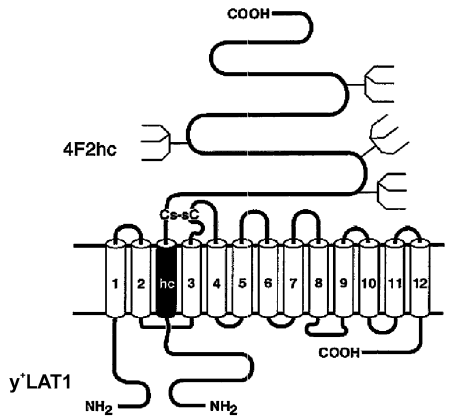

Figure 3. The predicted dimer of y^+LAT1 and 4F2hc.
4F2hc and y^+LAT1 are postulated to form a dimer with the help of a disulphide bridge connecting the two proteins. The cysteine residues on which the disulphide bridge is anchored (Cs-sC) are located in the extracellular neck of 4F2hc and, most probably, in the second extra-cellular loop of y^+LAT1. 4F2ch contains four N-linked glycosylation sites at p.264, p.280, p.323 and p.405, depicted by fork-like structures in the image (Quackenbush et al. 1987), potentially contributing to its interactions with other membrane proteins and those of the extra-cellular matrix. The image was modified from Verrey et al. (1999), with the kind permission of the copyright holder, Springer New York LLC (©Springer New York LCC).

The interaction of 4F2hc with y^+LAT2 requires the most C-terminal domain of 4F2hc, whereas the interaction required for forming the LAT1/4F2hc neutral amino acid transporter occurs at the 4F2hc N terminus. It has been suggested that the light chains form a double-barrelled structure with two separate translocation pores, one for efflux, one for influx. When the pore structures were discovered, it was assumed that the each pore would be specific to amino acids of a certain electrochemical charge (i.e. one pore for neutral, one for cationic amino acids). However, this is not how the structure functions since amino acids of different charges can compete with each other for transport (Chubb et al. 2006).

It has not yet been established whether the functional transporter units of the y^+LAT1/4F2hc transporters are formed as heterodimers [y^+LAT1/4F2hc] or heteromultimers [y^+LAT1/4F2hc]$^{2+}$, consisting of two or more of each subunit. Previous research on this has produced contradicting evidence: In 2006, Fernández and others discovered that *in vivo*, the anionic amino acid transporter light chain xCT forms a single dimer with 4F2hc and deduced from this that when the light chains are partnered with 4F2hc, they preferentially occur as single dimers. rBAT, on the other hand, preferentially formed multimers with $b^{0,+}$AT. When the light chains normally dimerising with 4F2hc were, in experimental conditions, dimerised with rBAT instead, they also formed multimers. Thus it was concluded that it is the heavy chain that defines the heteromerisation status of the holotransporter, and that for 4F2hc, it is a single heterodimer (Fernández et al. 2006).

The following year, however, the 3D structure of the 4F2hc ectodomain was crystalised successfully by Fort and others. Based on both the 3D modelling simulations and the expression studies of human 4F2hc *in vivo* in various cultured cell lines, it was concluded that 4F2hc has a strong tendency to form homodimers. The homodimerisation was thought to be triggered by parts of the protein other than the ectodomain, that is either the TM domain or the N terminus, and guided by shape and electrostatic complementarities rather than hydrophobic forces (Fort et al. 2007).

Further evidence supporting the multimerisation theory of this transporter was procured by Sperandeo and others in a study in which both of the y^+L transporters were experimentally co-expressed in the same cells (Sperandeo et al. 2005b) as discussed below.

2.2.5 The interplay of y^+LAT1 and y^+LAT2

The two y^+L type transporters, y^+LAT1 and y^+LAT2, share the same transport properties and role in the cell to a very great extent. Their tissue expression patterns *in vivo,* however, are largely complementary; therefore, y^+LAT1 and y^+LAT2 do not normally feature in the same tissues. The potential interaction between these transporters has puzzled researchers for quite a while, and two alternative theories have have been proposed.

Shoji and others measured the relative amounts of *SLC7A6* to *SLC7A7* mRNA in tissues affected / unaffected by LPI and reported a marked increase in the ratio from unaffected

to affected cells, leading them to speculate on the interrelation of the gene expression of these two genes and a possible modulatory effect of the *SLC7A6* expression on LPI symptoms (Shoji et al. 2002). As they observed no difference in the level of *SLC7A7* expression between the genotypes (unlike with the LPI_{Fin} mutant, which showed very low expression in a previous northern blot study (Toivonen et al. 2002)), they assumed the difference in the ratio must relate to the potentially compensatory increase of *SLC7A6* expression.

Another type of interaction between the transporter light chains was reported by Sperandeo and others when investigating the effect of an LPI mutation in y^+LAT1 on the observed y^+L transport activity in an experimental setting of cells also containing y^+LAT2. In the experiment, not only did the presence of certain mutations in y^+LAT1 affect the function of wild type y^+LAT1 (heterozygous state), but they also disturbed the function of y^+LAT2, leading the group to the hypothesis that instead of the single dimer expected of a transporter complex containing 4F2hc, the structure of the y^+L transporters could be a heteromultimer, formed by two or more heterodimers (dimers of the light and heavy subunit together) in the same complex (Sperandeo et al. 2005b).

2.2.6 The consequences of *SLC7A7* mutations for y^+LAT1

The *SLC7A7* mutations give rise to lysinuric protein intolerance (LPI) by obliterating the amino acid transport function of the y^+LAT1/4F2hc transporter in the basolateral plasma membrane. The apical surface of a cell usually serves as the entrance route of amino acids into the cell, whereas the efflux occurs through the basolateral side. A defect in a basolateral amino acid transporter is bound to have more serious consequences than an apical defect since there are alternative routes for amino acids into the cell: Only some of them enter the cell as free amino acids, the others as di- or tripeptides, which have their own transporters. The amino acid transporters in the basolateral plasma membrane, however, are the sole exit routes for amino acids from cells (Camargo, Bockenhauer & Kleta 2008).

In the experiments on the effects of different mutations on the y^+LAT1/4F2hc transport capacity in *Xenopus* oocytes, it was observed that all missense (for example, G54V), nonsense (for example, W242X) and frameshift mutations (for example, LPI_{Fin} and 1262delC) tested caused a complete loss of function for the amino acid transporter (Torrents et al. 1999, Mykkänen et al. 2000, Sperandeo et al. 2005a). However, there is an exception to this rule, as Sperandeo and others discovered a y^+LAT1 mutant (F152L) that retains some of its transport capacity when co-expressed in *Xenopus* oocytes with 4F2hc. Nevertheless, its activity was not strong enough to maintain CAA transport when another mutant y^+LAT1 was added. In the same expression study, they also observed a dose-dependent transport activity-suppressing effect of y^+LAT1 mutation e36del on the transport capacity of y^+LAT2 and wt y^+LAT1. This is interesting for two reasons: First, it provides an insight into the potentially tetrameric structure of the y^+L transporters as discussed above, and, second, it suggests a partially dominant-negative effect of the

y^+LAT1 mutations (Sperandeo et al. 2005b). This is puzzling since both the literature and the clinicians report the heterozygote carriers of LPI mutations to be phenotypically healthy (apart from some of the LPI heterozygotes disliking milk) (Norio et al. 1971, Sperandeo et al. 2005b).

The mutations in y^+LAT1, whether missense or frameshift, do not seem to prevent the heteromer formation between y^+LAT1 and 4F2hc (Toivonen et al. 2013). The effect of the mutation on transporter localisation, however, differs based on the type of mutation: The missense mutants, including G54V, when co-expressed with 4F2hc, reach the plasma membrane in a manner similar to that of the wt protein in *Xenopus* oocytes (Mykkänen et al. 2000) and transfected mammalian cells (Toivonen et al. 2002), whereas the frame shift mutants, including LPI_{Fin} and 1548delC, are retained in the cytoplasm.

2.3 Lysinuric protein intolerance (LPI)

2.3.1 The patho-mechanism of LPI

LPI is a rare primary aminoaciduria of autosomal recessive inheritance characterised by impaired absorption and reabsorption of cationic amino acids in the small intestine and kidney tubules, respectively. This results in excessive renal clearance and diminished plasma concentrations of arginine and ornithine and, in particular, lysine. Due to the diminished pools of arginine and ornithine, the function of the urea cycle is impaired, reducing the systemic nitrogen clearance and resulting in potential hyperammonaemia (a rise in the body ammonia concentrations to toxic levels) following protein intake. In addition to urea cycle dysfunction, the cationic amino acid starvation leads to variable clinical and biochemical symptoms, explored in greater detail in the chapters below and summed up in Table 2 (reviewed in, for example: Simell 2001, Sebastio, Sperandeo & Andria 2011, Ogier de Baulny, Schiff & Dionisi-Vici 2012).

LPI is caused by different *SLC7A7* mutations (Borsani et al. 1999, Torrents et al. 1999), 65 of which have been published previously (Table 1), all resulting in the same aminoaciduria when both copies of the gene carry a mutation. No genotype-phenotype correlation has been observed; on the contrary, the same mutational genotype can give rise to extremely diverse clinical symptoms, even within families. Different *SLC7A7* mutations have been identified in over 25 different populations (Cimbalistiene et al. 2007, Sperandeo, Andria & Sebastio 2008, Ko et al. 2012, Habib et al. 2013, Al-Shamsi et al. 2014). Most of these are sporadic individual cases, but three concentrations of patients have been detected, in Campania and Calabria in Southern Italy (Parenti et al. 1995), in Iwate, Northern Japan, and in Finland. The Italian LPI cases are due to several different mutations, but the North-Japanese all share the same founder mutation, R410X (Inoue et al. 2003). The Finnish LPI patients and their mutation are discussed below.

Table 1. LPI mutations based on the mutation type

Mutation type	N		References
Missense/nonsense	32	(49.2%)	(Torrents et al. 1999, Mykkänen et al. 2000, Noguchi et al. 2000, Sperandeo et al. 2000, Palacín, Borsani & Sebastio 2001, Shoji et al. 2002, Koizumi et al. 2003, Sperandeo et al. 2005b, Sperandeo et al. 2005a, Sperandeo, Andria & Sebastio 2008, Font-Llitjós et al. 2009, Shinawi et al. 2011, Barilli et al. 2012, Ben-Rebeh et al. 2012, Habib et al. 2013, Posey et al. 2014)
Deletion	19	(29.2%)	(Borsani et al. 1999, Torrents et al. 1999, Mykkänen et al. 2000, Sperandeo et al. 2000, Kamada et al. 2001, Palacín, Borsani & Sebastio 2001, Shoji et al. 2002, Sperandeo et al. 2005a, Font-Llitjós et al. 2009, Posey et al. 2014, Carpentieri et al. 2015)
Insertion	7	(10.8%)	(Borsani et al. 1999, Mykkänen et al. 2000, Sperandeo et al. 2000, Font-Llitjós et al. 2009, Güzel-Ozantürk et al. 2013)
Insertion + deletion	1	(1.5%)	(Al-Shamsi et al. 2014)
Splice site	6	(9.2%)	(Torrents et al. 1999, Borsani et al. 1999, Noguchi et al. 2000, Sperandeo et al. 2000, Sperandeo et al. 2005b, Cimbalistiene et al. 2007, Font-Llitjós et al. 2009, Ben-Rebeh et al. 2012)
Total	**65**	**(100%)**	

2.3.2 LPI in Finland

LPI was first reported in Finland in 1965 by Perheentupa and Visakorpi as familial protein intolerance (Perheentupa & Visakorpi 1965), and was subsequently renamed lysinuric protein intolerance (LPI), due to the massive secretion of lysine in urine (Norio et al. 1971) by Norio and others, who also discovered its recessive mode of inheritance. In 1997, the LPI gene was mapped to the long arm of chromosome 14 with linkage analysis (Lauteala et al. 1997), and in 1999 its identity was discovered to be *SLC7A7*, coding for the cationic amino acid transporter protein y^+LAT1 (Borsani et al. 1999, Torrents et al. 1999).

LPI has been included in the 'Finnish Disease Heritage' (a collection of 36 rare genetic diseases that, due to the Finnish population history, have a considerably higher prevalence in Finland than in any other country (Norio, Nevanlinna & Perheentupa 1973, Norio 2003a, Norio 2003b, Norio 2003c)); of the approximately 150 patients diagnosed in the world, about 50 are Finnish or of Finnish descent, and the reported prevalence of LPI in Finland is 1:76 000 (Norio 2003c). The overrepresentation of this disease in Finland may partly be a result of underdiagnosis of it in other countries as the clinical picture of LPI is very variable, and it is seen by clinicians so rarely.

In Finland, similarly to Northern Japan, only one mutation, c.895-2A>T ($LPI_{Fin,}$ previously known as 1136-2A>T, 1181-2A>T, IVS6AS, A-T, -2), has been detected. LPI_{Fin} is an intronic missense mutation that substitutes T for A as the second to last base of intron 6, thereby demolishing the splicing site in front of exon 7 and leading to cryptic splicing at the next AG site, 10 bp downstream in exon 7 (p.T299IfsX10). This disrupts the reading frame, potentially leading to a shortened polypeptide due to a new, premature stop codon (Torrents et al. 1999, Mykkänen et al. 2000).

2.3.3 The LPI mouse model

Creating a mouse model for LPI was a long and painstaking process undertaken by the Italian group in the mid-to-late 2000s (Sperandeo et al. 2007). Using an *Slc7a7* mutant clone from an embryonic stem cell library, they created heterozygous *Slc7a7*$^{+/-}$ mice which they bred in order to generate null *Slc7a7*$^{-/-}$ offspring. The first observation was that, inconsistently with the Mendelian segregation, only 3% of the over 600 pups were *Slc7a7*$^{-/-}$ homozygotes, which raised the issue of either high prenatal or neonatal mortality. Only two *Slc7a7*$^{-/-}$ mice, a male and a female, survived until adulthood. Their overall phenotype was very mild; the mice showed no symptoms of LPI apart from poor growth when maintained on a protein-restricted diet with a citrulline supplement. When, after 25 months, the citrulline supplement and dietary protein restriction of the male mouse were ceased and the mouse was fed normal fodder, it began to present neurological symptoms (hypotonia, tremors) and weight loss (65% of initial weight), until 15 days later, it died. The female mouse fared well until it cannibalised its prematurely born pups, presented severe neurological symptoms due to acute hyperammonaemia, and died a few hours later.

The clinical and biochemical symptoms of the *Slc7a7*$^{-/-}$ mice included intrauterine growth restriction (IUGR), poor growth and delay of bone development after birth, and elevated urinary concentrations of arginine, ornithine and orotic acid when maintained on the low-protein diet supplemented with citrulline. When the citrulline supplement was removed and the protein load increased on purpose or by accident, the mice showed signs of acute hyperammonaemia accompanied by increased urinary excretion of lysine.

The gene expression analysis of the liver of the *Slc7a7*$^{-/-}$ foetuses revealed a 7.4- and 4.0-fold downregulation of *Igf1* and *Igf2*, respectively, to be the reason for IUGR. Differential regulation of other Igf-related genes was also noted: *Igfbp1* and *Igfbp4* (down), and *Igfbp2*, *Igfbp5* and *Igfbp6* (up). In the intestines and livers of adult animals, differential expression of the Igfs was not altered. Instead, the largest group of differentially expressed genes were related to transport, the most strongly down-regulated gene being the Na^+-Pi co-transporter gene *Slc34a2* (the others were cationic amino acid transporter gene *Slc7a2*, and *Slc7a6*), and the strongest upregulation being displayed by *Igfbp1* (another being the $b^{0,+}$AT gene, *Slc7a9,* for instance). The genes of the urea cycle enzymes were also differentially expressed (arginase, argininosuccinate lyase and glutamate dehydrogenase 1, up-regulated; ornithine transcarbamylase, down-regulated) (Sperandeo et al. 2007).

2.3.4 The biochemical parameters/findings

LPI is characterised by the altered plasma concentrations of lysine, arginine, ornithine (decreased) and glutamine (increased) that contribute to changes in the levels of related bioactive agents, such as nitric oxide (NO), and those necessary in biological processes, such as the urea cycle. The concentrations of the cationic amino acids, particularly those of arginine and ornithine (and, in addition, citrulline and nitric oxide) are, to some extent, interdependent since they function as intermediaries in the same biological processes and can be converted into each other. Therefore, disturbances in the balance of one will invariably affect the other.

Before LPI patients began to receive lysine supplements, their measured plasma lysine concentrations were approximately 30-53% of the reference values (patients' range 34-154 µmol/L; reference range for adults, 114-289 µmol/L) and even with the supplement only about 41-57% (range 47-164 µmol/L) (Tanner et al. 2007a). Contributing to this, the urine concentrations of the cationic amino acids were greatly increased, resulting in selective amino acid starvation. The plasma concentrations of some neutral amino acids, mainly glutamine, glycine and alanine, however, were raised (Simell & Perheentupa 1974, Rajantie, Simell & Perheentupa 1980b, Rajantie, Simell & Perheentupa 1983, Simell 2001, Lukkarinen et al. 2003, Tanner et al. 2007a, Näntö-Salonen, Niinikoski & Simell 2012).

In order to counteract the lysine starvation, the patients currently receive an oral supplement of lysine administered to them with meals, according to their tolerance, despite the fact that in the late 1970s, Rajantie and others observed no increment of plasma lysine in LPI patients following oral doses of lysine. They experimentally administered lysine to the patients as a free amino acid or as a part of a dipeptide, but the form of the amino acid complex made no difference (Rajantie, Simell & Perheentupa 1980a) as although the dipeptides can be transported into the cell through a dipeptide transporter at the apical plasma membrane, the amino acids are only absorbed through the basolateral cell membrane as free amino acids (Boyd 2008). A *per os* lysine supplement, therefore, seems somewhat ineffective in a condition in which its basolateral transport in the small intestine is defective, but subsequent research has proven that the patients do nonetheless benefit from the tailored supplement, which improves their plasma lysine nutrition markedly (Lukkarinen et al. 2003, Tanner et al. 2007a).

As noted previously, arginine is a precursor of nitric oxide, and, therefore, changes in its bioavailability are also expected to have some effect on the levels of NO. The LPI patients suffer from hypertension, which suggests low plasma NO levels as NO is a vasodilating agent in vascular endothelial cells that adjusts them to blood pressure (Kamada et al. 2001). When it comes to the LPI patients, contradictory observations have been reported: the Japanese group reported decreased plasma NO_3^-/NO_2^- concentrations in one Japanese patient (Kayanoki et al. 1999, Kamada et al. 2001), whereas the Italian group measured elevated NO_3^- values in the plasma of three LPI patients, reflecting increased NO production (Mannucci et al. 2005). Differences were also found in the plasma arginine levels: the Japanese patient's arginine concentrations were decreased, whereas those of the Italian patients were normal. Manucci and others hypothesised that the availability of arginine was the key to the contradicting plasma NO levels as it is used as the substrate in NO production. The impaired arginine efflux traps it in cells and, by providing ample substrate for the NOS, promotes excess NO production (Mannucci et al. 2005).

Other biochemical markers of LPI include a raised serum concentration of ferritin with virtually no storage iron in the bone marrow and increased activity of lactate dehydrogenase, which is regarded as a reporter molecule for tissue damage (Rajantie et al. 1980, Rajantie, Rapola & Siimes 1981, Nagata et al. 1987). In the affected patients, urinary excretion of orotic acid is also increased. Orotic acid, also known as vitamin B13, is a compound overproduced in an alternative pathway when the urea cycle is not functioning properly. Excessive amounts

can therefore be measured in the urine in the hyperammonaemic conditions caused by urea cycle defects (Brosnan & Brosnan 2007). Defects of lipid metabolism (high cholesterol and plasma triglycerides) have also been observed in patients who display persistent hypocarnitinaemia (Tanner et al. 2008, Tanner et al. 2010).

Hyperammonaemia in LPI patients is currently prevented by using various nitrogen scavenging drugs and a citrulline supplement (urea cycle intermediate), both of which increase their protein tolerance. Citrulline is a neutral amino acid, and it can thus be readily absorbed in LPI in order to replenish the urea cycle as the transport defect has no adverse effect on its absorbance (Rajantie, Simell & Perheentupa 1980b). Hypertension and high plasma triglyceride and cholesterol levels are also medicated accordingly (Tanner et al. 2007a).

2.3.5 The classic clinical picture

When breast-fed, LPI babies usually remain asymptomatic and thrive well, which has been thought to derive from the low protein content of breast milk. In contrast, cow's milk and milk substitute formula, even when dilute, set off the symptoms; therefore, the tolerance is specific to human milk. Boyd and Shennan have a fascinating theory for this: They suggest that the breast milk carries micro-vesicles containing wt y^+LAT1 mRNA that is ingested by the suckling babies and then taken up by the cells in the gastrointestinal tract, protecting them against the onset of the disease (Boyd & Shennan 2010). The symptoms (Table 2) therefore only emerge after weaning: malaise, nausea, vomiting, voluminous diarrhoea, and fatigue or periods of unconsciousness after protein ingestion, abdominal pains and failure to thrive (Perheentupa & Visakorpi 1965, Simell et al. 1975, Perheentupa & Simell 1974, Simell 2001). When old enough to do so, the children quickly learn to refuse protein-rich food-stuffs (protein aversion) due to their diminished dietary protein tolerance. If maintained on a low-protein diet, the children fare reasonably well. However, if they are forced to intake larger amounts of protein, they exhibit the previous symptoms due to their inability to absorb the cationic amino acids (gastro-intestinal symptoms) and the rise in systemic ammonia levels (hyperammonaemia; symptoms of the central nervous system (CNS)) caused by the impaired function of the urea cycle (Simell 2001).

One of the symptoms of LPI is poor growth, usually ranging from a level of -2 to -6SD or less, resulting in a short stature. The bone age is often delayed by 1-5 years, resulting in prolonged growth, often lacking the pubertal growth spurt, with the patients reaching skeletal maturity later than at the age of 20 (Svedström et al. 1993). The growth hormone (GH) levels are low to deficient, and many young patients benefit from growth hormone treatment as, in addition to promoting growth, GH also promotes protein synthesis, prevents protein break-down and stimulates food and protein intake, thus increasing protein tolerance (Goto, Yoshimura & Kuroiwa 1984, Esposito et al. 2006, Niinikoski et al. 2011, Evelina et al. 2015a, Evelina et al. 2015b). The affected patients have structural skeletal abnormalities and abnormal bone structure (thin cortices, end-plate impressions of the vertebral bodies, metaphyseal changes and lamellar subcortical erosion (Svedström et al. 1993)), fragility fractures, osteopenia and osteoporosis (Carpenter et al. 1985, Parto et

al. 1993a, Posey et al. 2014), potentially caused by defective bone formation due to lack of cationic amino acids, as seen in chapter 2.1.2.

Table 2. Summary of the clinical and biochemical signs of LPI

Category of symptoms	Manifestations in LPI patients
Laboratory findings	Massive lysinuria
	Argininuria, ornithinuria
	Orotic aciduria
	Low plasma lysine, arginine, ornithine
	Raised plasma glutamine
	Raised plasma lactate dehydrogenase
	Raised plasma ferritin
	Raised plasma ammonia levels after protein intake (hyperammonaemia)
	Persisting anaemia
	Leukocytopaenia
	Thrombocytopaenia
Growth and well-being	Failure to thrive
	General malaise
	Severely short stature
	Sparse hair
Gastro-intestinal	Vomiting
	Diarrhoea
	Protein aversion
Abdominal	Hepato-splenomegaly
	Steatosis
	Pancreatitis
	Abdominal pain
Skeletal	Osteopenia, osteoporosis
	Delayed bone age
	Proneness to fractures
Muscular	Hypotonia
	Muscle weakness, muscle atrophy
Renal	Chronic renal insufficiency
	Hypertension
	Fanconi syndrome
Respiratory	Interstitial changes
	Pulmonary haemorrhages
	Pulmonary alveolar proteinosis
Neurological	Drowsiness to coma after protein intake
	Cognitive developmental delay if hyperammonaemia not under control
Haematological	Bleeding diathesis
Immunological	Poor vaccination response
	Impaired immunoresponse to viruses
	Impaired macrophage function
	Potential HLH*/ macrophage activation syndrome

* HLH = Haemophagocytic lymphohistiocytosis

The affected patients are small and thin, with sparse hair and little muscle mass as they are balancing between dietary protein starvation and hyperammonaemia. One of the typical features related to this is muscular hypotonia, frequently observed in LPI, and potentially contributing to osteoporosis and decrease in bone mass (Svedström et al. 1993). It has also been speculated that, in addition to the protein starvation, the chronic overproduction of TNFα seen in LPI patients may contribute to their general failure to thrive and muscle weakness (Duval et al. 1999).

Almost without exception, all the patients develop hepatosplenomegaly. It is particularly dramatic in children and those with fatal complications: for no obvious reasons, their spleens and livers are twice the weight of normal organs, the livers become hard and display pronounced steatosis and cholestasis (Parto et al. 1994a, McManus et al. 1996).

2.3.6 Complications

LPI has traditionally been regarded as a relatively benign disorder that is easily maintainable with a low-protein diet with a citrulline supplement and nitrogen scavenging medication. The main concern has been the functioning of the urea cycle as prolonged hyperammonaemia deteriorates the CNS, affecting cognitive development and leading to hepatic encephalopathy (HE) (Zielinska et al. 2012). During the course of recent research, however, it has become increasingly obvious that due to its life-threatening renal, pulmonary, and hepato-immunological dimensions, LPI is actually a more complex disorder than was previously envisioned.

Most of the adult patients suffer from a chronic renal insufficiency/nephropathy of varying severity (tubular or glomerular dysfunction, proteinuria, microscopic or macroscopic haematuria), and some develop an end-stage renal disease requiring dialysis and a kidney transplant (Tanner et al. 2007b). Without proper treatment, this would naturally be fatal. However, despite the severity of the chronic and gradual kidney disease, the largest threat to the life of LPI patients is posed by the pulmonary crises, beginning with a mild infection, developing into pulmonary alveolar proteinosis, and resulting in near-immediate multi-organ failure. Pulmonary alveolar proteinosis (PAP) is a disease state characterised by the accumulation of large amounts of phospholipids and proteinous material of unknown origin in the alveoli and distal airways of the lungs (Rosen, Castleman & Liebow 1958, Parto et al. 1993b). The progression of the disease is usually rapid: Patients present with a mild respiratory infection, which turns into respiratory insufficiency, unexplained septic fever with a severe bleeding tendency, and, in the end either fatal haemorrhage or proteinosis together with pathological renal (proteinuria, haematuria) and hepatological changes (enlarged, hard, fatty liver) (Parto et al. 1994a). Parto and others performed a chest-imaging survey of all the LPI patients in Finland at the time and discovered that even when clinically symptom-free, most of the patients show signs of pulmonary fibrosis or other pulmonary abnormalities (interstitial densities, for example (Parto et al. 1993b)).

It has been suggested that most of the unexplained findings in LPI, such as the serum hyperferritinaemia, hepatosplenomegaly, bleeding diathesis, PAP-related septic fever with no apparent infection and the unusual laboratory findings (hypoalbuminaemia, leukopaenia, severe anaemia, thrombocytopaenia and elevated LDH) could actually be chronic symptoms of hemophagocytic lymphohistiocytosis, HLH (Duval et al. 1999, Ogier de Baulny, Schiff & Dionisi-Vici 2012). HLH is a group of life-threatening immune disorders that are characterised by systemic inflammation, severe cytokine storm and immune-mediated multi-organ damage (Brisse, Wouters & Matthys 2014). In addition to the above list of unexplained chronic symptoms (each of which is regarded as a symptom of HLH), the chest radiographs of symptom-free patients who nonetheless had pathological findings support this theory, according to which LPI patients suffer from chronic HLH, which, prompted by a suitable pathogen, flares up as an acute multi-organ failure.

Various other immunological abnormalities have also been observed in LPI, which is only to be expected as *SLC7A7* is also expressed in immune-active cells, peripheral lymphocytes and monocytes/macrophages. The most common of these is leukocytopaenia/lymphopaenia (particularly CD4+ cells) or abnormal ratios of immune-active cells (T helper/suppressor ratio, (Dionisi-Vici et al. 1998)). Normal numbers of B lymphocytes, NK cells and HLA DR+ cells have been reported, although the B cell functions are deficient, resulting in low concentrations of IgG subclasses and poor vaccination response (Lukkarinen et al. 1999). In some cases, however, elevated serum levels of IgG, IgA, IgD, and/or IgM have been discovered (Nagata et al. 1987, Yoshida et al. 1995). In general, immunoreactions against viral pathogens seem to be impaired and, consequently, the outcomes of viral infections are generally more severe in LPI. *Varicella* infections have been life-threatening (Lukkarinen et al. 1998, Lukkarinen et al. 1999), and two patients are reported to have developed a PAP/multi-organ failure triggered by an Epstein-Barr virus (EBV) infection (Santamaria et al. 2004, Gordon et al. 2007) and one due to an intravascular *Aspergillus* infection (Parto et al. 1994a). Some patients present LE cells or systemic lupus erythematosus-like symptoms; therefore, it is likely that LPI also contains an autoimmunity-related aspect (Nagata et al. 1987, Parto et al. 1994b, Yoshida et al. 1995, Parsons et al. 1996, Dionisi-Vici et al. 1998, Barilli et al. 2012).

Barilli and others reported a macrophage dysfunction in which, despite the normal differentiation of LPI monocytes into macrophages, their phagocytotic activity was significantly reduced (Barilli et al. 2012). The inefficient clearing of apoptotic cells in a state of tissue damage could thus be one of the mechanisms contributing to the symptoms observed in the acute crises of LPI (for example, the accumulation of amorphous material in the lungs in PAP). Erythroblastophagocytosis has been observed (DiRocco et al. 1993, Parenti et al. 1995, Dionisi-Vici et al. 1998, Tanner et al. 2007b), and the patients tend to suffer from bleeding diathesis and severe anaemia, particularly during episodes of acute pulmonary crises. Other bone-marrow abnormalities include, for example, hyperplasia and dyserythropoiesis (Parto et al. 1994a), haemophagocytosis by myeloid precursor cells (Gordon et al. 2007), and decreased megakaryocytes (Nagata et al. 1987).

2.4 Fluorescence in research

2.4.1 Why fluorescence?

To a great extent, biomedical research focuses on examining and visualising the existence, cellular localisation, life-time or interaction of bioactive molecules. Currently, increasingly popular methods to achieve this are using fluorescent affinity reagents, such as antibodies with a fluorescent label, and fluorescent proteins fused together with the target of interest. The use of fluorescent reporter molecules has two benefits compared to the traditional molecular-genetic and protein analysis methods, such as co-immunoprecipitation, Western blotting and mass spectrometry. First, it renders possible the analysis of proteins in living, intact cells, which allows for real-time observation of biological processes. Second, it enables the visualisation of the proteins and processes in their natural cellular localisations. The more advanced applications also provide the opportunity to research protein-protein interaction or follow up protein lifetimes, for example. The use of FRET methods in microscopy greatly improves the resolution of microscopic imaging, making it possible to image distances of under 10 nm.

2.4.2 GFP and its spectral variants

The history of fluorescent reporter molecules began in 1962, when Shimomura and others extracted “Aequorin”, a bioluminescent compound in a deep-sea jellyfish, *Aequorea victoria* (Shimomura, Johnson & Saiga 1962). In experimental conditions, aequorin produces blue light, whereas the fluorescence of the jellyfish is green. The jellyfish was discovered to contain another protein responsible for the green fluorescence, which derives its excitation energy from aequorin. This green fluorescent protein, GFP, was extracted and purified in the early 1970s (Morin & Hastings, 1971). Its chromophore was discovered to be a hexapeptide beginning at amino acid 64, with the photo-adsorbing core formed by the cyclisation of the adjacent Ser65-dehydroTyr66-Gly67 amino acid residues (Shimomura 1979). In 1996, the crystal structure of GFP was solved. It was revealed to be a barrel-shaped structure formed by eleven beta-sheets with the chromophore buried in the middle, protected by the surrounding structure (Ormö et al. 1996, Yang, Moss & Phillips 1996).

In the mid-1990s, Chalfie and others published its use as a reporter protein for gene expression as they successfully expressed it in *Escherichia coli* and *Caenorhabditis elegans*. GFP is, indeed, excellent for this since the chromophore formation is not species-specific, nor does it require any other *A. victoria* product for its fluorescence (Chalfie et al. 1994). This is when scientific interest in GFP began to increase, for obvious reasons.

The GFP protein is 238 amino acids long, its absorbance maximum is at 395 nm (with a minor peak at 470 nm) and maximum emission at 509 nm, with a shoulder at 540 nm. Its fluorescence, as described by its name, is green (Chalfie et al. 1994). Heim and others

managed to improve its spectral properties by merely creating a point mutation: the resulting Ser65Thr mutant only had one excitation peak at 490 nm (emission at 510 nm) making it more photostable and more easily compatible with fluorescence imaging. The excitation peak also had amplitude six times greater than that of the wild type (Heim, Cubitt & Tsien 1995).

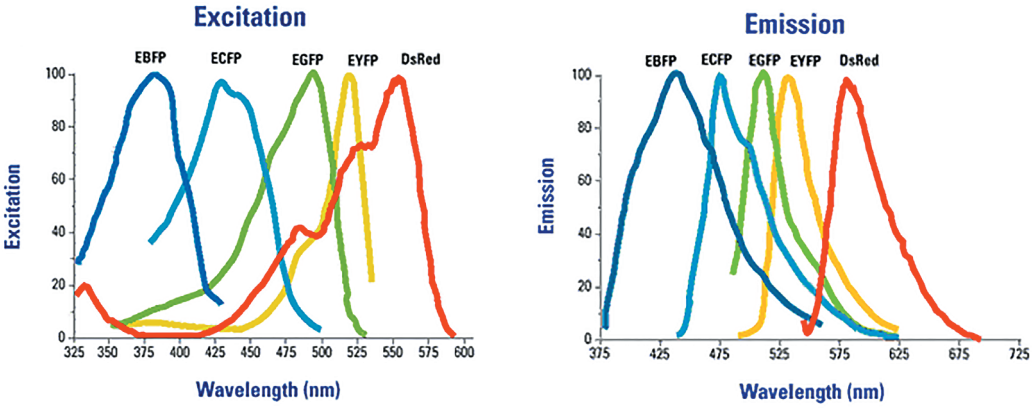

Figure 4. The excitation and emission spectra of assorted GFP variants and dsRed.
Image from www.clontech.com.

The creation of the enhanced green fluorescent protein, EGFP, gave rise to the idea that the spectral properties of GFP could easily be optimised for different purposes by site-targeted mutagenesis (for example, FACS-optimised variants of GFP, more suitable for excitation with the 488 nm argon laser (Cormack, Valdivia & Falkow 1996). The following years also saw the creation by mutation of several spectral variants of EGFP (Figure 4), the first of these being the blue fluorescent protein, BFP (Tyr66His/Tyr145Phe, excitation max 382 nm, emission max 448 nm (Heim, Prasher & Tsien 1994)), enhanced cyan fluorescent protein, ECFP (Phe64Leu/Ser65Thr/Tyr66Trp/Asn146Ile/Met153Thr/Val163Ala/His213Leu, excitation max 433 nm, emission max 476 nm (Heim, Tsien 1996)) and enhanced yellow fluorescent protein EYFP, excitation max 514 nm, emission max 527 nm (Ser65Gly/Val68Leu/Ser72Ala/Thr203Tyr (Wachter et al. 1998)). The mutations introduced in the original, wild-type *A. victoria* GFP in order to create these variations are summarised in Figure 5. Product development of the Aequorea-based fluorescent proteins still continues, and fluorescent proteins have also been extracted from other species (for instance, coral reef anemones, *Discosoma* sp.), which has extended the fluorescent colour palette to orange, red and the far-red spectral region, particularly useful for research on mammalian cells. Fluorescent proteins of varying colours and origins are widely used in all types of cells as internal labels in molecular biological research to report on the expression, interaction or cellular localisation of their target proteins (Kremers et al. 2011).

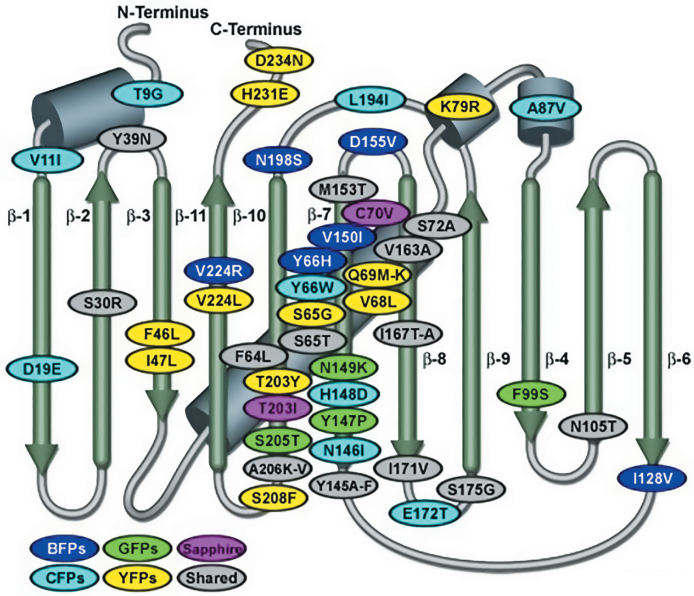

Figure 5. *Aequorea victoria* GFP mutation map.
The *A. Victoria* GFP protein has 11 beta sheets (green cylinders with arrows) surrounding the alpha-helix core (diagonal cylinder). It has been the target of numerous mutations in order to change and improve its folding and spectral properties, creating variants fluorescing in different colours. The mutations are depicted by the same colour ellipses as the fluorescence of the variant that the mutations apply to, whereas grey ellipses denote shared, folding and monomerising mutations (Image by Richard N. Day and Michael W. Davidson, http://zeiss-campus.magnet.fsu.edu/articles/probes/jellyfishfps.html).

2.4.3 FRET

The availability of several fluorescent proteins of different wavelengths also provided the opportunity to use them for fluorescence resonance energy transfer. The first fluorophore pair to be tested for this was BFP and EGFP (Heim, Prasher & Tsien 1994, Heim & Tsien 1996). The suitability of GFP for these kind of experiments was apparent from the start, as in native conditions in *A. victoria*, the excitation energy of GFP is transferred from aequorin "by a Förster-like mechanism" (Shimomura, 1979).

2.4.3.1 Förster resonance energy transfer

FRET, fluorescence resonance energy transfer, is a quantum physical phenomenon in which energy is transferred from one fluorescent molecule to another in a collision-free manner. In FRET, energy transferred from an excited donor molecule absorbing light on a shorter wavelength excites the acceptor with a longer wavelength absorbance (Figure 6), as long as their spectra are overlapping (Figure 7). If the acceptor is a fluorophore, the resulting FRET can be observed as acceptor fluorescence following donor excitation. No radiation or transfer of photons from the donor to the acceptor is involved, but the energy transfer is resonance-based and occurs between chromophores in a suitably close proximity of each other (commonly 1-10 nm): If the chromophores are too distant from each other, the FRET

mechanism does not work, whereas if they are too close to each other, the system becomes saturated. Lubert Stryer has, therefore, quite aptly called FRET a "Spectroscopic Ruler" reporting on distances 1-10 nm (Stryer & Haugland 1967). Other requirements for FRET to occur are a high quantum donor yield, overlapping donor emission and acceptor excitation spectra, and a suitable orientation of the donor and acceptor chromophores (Shrestha et al. 2015).

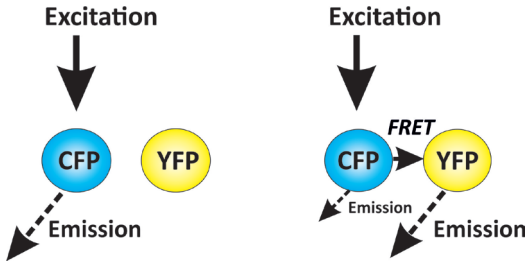

Figure 6. The FRET principle.
FRET occurs if two fluorophores with overlapping donor emission and acceptor excitation spectra, and in a suitable orientation and sufficiently close to each other. The transfer of energy from the donor to the acceptor fluorophore can be observed as the fluorescence of the acceptor and diminished donor fluorescence subsequent to donor excitation.

The principle and theory behind the FRET phenomenon was first described and published in 1946 by Theodor Förster, who named it "Inter-molecular energy migration with fluorescence" (Zwischenmolekulare Energiewanderung und Fluoreszenz (Förster 1948, Förster 2012)), and it was verified experimentally in the late 1960s (Stryer & Haugland 1967). It has been suggested that the name should be changed to 'Förster resonance energy transfer' to honour its inventor, as the energy transfer is photon-free and therefore does not involve fluorescence, which is based on the emission of photons.

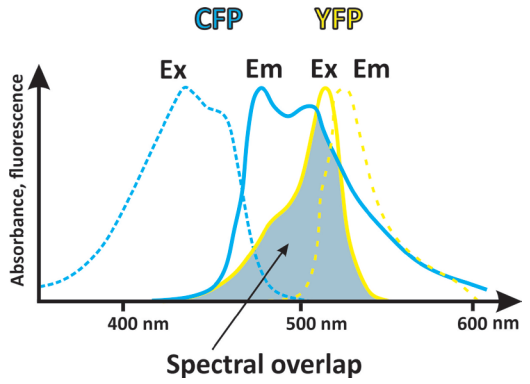

Figure 7. A requirement for FRET: overlap of the donor emission and acceptor excitation spectra.
In the image, the CFP excitation (Ex) and emission (Em) spectra are depicted in cyan, YFP in yellow. The spectral overlap of the fluorophores is illustrated with grey shading.

According to Förster, energy transfer, E, is a function of the distance separating the donor and the acceptor of the energy to the inverse power of six:

$$E = \frac{R_0^6}{R^6 + R_0^6}$$

in which R_0 is the Förster distance corresponding to the distance between the donor and acceptor at which the probability of the energy transfer is 50%, whereas R is the actual distance between the molecules. The Förster distance, R_0, is dependent on the dipole-dipole orientation of the donor and acceptor fluorophores (), the fluorescence quantum yield of the donor in the absence of the acceptor (), the refractive index of the medium () and the overlap of the donor emission and acceptor spectra (and can be determined with the following equation:

$$R_0 = 0.02108\,[\kappa^2\, x\, \phi_D x\, n^{-4} x\, J]^{\frac{1}{6}}\, nm$$

In order for FRET to occur, the emission spectrum of the donor and the excitation spectrum of the acceptor need to be overlapping; a larger overlap gives rise to a larger Förster distance, which extends the physical distance over which FRET can occur, and thus increases FRET power (Förster 1948).

Currently, there are several different applications of the FRET principle in practice. They are best suited for microscopy but can also be utilised in flow cytometry or other fluorescence-sensitive applications. Below, I describe a few of the best established FRET applications relevant for my work.

2.4.3.2 Assorted FRET applications

2.4.3.2.1 Donor quenching

The principle behind the donor quenching application is to image donor-acceptor pairs and donors only under the same conditions and to measure and compare their donor signal yield. In the case of FRET, some of the energy from the donor is transferred to the acceptor, resulting in partial donor quenching, which represents the amount of FRET in the imaged sample. The approach is straightforward and easy to carry out. The caveat in the approach is, however, that it assumes the diminishment of the donor signal to be entirely caused by FRET, and not by, for example, the smaller amount of donor in a double-labelled sample or by photo-damage to the donor (Shrestha et al. 2015).

2.4.3.2.2 Sensitised emission

This approach measures the FRET signal, the acceptor sensitised emission, directly and can be carried out using two or three channels. In the two channel emission ratio measurement, the sample containing the donor and acceptor is excited using the donor excitation wavelength, and the subsequent emission is collected in two channels, using the

donor emission filter and the acceptor emission filter. The signal measured on the acceptor emission channel is caused by FRET.

In the three-channel mode, the double-labelled specimen is exposed to both donor and acceptor excitation, and subsequently three different signals are measured on three separate channels: the donor channel uses donor emission filters to collect fluorescence induced by donor excitation, the acceptor channel collects acceptor excitation-induced signals with acceptor emission filters, and the FRET channel, on which acceptor emission filters are used subsequent to excitation on the donor wavelength.

This is a widely-used method, both in microscopy and flow cytometry. However, it has its drawbacks caused by the very spectral properties of the fluorophores essential for FRET, that is, the overlap of the donor and acceptor spectra. The biggest problem with sensitised emission is channel cross-talk, in other words spectral bleed-through of donor or acceptor fluorescence to the FRET channel. This is usually corrected by using donor-only and acceptor-only specimens to check for the ratio of spectral bleed-through in order to subsequently reduce the observed FRET channel yield by the correct amount (Sun et al. 2013, Shrestha et al. 2015).

2.4.3.2.3 Acceptor photobleaching

In the acceptor photobleaching application, FRET can be determined by quantifying donor intensities before and after photobleaching the acceptor. The photobleaching eradicates the light absorbing and emitting properties of the acceptor molecule, thereby dequenching the donor fluorescence (Figure 8). The increase in the donor fluorescence equals the amount of FRET (energy transferred to the acceptor) prior to photobleaching.

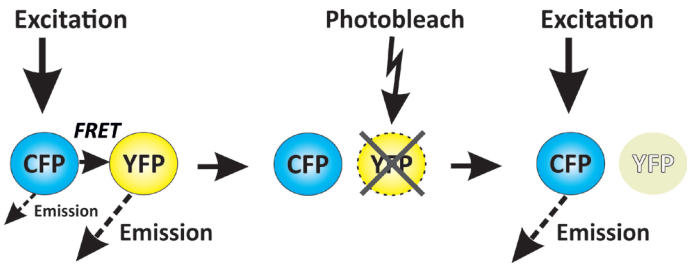

Figure 8. The principle of acceptor photobleaching.
In the acceptor photobleaching application, the light absorbing and emitting properties of the acceptor (YFP) are destroyed by photobleaching. Using this method, FRET can be observed as the dequenching of the donor fluorescence (CFP) as a consequence of photobleaching the acceptor (YFP).

This approach, too, has its problems. As the photo-destruction of the acceptor is permanent, it is only suitable for endpoint analysis. Photobleaching is a time-consuming process during which the specimen may move or be damaged by extensive exposure to laser light, which, in addition to the acceptor, may also bleach the donor, diminishing the donor intensity increase (Sun et al. 2013, Shrestha et al. 2015).

In addition to the above, in 2005, Valentin and others published a phenomenon that they called photoconversion, in which the spectral properties of EYFP are altered subsequent to photobleaching, creating an ECFP-like fluorescence. In acceptor photobleaching experiments in which the dequenching of the donor, ECFP, is measured, photoconversion thus causes erroneous FRET values as it artificially increases the donor fluorescence, which can readily be mistaken for increased FRET (Valentin et al. 2005). During the following years, contradictory reports of photoconversion were published (Thaler et al. 2006, Verrier & Soling 2006, Kirber, Chen & Keaney 2007) until recently, when Seitz and colleagues re-documented it and suggested an explanation for the controversy of its existence: According to their measurements, photoconversion is a true, ubiquitous phenomenon, linearly comparable to the concentration of EYFP in the region of interest (ROI). Therefore, it can only be measured in areas where the concentration of EYFP is high and its fluorescence bright; in areas of lower EYFP concentrations, it is too low to be measured, which is why some researchers could not observe it. They estimated that photoconversion would account for approximately 6% of the ECFP fluorescence increase perceived as donor dequenching and suggested a formula to correct it (Seitz et al. 2012).

2.5 Transcriptomics in biomedical research

Traditionally, the aim of molecular genetic investigations has been to link DNA mutations or polymorphisms with different traits or conditions. During the course of the years and with our deepening knowledge of the genome, however, it has become increasingly clear that, in addition to mutations, there remain a multitude of other factors defining the phenotypic outcome of the genes. Therefore, the focus of biomedical research has widened from the mutational study of genes downstream to the more functional approach of transcriptomics, the genome-wide analysis of gene expression.

A transcriptome contains the complete set of transcripts produced by a genome, including not only the messenger RNA (mRNA) molecules, but also all the other, noncoding RNAs of a given cell, tissue or organism, including transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs), small interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) (Wang, Gerstein & Snyder 2009, Veneziano, Nigita & Ferro 2015). As every cell of an organism contains the same DNA but only a defined set of genes are expressed in any given tissue, studying the transcriptome gives a considerably more realistic image of the actual processes carried out in that particular cell population. It has been pointed out, however, that studying the transcriptome does not necessarily directly reflect the ultimate protein content of the cell (Evans 2015), as the RNAs can be subjected to post-transcriptional regulation. Nevertheless, the RNA content of the cell is a clear indication of the genes that are active in the tissue at that particular moment.

The transcriptomic approach began with the introduction in biomedical research of microarrays (Fodor et al. 1991), which combined technologies previously established for manufacturing computer microchips with DNA hybridisation techniques. The method

revolutionised gene expression analysis by massively expanding the research capacity when compared to the traditional methods, such as northern blot. The evolving microarrays contained an ever-increasing number of oligonucleotide probes to hybridise with their targets. The extracted RNA of interest, reverse transcribed to cDNA and labelled with fluorescent dyes, is hybridised onto the microarrays together with similarly treated, differently labelled control RNA, which competes with it for the hybridisation with the probes. Based on the colour of the signal observed at each probe location after the hybridisation, the abundance of RNA of interest is determined in relation to the amount of control RNA. This yields the gene expression profile in the target cells, depicting the differences in gene expression in relation to the "normal" state or tissue (Shomaker & Ward 2006, Wiltgen & Tilz 2007). In 2004, microarrays were approved for medical use, after which they have been widely used for a variety of purposes (Shomaker & Ward 2006).

Recently, with the rise of high through-put next-generation sequencing technologies (NGS), massively parallel cDNA deep-sequencing (RNA-seq) has overtaken microarrays as the preferred method for transcriptomic analysis (Mortazavi et al. 2008, 't Hoen et al. 2008, Wang, Gerstein & Snyder 2009, Veneziano, Nigita & Ferro 2015). RNA-seq has a number of advantages over microarrays as it has higher resolution, sensitivity and accuracy. It is not dependent on prior knowledge of the sequence of the target transcript and can thus also be used for studying organisms whose genomic sequence is not yet fully known (Trapnell et al. 2010, Veneziano, Nigita & Ferro 2015).

Most recently, efforts in transcriptomics have been focussed on gene expression analysis combined with spatial information, single-cell transcriptomics (Shapiro, Biezuner & Linnarsson 2013, Crosetto, Bienko & van Oudenaarden 2015). In practice, this means the profiling of the transcriptome at the single cell level, either by imaging-based methods (such as single-molecule fluorescence *in situ* hybridisation (smFISH) (Kwon 2013)) or sequencing-based methods (RNA-seq, CytoSeq (Fan, Fu & Fodor 2015). The anatomical location of the target cell can either be defined at the sampling stage, involving either highly accurate isolation of cells from distinct locations or FACS (Shapiro, Biezuner & Linnarsson 2013), or retrospectively using known location-specific expression markers (Achim et al. 2015, Satija et al. 2015).

The comparison of transcriptomes, or gene expression profiling, involves determining patterns of differential gene expression in a specific cell population or under certain circumstances, relating to a disease state or response to medication, for example. It has proven a valuable tool in biomedical research in elucidating the genetic background of complex disease states, such as different types of cancer, for example breast cancers and B cell lymphoma (Alizadeh et al. 2000, Perou et al. 2000, van 't Veer et al. 2002, Dawson et al. 2013, Xu et al. 2014), multiple sclerosis (Habek, Borovecki & Brinar 2010), sepsis (Wong 2012), arterial thrombosis (Yan et al 2015), muscular dystrophies (De Arcangelis et al. 2015) and neurodegenerative disorders (Karim et al. 2014, Wes et al. 2015).

Transcriptomic methods have also been utilised for pharmacogenomics and personalised medicine. According to Spear and others, common medicines used for treating diseases are effective only for 50-75% of patients (Spear, Heath-Chiozzi & Huff 2001). Therefore, in order to improve the clinical outcome and cost effectiveness of the treatment, and potentially avoid the side effects of medicines, it has been deemed highly beneficial to explore the patient's gene variants affecting their drug response. In cancer research, gene expression analyses are used for discovering new drug targets in order to combat cancer (Walker 2001). Similarly, characterising cancer types based on specific signature genes can greatly assist in cancer treatment as it facilitates identifying drug-resistant forms of cancer (Shajahan-Haq, Cheema & Clarke 2015, Stäubert et al. 2015) and aids in selecting efficacious therapies for treatment-responding forms (Lazar et al. 2015). With regard to cancer treatment, transcriptomic methods aid in estimating the clinical outcome of the therapy and provide a basis for a realistic estimate of the prognosis.

Clinically, transcriptomic methods (mainly microarray) have also been widely used in detecting non-human transcripts in human samples in order to identify viral, bacterial and fungal pathogens, thereby facilitating accurate and specific treatment of the patient (Kumar 2009).

3. THE AIMS OF THE STUDY

In this work, my aim was to investigate the effects of LPI-causing mutations on the protein-protein interaction of the y^+LAT1/4F2hc transporter subunits and to clarify the structure of the transporter using FRET confocal microscopy and flow cytometry. Similarly, I sought to discover which molecular genetic factors, other than the *SLC7A7* mutation, contribute to the clinical picture of LPI and render it so variable, even in patients sharing the same genetic background and the same pathological mutation.

The specific aims of this work were:

- To establish FRET acceptor photobleaching as a method to investigate the interactions between y^+LAT1 and 4F2hc
- To explain the effect of different mutations on the interaction of the transporter subunits and the structure of the transporter complex
- To elucidate the heteromeric nature of the y^+LAT1/4F2hc holotransporter
- To explore the transcriptomic variance of LPI and the molecular agents behind the variable symptoms of the Finnish patients

4. MATERIALS AND METHODS

The detailed descriptions of the materials and methods used in the present study are given in original publications I-III.

4.1 The fluorescent constructs (I, II)

The plasmid constructs used for the experiments in I and II consist of the wild-type or mutated open reading frames (ORF) of y⁺LAT1 and 4F2hc, cloned into fluorescent expression vectors in four colours, pECFP-N1/C1, pEGFP-C1, pEYFP-N1/C1 and pDsRed1-C1 (plasmid vectors by Living Colors®, BD Biosciences Clontech, Palo Alto, CA). In addition to the wild-type DsRed1-C1 vector producing red fluorescence, a "dark" pDsRed1-N1-4F2hc was used with an introduced stop codon that prevented DsRed fluorescence and thus produced a non-fluorescing 4F2hc (Toivonen et al. 2002). Constructs cloned into the pEGFP and DsRed1-C1 plasmids were used for the analysis of the significance of the disulphide bridge for the y⁺LAT1/4F2hc dimer formation, whereas the constructs in the pECFP and pEYFP plasmids were used for the FRET studies on y⁺LAT1/4F2hc protein-protein interaction in both confocal microscopy and flow cytometry. The mutants of *SLC7A7* (y⁺LAT1) used for creating the constructs for the microscopy studies were either naturally-occurring (LPI mutations) or artificially created (site-directed mutagenesis on cysteine residues). The naturally-occurring LPI mutants used in the FRET microscopy are summarised in Table 3.

Table 3. The *SLC7A7* mutation constructs used in II

Nucleotide change	Effect on amino acid	Nomenclature used here	Described in
c.161G>T	p.G54V	G54V	(Mykkänen et al. 2000)
c.1262delC	p.P421RfsX98 (Introduction of 7 new codons)	1262delC	(Mykkänen et al. 2000)
c.895 -2A>T	p.T299IfsX10 (Acceptor splice site error, skipping first 10 bp in exon 7)	LPI_{Fin}	(Borsani et al. 1999, Torrents et al. 1999)

Different fluorescent plasmids were also used as controls for these visualising studies: A tandem plasmid with ECFP and EYFP fused together to produce obligatory "forced" FRET was used as the negative control in flow cytometric FRET assays, and the empty vectors pECFP-C1 and pEYFP-C1 were used as negative FRET controls in both FRET confocal microscopy and flow cytometry. When determining the y⁺LAT1/4F2hc interaction location in the cells, a commercial Golgi-targeted expression vector, pECFP-Golgi (Living Colors®, BD Biosciences Clontech) was used, while the endoplasmic reticulum was stained using the SelectFX® Alexa Fluor® 488 Endoplasmic Reticulum Labeling Kit

(Molecular Probes™, Invitrogen Corporation, Carlsbad, CA, USA) with an Alexa Fluor® 633-dye-labelled secondary antibody (for spectral reasons as imaged with ECFP and EYFP).

4.2 Site-directed mutagenesis (II)

In order to investigate the importance of the disulphide bridge for y^+LAT1/4F2hc we abolished its predicted docking point by substituting an alanine for the relevant cysteine residues, C151 and C174 in y^+LAT1 and C109 in 4F2hc. This was achieved by using site-directed mutagenesis (the QuickChange™ Site-Directed Mutagenesis Kit #200519, Stratagene, La Jolla, CA, USA).

The plasmids were first denatured in 95 °C for 30 sec, followed by a cycle of denaturation in 95° C for 30 sec, annealing 55 °C for 1 min and elongation in 68° C for 13.5 min (for EGFP-y^+LAT1) or 12.5 min (for DsRed1-4F2hc), repeated 16 times.

4.3 Cell culture and transfections

The main material for the investigations of the amino acid transporter subunit interactions (I, II) and transporter structure were transfected Human embryonal kidney cells, line 293 (HEK293; ATCC® CRL-1573™). In addition to these, transfections were performed also on Human colorectal adenocarcinoma cells, line 2 (ATCC® HTB-37™) and Madin Darby Canine Kidney cells (ATCC® CCL-34™), but the HEK293 cells were selected for the experiments due to their rapid proliferation and ease of use. The cells were cultured in standard conditions (more precisely described in I and II). For the FRET confocal microscopy, the cells were plated on poly-L-lysine-coated cover slips, transfected with varying fluorescent plasmid constructs (Table 4) using the FuGENE®6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) fixed with 4% paraformaldehyde and prepared for microscopy as described in I.

For the flow cytometric FRET, the HEK293 cells were plated onto 12-well plates and, having reached approximately 80% confluence, transfected the following day using the Hily Max Transfection reagent (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The DNA to transfection reagent ratio was 1 to 3. In the triple transfection reactions, the ratios of ECFP-labelled y^+LAT1 to EYFP-labelled y^+LAT1 to unlabelled 4F2hc were 1 : 1 : 1, yielding an overall y^+LAT1 to 4F2hc ratio of 2 to 1.

Table 4. Transfections performed on the HEK293 cells

Constructs	Transfection reagent	Publication
EGFP-y$^+$LAT1 + DsRed-4F2hc	FuGENE®6	II
EGFP-y$^+$LAT1$_{C151A}$ + DsRed-4F2hc	FuGENE®6	II
EGFP-y$^+$LAT1$_{C174A}$ + DsRed-4F2hc	FuGENE®6	II
EGFP-y$^+$LAT1$_{C151A}$ + DsRed-4F2hc$_{C109A}$	FuGENE®6	II
EGFP-y$^+$LAT1$_{C174A}$ + DsRed-4F2hc$_{C109A}$	FuGENE®6	II
EGFP-y$^+$LAT1 + DsRed-4F2hc$_{C109A}$	FuGENE®6	II
EGFP-y$^+$LAT1	FuGENE®6	II
y$^+$LAT1-ECFP	FuGENE®6	I
y$^+$LAT1-ECFP + 4F2hc (dark)	FuGENE®6	I
y$^+$LAT1-ECFP + 4F2hc-EYFP	FuGENE®6	I
y$^+$LAT1-ECFP + EYFP-4F2hc	FuGENE®6	I
ECFP-y$^+$LAT1	FuGENE®6 Hily Max	I II
ECFP-y$^+$LAT1 + 4F2hc (dark)	FuGENE®6	I
ECFP-y$^+$LAT1 + 4F2hc (dark) + pECFP-Golgi	FuGENE®6	I
ECFP-y$^+$LAT1 + 4F2hc-EYFP	FuGENE®6	I
ECFP-y$^+$LAT1 + EYFP-4F2hc	FuGENE®6	I, II
ECFP-y$^+$LAT1$_{Fin}$ + EYFP-4F2hc	FuGENE®6	II
ECFP-y$^+$LAT1$_{G54V}$ + EYFP-4F2hc	FuGENE®6	II
ECFP-y$^+$LAT1$_{1262delC}$ + EYFP-4F2hc	FuGENE®6	II
ECFP-y$^+$LAT1 + EYFP-y$^+$LAT1	Hily Max	II
ECFP-y$^+$LAT1 + EYFP-y$^+$LAT1 + 4F2hc (dark)	Hily Max	II
y$^+$LAT1-EYFP	FuGENE®6	I
y$^+$LAT1-EYFP + 4F2hc (dark)	FuGENE®6	I
y$^+$LAT1-EYFP + 4F2hc-ECFP	FuGENE®6	I
y$^+$LAT1-EYFP + ECFP-4F2hc	FuGENE®6	I
EYFP-y$^+$LAT1	FuGENE®6 Hily Max	I II
EYFP-y$^+$LAT1 + 4F2hc (dark)	FuGENE®6	I
EYFP-y$^+$LAT1 + 4F2hc-ECFP	FuGENE®6	I
EYFP-y$^+$LAT1 + ECFP-4F2hc	FuGENE®6	I, II
EYFP- y$^+$LAT1$_{Fin}$ + ECFP-4F2hc	FuGENE®6	II
EYFP- y$^+$LAT1$_{G54V}$ + ECFP-4F2hc	FuGENE®6	II
EYFP-y$^+$LAT1$_{1262delC}$ + ECFP-4F2hc	FuGENE®6	II
4F2hc-ECFP	FuGENE®6	I
4F2hc-EYFP	FuGENE®6	I
ECFP-4F2hc	FuGENE®6	I
EYFP-4F2hc	FuGENE®6	I
pECFP-EYFP (tandem)	FuGENE®6 Hily Max	I II
pECFP + pEYFP	FuGENE®6	I, II

4.4 Confocal imaging of the cysteine mutants

The cells expressing the EGFP-y$^+$LAT1 and DsRed1-4F2hc fluorescent constructs were studied with a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany) with a 63x NA 1.4 plan apochromat oil immersion objective. The 488 nm line of the argon laser was used for the excitation of EGFP, whereas DsRed was excited using the 543 nm HeNe laser. The fluorescence was detected with the BP 500-530 (EGFP) and LP560 (DsRed) filters.

4.5 FRET confocal microscopy (I, II)

4.5.1 Acceptor photobleaching the wild-type constructs (I)

Fluorescence resonance energy transfer (FRET) confocal microscopy was used to examine and image constructs in fluorescent ECFP and EYFP expression vectors. After testing the constructs for fluorescence, we proceeded to studying the interactions of the wild-type transporter subunits using acceptor photobleaching FRET microscopy, first at the whole-cell level and then in more detail with the aim of finding the pairs of fluorescent constructs best suited for documenting the transporter subunit interaction. The FRET microscopy research in its entirety was carried out using the above-mentioned Zeiss LSM510 META confocal microscope with the above-mentioned objective. For the excitation of ECFP and EYFP, the 405 nm laser diode and the 514 nm argon laser line were used, respectively, and the fluorescence emission was collected through the band pass filters of 435-485 nm (ECFP) and 535-590 nm (EYFP). First, entire cells were repeatedly scanned using the 514 nm line from the argon laser, thereby photobleaching EYFP in these areas. The changes in ECFP and EYFP fluorescence were documented by collecting images from these channels pre- and post-photobleaching. Subsequently, 20 suitable cells expressing both fluorophores were examined with a 2x zoom, and the photobleaching experiment was repeated, now targeting three parallel regions of interest (ROIs) – the cell membrane, the ER and the Golgi apparatus, where possible – scanning the EYFP 75 or 100 times with the 514 nm line from the argon laser. Again, pre- and post-photobleach images on both channels were collected for analysis.

The cellular localisation of the fluorescent fusion proteins in the ER and Golgi was verified by imaging their co-localisation with the ER-specific antibody stain and the fluorescent, Golgi-targeted commercial expression vector and imaging the ECFP-y$^+$LAT1 + EYFP-4F2hc + Alexa Fluor® 633 dye-positive (the ER) and the ECFP-Golgi + EYFP-y$^+$LAT1+ 4F2hc-dark-positive cells with appropriate additions to the laser and filter settings, described in detail in I.

The fluorescence intensities of ECFP and EYFP of the bleached and non-bleached cells before and after bleaching were quantified with the MetaMorph computing software (Universal Imaging Corporation, Downingtown, PA, USA). Using the donor (ECFP) intensity values, the fluorescence efficiency illustrating the degree of donor fluorescence dequenching following acceptor photobleaching was determined using the formula modified from the article by

Karpova and co-workers ($E_F = (I_{after} - I_{before}) \times 100 / I_{after}$ (Karpova et al. 2003)) This formula yielded the FRET energy transfer efficiency as a percentage. To allow comparison between the different construct combination, their net donor dequenching values (Table 1, I) were calculated by subtracting the mean of the E_F values of the control regions from the mean of the E_F values of the bleached regions.

4.5.2 Acceptor photobleaching, lambda mode

This method was used for the FRET confocal microscopic investigations in II: That is, the observation of the effects of LPI mutations for the y⁺LAT1/4F2hc interaction, and the multimerisation study aiming at uncovering the multimeric status of the transporter. The cells expressing the fluorescent fusion constructs were studied with the above-mentioned Zeiss LSM510 META confocal microscope using the 63x NA 1.4 plan apochromat oil immersion objective. For the acceptor photobleaching, the ECFP and EYFP were excited as previously, but the fluorescence emission was collected in the lambda mode from 411.3 nm to 625.3 nm in 10.7 nm steps. In every imaged cell (20 cells per construct combination for the y⁺LAT1 mutation study, 9-15 in the multimerisation study), three (mutation study) to six (multimerisation study) ROIs were subjected to acceptor photobleaching. EYFP was photobleached by scanning the ROIs 300 consecutive times with the 514 nm laser using zoom 2. Images were acquired before and after photobleaching to uncover the donor (ECFP) dequenching. The optical slice was set to <1.1 µm, and the detector gains were adjusted to an optimal signal level.

The donor dequenching caused by photobleaching was documented by examining the mean of ROI values using the Physiology option of the LSM 5 software. The pre- and post-bleach fluorescence intensity values in the bleached ROIs (three for the y⁺LAT1 mutation analysis and six for the dimerisation study) and corresponding unbleached control areas (five) at 427, 438, 449, 459 and 470 nm were documented to represent the fluorescence intensity change for ECFP reporting for FRET prior to acceptor photobleaching. The fluorescence efficiency values (E_F) were calculated for the above-mentioned wavelengths of the bleached and unbleached areas using the formula mentioned in 4.5.1. A mean of the E_F values was calculated first for each bleached and unbleached area and, second, for each bleached cell. The values for the unbleached regions were treated as background and subtracted from the E_F values of the bleached ROIs, thus yielding the net donor fluorescence change value of the given cell. Consequently, the mean E_F value was calculated for each of the four fluorescent construct combinations.

4.6 FRET flow cytometry (II)

For flow cytometry, the transfected cells were collected by trypsination two days post-transfection and fixed using 4% paraformaldehyde in PBS. After fixing, the cells transfected with 4F2hc expression plasmids were permeabilised using TTBS and antibody incubated overnight with a mouse-anti-human CD98 antibody (Ma1-34546 by Thermo Fisher

Scientific, MA, USA). The primary antibody was detected using a goat-anti-mouse secondary antibody with an Alexa Fluor 647 label, suitable for flow cytometry (#4410, Anti-mouse IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 647 Conjugate) Cell Signalling Technology, Leiden, The Netherlands). The cells were then re-suspended in PBS for immediate flow cytometric assay.

Using the BD LSR II flow cytometer (Becton Dickinson, CA, USA), the transfected, fixed and antibody stained cells were exposed to a 405 nm (for CFP and FRET) and 488 nm (for YFP) laser, followed by the collection of signals using the following emission filters: 440/40 for CFP, 530/30 for YFP, 585/42 for FRET and 660/20 for Alexa Fluor® 647. Thus, FRET was observed as emission detected through the 585/42 filter after the exposure of cells to the 405 nm (ECFP) laser light. 30 000 gated events were collected per sample. Using Flowing Software ver. 2.5 (Mr Perttu Terho, Turku Centre for Biotechnology, Turku, Finland, www.flowingsoftware.com), the FRET-positive cells were first rigorously gated using the ECFP-only- and EYFP-only-positive control cells in order to remove any false positives from the FRET gate. In addition, the resulting percentages of the FRET-positive cells (FRET-positive cells expressing the tandem plasmid, those expressing y^+LAT1+ECFP and y^+LAT1+EYFP without 4F2hc, and those expressing y^+LAT1+ECFP and y^+LAT1+EYFP with 4F2hc) were further corrected for any bleed-through of the signal of only-ECFP- or only-EYFP-positive cells into the FRET gate by subtracting their percentages from those of the FRET-positive cells. The percentages of 4F2hc-positive cells in the FRET-positive cell populations were calculated with the help of Alexa Fluor® 647 signals.

4.7 The microarray analysis of the gene expression

The microarray gene expression study of the Finnish LPI patients was carried out using the RNA samples of 13 Finnish patients (6 male, 7 female) chosen from the cohort of 38 patients in follow-up at Turku University Hospital and those of 10 healthy age- and sex-matched volunteers (5 male, 5 female). The detailed information of the patients' symptoms and medication is described in Table 1 and Supplementary Tables 1A and 1B of original publication III. The age of the patients and control subjects chosen for this study ranged from 7 to 47 years (mean 29.9) and 9 to 48 years (mean 30), respectively. The inclusion criterion of the patients was the "classic" clinical picture of LPI with moderate symptoms and no end-stage renal disease or active PAP. The control subjects, not genotyped for *SLC7A7*, were unrelated to the LPI patients and thus represented the general Finnish population.

For the gene expression analysis, peripheral whole blood samples of patients and control subjects were collected into PAXgene Blood collection tubes (PreAnalytiX, Hombrechtikon, Switzerland) and the total RNA was extracted using the PAXgene Blood RNA Kit (PreAnalytiX GmbH) as described in III. Individual patient samples and the pooled control samples were amplified using the RiboAmp® OA1 Round RNA Amplification Kit (Arcturus, Sunnyvale, CA), and the cDNAs were *in vitro* transcribed into cRNA then labelled with biotine 11 dUTP (PerkinElmer, Wellesley, MA) as described in III. The labelled samples were hybridised on the Sentrix® HumanRef-8 Expression BeadChip Array (Illumina, San Diego, CA) containing 24526

probes (assigned to 18415 genes), according to the instructions for Illumina® BeadStation 500X Revision D. Hybridisation was detected with cyanine3-streptavidine (GE Healthcare Europe, Munich, Germany). The arrays were scanned with the Illumina BeadArray Reader, and the results were converted into numerical data using the Bead Studio v1.5.1.34 Data Analysis Software (Illumina).

The hybridisation data were normalised and subjected to statistical analysis as described in III. The resulting genes were filtered using a log 2 fold change limit of 0.8, and a p-value limit of 0.05 from the t-test (the results were not corrected for the number of probes at this point but the correction was performed in subsequent analysis steps). In this way, 501 up-regulated and 461 down-regulated genes were discovered. The functional distribution of the genes with differential expression was studied by counting and plotting the frequencies of their gene ontology annotations (GOA). In addition, the ratio of the GOA frequency of the genes with altered expression to those of the genes with unaltered expression was plotted for each GOA category in order to pursue the change of gene functions observed in LPI.

The functional annotations were studied, and a clustering of functional annotations was performed with the online Functional Annotation Tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID); the canonical pathway analyses were carried out with Ingenuity Pathway Analysis (IPA) software version 9.0 (Ingenuity Systems, Redwood City, CA, USA). The results were statistically controlled/corrected by the Fisher Exact test (EASE < 0.05), and Benjamini-Hochberg procedure (FDR < 2%). The hierarchical clustering of the differentially expressed genes was performed with the R/Bioconductor software, as described in Publication III. The microarray data were deposited in the EMBL-EBI microarray database (accession number A-MEXP-525).

In order to verify the microarray results reporting for differential gene expression, the expression levels of four SLC7 family genes (*SLC7A1*, *SLC7A5*, *SLC7A6*, *SLC7A7*) and two other relevant amino acid transporters (*SLC3A2*) were measured for each patient and control, and an additional 22 LPI patients, using quantitative real-time PCR and employing *GNB2L1* and *TRAP1* as reference genes, as described in III. The expression changes of the transporter genes were tested for correlation (Pearson's correlation) using the SPSS11.0.1 software (IBM, Armonk, NY, USA).

4.8 The lymphocyte subpopulation analysis

The LPI patients' lymphocyte subpopulations were analysed by staining their blood samples with the Simultest™ IMK Plus immuno-staining kit for flow cytometry (BD Becton Dickinson UK, Oxford, United Kingdom) according to the manufacturer's instructions and running them with the BD FACScan flow cytometry analyser. The percentages of the lymphocyte subpopulations were compared to the reference values in use nationally in Finnish university hospitals.

5. RESULTS AND DISCUSSION

5.1 The localisation of the cysteine mutants (II)

When this thesis work was begun, there was no functioning antibody to specifically identify the human y$^+$LAT1, and due to the low expression of at least the Finnish mutant, its detection with northern blot was difficult (Mykkänen et al. 2003). Our group therefore began to look into employing fluorescence-based research methods in order to visualise y$^+$LAT1, which, as I joined the group, had been successfully cloned into the pEGFP vector and imaged using confocal microscopy (Toivonen et al. 2002).

Since 1999, y$^+$LAT1 and 4F2hc have been known to dimerise to form an amino acid transporter whose function in different tissues is well characterised and documented in the literature. Its structure and the factors governing the dimer formation and maintenance, however, are still largely unclear. One of such features is the disulphide bond formed between cysteine residues C109 of 4F2hc, and C151 or C174 of y$^+$LAT1, the significance of which was explored by Torrents and others. They discovered that compromising the disulphide bond affects both the form and function of this transporter, as mutating 4F2hc C109 leads to the separation of y$^+$LAT1 and 4F2hc in an immunoprecipitation experiment and obliterates their transport function in a test setting (Torrents et al. 1998). Subsequently, the research conducted on the ectodomain of 4F2hc has suggested that the interaction between the subunits may not be tied to any particular domain of the proteins but occurs through non-covalent bonds at the whole-protein level (Franca et al. 2005), and that while non-essential for the dimer formation, the disulphide bridge may, in addition to regulating the transport channel, stabilise the transport complex in the plasma membrane, thus preventing its disassembly *in vivo* (Bröer et al. 2001, Wagner, Lang & Bröer 2001, Chubb et al. 2006).

In order to visualise the role of the y$^+$LAT1$_{C151}$-4F2hc$_{C109}$ or y$^+$LAT1$_{C174}$-4F2hc$_{C109}$ disulphide bridge in the maintenance of the heteromeric structure of the y$^+$LAT1/4F2hc transporter, we used site-directed mutagenesis to create a cysteine to alanine mutant C109A in 4F2hc, cloned it in a DsRed1 expression vector, and paired it up with a respective cysteine to alanine mutant of y$^+$LAT1 (either C151A or C174A) cloned in an EGFP expression vector. These fluorescent constructs were co-transfected into the HEK293 cells and imaged with a confocal microscope in order to document the localisation of the fluorescent signals.

Both fluorescent signals could be detected in various cellular compartments (the ER, Golgi and plasma membrane), and the cells had the general appearance of those expressing wild-type y$^+$LAT1 and 4F2hc (Figure 9) irrespective of whether one or both of the subunits had a cysteine to alanine substitution. No visual effect on the dimer formation could be observed. This allows us conclude that the sulphur bridge between the transporter subunits is not critical for their dimer formation since abolishing the cysteine residues serving as anchoring points for the sulphur bridge binding sites does not disrupt the dimer, and y$^+$LAT1 continues

to be transported to the plasma membrane in a wild-type-like fashion. This supports the theory according to which the subunit interaction is not tied to any individual element or structure of the subunits but occurs based on non-covalent bonds and electrostatic complementarities with the disulphide bridge serving a supplementary, stabilising role, and perhaps being more involved in forming the transport channel.

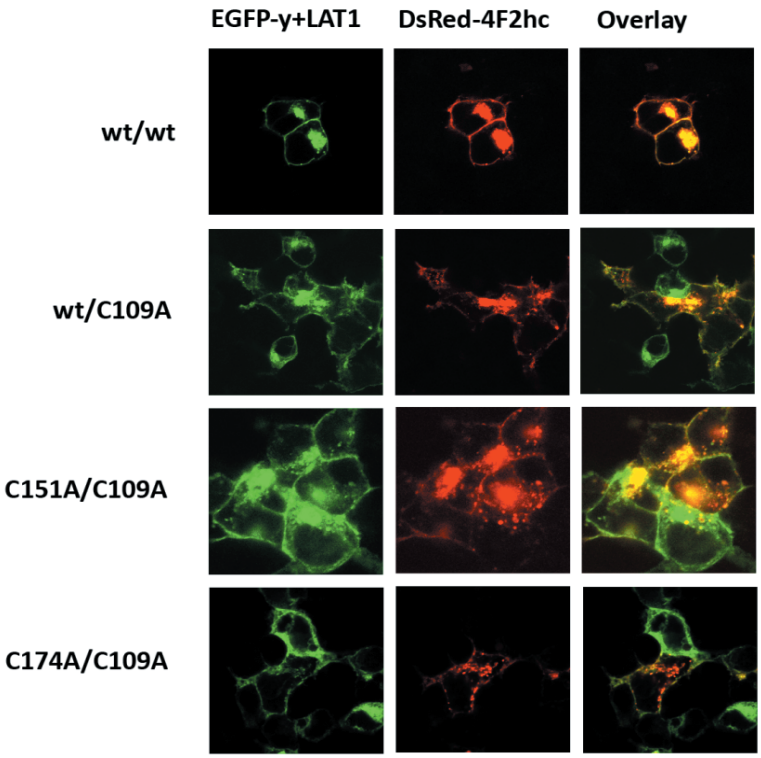

Figure 9. The effect of cysteine residue omission on y⁺LAT1/4F2hc dimerisation.
This image contains representative confocal microscopic images displaying cells with y⁺LAT1 and 4F2hc with cysteine mutations. The disulphide bond-forming cysteine residues, C109 in 4F2hc, and either C151 or C174 in y⁺LAT1, were substituted with alanine using site-directed mutagenesis, and the constructs were transfected into the HEK293 cells in fluorescent expression plasmids, EGFP (y⁺LAT1, left column) and DsRed1 (4F2hc, middle column). The fluorescent cells were imaged with confocal microscopy. The cysteine mutants (the second row of this panel illustrating the 4F2hc cysteine mutant, and the bottom two rows of double mutants) interacted together in a manner similar to the wild-type constructs (top row of this panel). In the figures of this panel, EGFP is illustrated in green, DsRed in red, and the co-localisation of these fluorophores in yellow. Figure from II.

5.2 Setting up FRET (I)

The following step was to document the interaction of y⁺LAT1 and 4F2hc by using the less-conventional fluorescent visualisation methods. Thus, the aim of this work was to establish and optimise the acceptor photobleaching application of FRET confocal microscopy as a new method for this purpose. Due to their spectral properties, ECFP and EYFP were selected as

the FRET donor and acceptor, respectively. y^+LAT1 and 4F2hc were cloned into fluorescent plasmids (pECFP and pEYFP), transfected into HEK293 cells, and imaged with a confocal microscope to examine the cells for FRET reporting of the interaction of the proteins of interest. Eight different combination of the fluorescent construct were tested for FRET, varying by the localisation and colour of the fluorescent label: N-terminal vs. C-terminal, ECFP vs. EYFP.

The interaction of the protein constructs was evaluated by calculating the fluorescence efficiency values (E_F) representing the donor dequenching in the imaged cells. Similarly, the different FRET construct combinations were evaluated based on their FRET potential. Due to the extracellular localisation of the 4F2hc C terminus, the C-terminal tags of 4F2hc produced no FRET and were thus discarded first. The strongest FRET-producing construct combination was identified to be the combination of pECFP-C1-y^+LAT1 and pEYFP-C1-4F2hc (N-terminal label in both proteins), subsequently used as the positive control in a later study. Consequently, the acceptor photobleaching application of FRET confocal microscopy was proven to be a suitable tool for studying amino acid transporter subunit interaction. The images and E_F values of different fluorescent construct combinations can be studied in Original Publication I (Figures 3, 4 and 5).

At the time of my first experiments, no knowledge of photo-conversion had reached the awareness of FRET confocal microscopists, and by the time that all the FRET experiments had been performed, the anti-photoconversion statements were still more numerous that those reporting in its favour; therefore, it was not accounted for in my FRET efficiency calculations. In 2012, however, Seitz and others documented this much-debated phenomenon of EYFP and stated that following photobleaching, the converted acceptor fluorescence could be measured in the donor channel in the non-ECFP-containing cells only when the pre-photobleach EYFP fluorescence had been high (Seitz et al. 2012). No donor-resembling fluorescence increase could be measured in the EYFP-only cells with low or moderate EYFP fluorescence as the photoconversion is a linear process, proportional to initial EYFP brightness. In order to obtain true FRET, devoid of EYFP fluorescence photoconverted to wavelengths resembling that of ECFP, one should choose areas of only moderately bright EYFP fluorescence for photobleaching and subsequent measurements of donor fluorescence un-quenching. For my experiments, this conclusion is fortunate: in the initial tests, the cells with high EYFP content had yielded “odd” and “unexplained” results and to avoid these I had focussed my experiment design on the cells with low to moderate EYFP fluorescence. Thus, I can confidently conclude that the FRET results are not rendered false by photoconversion but convey true donor dequenching resulting from acceptor photobleaching.

5.3 The effect of LPI mutations on subunit interaction (II)

Given that the light subunits of heteromeric amino acid transporters cannot localise at the plasma membrane without the assistance of the heavy subunit, and that the frame shift mutations of *SLC7A7* cause the resulting y^+LAT1 to be retained in the cytoplasm, the

hypothesis for the FRET study of the effects of *SLC7A7* mutations on the dimer formation was that the mutations disrupt the dimer formation, thereby preventing the mutant y⁺LAT1 from reaching its cellular target. In a simultaneous flow cytometric FRET study by our group, it was documented that, contrary to expectations, y⁺LAT1 and 4F2hc displayed interaction despite the LPI-causing mutations in y⁺LAT1 (Toivonen et al. 2013). Thus, the aim of the FRET confocal imaging approach was to visualise the intra-cellular interaction between mutated y⁺LAT1 and 4F2hc in greater detail.

For the FRET microscopy analysis, I selected the same three different mutations used in the flow cytometric FRET study; one missense mutation (G54V), and two frameshift mutations, one of which was predicted to yield a truncated protein (LPI_{FIN}), the other an extended one (1262delC, called 1548delC in Toivonen et al. (2013)). The y⁺LAT1 molecules carrying these mutations were cloned into pECFP-C1 and pEYFP-C1 expression vectors. They were then transfected into the HEK293 cells together with wild-type 4F2hc in either pECFP-C1 or pEYFP-C1 plasmids, forming ECFP-EYFP pairs appropriate for acceptor photobleaching.

In the FRET confocal imaging experiments, interactions between 4F2hc and y⁺LAT1 with different mutations were observed as expected based on the flow cytometric FRET experiments, yielding wild-type-like fluorescence efficiency values (Table 4 and Table 2, II). In the cells carrying the missense mutation, the distribution of the interaction was wild-type-like, extending throughout the biosynthetic pathway, from the ER to the plasma membrane, with the cells displaying a wild-type-like appearance. In the cells transfected with the frame shift mutations of y⁺LAT1, the fluorescence was dispersed into the entirety of the extra-nuclear space of the cell, with some visible densification in the Golgi area, all yielding FRET. The y⁺LAT1/4F2hc interaction could be measured wherever y⁺LAT1 molecules were present. Due to the vast difference in the cellular distribution of FRET in the cells with different LPI mutations, however, no numerical comparison of FRET per cellular compartment between the mutation types could be achieved.

Table 5. The mean FRET acceptor photobleaching fluorescence efficiency (E_F) values in the fluorescence intensity measurements in the FRET confocal microscopy study

y⁺LAT1 construct	Bleached regions		Control Regions		NET
	E_F [a] ± SD	(n) ROI	E_F [a] ± SD	(n) ROI	E_F [b] (%)
wt	2.9 ± 1.6	60	0.5 ± 0.6	100	2.4 ± 1.4[c]
G54V	3.7 ± 2.0	60	0.5 ± 0.6	100	3.2 ± 1.7[c]
FIN	2.9 ± 1.6	60	0.6 ± 0.5	100	2.3 ± 1.3[c]
1262delC	2.4 ± 1.6	60	0.5 ± 0.6	100	1.9 ± 1.4[c]

[a] The mean donor fluorescence intensity change (fluorescence efficiency, E_F) was calculated as an average of the number of bleached or control regions of interest (ROI) indicated in the table. The numbers of bleached and control regions per cell were 3 and 5, respectively. The standard deviations were calculated using the average value of each bleached cell containing the three bleached regions and five control regions (20 cells per construct).

[b] The net donor fluorescence intensity change was calculated by subtracting the mean E_F values of the control regions from those of the bleached regions.

[c] The net fluorescence intensity values of the mutant constructs show no statistically significant differences when compared to the wild type construct. $p = 0.12$.

5.4 The y⁺LAT1/4F2hc transporter – dimer or tetramer? (II)

5.4.1 Flow cytometry

Despite the extensive research on the function of the heteromeric amino acid transporters in the late 1990s and early 2000s, relatively little is known about their structure and formation. Deducing the heteromeric structure of the y⁺LAT1/4F2hc based on previous publications has proven impossible as their results provide contradicting evidence regarding their multimerisation. Based on the previous reports, when in liquid, 4F2hc appears as a monomer (Turnay et al. 2011), when attached alone in the plasma membrane it forms homodimers (Fort et al. 2007), whereas when dimerised with a light chain, the preferred form is a single heterodimer (Fernández et al. 2006). However, it has been reported that dimerisation with different light chains requires different domains of 4F2hc, which makes the interaction with the particular light chain important and potentially the decisive element in determining the heteromerisation properties of 4F2hc (Bröer et al. 2001).

This is particularly of interest concerning the y⁺LAT1/4F2hc transporter properties as a previous article reported on the interference of mutated y⁺LAT1 on the function of not only the wild-type protein but also that of the sister transporter y⁺LAT2, when experimentally expressed in the same cell (Sperandeo et al. 2005b). The effect was dose-dependent and could already be seen with ratios as low as 1:3 (mutant:wild-type). This dominant negative effect can be considered a strong suggestion in favour of the transporter being formed as a heterotetramer of two y⁺LAT1 and two 4F2hc molecules. However, another contradiction lies here: the heterozygote carriers of the LPI mutation are phenotypically and biochemically healthy, exhibiting no aminoaciduria.

Inspired by the foregoing, my aim was to clarify the nature of the y⁺LAT1/4F2hc transporter structure using FRET-based imaging methods; FRET in flow cytometry and in confocal microscopy. The cultured HEK293 cells were transfected with y⁺LAT1 in both ECFP and EYFP vectors, either in the presence or absence of non-fluorescent 4F2hc, the hypothesis being that the potential FRET representing the interaction of the light subunits can be considered to testify for the tetrameric form of the transporter as the y⁺LAT1 molecules are known to primarily/preferentially dimerise with 4F2hc. That is, the light subunit interaction represents, in reality, the interaction of two dimers consisting of a fluorescent y⁺LAT1 and a dark 4F2hc.

As a mean result of three (of both positive and negative controls), four (y⁺LAT1^{2,} without 4F2hc) or six (y⁺LAT1^{2} with 4F2hc) individual transfections, 9.9 ± 4.1% of all live cells that contained the y⁺LAT1 subunits but no 4F2hc were FRET-positive, whereas when the 4F2hc was also present, the proportion of FRET-positive cells was 15.6 ± 5.6% (the respective figure for the positive control was 29.3 ± 5.1%). When normalised regarding the positive control, the proportion of the FRET-positive cells of all live cells, if the positive control is considered to equal 1, was 0.34 for cells with no 4F2hc (in addition to the ECFP-y⁺LAT1 and EYFP-y⁺LAT1) and 0.53 when the cells were also expressing 4F2hc. According to these calculations, 34% of the cell population randomly transfected with ECFP-y⁺LAT1 and EYFP-y⁺LAT1, and no 4F2hc, and 53% of those transfected with all three constructs,

had successfully internalised both fluorophore-associated forms of the light subunit (ECFP-y$^+$LAT1 and EYFP-y$^+$LAT1), and displayed their interaction. In addition, 18.4% of the FRET-positive cells that had been exposed to transfection of all three constructs, were expressing 4F2hc in addition to both of the light chain types. This was taken as a strong indication for the occurrence of the tetrameric form of the transporter, [y$^+$LAT1/4F2hc]^2.

A potential error in FRET measurements can occur when interpreting the FRET signal and gating FRET-positive cells. Since in flow cytometry, the FRET application used is sensitised emission, the signal interpreted as 'FRET' is the fluorescence obtained through the EYFP filters subsequent to ECFP excitation. As both the excitation and emission spectra of ECFP and EYFP overlap, some spectral bleed-through of ECFP and EYFP can occur on the FRET channel. Therefore, it is essential to define the FRET collection gate using negative controls of ECFP-only and EYFP-only to exclude the non-FRET signals. Finally, we also corrected the percentages of the FRET-positive cells by subtracting the remaining ECFP and EYFP bleed-through to the FRET channel (mean ECFP 0% and EYFP 0.874 ± 0.44%, seen also in panel C of Figure 10, illustrating a representative FRET experiment in flow cytometry). The flow cytometric FRET values presented in the text above are EYFP-corrected, eliminating false FRET.

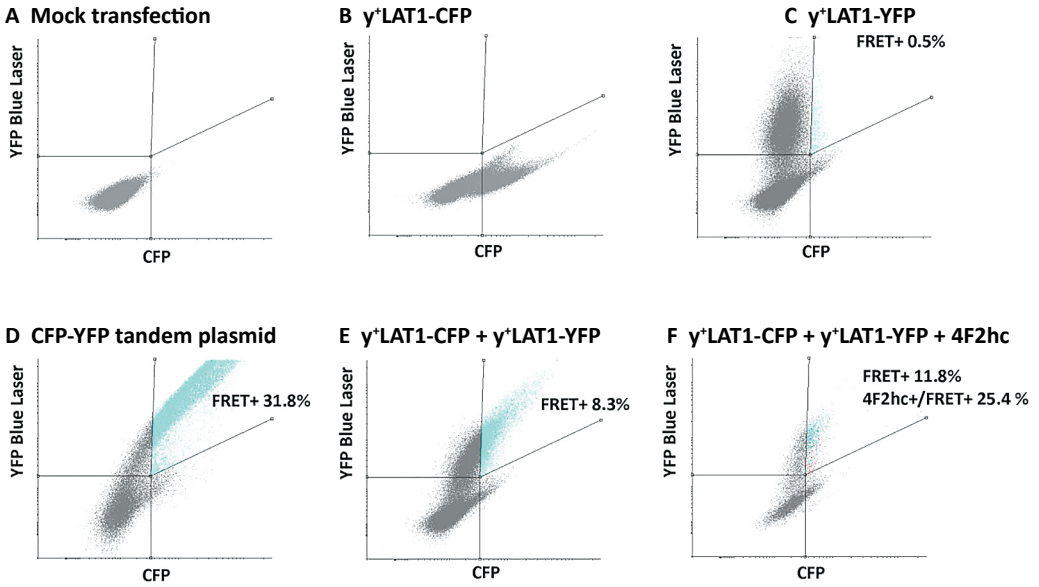

Figure 10. FRET measurements of the y$^+$LAT1-y$^+$LAT1 interaction in flow cytometry.
This figure shows FACS images of one representative FRET measurement demonstrating the interaction between ECFP-y$^+$LAT1 and EYFP-y$^+$LAT1. On the top row of this panel, the negative control measurements of A) mock transfection (no DNA), B) ECFP-only and C) EYFP-only are displayed. The bottom row shows the results for D) the positive control (ECFP-EYFP tandem plasmid producing forced FRET), E) the ECFP- and EYFP-labelled y$^+$LAT1s without 4F2hc and F) with 4F2hc present. The FRET-positive cells (turquoise) appear in the top right quadrate, adjusted for ECFP and EYFP bleed-through to the FRET channel. The percentages stated are those of FRET-positive cells out of all live cells and, in addition in F) those of 4F2hc-positive cells of FRET-positive cells.

Using the tandem plasmid-transfected cells (positive control) as a basis for normalising the FRET is justifiable since it produces forced FRET in which every successfully transfected cell produces a signal; it represents the ideal of the FRET fluorescence in relation to transfection efficiency – that is, 100%. The double- or triple-transfected cells used in this work, however, had two (ECFP-y$^+$LAT1 and EYFP-y$^+$LAT1) or three (the former and non-fluorescing 4F2hc) independent exogenous elements for the cells to internalise, and we had no way of controlling or monitoring the process of internalisation of the exogenous DNA by the cells. Thus, we could only observe its success as FRET (two differently-labelled y$^+$LAT1 molecules present) and as co-localisation of the 4F2hc antibody-staining in the FRET-positive cells (all three constructs present). Bearing this in mind, the cells that failed to be transfected or those that internalised only one type of y$^+$LAT1 construct (with or without 4F2hc) can thus be held responsible for the "missing FRET", that is about 47% of the supposedly triple-transfected cell population.

5.4.2 FRET confocal microscopy

FRET reporting for the interaction of the ECFP- and EYFP-labelled y$^+$LAT1 in the presence and absence of exogenous 4F2hc was also examined in greater detail using acceptor photobleaching FRET in confocal microscopy. Despite the low fluorescence efficiency values, the acceptor photobleaching FRET microscopy results, displayed in Table 6, confirm those obtained from the flow cytometric experiments and thus provide evidence for the existence of complexes containing two or more y$^+$LAT1/4F2hc dimers.

Table 6. FRET acceptor photobleaching fluorescence efficiency (E_F) values in the multimerisation study

Constructs	Bleached regions		Control regions		NET
	E_F ± SD	(n) ROI	E_F ± SD	(n) ROI	E_F (%)
ECFP and EYCP vectors (negative control)	0.2 ± 2.4	92	0.0 ± 0.6	90	0.2
ECFP-y$^+$LAT1 + EYFP-4F2hc (positive control)	4.6 ± 4.9	42	0.6 ± 1.1	35	4.1
ECFP-y$^+$LAT1 + EYFP-y$^+$LAT1 + 4F2hc	4.2 ± 5.5	84	0.2 ± 0.7	70	3.9
ECFP-y$^+$LAT1 + EYFP-y$^+$LAT1 no 4F2hc	2.4 ± 3.5	69	0.3 ± 1.3	60	2.1

As the positive control in this experiment, we used the construct combination shown to yield the highest FRET in subproject I. The fluorescence efficiency values obtained in this experiment were consistently considerably lower than in I. Despite this, a clear difference between the positive versus negative control could be obtained, facilitating the qualitative evaluation of the interaction between the construct combinations tested, ECFP-y$^+$LAT1/ EYFP-y$^+$LAT1 in the presence or absence of 4F2hc. The net E_F obtained from the triple-transfected (y$^+$LAT1^2 + 4F2hc) cells was 95.1% of that of the positive control whereas the cells with only the y$^+$LAT1 subunits and no 4F2hc, yielded a net E_F of only 51.2% of that of the positive control (cf. negative control, 4.9%).

The same effect can also be observed when E_F was quantified for different construct combinations and different parts of the biosynthetic pathway (the ER, Golgi and the target location, the plasma membrane, see Table 7) to those of the triple-transfected cells (4F2hc present) closely resemble those in all areas measured (88.6% for the ER, 105.5% for the Golgi complex and 80% for the plasma membrane), whereas the E_F of the double-transfected cells (no 4F2hc present) are only 45.7 % of those of the positive control for the EF and 70.9% for the Golgi complex. As expected, based on the fact that the light subunits are transported to the plasma membrane by the heavy subunits, the interaction of the y⁺LAT1 subunits at the plasma membrane in the absence of 4F2hc was negligible. Due to the shape of the ROI (square), the residual interaction seen at the plasma membrane between the y⁺LAT1 subunits (no 4F2hc) is the result of their interaction in the near-plasma membrane compartment of the cytoplasm.

Table 7. Mean donor fluorescence intensity change (fluorescence efficiency, E_F) in bleached regions by cellular compartment

Construct combinations	Plasma membrane		Endoplasmic reticulum		Golgi apparatus	
	E_F	(n) ROI	E_F	(n) ROI	E_F	(n) ROI
ECFP-y⁺LAT1 + EYFP-4F2hc	4.0 ± 4.0	14	3.5 ± 5.2	14	5.5 ± 5.5	14
ECFP-y⁺LAT1 + EYFP-y⁺LAT1 + 4F2hc	3.2 ± 3.3	29	3.1 ± 5.6	29	5.8 ± 6.6	29
ECFP-y⁺LAT1 + EYFP-y⁺LAT1 no 4F2hc	0.9 ± 1.5	20	1.6 ± 3.5	19	3.9 ± 3.8	28

The reason for the weaker interaction between the y⁺LAT1 subunits appearing alone in the cells can only be speculated upon. Potentially, the y⁺LAT1 molecules form complexes readily, but the nature of the complexes is transient in the absence of the stabilising forces provided by 4F2hc.

5.5 The effect of the LPI_{Fin} mutation at the systemic level – analysis of transcriptomic variance in LPI (III)

In Finland, all the approximately 40 patients share the same lysinuric protein intolerance-causing mutation c.895-2A>T ($LPI_{Fin,}$ 1181-2A>T, IVS6AS, A-T, -2), but the variability of their clinical picture is extensive, ranging from a mild protein aversion to fatal multi-organ complications. This controversy between the genetic homogeneity and phenotypic variance of the patients prompted us to explore their transcriptomes in greater detail. The aim of this project was to identify some genetic factors contributing to the variability of the clinical picture. One of the relevant candidates was *SLC7A6,* encoding the sister y⁺L transporter, y⁺LAT2, the potential compensatory effect of which had been speculated upon since the implication of y⁺LAT1 for lysinuric protein intolerance in 1999. Increased expression of *SLC7A6* had even been detected in the lymphoblast extracted from LPI patients, which suggested that the expression of these genes may be connected (Shoji et al. 2002).

The result of the Illumina microarray analysis for the differentially expressed genes in the 13 selected Finnish LPI patients with the "classic" clinical picture compared to the 10 healthy age- and sex-matched volunteers revealed a total of 962 genes with altered expression, meeting our criteria of the degree of expression alteration (log 2 fold change limit of ± 0.8) and the statistical significance of the change (p-value limit 0.05). 501 of these were up- and 461 down-regulated. The most down-regulated gene of all was *SLC7A7* with a log 2 fold change of -2.37, translating into a residual expression of 19% of normal expression. With the qRT-PCR verification, the down-regulation of *SLC7A7* was fine-tuned to be -2.66 (the array patients) or -3.05 (all patients).

The transcriptome analysis revealed to us that the effects of the LPI mutation on amino acid transport are not restricted to the down-regulation of *SLC7A7*, but the resulting interplay of amino acid transporter gene expression changes creates a far more complicated picture. Based on the microarray results, in addition to *SLC7A7*, two other *SLC7* family members, *SLC7A1* (CAT1) and *SLC7A5* (LAT1) had differential expression (upregulation by a fold-change of 1.05 and 0.88, respectively). Similarly, another y^+LAT1-related transporter gene, *SLC1A5*, coding for neutral amino acid transporter ASCT2, was upregulated by a fold-change of 1.10. To our surprise, *SLC3A2* (4F2hc) and *SLC7A6* (y^+LAT2) displayed no change in their expression. In a subsequent qRT-PCR survey of the gene expression levels of the transporter genes of interest in the array patients and also the entire Finnish patient population we had collected RNA samples from, we also discovered a slight upregulation of *SLC3A2* in addition to verifying the gene expression changes stated above. No compensatory change in the expression of *SLC7A6* could be detected when the *SLC7A6* expression changes were calculated as an average of the patient population. In individual patients, the expression of *SLC7A6* varied considerably, potentially conveying some compensatory effect on the y^+L transport. However, no parallel between *SLC7A6* up-regulation and a milder clinical picture of the patient could be drawn, nor did we detect any correlation between the expression changes of *SLC7A7* and *SLC7A6*. In contrast to this, the expression changes of several other amino acid transporters did display significant correlation (Pearson's correlation): *SLC3A2* correlated significantly with *SLC7A7*, *SLC7A5* and *SLC1A5*, *SLC1A5* with *SLC7A5* and *SLC7A1*, and *SLC7A5* with *SLC7A6* (Table 8).

Table 8. Correlations (Pearson) of the amino acid transporter genes in the gene expression analysis

	SLC3A2	*SLC7A7*	*SLC7A6*	*SLC7A5*	*SLC7A1*	*SLC1A5*
SLC3A2	1	0.501**	-0.090	0.470**	0.279	0.439**
SLC7A7	0.501**	1	0.166	0.320	0.194	0.231
SLC7A6	-0.090	0.166	1	0.394*	-0.231	0.101
SLC7A5	0.470**	0.320	0.394*	1	0.230	0.702***
SLC7A1	0.279	0.194	-0.231	0.230	1	0.577**
SLC1A5	0.439**	0.231	0.101	0.702***	0.577**	1

* = P<0.05, ** = P<0.01, *** = P<0.001

The upregulation of the cationic amino acid transporter CAT1 gene, *SLC7A1*, may be the result of an attempt to maintain normal cationic amino acid influx, particularly of arginine, into cells in an environment of reduced availability of extra-cellular arginine. This could be the consequence of two mechanisms, both debilitated by the impaired arginine transport in LPI: the reduced renewal of the arginine pools in the body by absorbance in the small intestine, or the trapping of arginine into cells due to the lack of efflux of cationic amino acids from cells. The latter mechanism would be of importance in monocytes and macrophages, in which the efflux of cationic amino acids is dependent upon y^+LAT1 (Barilli et al. 2010). The efflux of arginine from the monocytes has been recognised as a delicate and highly controlled method of regulating the division of the pool of free arginine in the body into the intra-cellular reserve and the extra-cellular, bioavailable arginine (Rotoli et al. 2009). In LPI, the efflux is defective, and as a consequence this regulation mechanism no longer works, depleting the extra-cellular reserves as arginine becomes trapped inside the monocytes. This also has a second consequence for macrophages as they express NOS that converts arginine into nitric oxide. Nitric oxide in large quantities is cytotoxic, impairing cellular function and promoting apoptosis. Possibly for this reason, the phagocytotic ability of the LPI macrophages is reduced, rendering them inefficient in their task of pathogen removal (Barilli et al. 2012). Barilli and colleagues speculate that when considered together, the increased apoptosis combined with a defective phagocytotic function of LPI macrophages results in incomplete clearance of apoptotic material from tissues, which can be seen, for example, in the gradual build-up of amorphous protein material in the lungs during a PAP crisis. However, given that in human macrophages, the y^+LAT1/4F2hc transporter can mediate either CAA influx or efflux, depending on the conditions and the availability of its intra- and extra-cellular substrates (Barilli et al. 2012), it also seems possible that in LPI, the transport defect deprives monocytes of arginine by preventing its influx, thus having the opposite, reducing effect on the production of NO in these cells (Kurko et al. 2015). The actual direction of transport in these cells in biological conditions nevertheless remains to be clarified.

The increase of apoptosis reported in LPI also manifested itself in study III as the differential regulation of genes grouping under gene ontologies such as ‘negative regulation of programmed cell death’, ‘negative regulation of cell death’, ‘apoptosis’, ‘death’, ‘programmed cell death’ and ‘regulation of apoptosis’ (DAVID), and involved in canonical pathways such as ‘hepatic fibrosis’, ‘hepatic cholestasis’ and ‘renal necrosis’ (IPA). One reason for the increased apoptosis in the Finnish LPI patients may be provided by their mutation. The Finnish LPI mutation, being a frame shift mutation, retains the y^+LAT1 in the cytoplasm. As the interaction of y^+LAT1 and 4F2hc occurs irrespective of the mutations in y^+LAT1, the 4F2hc coupled to the mutant y^+LAT1 molecules are also prevented from featuring in the plasma membrane, which may lead to a scarcity of 4F2hc in the plasma membrane, particularly, given that the expression of 4F2hc is not upregulated. Since 4F2hc can be regarded as an anti-apoptotic factor, its shortage may manifest itself as increased apoptosis.

The second cell type in which the impaired efflux and the subsequent trapping of arginine is of systemic importance is the kidney cells. As the kidney is the main location for arginine

de novo synthesis from citrulline (which has been synthesised from glutamine in the small intestine), trapping arginine into the kidney cells efficiently depletes the systemically bioavailable extracellular arginine reserves, reducing systemic NO production, for example. In addition, the presence of extra amino acids in the kidney encumbers the renal cells, predisposing them to kidney damage (also seen as gene expression changes relating to 'renal necrosis' (IPA)).

The reduction in nitric oxide production also contributes to the renal involvement in LPI. The mechanism for this has been demonstrated in the chronic kidney disease, in which arginine synthesis in the kidney is inefficient, resulting in impaired NO production. This leads to a vicious cycle as the NO deficiency causes endothelial dysfunction, cardiovascular events and hypertension, which contribute to kidney damage (Baylis 2006). The arginine transport defect in LPI leads to a systemic arginine deficiency in the patients, and low plasma nitric oxide levels (Kamada et al. 2001), resulting in endothelial dysfunction and hypertension, exacerbating the renal insufficiency and nephropathy. A large percentage of the LPI patients (currently 70% of the Finnish patients) develop chronic renal insufficiency, potentially developing into end-state renal disease requiring kidney transplantation.

Among the transporter genes whose upregulation was discovered in study III is *SLC1A5,* the gene for glutamine transporter ASCT2. As the plasma glutamine levels in LPI are elevated, one can speculate that the upregulation is a substrate-driven acclimation to an altered amino acid environment with the aim of creating a new amino acid homeostasis. However, the expression change of *SLC1A5* is significantly correlated with that of *SLC7A5*, coding for LAT1, which also transports neutral amino acids, mainly leucine. These transporters have been linked to an amino acid transport "shuttle", which drives the influx of leucine (ASCT catalyses the influx of glutamine which LAT1 exchanges with leucine, (Verrey 2003)). The essential amino acids, including leucine, imported through this method, have been reported to activate the mTOR pathways linked to the suppression of autophagy, which in turn is known to have a role in programmed cell death (Shintani & Klionsky 2004, Levine & Kroemer 2008, Nicklin et al. 2009). The gene expression analysis (III) revealed, as mentioned above, several differentially expressed genes belonging to the apoptosis and cell death-related categories based on their gene ontologies and the canonical pathways they function within.

The functional analysis of the microarray data revealed a substantial immunological side to LPI: When the over-expressed genes were divided into gene ontology categories (GO) based upon the biological processes in which they were involved, the largest was 'immunological processes', comprising 19% of the over-expressed genes. Similarly, the most prominent groups of differentially expressed genes according to the DAVID gene ontology analysis were 'immune response' and 'inflammatory response', and the IPA pathway analysis revealed significant changes in canonical pathways including 'interferon signalling' and 'antiviral immune response'. The differential expression of the genes related to immunological processes is supported by the various immunological abnormalities observed in the LPI patients (including low proportions of activated T cells detected in III, lymphocytopaenia,

impaired vaccination response, impaired macrophage function and inadequate responses to viral infections). The LPI patients in other countries have been linked to hemophagocytic lymphohistiocytosis (HLH) which has not been officially reported in connection with the Finnish patients. Considering the symptoms listed in sections 2.2.4 and 2.2.5 (such as hepatosplenomegaly, thrombocytopaenia, bleeding diathesis, erythrophagocytosis, elevated plasma ferritin and LDH levels, etc.), and the significant number of differentially expressed immunology-related genes gives rise to the hypothesis that in addition to their weakened immunological functions described above, the Finnish patients may suffer from a chronic sub-acute version of HLH which, triggered by a viral cue (for example, EBV or other respiratory infection diagnosed in patients preceding a PAP crisis (Santamaria et al. 2004, Gordon et al. 2007)), flares up to produce the full clinical manifestations of HLH, leading to a fatal multi-organ complication.

Indications for the potential connection of LPI and HLH were also observed in our gene expression analysis, as the microarray analysis detected differential expression of a number of genes previously implicated in disease states mapping under the HLH umbrella. The differential expression of these genes, however, still remains to be verified by quantitative methods.

The immunological connection was also demonstrated by the altered proportions of the LPI patients' lymphocyte populations (T lymphocytes, B lymphocytes, helper/inducer T cells, suppressor/cytotoxic T cells, NK cells and activated T cells) in the flow-cytometric measurements. The proportions of all the lymphocyte subpopulations were mostly low normal to slightly subnormal. In contrast to this, the proportions of the activated T cells ($CD3^+$, HLA DR^+) were remarkably low in all patients but three. This likely reflects abnormalities in the activation and function of T cells (for instance antigen presentation to the phagocyting cells) and thus contributes to the immunological abnormalities observed in the patients. Interestingly, low values of $CD3^+$, HLA DR^+ T cells have been linked to hyperferritinemia (Shiota et al. 2011), also manifested by the LPI patients.

6. SUMMARY AND CONCLUSIONS

The first of the three substudies included in this thesis aimed at optimising a new, visual research method for our study of the y^+LAT1/4F2hc amino acid transporter in order to clarify the protein-protein interactions between the transporter subunits and to establish its heteromeric structure. Acceptor photobleaching FRET confocal microscopy and, subsequently, FRET in flow cytometry proved to be useful and, with stringent controls, reliable tools for obtaining visual data on the interactions of y^+LAT1 and 4F2hc.

The interaction between the transporter subunits seems to be of a primary nature as y^+LAT1 and 4F2hc dimerise unperturbed by LPI-causing mutations, or those abolishing the cyteines supporting the disulphide bridge thought to unite the two proteins. However, our investigations did not cover the function of the transporter, and therefore the significance of the disulphide bridge for the function of the transporter remains undefined and requires further research.

The results obtained from the interaction experiments measured by FRET between the y^+LAT1 subunits tagged with FRET-appropriate fluorophores strongly indicate that the y^+LAT1/4F2hc transporter is formed as a heterotetramer consisting of two of each subunit, y^+LAT1 and 4F2hc. This is consistent with the results from Sperandeo and colleagues, reporting on the interference of mutated y^+LAT1 with the function of the wild-type protein (Sperandeo et al. 2005b). This poses an interesting biological question with regard to the heterozygous carriers of LPI: In the heterotetrameric random combination of the wild-type and LPI-mutated proteins of the heterozygotes, one could expect to observe the same dominant-negative effect as in the experimental setting in *Xenopus* oocytes. The heterozygote carriers, however, are phenotypically normal, exhibiting no clinical or biochemical symptoms of LPI. In the analysis of the transcriptomic variance in the Finnish LPI patients, we discovered the *SLC7A7* gene to be the most down-regulated of all differentially regulated genes. Potentially, this is the result of an allele-specific down-regulation mechanism that also silences the mutant allele of the heterozygotes, thus preventing the interference pathological to the function of the wild-type allele. Thus far, our research interest has been focussed, for obvious reasons, on the LPI patients, but now, perhaps, it would be beneficial to widen the research to also cover the heterozygous carriers of LPI.

In this work, I examined the structure of the transporter in one cell line, using confocal microscopy and flow cytometry. It is generally customary to use immunohistochemical methods in detecting proteins, and, indeed, demonstrating the structure and size of the y^+LAT1/4F2hc transporter using, for example, co-immunoprecipitation and Western blotting, in addition to the fluorescence-based detection methods, would naturally increase the reliability of the results. Furthermore, using antibody-based methods, the transporter could be visualised in primary patient cells, thereby obtaining valuable information on the native state of the mutant transporter *in vivo.* However, due to the continued lack of

a functioning *SLC7A7* antibody capable of detecting the Finnish y⁺LAT1 this could not be achieved, and verification therefore requires further product development. In addition to the non-polar HEK293 cells, repeating the experiments in a polarised cell line, for example MDCK cells, could provide further spatial information on the location of the transporter subunit interaction and the formation of the holotransporter. This would be of particular biological interest as the main target tissues of the y⁺LAT1/4F2hc transporter are polarised epithelial cells.

LPI has traditionally been regarded as a relatively benign condition that can be easily contained with a protein-restricted diet, citrulline supplement and nitrogen-scavenging medication. The analysis of the transcriptomic variance caused by the Finnish LPI mutation proved that the disease is actually considerably more complex than initially believed, including changes in various biological processes, such as inflammatory response, immune system processes and apoptosis, thus indicating that the amino acid transport defect in LPI also has serious consequences for immune defence. The effects of the mutation on amino acid transport were not limited to cationic amino acid transport but also involved several other transporters (summarised in Figure 11), further complicating the transport-related picture of this disease. However, no compensatory effect in *SLC7A6* expression was detected.

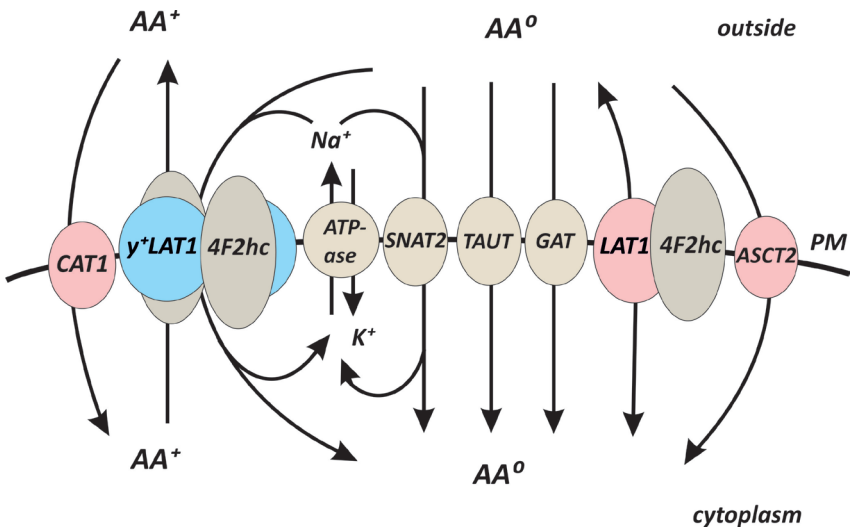

Figure 11. Summary of the amino acid transporter results.
This figure illustrates the structure of the y⁺LAT1/4F2hc transporter (tetramer) and the altered balance of cationic and neutral amino acid transporters detected in peripheral blood cells of the LPI patients. The expression of y⁺LAT1 was discovered to be down-regulated (depicted in blue), and the expression of cationic amino acid transporter CAT1 and neutral amino acid transporters LAT1 and ASCT2 were up-regulated (depicted in red). The expression of the other amino acid transporters (brown) were either unchanged (4F2hc), or not assessed in the real-time PRC verification step.
AA^+ =cationic amino acids, AA^0 =neutral amino acids, SNAT2 = transporter of small neutral amino acids, TAUT = taurine transporter, GAT = GABA transporters 1 and/or 2, PM = plasma membrane

Although providing new foci for LPI research by uncovering novel gene expression changes in the LPI patients, the transcriptomic analysis was still rather limited in sample size (13 patients in the microarray and 35 in the verification step). However, as the study population contains approximately 80% of the Finnish LPI patients, obtaining a meaningfully larger study population would require inclusion of LPI patients from other countries. The benefits gained in increasing statistical power would be lost in the diversification of the genetic background as that of an internationally pooled patient population would be considerably more heterogeneous than that of an all-Finnish patient population.

The transcriptomic analysis, instead of providing an all-encompassing explanation for the variable symptoms of LPI, opened up a multitude of questions, including a potential connection between LPI and HLH which remains to be validated. One interesting way to seek answers for these questions would be to perform a comparative transcriptomic assay that includes LPI patients with different mutations in order to observe whether some of the expression changes are caused specifically by the Finnish frame shift mutation. Similarly, bearing in mind the heteromeric form of the transporter, it would be of particular interest to compare our transcriptomic data with the gene expression profiles of phenotypically healthy, heterozygotic carriers. Furthermore, as the transcriptomic data revealed the gene expression profiles of individual patients, it provides a tool for the assessment of the correlation between the symptoms and gene expression levels of individual patients. LPI being a genetic disorder, the options for treatment are relatively few (lysine and citrulline supplements, nitrogen scavengers). Nonetheless, the gene expression profiles generated by this transcriptomic analysis serve as an excellent starting point for personalising the treatment and medication of the patients.

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