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MOLECULAR PATTERNS BEHIND IMMUNOLOGICAL AND METABOLIC ALTERATIONS IN LYSINURIC PROTEIN INTOLERANCE

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'Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life.'

Marcus Aurelius

To my family

ABSTRACT

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Molecular patterns behind immunological and metabolic alterations in lysinuric protein intolerance

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Lysinuric protein intolerance (LPI) is a recessively inherited disorder characterised by reduced plasma and increased urinary levels of cationic amino acids (CAAs), protein malnutrition, growth failure and hyperlipidemia. Some patients develop severe immunological, renal and pulmonary complications. All Finnish patients share the same LPI_{Fin} mutation in the *SLC7A7* gene that encodes CAA transporter y^+LAT1.

The aim of this study was to examine molecular factors contributing to the various symptoms, systemic metabolic and lipid profiles, and innate immune responses in LPI. The transcriptomes, metabolomes and lipidomes were analysed in whole-blood cells and plasma using RNA microarrays and gas or liquid chromatography-mass spectrometry techniques, respectively. Toll-like receptor (TLR) signalling in monocyte-derived macrophages exposed to pathogens was scrutinised using qRT-PCR and the Luminex technology.

Altered levels of transcripts participating in amino acid transport, immune responses, apoptosis and pathways of hepatic and renal metabolism were identified in the LPI whole-blood cells. The patients had increased non-essential amino acid, triacylglycerol and fatty acid levels, and decreased plasma levels of phosphatidylcholines and practically all essential amino acids. In addition, elevated plasma levels of eight metabolites, long-chain triacylglycerols, two chemoattractant chemokines and nitric oxide correlated with the reduced glomerular function in the patients with kidney disease. Accordingly, it can be hypothesised that the patients have increased autophagy, inflammation, oxidative stress and apoptosis, leading to hepatic steatosis, uremic toxicity and altered intestinal microbe metabolism. Furthermore, the LPI macrophages showed disruption in the TLR2/1, TLR4 and TLR9 pathways, suggesting innate immune dysfunctions with an excessive response to bacterial infections but a deficient viral DNA response.

Keywords: lysinuric protein intolerance (LPI), amino acid transport, cationic amino acid (CAA), kidney, liver, macrophage, toll-like receptor (TLR), transcriptome, metabolome, lipidome

TIIVISTELMÄ

Johanna Kurko

Lysinuurisen proteiini-intoleranssin immunologisten ja metabolisten muutosten molekulaarinen tausta

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia ja genetiikka, Turun molekyylilääketieteen tohtoriohjelma (TuDMM)

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Lysinuurinen proteiini-intoleranssi (LPI) on peittyvästi periytyvä sairaus, jossa kationisten aminohappojen pitoisuudet ovat plasmassa matalat ja virtsassa korkeat ja potilailla esiintyy proteiinialiravitsemusta, kasvuhäiriöitä ja hyperlipidemiaa. Joillekin potilaille kehittyy lisäksi immunologisia sekä munuais- ja keuhkotoimintojen komplikaatioita. Kaikilla suomalaispotilailla on sama LPI_{Fin}-mutaatio *SLC7A7*-geenissä, joka koodaa kationisten aminohappojen kuljetinta y^+LAT1:tä.

Tämän tutkimuksen tarkoituksena oli selvittää molekulaaristen tekijöiden vaikutusta taudin moninaisiin oireisiin, systeemisiä metabolia- ja lipiditason muutoksia sekä synnynnäisen immuniteetin vasteita LPI-potilailla. Kokoveren soluista ja plasmasta analysoitiin RNA-mikrosiruja ja kaasu- tai nestekromatografia-massaspektrometriatekniikoita käyttämällä transkriptomit, metabolomit ja lipidomit. Monosyyteistä erilaistettujen ja patogeeneille altistettujen makrofagien tollinkaltaisten reseptorien (TLR) signalointia tarkasteltiin käyttämällä qRT-PCR:ää ja Luminex-teknologiaa.

Kokoveren soluista löydettiin aminohappokuljetukseen, immuunivasteisiin, apoptoosiin sekä maksa- ja munuaismetaboliareitteihin liittyviä transkripteja, joiden tasot olivat muuttuneet potilailla. Potilaiden ei-välttämättömien aminohappojen, triasyyliglyserolien ja rasvahappojen plasmapitoisuudet olivat kohonneet, kun taas fosfatidyylikoliinien ja käytännössä kaikkien välttämättömien aminohappojen pitoisuudet olivat alentuneet. Lisäksi kahdeksan metaboliitin, pitkäketjuisten triasyyliglyserolien, kahden kemoatraktantin kemokiinin ja typpioksidin kohonneet plasmapitoisuudet korreloivat heikentyneen glomerulustoiminnan kanssa munuaistautia sairastavilla potilailla. Tulosten perusteella näyttää siltä, että LPI-potilailla on lisääntynyt autofagia, tulehdustila, oksidatiivinen stressi ja apoptoosi, jotka voivat johtaa maksan steatoosiin, toksisten aineiden kerääntymiseen veressä ja muuttuneeseen suolistomikrobien metaboliaan. Lisäksi LPI-makrofagien TLR2/1-, TLR4- ja TLR9-signaalivälitysreiteissä havaittiin muutoksia, jotka saattavat aiheuttaa synnynnäisen immuniteetin toimintahäiriöitä ja liiallisen vasteen bakteeri-infektioille mutta heikentyneen vasteen virus-DNA:lle.

Avainsanat: lysinuurinen proteiini-intoleranssi (LPI), aminohappokuljetus, kationinen aminohappo, munuainen, maksa, makrofagi, tollinkaltainen reseptori (TLR), transkriptomi, metabolomi, lipidomi

TABLE OF CONTENTS

ABBREVIATIONS

4F2hc	surface antigen 4F2 heavy chain
α-KG(DH)	alpha-ketoglutarate (dehydrogenase)
AM	alveolar macrophage
AP-1	activator protein 1
APC	amino acid-polyamine-choline
asc1	system asc amino acid transporter 1
ATP	adenosine triphosphate
$b^{0,+}$AT	system $b^{0,+}$ amino acid transporter
BAIBA	beta-aminoisobutyric acid
BCAA	branched-chain amino acid
CAA	cationic amino acid
CAT(1-4)	cationic amino acid transporter, member (1-4)
cDNA	complementary deoxyribonucleic acid
CE	capillary electrophoresis
cGMP	cyclic guanosine monophosphate
CKD	chronic kidney disease
c/mRNA	complementary/messenger ribonucleic acid
CNS	central nervous system
CoA	coenzyme A
CP	carbamoyl phosphate
CpG	cytidine-phosphate-guanosine
(e)GFR	(estimated) glomerular filtration rate
e/i/nNOS	endothelial/inducible/neuronal nitric oxide synthase
ESI	electrospray ionisation
FC	fold change
FDCA	2,5-furandicarboxylic acid
FDH	Finnish disease heritage
G3P	glycerol-3-phosphate
GH	growth hormone
(G)M-CSF	(granulocyte)-macrophage colony stimulating factor
GO	gene ontology
GS	gas chromatography
GSH	glutathione
HAT	hetero(di)meric amino acid transporter
HDL	high-density lipoprotein
HLH	haemophagocytic lymphohistiocytosis
HMDB	Human Metabolome Database
HPA	4-hydroxyphenylacetic acid
HSHAT	heavy subunit HAT
IAA	indole-3-acetic acid
IFN-α/β/γ	interferon alpha/beta/gamma
Ig	immunoglobulin
IGF1	insulin-like growth factor 1
IGFBP	insulin-like growth factor binding protein
IL-1RA	interleukin 1 receptor antagonist
IPA	Ingenuity pathway analysis

IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
IUGR	intrauterine growth restriction
LAT(1-2)	system L amino acid transporter, member (1-2)
LBP	lipopolysaccharide binding protein
LC	liquid chromatography
LDH	lactate dehydrogenase
LP	lipopeptide
LPI	lysinuric protein intolerance
LPI_{Fin}	LPI Finnish mutation, IVS6AS, A-T, -2, c.895-2A>T
LPS	lipopolysaccharide
LSHAT	light subunit HAT
MALDI	matrix-assisted laser desorption ionisation
MAPK	mitogen-activated protein kinase
MAS	macrophage activation syndrome
MD-2	myeloid differentiation protein 2
MDM	monocyte-derived macrophage
MS	mass spectrometry
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation factor 88
m/z	mass/charge
NAA	neutral amino acid
NADH	nicotinamide adenine dinucleotide (reduced)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NO	nitric oxide
ODN	oligodeoxynucleotide
Pam_3CSK_4	Pam_3(tripalmitoylated)$CysSerLys_4$
PAMP	pathogen-associated molecular pattern
PAP	pulmonary alveolar proteinosis
PBMC	peripheral blood mononuclear cell
PC	phosphatidylcholine
(p)DC	(plasmacytoid) dendritic cell
PE	phosphatidylethanoamine
PIA	primary inherited aminoaciduria
PRR	pattern recognition receptor
QqQ	triple quadrupole
qRT-PCR	quantitative real-time PCR
rBAT	related to $b^{0,+}$ -type amino acid transporter
RNA-Seq	RNA sequencing
RNS	reactive nitrogen species
ROS	reactive oxygen species
SLC7A(1-14)	solute carrier family 7, member (1-14)
SLE	systemic lupus erythematosus
SM	sphingomyelin
TAK1	TGF-β-activated kinase 1
TCA	tricarboxylic acid
TG	triacylglycerol

TGF-β	transforming growth factor beta
TIRAP	TIR domain-containing adaptor protein
TLR(1-13)	toll-like receptor (1-13)
TM	transmembrane
TNF-α	tumour necrosis factor alpha
TOF	time-of-flight
TRAF	tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adapter molecule
TRIF	Toll/IL-1R (TIR) domain-containing adapter inducing IFN-β
UHPLC	ultrahigh performance liquid chromatography
(V)LDL	(very) low-density lipoprotein
xCT	system x_c^- transporter
y^+LAT(1/2)	system y^+L amino acid transporter, member (1-2)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles referred to in the text by their Roman numerals I-III.

I Tringham M, **Kurko J**, Tanner L, Tuikkala J, Nevalainen OS, Niinikoski H, Näntö-Salonen K, Hietala M, Simell O, Mykkänen J. Exploring the transcriptomic variation caused by the Finnish founder mutation of lysinuric protein intolerance (LPI). Molecular Genetics and Metabolism. Mol Genet Metab. 2012. 105(3):408-15.

II **Kurko J**, Vähä-Mäkilä M, Tringham M, Tanner L, Paavanen-Huhtala S, Saarinen M, Näntö-Salonen K, Simell O, Niinikoski H, Mykkänen J. Dysfunction in macrophage toll-like receptor signaling caused by an inborn error of cationic amino acid transport. Mol Immunol. 2015. 67(2 Pt B):416-25.

III **Kurko J**, Tringham M, Tanner L, Näntö-Salonen K, Vähä-Mäkilä M, Nygren H, Pöhö P, Lietzen N, Mattila I, Olkku A, Hyötyläinen T, Orešič M, Simell O, Niinikoski H and Mykkänen J. Imbalance of plasma amino acids, metabolites and lipids in patients with lysinuric protein intolerance (LPI). Manuscript.

In addition, some unpublished data are presented in this thesis.

1 INTRODUCTION

Lysinuric protein intolerance (LPI) is an aminoaciduria first described in 1965 by Perheentupa and Visakorpi (Perheentupa and Visakorpi 1965) and later established as a disorder of the Finnish disease heritage. Torrents and coworkers (Torrents *et al.* 1998) identified a novel cationic amino acid (CAA) transporter, the contribution of which in LPI pathogenesis was confirmed a year later by two separate groups (Borsani *et al.* 1999, Torrents *et al.* 1999) who discovered that mutations in the *SLC7A7* gene encoding y^+LAT1 cause LPI. LPI is characterised by defective CAA transport in the small intestine and proximal kidney tubules, causing depletion of lysine, arginine and ornithine in the blood and their increased excretion in the urine. The wide range of symptoms in LPI include protein aversion after weaning leading to malnutrition, failure to thrive, growth failure, hyperammonaemia due to urea cycle dysfunction, combined hyperlipidemia, haematological and immunological defects, and renal and pulmonary complications. The patients are treated with a low-protein diet, citrulline and lysine supplements and nitrogen scavengers in order to prevent hyperammonaemia and to improve their urea cycle function, protein tolerance and nutritional status.

Since the discovery of the LPI gene, considerable effort has been put into gaining knowledge of the pathophysiology of LPI and improving the treatment of the patients. However, much still remains uncovered. Over half of the Finnish LPI patients suffer from renal insufficiency, some of the patients have experienced severe pulmonary alveolar proteinosis and most of the patients suffer from hepatosplenomegaly, combined hyperlipidemia and immunological complications with severe viral and bacterial infections, for all of which the aetiology remains unknown. Some patients have had a multiorgan failure, which has proven to be life-threatening. A hypothesis of a high intracellular arginine level due to the CAA export defect causing an inflammatory and apoptotic state in the target cells such as macrophages and renal tubular cells has been proposed to explain some LPI-related complications (Sebastio *et al.* 2011, Ogier de Baulny *et al.* 2012). In addition, an observed B cell dysfunction affecting antibody production may impair microbial clearance (Lukkarinen *et al.* 1999).

In this study, the effect of the Finnish LPI mutation on the whole-blood genome-wide gene expression patterns, macrophage innate immune responses and systemic metabolic and lipid profiles was scrutinised in order to produce new hypotheses for the molecular mechanisms behind various symptoms and complications in the patients.

2 REVIEW OF THE LITERATURE

2.1 Amino acids and their transport systems

2.1.1 Classification of amino acids

Amino acids are vital in biological and biochemical systems. In addition to being building blocks in proteins, amino acids are important substrates and intermediates of numerous metabolites in various biochemical pathways. They are precursors for the synthesis of neutrotransmitters, catecholamines, purines, pyrimidines and haem (Elliot and Elliot 2001). In general, amino acids are crucial for energy metabolism, normal cell growth, maturing, activation, differentiation and proliferation of cells, and particularly so for the immunological processes (Daly *et al.* 1990, Evoy *et al.* 1998, Li *et al.* 2007). It is well established that an amino acid deficiency may predispose to inflammatory or immune-related diseases (Li *et al.* 2007, Grohmann and Bronte 2010, Ghesquière *et al.* 2014).

Amino acids are classified into different groups by their properties and structure. In eukaryotes, there are twenty standard amino acids encoded by the genetic code. These amino acids are the building blocks of proteins; thus, they are defined as proteinogenic amino acids. The main roles of the twenty standard amino acids are listed in Table 1. Selenocysteine has also been added to the list of proteinogenic amino acids, but, in contrast to the standard amino acids, it is incorporated into proteins by a co-translational process (Xu *et al.* 2007). In total, there are more than 140 naturally occurring amino acids (Ambrogelly *et al.* 2007), which may be either intermediates in metabolic pathways, such as ornithine and citrulline, post-translationally added into proteins, or they may even be present only in extra-terrestrial meteorites (Cronin and Pizzarello 1983). Amino acids are divided into positive (cationic/basic), negative (anionic/acidic) and neutral (zwitterionic) by their side-chain charge, and into basic polar, acidic polar, uncharged polar and nonpolar amino acids based on their side-chain polarity (Alberts *et al.* 2002). Amino acids can occur both in L and D isomer forms, but in proteins they exist only in their L forms. Based on their side-chains, amino acids can also be grouped into aliphatic (according to their hydrophobicity), hydroxyl or sulfur/selenium-containing, cyclic, aromatic or branched. These characteristics facilitate the appropriate folding of proteins, with the hydrophobic groups escaping water and the polar ones facing it, in order to, for example, form protein-protein interactions or active sites of enzymes. In addition, amino acids are also divided into essential and non-essential amino acids according to whether their deficit in the diet causes a deficiency disease or has no effect. Essential amino acids are exclusively received from the diet whereas the non-essential ones can be synthesised *de novo*. Some amino acids are semi-essential, such as arginine: under normal conditions their synthesis is perfectly sufficient. However, at certain stages of development or disease conditions their deficit in the diet may upset the system. Other semi-essential amino acids, such as cysteine and tyrosine, are dependent on essential amino acids for their synthesis. (Elliot and Elliot 2001.)

Amino acids are also grouped into glucogenic or ketogenic amino acids according to whether they may be broken down into products either entering gluconeogenesis or are able to be converted into ketone bodies and fatty acids during fasting and starving. After deamination of amino acids,

the remaining carbon skeleton, the keto acid, is either converted to pyruvate or some of the following tricarboxylic acid (TCA) cycle intermediates: alpha-ketoglutarate (α-KG), succinyl-CoA, fumarate or oxaloacetate, and further to phosphoenolpyruvate and glucose (glucogenic amino acids), or into acetyl-CoA or acetoacetyl-CoA for use as ketone bodies or fatty acids (ketogenic amino acids). (Elliot and Elliot 2001, Berg *et al.* 2002.) However, in normal nutritional conditions, all of the above-mentioned amino acid metabolites participate in the TCA cycle in order to produce energy. Subsequently, toxic ammonia generated in the process is transferred to the liver to be used in the urea cycle and excreted as urea. (Elliot and Elliot 2001.)

In the next chapter, the biological roles of cationic amino acids lysine, arginine and non-proteinogenous ornithine relevant in this thesis are discussed in detail.

Table 1. The main features and biological functions of the twenty proteinogenic amino acids.

Amino acid	Side-chain charge	Side-chain polarity	Side-chain features	Dietary essentiality	TCA cycle intermediate	Main functions	References
Alanine	neutral	nonpolar	aliphatic	non-essential	glucogenic/ pyruvate	[a]A role in a glucose-alanine cycle in the liver and muscle, [b]a constituent of collagen	[a](Elliot and Elliot, 2001), [b](Barbul, 2008)
Arginine	positive/ cationic	basic polar		semi-essential	glucogenic/ α-ketoglutarate	A precursor of [a]nitric oxide, [b]creatine, [c]agmatine, [d]ornithine, [e]polyamines and urea	[a](Hibbs *et al.* 1987), [b](Brosnan *et al.* 2011), [c](Satriano 2003), [d](Bommarius and Drauz 1994), [e](Wei *et al.* 2001)
Asparagine	neutral	polar	amine group	non-essential	glucogenic/ oxaloacetate	[a]A precursor for NH_3, [b]required for the development and function of the brain, [c]residue is the main site for N-linked protein glycosylation	[a](Elliot and Elliot, 2001), [b](Ruzzo *et al.* 2013), [c](Schwarz and Aebi 2011)
Aspartic acid	negative/ anionic	acidic polar	carboxyl group	non-essential	glucogenic/ fumarate/ oxaloacetate	A precursor of [a]neurotransmitter D-aspartic acid and [b]purines, a metabolite of the urea cycle, a role in malate-aspartate shuttle (ATP production)	[a](D'Aniello *et al.* 2011), [b](Elliot and Elliot, 2001)
Cysteine	neutral	nonpolar	sulphur-containing	semi-essential	glucogenic/ pyruvate	A precursor for [a]glutathione, [b]taurine and sulphate	[a](Meister and Tate 1976), [b](Stipanuk and Ueki 2011)
Glutamic acid	negative/ anionic	acidic polar	carboxyl group	non-essential	glucogenic/ α-ketoglutarate	A precursor for [a]ornithine, proline, [b]glutathione and [c]GABA, [d]a neurotransmitter, [e]byproduct in amino acid degradation, an NH_3 carrier	[a](Jones 1985), [b](Meister and Tate, 1976), [c](Petroff 2002), [d](Meldrum 2000), [e](Elliot and Elliot, 2001)
Glutamine	neutral	polar	amine group	semi-essential	glucogenic/ α-ketoglutarate	A precursor of [a]purines, pyrimidines, [b]citrulline and [c]NH_3, a carrier of NH_3	[a](Cory and Cory 2006), [b](van de Poll *et al.* 2007), [c](Elliot and Elliot, 2001)
Glycine	neutral	nonpolar	smallest amino acid	non-essential	glucogenic/ pyruvate	A precursor of [a]serine, creatine, purines, porphyrins such as haem, bile acids and glutathione, a constituent of collagen, [b]neurotransmitter	[a](Cook 2000), [b](López-Corcuera *et al.* 2001)
Histidine	positive/ negative	basic polar	aromatic	essential	glucogenic/ α-ketoglutarate	A precursor for [a]histamine, [b]carnosine, homocarnosine and anserine biosynthesis	[a](Reilly and Schayer 1968), [b](Kohen *et al.* 1988)
Isoleucine	neutral	nonpolar	branched aliphatic	essential	glucogenic/succinyl-CoA, ketogenic/ acetyl-CoA	[a]Stimulates muscle protein synthesis and prevents muscle protein breakdown, [b]regulates glucose uptake in the muscle, [c]an amino group donor in the brain	[a](Shimomura *et al.* 2006), [b](Doi *et al.* 2005), [c](Yudkoff 1997)
Leucine	neutral	nonpolar	branched aliphatic	essential	ketogenic/ acetyl-CoA/ acetoacetyl-CoA	[a]A precursor of sterol in adipose and muscle tissue, [b]stimulates muscle protein synthesis and prevents muscle protein breakdown, [c]an amino group donor in the brain, [d]an activator of the mTOR pathway	[a](Rosenthal et al. 1974), [b](Shimomura et al. 2006), [c](Yudkoff 1997), [d](Lynch 2001)

Amino acid	Side-chain charge	Side-chain polarity	Side-chain features	Dietary essentiality	TCA cycle intermediate	Main functions	References
Lysine	positive/ cationic	basic polar		essential	ketogenic/ acetoacetyl-CoA	A precursor of derivatives needed for [a]collagen and elastin cross-linking, and [b]hemin and [c]carnitine synthesis, [d]a target for post-translational modifications in proteins	[a](Eyre *et al.* 1984), [b](Altman *et al.* 1952), [c](Feller and Rudman 1988), [d](Zencheck *et al.* 2012)
Methionine	neutral	nonpolar	sulphur-containing	essential	glucogenic/ succinyl-CoA	A precursor for [a]cysteine, [b]carnitine, [c]creatine and [d]phosphatidylcholine	[a](Brosnan and Brosnan 2006), [b](Feller and Rudman 1988), [c](Brosnan *et al.* 2011), [d](Visioli *et al.* 1998)
Phenylalanine	neutral	nonpolar	aromatic	essential	glucogenic/fumarate, ketogenic/ acetoacetyl-CoA	A precursor of [a]tyrosine and [b]neuromodulator phenylethylamine	[a](Matthews 2007), [b](Davis *et al.* 1991)
Proline	neutral	nonpolar	cyclic	semi-essential	glucogenic/ α-ketoglutarate	[a]A precursor of glutamate, [b]endogenous excitotoxin, [c]constituent of collagen, [d]an alpha-helix and beta-sheet breaker in proteins	[a](Jones 1985), [b](Henzi *et al.* 1992), [c](Barbul, 2008), [d](Li *et al.* 1996)
Serine	neutral	polar	hydroxyl group	semi-essential	glucogenic/ pyruvate	A precursor of [a]glycine, cysteine, purines, sphingolipids, folate and [b]neuromodulator D-serine, [c]mediates catalytic function in serine proteases, [d]phosphorylated by serine/threonine protein kinase during signal transduction	[a](Cook 2000), [b](Wolosker *et al.* 1999), [c](Di Cera 2009), [d](Josso and di Clemente, 1997)
Threonine	neutral	polar	hydroxyl group	essential	glucogenic/pyruvate/ succinyl-CoA, ketogenic/acetyl-CoA	[a]The residue phosphorylated by the serine/threonine protein kinase during signal transduction, [b]needed in the synthesis of intestinal mucin	[a](Josso and di Clemente 1997), [b](Nichols and Bertolo 2008)
Tryptophan	neutral	nonpolar	aromatic	essential	glucogenic/pyruvate, ketogenic/acetyl-CoA/ acetoacetyl-CoA	A precursor of [a]neurotransmitter serotonin, melatonin and [b]niacin	[a](Yao *et al.* 2011), [b](Goldsmith 1958)
Tyrosine	neutral	polar	aromatic/ hydroxyl group	semi-essential	glucogenic/fumarate, ketogenic/ acetoacetyl-CoA	A precursor of [a]melanin, [b]coenzyme Q10, [c]catecholamine neurotransmitters dopamine, epinephrine and norepinephrine, [d]triiodothyronine (T3) and thyroxine (T4), phosphorylated by tyrosine kinase during signal transduction	[a](Słominski *et al.* 1988), [b](Willis *et al.* 1999), [c](Fernstrom and Fernstrom 2007), [d](Elliot and Elliot, 2001)
Valine	neutral	nonpolar	branched aliphatic	essential	glucogenic/ succinyl-CoA	[a]Stimulates muscle protein synthesis and prevents muscle protein breakdown, [b]an amino group donor in the brain	[a](Shimomura *et al.* 2006), [b](Yudkoff 1997)

TCA, tricarboxylic acid; NH_3, ammonia; GABA, gamma-aminobutyric acid; CoA, coenzyme A

2.1.2 The biological roles of lysine, arginine and ornithine

2.1.2.1 Lysine and protein modifications

Lysine is an essential amino acid which is needed for the synthesis of all proteins. Lysine residues in proteins undergo post-translational modifications such as methylation, acetylation, acylation, deamination, sumoylation and ubiquitination, which are the major regulators of gene expression, protein-protein interactions, and protein processing and degradation (Zencheck *et al.* 2012). Zencheck and others (2012) have shown that lysine modifications are particularly crucial in the regulation of the cell cytoskeleton which is responsible for maintaining cell structure, intracellular trafficking and cell motility. Lysine derivatives allysine and hydroxyallysine are crucial in the cross-linking of elastin and collagen molecules (Eyre *et al.* 1984), and lysine is suggested to be important in the prevention and therapeutics of osteoporosis due to its enhanced capacity for intestinal calcium absorption and renal reabsorption (Civitelli *et al.* 1992). It may also have beneficial effects in the treatment of cardiovascular disease (Pauling 1993, Flodin 1997). However, excess plasma concentrations of lysine may inhibit the urea cycle and increase hyperammonaemia (Kato *et al.* 1987), and in rats additional lysine has been demonstrated to cause an increased orotic acid synthesis and a decreased production of urea; however, arginine supplementation has been shown to overcome the action of lysine (Fico *et al.* 1982). Lysine is also a precursor for haem in haemoglobin synthesis (Altman *et al.* 1952).

Lysine deficiency may limit cytokine production, proliferation of lymphocytes and immune responses during infections (Petro and Bhattacharjee 1981, Li *et al.* 2007). Oral lysine supplementation is known to weaken *Herpes simplex* virus infections (Griffith *et al.* 1978, Griffith *et al.* 1987) by depleting the polyamines necessary for the virus's survival via decreased arginine transport into the virus and an inhibition of arginase activity (Griffith *et al.* 1981). Lysine, along with another essential amino acid, methionine, is needed for the synthesis of carnitine, which has an essential role in fatty acid energy metabolism, as carnitine transports fatty acids into mitochondria for β-oxidation (Borum and Broquist 1977, Feller and Rudman 1988). Therefore, carnitine may function in protecting cells against toxic accumulation of acyl-CoA compounds; its deficit in turn causes muscular weakness (Feller and Rudman 1988).

2.1.2.2 Arginine and nitric oxide

Arginine is an intriguing amino acid, being a precursor for several important metabolites. It is not an essential amino acid, but it is classified as semi-essential or conditionally essential as its dietary demand may increase in different developmental and disease states, such as stress, infections and dysfunction of the small intestine or kidneys (Morris 2007), whereupon the endogenous arginine synthesis does not meet its increased consumption (Popovic *et al.* 2007). Arginine is synthesised from citrulline through the intestinal-renal axis (Morris 2007). First, dietary-derived glutamine is converted into citrulline in enterocytes, after which citrulline is released into the blood in which it is transported to the kidney and converted into arginine. Subsequently, the arginine is released from the kidney into the circulation, where it is available for the use of the entire body. (Wu and Morris 1998, Brosnan and Brosnan 2004, van de Poll *et al.* 2007.) Although the kidney is the most important site for arginine synthesis, arginine is also formed at a low level in many other

cells. However, *de novo* arginine synthesis accounts for only 5-15% of endogenous arginine production, thus the major provider of arginine is protein degradation at the systemic level (Wu and Morris 1998).

Arginine metabolism is characterised by the balance of two enzymes, NOS (nitric oxide synthase) and arginase. The main product of the arginine metabolism is nitric oxide (NO), the increased production of which is directly followed by an accelerated arginine formation (Hibbs *et al.* 1987, Iyengar *et al.* 1987, Marletta *et al.* 1988). Further, citrulline, which is a byproduct in NO formation (Hibbs *et al.* 1987, Iyengar *et al.* 1987, Marletta *et al.* 1988), can be recycled back to arginine in the citrulline-NO pathway and, thus, be re-exploited in NO synthesis (Nussler *et al.* 1994, Morris 2007). NO synthesis is mediated by three different NOS isoforms in different tissues: nNOS (neuronal NOS) or NOS1 (Nakane *et al.* 1993), iNOS (inducible NOS) or NOS2 (Lyons *et al.* 1992, Geller *et al.* 1993) and eNOS (endothelial NOS) or NOS3 (Janssens *et al.* 1992, Marsden *et al.* 1992). In the nervous tissue, NO, synthetised by constitutively expressed nNOS, is involved in the synaptic plasticity and memory formation, and acts as an unorthodox neurotransmitter that decreases the tone of various types of smooth muscle (Förstermann *et al.* 1994). eNOS, also constitutively expressed, is an effective vasodilator increasing blood flow and decreasing blood pressure by smooth muscle cell relaxation (Palmer *et al.* 1987, Förstermann *et al.* 1994). In addition, it plays a role in protecting blood vessels by inhibiting smooth muscle cell proliferation, platelet aggregation (de Graaf *et al.* 1992) and leukocyte adhesion (Kubes *et al.* 1991, Ouedraogo *et al.* 2007).

NO synthesis in macrophages is induced by iNOS which, in contrast to nNOS and eNOS, is expressed only after an induction with lipopolysaccharide (LPS) or cytokines such as IFN-γ (Li *et al.* 2002, Tötemeyer *et al.* 2006). In addition to monocyte-derived macrophages (MDMs) (Denis 1991, MacMicking *et al.* 1997), iNOS expression has also been detected in human alveolar macrophages (AMs) (Thomassen and Kavuru 2001), hepatocytes (Geller *et al.* 1993), kidney proximal tubule cells (Heemskerk *et al.* 2006), the pulmonary epithelium (Asano *et al.* 1994, Guo *et al.* 1995) and the colon epithelium (Perner *et al.* 2002). However, most of the studies concerning the detection and function of NO and iNOS have been carried out using murine macrophages (Kakuda *et al.* 1999, Nicholson *et al.* 2001, Yeramian *et al.* 2006a) which seem to contradict human studies (Venketaraman *et al.* 2003). Firstly, in human macrophages, NO and iNOS expression have been scarcely or not at all detectable in healthy subjects (Schneemann *et al.* 1993, Albina 1995, Fang and Vazquez-Torres 2002, Rotoli *et al.* 2007, Thomas and Mattila 2014); however, NO and iNOS have been observed in patients with infections and inflammatory diseases (MacMicking *et al.* 1997, Thomas and Mattila 2014) in which NO has an important role in the pathogen clearance (James 1995, MacMicking *et al.* 1997, Fang and Vazquez-Torres 2002). Secondly, in humans, the classical activation of macrophages with LPS and cytokines may not be effective to induce NO production (Albina 1995, Rotoli *et al.* 2007).

NO acts as a paracrine mediator by diffusing across cell membranes, migrating either in a tissue or the circulation bound to erythrocytes (Kelm 1999). The action through which NO functions, for example increasing vasodilation in smooth muscles, is mediated via stimulating the soluble guanylate cyclase to generate cyclic GMP, and further activating the K channels by a cGMP-dependent protein kinase (Archer *et al.* 1994). However, NO is a radical with the very short half-life

of a few seconds. In erythrocytes, NO may react with either oxyhaemoglobin to form nitrate, haemoglobin to form nitrosylhaemoglobin, or the 93-cysteine residue of the β-subunit of haemoglobin to form *S*-nitrosohaemoglobin. In the aqueous phase of plasma, NO may react with molecular oxygen to form nitrite, but also with the reactive oxygen species (ROS) such as superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) and further nitrogen dioxide (NO_2) or dinitrogen trioxide (N_2O_3), and also nitrosylated proteins of impaired function. (Kelm 1999, Rath *et al.* 2014.)

Arginase is another important enzyme in arginine metabolism pathways. Two isozymes of arginase catalyse the hydrolysis of arginine into ornithine and urea in the cytosol of hepatocytes (arginase I) and in the mitochondria of peripheral cells, such as macrophages (arginase II) (Munder 2009, Rath *et al.* 2014). However, in addition to iNOS expression, some groups claim to have detected arginase activity in human macrophages, whilst other groups argue the opposite (Schneemann *et al.* 1993, Raes *et al.* 2005, Thomas and Mattila 2014).

In addition to the NO synthesis, arginine is essential in the proliferation, activation and function of T cells (Bronte *et al.* 2003, Rodriguez *et al.* 2007, Choi *et al.* 2009). It has also been shown to stimulate the secretion of hormones, such as insulin, insulin-like growth factor 1 (IGF1), glucagon and prolactin (Vierhapper *et al.* 1980, Chevalley *et al.* 1998). Arginine induces collagen synthesis in osteoblast-like cells (Chevalley *et al.* 1998), and it enhances wound healing by collagen deposition (Barbul *et al.* 1990). In addition, it is necessary in macrophage-mediated tumour cell cytotoxicity (Evoy *et al.* 1998). Arginine, along with glycine and methionine, is a precursor for creatine (Wu and Morris 1998), which provides energy for the muscles (Elliot and Elliot 2001) and eventually dehydrates to yield creatinine which is then excreted by the kidney (Wu and Morris 1998). Agmatine, the end product of decarboxylated arginine, functions as a cell signalling molecule triggering innate immune responses (Jones *et al.* 2010); it may also be further converted into polyamine putrescine and urea by agmatinase (Morris 2007). The arginine metabolism pathways are described in Figure 1.

2.1.2.3 Ornithine and the urea cycle

Ornithine and arginine are intermediates of the urea cycle, which metabolises toxic nitrogenous ammonia after dietary protein loads. First, deaminated amino groups of amino acids are transferred to α-KG by transamination, giving rise to glutamate, which is, by the inclusion of ammonia formed from amino groups, further converted into glutamine. The glutamine is then transported in the blood to the liver, where it is hydrolysed and the released ammonia is bound to bicarbonate, producing carbamoyl phosphate (CP). CP reacts with ornithine, forming citrulline, which further reacts with nitrogen derived from aspartate to form argininosuccinate. Subsequently, argininosuccinate is degraded into arginine and fumarate. Finally, arginine is hydrolysed into ornithine and urea. Urea is further transported from the liver to the kidney to be excreted in the urine, whereas ornithine is recycled in the urea cycle. (Elliot and Elliot 2001.) The steps of the urea cycle are summarised in Figure 1. Five different disorders caused by mutations affecting the synthesis of five urea cycle enzymes have been described. They are all characterised by hyperammonaemia, and, for example, in patients with ornithine transcarbamoylase deficiency, increased orotic acid levels are also observed (Brosnan and Brosnan 2007).

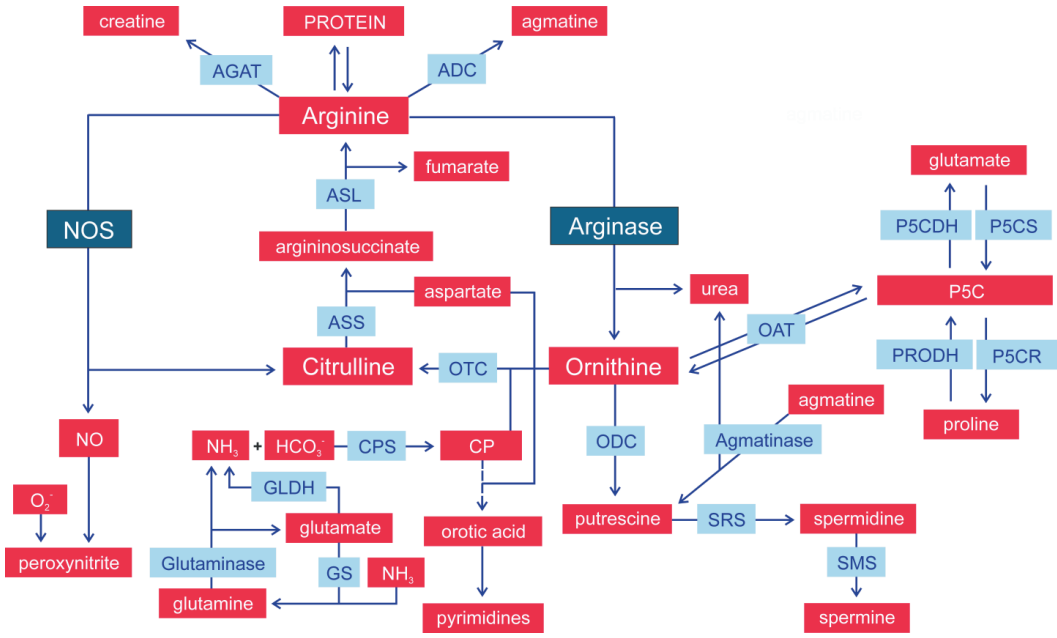

Figure 1. Arginine and ornithine metabolisms induced by NOS- and arginase-mediated pathways. ADC, arginine decarboxylase; AGAT, arginine:glycine amidinotransferase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; CP, carbamoyl phosphate; CPS, CP synthase; GLDH, glutamate dehydrogenase; GS, glutamine synthase; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamylase; P5C, pyrroline-5-carboxylate; P5CDH, P5C dehydrogenase; P5CR, P5C reductase; P5CS, P5C synthase; PRODH, proline dehydrogenase; SMS, spermine synthase; SRS, spermidine synthase. Modified from Rath *et al.* 2014.

In addition to its role in the urea cycle, ornithine also plays an important part in other biochemical pathways (Figure 1). The first step in the synthesis of polyamines, molecules crucial for normal cell growth, development and tissue repair, is the decarboxylation of ornithine by ornithine decarboxylase into putrescine, which is a precursor used in spermidine and spermine biosynthesis (Gerner and Meyskens 2004). Ornithine is also an intermediate in proline and glutamate synthesis (Wu and Morris 1998). As proline and its derivative hydroxyproline are the second largest amino acid components of the collagen protein after glycine, the positive effect of arginine on wound healing is proposed to be mediated through the ornithine-proline pathway (Barbul 2008). Ornithine supplementation has been demonstrated to have an antifatigue effect by promoting lipid metabolism and energy production (Sugino *et al.* 2008).

2.1.3 Solute carrier (SLC) families

The amino acid balance across the cell membrane is regulated by different transporters defined by their structure, function, and substrate and cell specificity. The known amino acid transporters are dived phylogenetically into five superfamilies encoded by the solute carrier (SLC) genes: the amino acid-polyamine-choline (APC) superfamily, sodium-dicarboxylate symporter (SDS) superfamily, neurotransmitter superfamily (NTS), amino acid transporter superfamily 1 (ATF1) and ma-

jor facilitator superfamily (MFS), which together include 19 identified transport systems in mammalian cells (Wipf *et al.* 2002). The APC family consists of the solute carrier 7 (SLC7) transporters which are divided into CATs (CAA transporters) and LATs (L-type amino acid transporters). LATs are the catalytic subunits, also called LSHATs (light subunit hetero(di)meric amino acid transporters) or gpaATs (glycoprotein-associated amino acid transporters), of the heteromeric amino acid transporters (HATs) (Verrey *et al.* 1999, Wipf *et al.* 2002, Fotiadis *et al.* 2013). The amino acid transporter families and transport systems are presented in Table 2.

2.1.3.1 The CAT family

The CAT transporters, mediating the y^+-type transport of CAAs, arginine, lysine and ornithine, are encoded by four genes (*SLC7A1-4*). These glycosylated transporter family members have 14 putative transmembrane (TM) segments. **CAT1,** encoded by *SLC7A1*, is expressed constitutively and almost ubiquitously, with the exception of the liver (Closs *et al.* 2004). In epithelial cells, it is localised basolaterally (Cariappa *et al.* 2002, Kizhatil and Albritton 2002), and in endothelial cells it colonises the caveolae membrane (Mann *et al.* 2003). CAT1 protein production is known to increase during glucose deprivation (Fernandez *et al.* 2002).

SLC7A2-encoded **CAT2** exists as two splice variants, CAT2A and 2B, with distinct expression patterns. CAT2A is most abundant in the liver, but is also expressed in other tissues, such as skeletal muscle, vascular smooth muscle and the pancreas (Verrey *et al.* 2004). CAT2B, however, is generally expressed together with CAT1 and induced mainly after cytokine or LPS stimulation in murine macrophages, where it is thought to provide arginine in order to activate iNOS for NO synthesis (Kakuda *et al.* 1999, Nicholson *et al.* 2001, Closs *et al.* 2004, Yeramian *et al.* 2006a, Yeramian *et al.* 2006b). Accordingly, iNOS would thus have access to the arginine pool nourished by CAT2B, but not the pool fed by CAT1 (Closs *et al.* 2004).

CAT3 expression in humans has been detected predominantly in the peripheral tissues, mostly in the thymus, but also in the uterus, testis, mammary gland, ovary and stomach, and, in addition, in the brain (Vékony *et al.* 2001). The fourth identified CAT family member, expressed in the brain, testis and placenta, is **CAT4,** encoded by *SLC7A4*, the deletion of which has been associated with the velocardiofacial syndrome (Sperandeo *et al.* 1998). However, it was later demonstrated that CAT4 does not mediate amino acid transport activity, either because it is not an amino acid transporter after all or it needs additional cofactors to be functional (Wolf *et al.* 2002).

The newest family member of the CAT family is an amino acid transporter encoded by *SLC7A14* (Closs *et al.* 2006), which is expressed in the central nervous system (CNS) in rat (Sreedharan *et al.* 2011), and mediates lysosomal CAA transport in human skin fibroblasts (Jaenecke *et al.* 2012). *SLC7A14* has also been linked to autosomal recessive retinitis pigmentosa (Jin *et al.* 2014).

Table 2. Amino acid transporter superfamilies and their associated amino acid transport systems.

Superfamily	Transport system	Example transporter	HUGO SLC series	Transport specificity
SDS	ASC	ASCT1	SLC1	Ubiquitous, prefers NAAs without bulky or side-branched chains, Na^+-dependent
SDS	B^0	B^0AT1	SLC1	Brush border membrane of epithelia, broad substrate specificity, does not accept N-methyl amino acids, Na^+-dependent
SDS	X^-_{AG}	EAAT1	SLC1	Brain and epithelial tissues, for anionic amino acids, Na^+-dependent
NTS	$B^{0,+}$	$ATB^{0,+}$	SLC6	In blastocysts and probably also in brush-border membrane, broad specificity for NAAs, CAAs and β-alanine, Na^+-dependent
NTS	BETA	GAT1	SLC6	Widespread, transports β-alanine, taurine and GABA, Na^+-dependent
NTS	GLY	GLYT1	SLC6	Present in several tissues, transports glycine, Na^+-dependent
NTS	PROT	PROT	SLC6	Proline-specific carriers, Na^+-dependent
NTS	IMINO	IMINO/XT3/SIT1	SLC6	In intestinal and kidney brush-border membrane, imino proline and hydroxyproline transporter, Na^+-dependent
APC	y^+	CAT1	SLC7	Widespread, for CAAs
APC + 4F2hc	asc	HAT (asc1 + 4F2hc)	SLC7 + SLC3	Specific for small NAAs
APC + rBAT	$b^{0,+}$	HAT ($B^{0,+}AT$ + rBAT)	SLC7 + SLC3	Widespread, brush border membrane of epithelia, for CAAs and some NAAs
APC + 4F2hc	L	HAT (LAT1 + 4F2hc)	SLC7 + SLC3	Widespread, for branched-chain and aromatic NAAs
APC + 4F2hc	X^-_c	xCT	SLC7 + SLC3	Glutamate-cystine exchanger
APC + 4F2hc	y^+L	HAT (y^+LAT1 + 4F2hc)	SLC7 + SLC3	Widespread, basolateral membrane of epithelia, transports CAAs and NAAs, Na^+-dependent
Monocarboxylate	T	TAT1	SLC16	Aromatic amino acids
VGT	VGT	BNP1	SLC17	Vesicular glutamate transport
ATF1	PAT/Imino acid	PAT1	SLC36	1:1 symport of protons and small NAAs, such as glycine, alanine, proline and hydroxyproline
ATF1	A	ATA3	SLC38	Widespread, mediates transport of NAAs with broad selectivity including N-methyl derivatives, Na^+-dependent
ATF1	N	SN1	SLC38	Cotransports glutamine and asparagine (and in some instances histidine) with Na^+, but, in contrast to system A, additionally countertransports protons

NAA, neutral amino acid; CAA, cationic amino acid; GABA, gamma-aminobutyric acid
Modified from Wipf *et al.* 2002, Boll *et al.* 2004, Takanaga *et al.* 2005, Bröer 2008.

2.1.3.2 The HAT family

The HAT family comprises seven known LSHATs: LAT1, y^+LAT2, y^+LAT1, LAT2, $b^{0,+}$AT, asc1 and xCT, all containing twelve TM segments and encoded by the *SLC7A5-11* genes, respectively (Wagner *et al.* 2001, Bröer and Wagner 2002, Fotiadis *et al.* 2013). LSHATs associate with the SLC3 family-encoded heavy subunits (HSHATs), facilitating the transport of the heteromers to the plasma membrane (Nakamura *et al.* 1999, Bröer and Wagner 2002, Fotiadis *et al.* 2013). The HSHATs binding with LSHATs are either 4F2hc (CD98hc) (Bertran *et al.* 1992a, Wells *et al.* 1992) or rBAT (related to $b^{0,+}$ -type amino acid transporter) (Bertran *et al.* 1992b, Tate *et al.* 1992, Wells and Hediger 1992, Bertran *et al.* 1993), encoded by the *SLC3A2* and *SLC3A1* genes, respectively. Six LSHATS (LAT1, LAT2, y^+LAT1, y^+LAT2, xCT and asc1) are known to heteromerise with 4F2hc, and only one light chain, $b^{0,+}$AT, associates with rBAT (Wagner *et al.* 2001, Fotiadis *et al.* 2013). 4F2hc was found in 1981 (Haynes *et al.* 1981) as a surface antigen in human activated lymphocytes and peripheral blood monocytes. It is ubiquitously expressed and found in all established cultured human cell lines tested at the time (Haynes *et al.* 1981, Quackenbush *et al.* 1987). In addition to amino acid transport, 4F2ch also participates in other important cellular functions, such as the proliferation, differentiation and fusion of cells (Devés and Boyd 2000), the clonal expansion of T and B cells and antibody responses (Cantor *et al.* 2009, Cantor *et al.* 2011, Cantor and Ginsberg 2012), and it also posses oncogenic properties (Devés and Boyd 2000). Moreover, it is essential in integrin mediated adhesion, thus determining the membrane domain polarity of the heteromeric transporter complexes (Fenczik *et al.* 1997, Fenczik *et al.* 2001, Feral *et al.* 2005). However, in addition to the LSHATs associating with either 4F2hc or rBAT, AGT1 encoded by *SLC7A13* has an as yet unknown HSHAT partner (Matsuo *et al.* 2002, Fotiadis *et al.* 2013).

The system L transporters

System L refers to leucine (L) transport without sodium (Verrey 2003). Its transporters LAT1 and LAT2 are encoded by the *SLC7A5* and *SLC7A8* genes, respectively. **LAT1**, the first identified LSHAT (Mastroberardino *et al.* 1998), is specialised in transporting large aromatic (tryptophan, phenylalanine, histidine and tyrosine) and branched-chain (valine, leucine and isoleucine) neutral amino acids (NAAs) (Kanai *et al.* 1998, Prasad *et al.* 1999). It is expressed in almost all tissues, where it mediates both influx and efflux of amino acids (Prasad *et al.* 1999, Wagner *et al.* 2001, Yanagida *et al.* 2001). LAT1-mediated simultaneous essential amino acid, especially that of leucine, import and glutamine export, is required for normal cellular growth and homeostasis as it induces the activation of the mTOR (mammalian target of rapamysin) kinase and subsequently inhibits autophagy. In an amino acid-rich environment, mTOR is active and regulates protein translation, but when the availability of extracellular amino acids is limited autophagy starts to break down cellular components in order to maintain cellular energy levels. The crucial step in the mTOR activation enabling LAT1-promoted glutamine/leucine exchange is the uptake of glutamine by the *SLC1A5*-encoded ASCT2 transporter. (Nicklin *et al.* 2009.) Further, it has been demonstrated that the actual mTOR activation takes place at the lysosomal membrane to which the LAT1/4F2hc complex is recruited in order to import essential amino acids into lysosomes (Milkereit *et al.* 2015). In addition, in rodents, LAT1 is an important amino acid transporter across the blood-brain barrier,

where it also allows the permeation of L-DOPA, a neurotransmitter precursor (Kageyama *et al.* 2000, Matsuo *et al.* 2000).

LAT2 has a wider substrate specificity than LAT1, as it transports both large and small NAAs at the basolateral membrane of the epithelial cells in the small intestine and kidney proximal tubules (Pineda *et al.* 1999, Rossier *et al.* 1999, Segawa *et al.* 1999, Bauch *et al.* 2003). In the placenta, LAT2 is located at the basal membrane side of the syncytiotrophoblast, mediating efflux towards the foetus (Verrey 2003). In the brain, hepatocytes, spleen and skeletal muscles, LAT2 is particularly important as it releases glutamine in the blood to be used for different metabolic purposes (Pineda *et al.* 1999). LAT2 is also an efficient outwardly-directed transporter of cysteine in epithelial cells (Fernández *et al.* 2003, Verrey 2003).

The system y^+L transporters

y^+LAT1 and y^+LAT2 are the transporters of the y^+L system first described in human erythrocytes (Devés *et al.* 1992). The y^+L system mediates the transport of CAAs (y^+) arginine, lysine and ornithine, in exchange for NAAs (L, leucine) with Na^+ or, in the absence of sodium, other inorganic cations such as Li^+ or H^+ (Kanai *et al.* 2000). However, in the absence of cations, exchange of intracellular CAAs for extracellular ones occurs (Devés *et al.* 1992). The **y^+LAT1** cDNA was first identified by the group of Torrents and others (Torrents *et al.* 1998), and its association with 4F2hc was further confirmed by Pfeiffer and others (Pfeiffer *et al.* 1999). The transport of CAAs by y^+LAT1 is outwardly directed at the basolateral membrane of the epithelial cells in the small intestine, kidney proximal tubules (Bauch *et al.* 2003, Verrey *et al.* 2004) and the airways (Rotoli *et al.* 2005), but in human monocytes and macrophages, where y^+LAT1 is the most important transporter of arginine, the CAA transport is bidirectional (Rotoli *et al.* 2004, Rotoli *et al.* 2007, Barilli *et al.* 2011). In the kidney, the arginine transport by y^+LAT1 is particularly vital in order to release the newly formed arginine into the circulation (Wagner *et al.* 2001, Brosnan and Brosnan 2004, Morris 2007). In addition to the kidney, intestine and blood leukocytes, y^+LAT1 expression has also been detected in the lung, placenta, spleen, liver, pancreas, epididymis, testis, ovary and thyroid (Torrents *et al.* 1998, Pfeiffer *et al.* 1999, Wagner *et al.* 2001). In the human umbilical vein endothelial cells (HUVECs), y^+LAT1 activity is required for arginine transport for NO synthesis (Arancibia-Garavilla *et al.* 2003). Interestingly, upregulation of *SLC7A7* and its protein product y^+LAT1 has been demonstrated to be a marker of poor prognosis for glioblastoma patients (Fan *et al.* 2013). Recently, it has been reported that *SLC7A7* expression is necessary for microglial, a subset of brain macrophages, colonization in the zebrafish brain (Rossi *et al.* 2015). Hence, the findings of the new roles of *SLC7A7* have widened the relevance of CAA transport further.

y^+LAT2, which is encoded by *SLC7A6*, was first detected in human erythrocytes (Devés *et al.* 1992). It is widely expressed in different non-epithelial and epithelial tissues such as brain astrocytes and neurons, testes, skin fibroblasts, and to a lesser degree in the small intestine, kidney and heart. Similarly to its sister transporter y^+LAT1, y^+LAT2 exchanges CAAs and large NAAs with Na^+. It is particularly important in the blood-brain barrier, releasing arginine in the brain in exchange for glutamine to maintain nitrogen balance, especially to secure arginine supply for the NO produc-

tion. On the other hand, y^+LAT2 may have a role in neurons taking up glutamine in order to synthesise glutamate. (Bröer *et al.* 2000, Wagner *et al.* 2001.) Recently, it has been shown that y^+LAT2 mediates arginine influx in rat astrocytes for increased NO synthesis and oxidative/nitrosative stress during hyperammonaemia (Zielińska *et al.* 2012).

The CAT, LAT and y^+LAT transporters and their associated genes and expression patterns are summarised in Table 3.

Table 3. The y^+, L and y^+L transport systems and their associated amino acid transporters.

System	Transporter	Gene	Tissue expression	Reference
y^+	CAT1	*SLC7A1*	Ubiquitous (except liver)	(Yoshimoto *et al.* 1991, Albritton *et al.* 1992, Closs *et al.* 2004)
y^+	CAT2 (A and B)	*SLC7A2*	Skeletal muscle, placenta, ovary, liver, vascular smooth muscle, pancreas, kidney and heart	(Hoshide *et al.* 1996, Closs *et al.* 1997, Lauteala *et al.* 1997a, Verrey *et al.* 2004)
y^+	CAT3	*SLC7A3*	Thymus, uterus, testis, mammary gland, ovary, stomach and brain	(Vékony *et al.* 2001)
y^+	CAT4 (not active)	*SLC7A4*	Brain, testis and placenta	(Sperandeo *et al.* 1998)
y^+	SLC7A14	*SLC7A14*	Skin fibroblasts	(Jaenecke *et al.* 2012)
L	LAT1/ 4F2hc	*SLC7A5/ SLC3A2*	Almost ubiquitous (e.g. placenta, brain, skeletal muscle, heart, colon, thymus, spleen, kidney, liver, testis, bone marrow, lymph node, lung and leukocytes)	(Mastroberardino *et al.* 1998, Kanai *et al.* 1998, Prasad *et al.* 1999, Yanagida *et al.* 2001)
L	LAT2/ 4F2hc	*SLC7A8/ SLC3A2*	Small intestine, kidney, placenta, brain, liver, spleen and skeletal muscles	(Bassi *et al.* 1999, Pineda *et al.* 1999, Rossier *et al.* 1999, Segawa *et al.* 1999, Verrey 2003)
y^+L	y^+LAT1/ 4F2hc	*SLC7A7/ SLC3A2*	Small intestine, kidney, leukocytes, lung, placenta, spleen, liver, pancreas, epidymis, testis, ovary and thyroid	(Torrents *et al.* 1998, Pfeiffer *et al.* 1999, Wagner *et al.* 2001)
y^+L	y^+LAT2/ 4F2hc	*SLC7A6/ SLC3A2*	Almost ubiquitous (e.g. erythrocytes, brain astrocytes and neuron, testis, skin fibroblasts, small intestine, kidney and heart)	(Devés *et al.* 1992, Bröer *et al.* 2000)

2.1.4 Amino acid transport defects – primary inherited aminoacidurias (PIAs)

Primary inherited aminoacidurias (PIAs) are a group of rare diseases caused by defective amino acid transport through renal epithelia (reabsorption), leading to an excess excretion of amino acids into the urine. In many cases, epithelial transport in the small intestine (absorption) is also affected. These diseases are derived from mutations in the amino acid transporter genes and, therefore, they differ from other aminoacidurias caused by secondary defects in enzyme functions in metabolic pathways, such as tyrosinemia or phenylketonuria. Currently, five PIAs have been identified: lysinuric protein intolerance (LPI) (MIM#222700), cystinuria (MIM#220100), Hartnup disorder (MIM#234500), dicarboxylic aminoaciduria (MIM#222730) and iminoglycinuria (MIM#242600). All of these PIAs, with the exception of LPI, are characterised by an impaired amino acid transport at the apical membrane of epithelial cells; no defective conditions affecting the basolateral transport have been found thus far, other than LPI. (Camargo *et al.* 2008.)

Cystinuria is the most common PIA with a global incidence of 1:7 000 births (Camargo *et al.* 2008, Näntö-Salonen *et al.* 2012). However, the incidence varies extremely between 1:2 500 neonates in Libyan Jews and 1:100 000 in Sweden (Barbosa *et al.* 2012). Cystinuria is characterised by a defect in the reabsorption of cystine and CAAs in the kidney tubules, leading to the precipitation of cystine and, further, to kidney stones causing infections and renal insufficiency (Palacín *et al.* 2001). Mutations in *SLC3A1* (rBAT) (Calonge *et al.* 1995) and *SLC7A9* ($b^{0,+}$AT) (Feliubadaló *et al.* 1999) are known to cause three different types of cystinuria, type A and type B, respectively, but type AB is also possible if both genes are affected (Dello Strologo *et al.* 2002). In the autosomal recessive type A cystinuria, the mutations cause a delay in the rBAT transport to the plasma membrane (Chillarón *et al.* 1997, Palacín *et al.* 2000), but some mutations altering the actual transport activity have also been observed (Wagner *et al.* 2001). In contrast, the type B cystinuria results from a defect in the function of the transporter complex (Font *et al.* 2001). A genotype-phenotype correlation is seen in type B cystinuria since the heterozygotes also suffer from varying levels of cystinuria and excretion of CAAs (Font *et al.* 2001, Palacín *et al.* 2001).

The transporter affected in the autosomal-recessive Hartnup disease (estimated incidence of 1:14 000 to 1:45 000 births) (Näntö-Salonen *et al.* 2012) is NAA transporter B^0AT1, encoded by *SLC6A19* (Camargo *et al.* 2008). The autosomally recessively inherited dicarboxylic aminoaciduria (incidence of 1:35 000 births in Canada) is caused by a defect in the transport of aspartate and glutamate (Camargo *et al.* 2008, Näntö-Salonen *et al.* 2012), but it was only in 2011 that *SLC1A1,* encoding EAAT3, could be pin-pointed as the causative gene of this disease (Bailey *et al.* 2011). Iminoglycinuria (incidence 1:10 000 births) is an autosomal recessive abnormality of the renal transport of glycine, and the imino acids proline and hydroxyproline (Näntö-Salonen *et al.* 2012). Homozygous mutations in the *SLC36A2* gene encoding proton transporter PAT2 were discovered to cause the iminoglycinuria phenotype, while heterozygous mutations induced hyperglycinuria (MIM#138500) without iminoaciduria. Mutations in *SLC36A2* that retain partial transport activity result in the iminoglycinuria phenotype when combined with the mutations in gene *SLC6A20* encoding the imino acid transporter XT3. Even more complexity in the genetics of iminoglycinuria is provided by additional mutations in *SLC6A18* encoding the glycine transporter XT2, and mutations in *SLC6A19* encoding the NAA transporter B^0AT1 in families with either iminoglycinuria or hyperglycinuria. (Bröer *et al.* 2008.)

In the next chapter, lysinuric protein intolerance, the disease scrutinised in this thesis, is discussed in detail.

2.2 Lysinuric protein intolerance (LPI)

2.2.1 Background

Lysinuric protein intolerance (LPI, MIM#222700), also known as hyperdibasic aminoaciduria type 2 or familial protein intolerance, is an autosomal recessive aminoaciduria belonging to the Finnish disease heritage (FDH). LPI was first described by Perheentupa and Visakorpi in 1965 (Perheentupa and Visakorpi 1965) in three poorly growing infants suffering from protein intolerance and defective intestinal and renal CAA transport, mainly that of lysine. As Dr. Perheentupa

has described most of the FDH diseases, 'Perheentupa's steps', an illustration representing the process and timeline of the discovery of the FDH diseases beginning in the 1950s was named after him. The FDH is a group of rare hereditary diseases that are overrepresented in Finland and appear less frequently elsewhere in the world (Norio 2003a). It currently comprises 36 monogenic diseases, the majority of which are autosomally recessively inherited, caused by one or few founder mutations and manifested mostly in eastern and northern Finland (Norio 2003b).

It has been believed that the colonization of Finland occured in two separate waves: the southern and western areas after the last glacial period ("early settlement") and the eastern and northern parts as late as in the 16th century ("late settlement") (Peltonen *et al.* 2000). However, the archeological data indicate that all Finland was initially colonised after deglaciation (Bergman *et al.* 2004, Palo *et al.* 2009). Similarly, the idea of genetic "bottlenecks" by late migration wave causing the enrichment of the FDH alleles in small founder population isolates was questioned by Palo and coworkers (Palo *et al.* 2009). Instead, they suggested that the random enrichment of the FDH diseases is due to a gene flow and long-term genetic drift which is more prominent in the isolates in sparsely inhabited areas. Further genetic divergence is caused by the male-biased gene flow from Scandinavia to the western parts of Finland seen as different Y-chromosomal marker distribution between western and eastern Finland (Palo *et al.* 2009). The geographical division is also marked in LPI, in which the ancestors of patients inhabited the south-eastern, eastern and northern parts of Finland, which is still apparent in the geographical distribution of the birthplaces of the LPI family grandparents in the Säkkijärvi-Lemi-Savitaipale, Suomussalmi and Kittilä regions, respectively (Norio 2003b). The estimated age of the ancestor LPI_{Fin} founder mutation carried by all the Finnish patients (Borsani *et al.* 1999, Torrents *et al.* 1999) and not found anywhere else is 50 generations based on the birthplaces of the LPI grandparents (Lauteala *et al.* 1997b). Based on the proposal by Palo and coworkers, it can be assumed that the LPI allele enriched in these regions by random long-term drift. This disequilibrium in allelic distribution is seen in notable differences in the carrier frequencies of the Finnish founder mutation in the different parts of Finland; frequencies being 1:91 in Oulu and 1:194 in Helsinki (Pastinen *et al.* 2001).

LPI is a rare disease; there are only approximately 50 LPI patients in Finland and 150 others worldwide, in at least 24 countries on every continent (Norio 2003b, Sperandeo *et al.* 2008, Näntö-Salonen *et al.* 2012). In Finland, the incidence of LPI is approximately 1:60 000 newborns (Näntö-Salonen *et al.* 2012). There is also an LPI cluster in the northern part of Iwate in northern Japan, where the incidence is estimated to be 1:52000 newborns and the carrier frequency of the R410X founder mutation is 1:114 (Koizumi *et al.* 2003). In Campania, in southern Italy, there is another, smaller LPI cluster in which patients in four families share the same mutation, 1625insATCA (Borsani *et al.* 1999, Sperandeo *et al.* 2000, Sperandeo *et al.* 2008). The low prevalence of LPI in other countries may be due to its mis- or underdiagnosis by clinicians unfamiliar with this disease with its highly variable and nonspecific phenotype, especially those working with laboratories that lack readily accessible tests (Sperandeo *et al.* 2008, Ogier de Baulny *et al.* 2012).

2.2.2 The CAA transport defect

LPI is characterised by a low level of CAAs, lysine, arginine and ornithine, in the plasma and their increased excretion in the urine due to a defect in the (re)absorption of CAAs at the basolateral membrane of epithelial cells in the small intestine and proximal kidney tubules (Perheentupa and Simell 1974). The transport defect was first detected *in vivo* in the LPI kidney tubules where CAAs were not reabsorbed into the blood but remained in the tubular urine (Simell and Perheentupa 1974). The transport defect was suggested to be basolateral since after oral administration of lysylglycine, the plasma glycine level increased normally, but the lysine level remained low (Rajantie *et al.* 1980a). Impaired CAA transport was further detected in the intestine (Rajantie *et al.* 1980b) and localised to the basolateral membrane of epithelial cells in the jejunum *in vitro*; however, the transport was seen to be intact at the luminal membrane (Desjeux *et al.* 1980). Interestingly, at that time, it was demonstrated by Rajantie and coworkers (Rajantie *et al.* 1980b) that the plasma lysine levels were actually intermediate in heterozygotes for the LPI mutation. A year later, the defect was confirmed *in vivo* to localise to the basolateral membrane of the renal tubuli (Rajantie *et al.* 1981b). The intestinal transport was shown to be normal for citrulline, a NAA, at both membranes (Rajantie *et al.* 1980b), but, after its oral dose, it was shown to be excreted excessively from the kidney into the urine along with arginine and ornithine (Rajantie *et al.* 1981b). This may be due to the partial intracellular conversion of citrulline into arginine and further into ornithine; therefore, an accumulation of citrulline's conversion products may inhibit its metabolic disposal, resulting in its high cellular concentration and increased luminal backflux, along with arginine and ornithine (Rajantie *et al.* 1981b).

The transport defect was also studied in the liver slices where the uptake of arginine was clearly impaired (Simell 1975). However, when studied in hepatocytes, the concentrations of arginine, ornithine and citrulline was revealed to be normal or even elevated rather than repressed (Rajantie *et al.* 1983). Rajantie and coworkers (1983) hypothesised that the CAAs accumulate in the cytoplasm as a result of their impaired export from hepatocytes and weakened import into the mitochondria, thus leading to the depletion of these amino acids in the mitochondria where ornithine is needed in the urea cycle. The transport in cultured fibroblasts is known to be normal due to the compensating effect of another CAA transporter, y^+LAT2 (Dall'Asta *et al.* 2000). In granulocytes (Simell 1975) and erythrocytes (Smith *et al.* 1988, Boyd *et al.* 2000), the CAA transport is normal probably thanks to a CAA transporter other than y^+LAT1 or y^+LAT2. However, the transport defect is detected in monocytes, MDMs and AMs (Barilli *et al.* 2010, Barilli *et al.* 2012).

In addition to a genetic test, LPI can be verified biochemically as a vast excretion of lysine together with moderately increased excretion of arginine and ornithine in the urine. The plasma levels of these amino acids are from one third to a half of their normal concentrations; however, sometimes they are within the normal range. The levels of other amino acids than CAAs are also regularly monitored in LPI: the plasma and urine concentrations of serine, alanine, glycine, proline and citrulline are slightly elevated, and the plasma levels of glutamine and glutamic acid are also moderately increased. (Tanner 2007.)

2.2.3 The *SLC7A7* gene

Lauteala and others (Lauteala *et al.* 1997b) mapped the LPI gene to the proximal long arm of chromosome 14 by a linkage analysis of 20 Finnish LPI families. The possible founder effect in LPI in Finland was suggested a year later, and also the same 14q11 area was linked to the non-Finnish LPI patients (Lauteala *et al.* 1998). Torrents and coworkers (Torrents *et al.* 1998) identified the y^+LAT1 cDNA that, together with 4F2hc, induces y^+L-type transport, and located it to the same chromosomal area as the LPI locus. Hydrophobicity studies predicted the y^+LAT1 protein to have twelve TM domains with cytoplasmic C- and N-terminal segments, a structure quite similar to that detected in other identified transporters. The gene encoding y^+LAT1 was suggested to be a candidate gene in LPI due to its promising chromosomal location and expression pattern. In addition, the knowledge that the 4F2hc-induced y^+L transport mediates efflux of CAAs in oocytes and 4F2hc is expressed at the basolateral membrane of the renal proximal tubule epithelial cells further supported the hypothesis. Finally, mutations in the *SLC7A7* gene [solute carrier family 7 (amino acid transporter light chain, y^+L system), member 7] encoding y^+LAT1 were confirmed to cause LPI by two separate groups (Borsani *et al.* 1999, Torrents *et al.* 1999) by cDNA identification, mutation analysis, mRNA tissue analysis and transport activity assays. It was discovered that all the Finnish patients are homozygous for an acceptor splice-site mutation (LPI_{Fin} IVS6AS, A-T, -2, c.895-2A>T) in intron six. The mutation results in cryptic splicing 10 base pairs downstream in the following exon, causing a frameshift and formation of a premature stop codon then resulting in a protein that is putatively truncated by one third (Borsani *et al.* 1999, Torrents *et al.* 1999). At the same time, the first Spanish (Borsani *et al.* 1999, Torrents *et al.* 1999) and Italian (Borsani *et al.* 1999) LPI mutations were described.

Currently, 65 mutations of *SLC7A7* have been detected according to the HGMD® Professional 2015.1 database (http://www.hgmd.org). Most of the mutations are missense/nonsense mutations (32), but small deletions (10), large deletions (9), small insertions (7), splice-site mutations (6) and small indels (1) also occur in every coding exon of *SLC7A7*. To date, regulatory mutations or chromosome abnormalities have not been reported as associating with LPI. No genotype-phenotype correlation has been established even in those patients with the same mutation within the same family, thus suggesting that genetic factors other than *SLC7A7* and environmental modifiers may contribute to the phenotype (Sperandeo *et al.* 2008).

The *SLC7A7* gene is 2186 base pairs long with a 1536-base pair open reading frame (GenBank: Y18474), and the y^+LAT1 protein is 511 amino acids long (UniProtKB: Q9UM01). *SLC7A7* consists of eleven exons, but the first two are untranslated (Mykkänen *et al.* 2000, Noguchi *et al.* 2000, Sperandeo *et al.* 2000). Expression of the *SLC7A7* mRNA has been confirmed in the kidney and small intestine, and also to a lesser extent in the peripheral blood leukocytes, erythrocytes, heart, placenta, lung, liver, spleen, pancreas, epididymis, testis, ovary and thyroid (Borsani *et al.* 1999, Torrents *et al.* 1999, Boyd *et al.* 2000, Wagner *et al.* 2001). The regulation of *SLC7A7* expression appears to be mediated by two alternative tissue-specific promoters: the first proximal to exon 2 is active in the kidney and small intestine where the primary defect is manifested and the highest *SLC7A7* expression is detected, and the second proximal to exon 1 was detected to be active in the brain where *SLC7A7* expression is low (Puomila *et al.* 2007).

2.2.4 The functional defect of the y⁺LAT1 protein

Functional analyses of the y⁺L transport activity have been performed on five mutations, including the LPI_{Fin} mutation, using *Xenopus laevis* oocytes (Mykkänen *et al.* 2000). When expressed with 4F2hc, all the mutants failed to induce CAA transport, although for different reasons. The frameshift mutants remained intracellular, sequestring the transporters from the plasma membrane. Instead, the missense mutants reached the plasma membrane, resulting in inactivation of the transport and, thereby, indicating that the affected amino acids are concerved and crucial for the transport. Later, Toivonen and others (Toivonen *et al.* 2002) studied the trafficking of the LPI_{Fin} and three other y⁺LAT1 mutant proteins in the HEK293 (human embryonic kidney 293) and CaCO2 (human colorectal adenocarcinoma) cell lines. Again, the frameshift and nonsense mutant proteins failed to reach the plasma membrane, but the missense mutant protein successfully localised to the plasma membrane (Toivonen *et al.* 2002). It has been shown by our group that the mutant y⁺LAT1 proteins are expressed at a lower than normal level in the HEK293 cells, and that they induce an increased cellular mortality when compared to the wild type protein (Toivonen *et al.* 2013).

Interestingly, when our group studied the dimerisation of the mutant y⁺LAT1 proteins, including LPI_{Fin}, with 4F2hc, it was seen that 4F2hc is able to form heterodimers with those mutant y⁺LAT1 proteins incapable of reaching the plasma membrane (Toivonen *et al.* 2013). This indicates that the cellular quality control recognising defective transporters takes place only after heteromer formation.

2.2.5 Symptoms, signs and clinical findings

2.2.5.1 General clinical picture

Most LPI newborns and infants are symptom-free during breast-feeding but hyperammonaemic episodes arise after the children begin to be fed with high-protein food. Hyperammonaemia may appear as nausea, vomiting, mild diarrhoea and even unconsciousness. At approximately 1 year of age, the patients develop a natural aversion to protein-rich food and spontaneously begin to follow a protein-restricted diet. Protein malnutrition leads to a failure to thrive and growth failure; the patients have a short stature and weak limbs and muscles. (Simell 2001, Näntö-Salonen *et al.* 2012.) Severe osteoporosis, increased incidence of fractures, osteopaenia, decreased collagen synthesis and delayed skeletal maturation are observed in the untreated patients (Parto *et al.* 1993a, Svedström *et al.* 1993, Posey *et al.* 2014). The growth failure in LPI also associates with growth hormone (GH) and IGF1 deficiencies, which are treated with a GH replacement therapy (Esposito *et al.* 2006, Niinikoski *et al.* 2011). It has been shown that long-term GH therapy is beneficial in improving low IGF1 values and height in LPI patients even with normal GH levels (Niinikoski *et al.* 2011). However, in one patient, the GH replacement therapy failed to ameliorate the growth failure (Evelina *et al.* 2015). Earlier, arginine supplementation has been shown to improve the GH response to insulin, indicating that arginine may be sufficient to improve growth retardation (Goto *et al.* 1984). Serum thyroxine (T4), triiodothyronine (T3) and thryroxine-binding globulin (TBG) levels are elevated in some LPI patients (Lamberg *et al.* 1981).

The patients are also at risk of many nutritional deficiencies such as calcium, vitamin D and iron (Tanner *et al.* 2007c); however, their plasma zinc levels become inappropriately high, especially during pregnancy (Tanner *et al.* 2006). Hepatosplenomegaly is consistently observed in LPI (Simell 2001, Näntö-Salonen *et al.* 2012), and expanded and vesicular smooth endoplasmic reticulum, glycogen particles and extensive fatty degeneration (steatosis) in hepatocytes, cirrhosis and cholestasis have also been detected (Kekomäki *et al.* 1968, Simell *et al.* 1975, Rajantie *et al.* 1980d, McManus *et al.* 1996). In pregnancies of LPI patients, intrauterine growth retardation is common. Pregnant women with LPI have also been shown to be at an increased risk of anaemia and toxemia, and deliveries are associated with bleeding complications. However, children of LPI mothers develop generally normally. (Tanner *et al.* 2006.) The patients suffer from carnitine deficiency due to the deficit of its building block, lysine, and protein malnutrition (Takada *et al.* 1987, Tanner *et al.* 2008) since protein-rich food, especially red meat and dairy products, is the most important source for exogenous carnitine (Feller and Rudman 1988). Hypocarnitinemia seems to occur more frequently in women, in the patients with renal disease and those who use ammonia-scavenging medication (Tanner *et al.* 2008). Mental capacity is normal in LPI but moderate retardation may occur due to previous episodes of hyperammonaemia (Simell 2001, Näntö-Salonen *et al.* 2012). The most common signs, symptoms, clinical findings and dysfunctions detected in LPI are described in Table 4.

2.2.5.2 Haematological and immunological abnormalities

Many LPI patients suffer from haemorrhagic diathesis, mild normochromic or hypochromic anaemia, poikilocytosis, anisocytosis, thrombocytopaenia and leukopaenia due to a decreased number of neutrophil granulocytes. The patients have a slightly elevated reticulocyte count, subnormal haemoglobin concentration, highly elevated lactate dehydrogenase (LDH) levels and low haptoglobin levels, indicating intravascular haemolysis. (Rajantie *et al.* 1980c, Yoshida *et al.* 1995, Lukkarinen *et al.* 1999, Tanner *et al.* 2007b.) In addition, the serum ferritin levels are highly elevated, although serum iron concentration is normal and no stainable bone marrow and liver iron storages are detected (Rajantie *et al.* 1980c, Rajantie *et al.* 1981a). Some cases of autoimmune diseases such as rheumatoid arthritis (Parto *et al.* 1993b) and systemic lupus erythematosus (SLE) (Parto *et al.* 1993b, Kamoda *et al.* 1998, Aoki *et al.* 2001) have also been observed in LPI patients. Bone marrow abnormalities of erythroblastophagocytosis (DiRocco *et al.* 1993, Parenti *et al.* 1995, Tanner *et al.* 2007b) and haemophagocytic lymphohistiocytosis (HLH) with macrophage activation syndrome (MAS) (Duval *et al.* 1999) have been described in LPI. A highly increased secretion of soluble IL-2R and soluble CD8, products of activated T cells, and moderately increased levels of IL-1RA, IL-6, IL-10 and TNF-α, indicating monocyte-macrophage activation, have been seen in the serum of non-Finnish LPI patients suffering from HLH (Duval *et al.* 1999). HLH is a syndrome characterised by hepatosplenomegaly, cytopaenia, haemophagocytosis, increased levels of ferritin and LDH, activation of T lymphocytes and macrophages with high secretion levels of proinflammatory cytokines and multiorgan dysfunction (Osugi *et al.* 1997, Canna and Behrens 2012). Its significance in the Finnish patients is yet to be evaluated since they seem to manifest several markers of HLH as a chronic form, not an acute phase disorder (L. Tanner, personal communication).

Table 4. Signs, symptoms, clinical findings and dysfunctions of the Finnish LPI patients.

General signs and symptoms	
	Nausea, vomiting, diarrhoea
	Hepatosplenomegaly
	Protein aversion/malnutrition
	Failure to thrive, growth failure
	Hypotonia
	Osteopaenia, osteoporosis
Biochemical metabolic findings	
	Lysinuria, argininuria, ornithinuria
	Low plasma lysine, arginine and ornithine
	Moderately increased plasma glutamine and glutamic acid
	Slightly elevated plasma and urine serine, alanine, glycine, proline and citrulline
	Hyperammonaemia
	Orotic aciduria
	Proteinuria, albuminuria, haematuria
	Combined hyperlipidemia
	Hypocarnitinemia
Haematological and immunological findings	
	High ferritin
	High LDH
	Low haptoglobin
	High zinc
	Haemorrhagic diathesis, easy bruising
	Anaemia, poikilocytosis, anisocytosis, reticulocytosis
	Erythroblastophagocytosis
	Thrombocytopaenia, leukopaenia
	Impaired B cell functions; low IgG subclasses, poor response to vaccines
	SLE
	Severe viral and bacterial infections
Organ dysfunctions	
	CKD (tubular and glomerular dysfunction)
	Lung disease (PAP, pulmonary haemorrhages, cholesterol granulomas)

LDH, lactate dehydrogenase; SLE, systemic lupus erythematosus; CKD, chronic kidney disease; PAP, pulmonary alveolar proteinosis

The patients also suffer from other immunological complications, with recurrent or chronic infections such as pneumonia, sepsis, bacterial meningitis, sinusitis (Lukkarinen *et al.* 1999), *Herpes simplex* and tuberculosis (Tanner *et al.* 2007b). The LPI patients may develop severe *Varicella* infections which are similar to corresponding infections observed in immunocompromised children (Lukkarinen *et al.* 1998). *Varicella* and other severe microbial infections may derive from deficient B cell functions with low concentrations of IgG1-4 subclasses detected in LPI (Lukkarinen *et al.* 1999). As viral infections are known to induce IgG1 and IgG3 antibodies (Vidarsson *et al.* 2014), exceptionally low IgG3 levels in LPI may impair anti-viral defence and prolong the infection as virus neutralization and antibody-dependent cellular cytotoxicity depend mostly on IgG3. The B cell dysfunctions in LPI lead further to humoral immune deficiency and poor vaccination response with decreased antibodies against commonly used vaccines. (Lukkarinen *et al.* 1999.) In contrast, elevated serum levels of IgG, IgA and IgD have been detected in non-Finnish patients. In addition to this, an impaired function of lymphocytes, an elevated level of the immune complexes, pres-

ence of antinuclear antibodies, a high ratio of $CD4^+$ (helper inducer) to $CD8^+$ (suppressor/cytotoxic) lymphocytes, a low level of phagocytic and cytotoxic leukocyte and natural killer (NK) cell activities and impaired phagocytosis in macrophages have been observed in non-Finnish patients. (Nagata *et al.* 1987, Yoshida *et al.* 1995, Barilli *et al.* 2012.) In the Finnish patients, however, the $CD4^+$ to $CD8^+$ ratio has been shown to be decreased, mostly due to high $CD8^+$ levels (Lukkarinen *et al.* 1999).

2.2.5.3 Chronic kidney disease (CKD) and pulmonary alveolar proteinosis (PAP)

In LPI, involvement of nephropathy and renal insufficiency seems to be almost an inseparable part of the disease manifestation. Over half of the Finnish patients suffer from chronic kidney disease (CKD), the aetiology of which is unknown. The patients develop either tubular or glomerular dysfunction, and they have proteinuria, albuminuria and microscopic or macroscopic haematuria (Tanner *et al.* 2007b, Kärki *et al.* 2015). The levels of serum creatinine and cystatin C (Tanner *et al.* 2007b, Kärki *et al.* 2015) and urine β2-microglobulin (Kärki *et al.* 2015) are inappropriately high, marking a decreased filtration rate of the glomerulus, and tubular damage, respectively. Hypertension, tubular hypophosphatemia, decreased bicarbonate levels and base excess are detected in the patients (Tanner *et al.* 2007b, Kärki *et al.* 2015). In Finland, six LPI patients in total have been treated with peritoneal dialysis and five of them have had a kidney transplant; however, one transplant was subsequently lost (Kärki *et al.* 2015). In addition, three patients have had a rejection of their kidney transplant, and one patient is still waiting for a transplant (M. Kärki, personal communication). Kidney biopsy findings of the Finnish patients have revealed both glomerular and tubular dysfunctions, including glomerular amyloidosis, mild mesangial sclerosis, hyalinous hyperplasia of the arterioles, atrophy of renal tubules and interstitial fibrosis (Tanner *et al.* 2007b). In addition, immune complex-mediated (membranous or mesangial) glomerulonephritis (Parto *et al.* 1994a, McManus *et al.* 1996) and Fanconi syndrome-type tubular dysfunction (Parenti *et al.* 1995, Benninga *et al.* 2007, Riccio and Pisani 2014) have been observed in the patients. The aetiology of the CKD is still poorly understood in LPI; nevertheless, an elevated urine β2-microglobulin level at an early stage in CKD, before any sign of decreased glomerular filtration rate (GFR), suggests that tubular dysfunction may be the first step in the kidney disease and that the β2-microglobulin level should be monitored regularly in LPI patients (Kärki *et al.* 2015).

The patients may also develop an acute respiratory insufficiency, including pulmonary haemorrhages, cholesterol granulomas and pulmonary alveolar proteinosis (PAP) (Parto *et al.* 1993b, Parto *et al.* 1994a). PAP is mainly characterised by the accumulation of proteinaceous material in the alveoli leading to dyspnea and cough (Rosen *et al.* 1958), but morphologic abnormalities including excessive lipid accumulation and giant secondary lysosome formation in macrophages are also detected (Golde *et al.* 1976). In LPI with secondary PAP, a large amount of cholesterol, a large number of cholesterol crystals and dying cells and a low level of surfactant protein D in the airways have been observed (Douda *et al.* 2009). Especially, AM function and morphology are affected possibly due to their ingestion of proteinaceous alveolar fluid, leading to an excessive lipid accumulation that gives rise to foamy macrophages, multilamellar structures and excess iron, indicating alveolar haemorrhage (Parto *et al.* 1994b, Douda *et al.* 2009). PAP has been observed both in Finnish and non-Finnish patients (Parto *et al.* 1993b, Parenti *et al.* 1995, Valimahamed-

Mitha *et al.* 2015), who have benefitted from whole-lung lavage as a treatment for PAP (Ceruti *et al*. 2007, L. Tanner, personal communication). In addition, lung transplantation has been performed on an LPI patient who had experienced an unsatisfactory result from a lavage (Santamaria *et al.* 2004). Recently, at least six Finnish patients have experienced pulmonary insufficiency, as a result of which two have died (L. Tanner, personal communication).

PAP in LPI may be associated with other organ dysfunctions, such as renal insufficiency or hepatic insufficiency with fatty degeneration and cirrhosis, leading to a fatal multiple-organ dysfunction syndrome (DiRocco *et al.* 1993, Parto *et al.* 1994a, McManus *et al.* 1996). Similarly, acute pancreatitis (Parenti *et al.* 1995) and amyloid depositions in the lymph nodes and spleen (Parto *et al.* 1994a) have been observed together with renal and respiratory complications. During the years 2009-2015, five Finnish patients in total have died due to multiorgan failure (L. Tanner, personal communication).

2.2.5.4 Combined hyperlipidemia

Almost all Finnish LPI patients suffer from a combined hyperlipidemia with high serum triacylglycerol (TG) and total and low-density lipoprotein (LDL) cholesterol levels. The high-density lipoprotein (HDL) cholesterol level is subnormal, although within reference range. However, statin medication has markedly improved serum lipid values. The mechanism behind hyperlipidemia is still unknown and is not explained merely by dietary fat consumption. Hyperlipidemia is progressive with age and even more prominent in patients with renal dysfunction. (Tanner *et al.* 2010.)

Vascular endothelial function was studied in a Japanese LPI patient by Kamada and co-workers (Kamada *et al.* 2001), who detected dysfunction in the vascular endothelium and ischemic changes in the coronary arteries. This condition was improved by an arginine supplementation, indicating that a low plasma arginine level decreases the production of the endothelial NO needed for vasodilation.

2.2.6 Pathophysiology

LPI has proven to be a complex and severe disease affecting multiple organs, and it may even lead to life-threatening conditions. Although many parts of the pathophysiology of LPI still remain unclear, the main course of events causing the disease has been unraveled. Figure 2 combines the following summary of the LPI pathophysiology. Defective intestinal and renal (re)absorption of lysine, arginine and ornithine leads to their low plasma and increased urine levels, respectively. It is not exactly clear how arginine and ornithine are depleted from the urea cycle in the liver. Decreased plasma levels of CAAs and, further, their impaired influx in the liver may be one possible explanation (Simell 1975). Additionally, the trapping of CAAs in the hepatocyte cytosol and their decreased importation into the mitochondria has been suggested (Rajantie *et al.* 1983). Nevertheless, a low supply of the urea cycle intermediates ornithine and arginine leads to urea cycle dysfunction and to decreased ammonia detoxification. Subsequently, the ammonia level increases in the blood leading to hyperammonaemia. In addition, a decreased level of the urea cycle end-product, urea, and its abnormally slow increase after dietary nitrogen loads are detected in

the serum (Tanner 2007). The high ammonia level in LPI is also known to increase CP concentration due to its blocked metabolism with ornithine in the urea cycle and its following leakage into the cytoplasm from the mitochondria (Rajantie 1981). An accumulation of CP and aspartate results in an accelerated activation of the pyrimidine pathway and increased synthesis of its intermediate, orotic acid, leading to orotic aciduria (Brosnan and Brosnan 2007). The orotic acid level increases more readily than blood ammonia, which makes orotic aciduria an efficient indicator of hyperammonaemia in LPI (Rajantie 1981). It is known that increased ammonia levels due to high protein intake or starvation lead to increases in urea cycle enzyme activities (Morris 1992, Takiguchi and Mori 1995); however, in LPI, the enzyme activities in the urea cycle have been confirmed to be normal (Kekomäki *et al.* 1967).

To avoid the hyperammonaemia caused by a dietary protein load, patients are on a permanent low-protein diet. Since their protein nutrition is diminished, the patients develop protein energy malnutrition and, likely, a deficiency of essential amino acids. A deficit of proteins and amino acids has a direct impact on the patients' growth development, which manifests itself in a short stature, osteoporosis and weak muscles.

Arginine in particular has been strongly suggested to have a role in the pathophysiology of LPI. The export defect of CAAs has been proposed to lead to the increased level of arginine in the proximal kidney tubule cells, macrophages and other target cells of the mutated transporter. Since the kidney is the most important site for arginine synthesis from citrulline, an exogeneous citrulline supply may even accelerate arginine production. The transport defect, in addition to the poor intestinal supply, results in the depletion of arginine in the circulation, and probably to the increased production of NO from the arginine trapped inside the kidney cells. Excess NO in glomerular mesangial and tubular cells is toxic and, thus, may cause apoptosis and damage in the glomerulus and tubular cells leading into glomerulonephritis and tubulopathy, respectively (Sebastio *et al.* 2011, Ogier de Baulny *et al.* 2012). Interestingly, kidney glomerular mesangial cells are known to have macrophage-like phagocytic properties in glomerulonephritis (Watanabe *et al.* 2001) and thereby their dysfunction in LPI may be especially deleterious. Subsequently, the damage to the glomerulus and tubules may result in decreased glomerular filtration and tubular reabsorption, causing increased metabolite levels in the plasma and urine, such as creatinine and β2-microglobulin, respectively.

In macrophages and lymphocytes, the entrapping of arginine due to the CAA transport defect is believed to lead to an enhanced NO synthesis and result in toxicity and an impaired function of immune cells. The macrophage cell functions weakened by the hampered arginine efflux would lead to increased cytokine secretion and activation of the $CD8^+$ lymphocytes. This condition may result in a system-wide inflammation state and general immune dysfunction characterised by HLH and MAS, autoimmune reactions, and severe viral and bacterial infections. Further, macrophages and lymphocytes would perpetuate inflammatory processes in the target organs, such as the kidney, already suffering from excess CAAs. Another consequence of HLH may be the hepatosplenomegaly consistently detected in LPI patients. (Sebastio *et al.* 2011, Ogier de Baulny *et al.* 2012.)

The increased predisposition to the development of lung diseases in LPI, particularly PAP, may be explained by the increased CAA concentration in the alveolar lining, which leads to disturbance

of the cell membrane and surfactant turnover, and results in decreased clearance of lipoproteinaceous material by macrophages (Ceruti *et al.* 2007). Alternatively, PAP may be caused by defective bone marrow-derived monocytes since erythroblastophagocytosis and abnormal AMs are detected in LPI patients (Parto *et al.* 1994b). Therefore, bone marrow transplantation is considered potentially beneficial in treating PAP in LPI (Santamaria *et al.* 2004). The increased arginine concentration could also intensify NO production either in AMs (Santamaria *et al.* 2004) or in the airway epithelium, where y^+LAT1 is the main basolateral transporter for CAAs (Rotoli *et al.* 2005), and promote chronic inflammation. Anti-GM-CSF (granulocyte-macrophage colony stimulating factor) auto-antibodies do not seem to contribute to secondary PAP, such as in LPI, despite their known role in acquired PAP (Ceruti *et al.* 2007). PAP is often associated with other severe organ dysfunctions in LPI, thus the cause of death in many PAP patients has been suspected to be multiorgan failure.

In addition, low levels of circulating plasma arginine may reduce its intracellular availability in vascular endothelial cells leading to attenuated NO synthesis in those cells (Kamada *et al.* 2001). Since NO is an important vasodilator, its decreased level may have an impact on vascular function and associate with the combined hyperlipidemia detected in LPI.

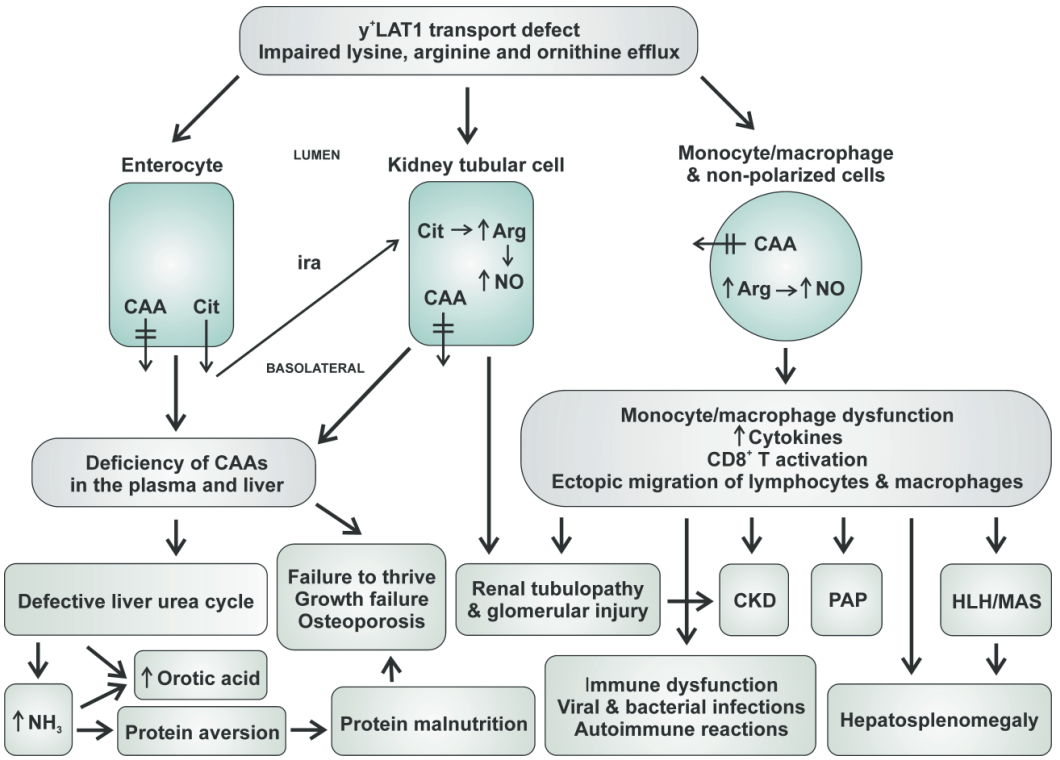

Figure 2. The current knowledge of the model and pathways in the pathophysiology of LPI. CAA, cationic amino acid; Cit, citrulline; ira, intestinal-renal axis; Arg, arginine; NO, nitric oxide; NH_3, ammonia; CKD, chronic kidney disease; PAP, pulmonary alveolar proteinosis; HLH, haemophagocytic lymphohistiocytosis; MAS, macrophage activation syndrome. Modified from Sebastio *et al.* 2011 and Ogier de Baulny *et al.* 2012.

2.2.7 Treatment

There is no cure for LPI, but the aim of the available treatment is to prevent the manifestation of the symptoms. The patients are restricted to a permanent low-protein diet in order to prevent hyperammonaemia after meals. In order to improve protein tolerance and to prevent hyperammonaemia, the patients also receive oral citrulline supplementation (Awrich *et al.* 1975, Rajantie *et al.* 1980d), at doses of 50-100 mg/kg/day, either alone or in a combination with ammonia-scavenging drugs, sodium benzoate or sodium phenylbutyrate. Citrulline, whose transport is not defective in LPI, is a urea cycle intermediate, converted to arginine and, further, to ornithine, replenishing the deficit of arginine and ornithine. Since the patients suffer from a chronic deficiency of lysine and as it is an essential building block of proteins, a low-dose L-lysine hydrochloride supplement at mealtimes is used for improving fasting plasma lysine concentrations, and it is well tolerated by the patients (Tanner *et al.* 2007a) at doses of 20-30 mg/kg/day. The patients' diets are also supplemented with calcium, vitamin D and multivitamin. For the carnitine deficiency, some of the patients take carnitine supplementation (Tanner *et al.* 2008). The patients receive oral phosphate supplementation for tubular hypophosphatemia and one of bicarbonate to maintain acid-base balance (Tanner *et al.* 2007b, Kärki *et al.* 2015). The patients with combined hyperlipidemia are treated with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, either simvastatin or atorvastatin, which have markedly improved serum lipid values (Tanner *et al.* 2010). The elevated blood pressure is medicated with antihypertensive drugs (Tanner *et al.* 2007b).

2.2.8 The LPI mouse model

The only animal model for LPI so far has been introduced by Sperandeo and coworkers (Sperandeo *et al.* 2007), who generated a *Slc7a7* deficient mouse by using high-throughput retroviral gene trapping in embryonic stem cells. In contrast to human LPI, the *Slc7a7*$^{-/-}$ mouse displayed intrauterine growth restriction (IUGR) which led to neonatal lethality. Of all the *Slc7a7*$^{-/-}$ pups, only two survived, appearing at birth clearly smaller and less vital than their wild-type siblings. After weaning, the mice were fed a low-protein diet with citrulline supplementation. They manifested growth retardation compared to the wild-type siblings kept on the same diet as the mutant ones, and after a heavy protein ingestion the animals presented similar metabolic derangements to those observed in human LPI. IUGR was explained by the downregulation of the IGF genes, *Igf1* (3.2-fold) and *Igf2* (1.7-fold), and their binding protein gene, *Igfbp1* (2.6-fold), in the foetal liver. These gene expression level changes were particularly prominent in the smallest foetus. Since arginine has been shown to stimulate IGF1 production and collagen synthesis in osteoblast-like cells, it could be that an arginine deficiency may cause growth failure in LPI mouse foetuses due to impaired bone formation (Chevalley *et al.* 1998). In contrast to this, in human LPI, the maternal serum IGF1 level has been observed to increase normally during pregnancy (Tanner *et al.* 2006).

Gene expression profiling of adult *Slc7a7*$^{-/-}$ mice was carried out using DNA microarray technology, revealing that at least twofold upregulation or downregulation was observed in 488 genes in the intestine and in 521 genes in the liver, most of which were related to transport but also to

metabolism and apoptosis. The highest upregulation (71.1-fold) in the liver was detected in the *Igfbp1* gene, which may cause the growth inhibition and bone developmental delay in the mutant mice through the inhibitory role of its protein product on IGF-stimulated growth and differentiation (Verhaeghe *et al.* 2001). CAA transporter genes *Slc7a2* (4.3-fold) and *Slc7a6* (5.8-fold) were upregulated in the liver, and *Slc7a9* was downregulated (7.0-fold) in the intestine. The upregulation of genes encoding the urea cycle-related enzymes in the liver may indicate the systems' attempt to reduce hyperammonaemia (Takiguchi and Mori 1995).

The mouse model provided an excellent opportunity to examine the defective LPI target tissues which would be impossible in the human patients due to the unavoidable use of invasive techniques. However, the manifestation of the disease in mice compared to humans seems to be more severe since they experience severe growth retardation and neonatal death. Thus, the LPI mouse does not fully correspond to the human disease, and, therefore, it is not suitable for the modelling of LPI in the patients as such. This is in contrast to the mouse models of the two types of cystinuria (Feliubadaló *et al.* 2003, T. Peters *et al.* 2003) which mimic well the phenotypes observed in humans and thus provide suitable models for the study of the pathophysiology and treatment of cystinuria (Font-Llitjós *et al.* 2007, Ercolani *et al.* 2010, Goldfarb 2011, Livrozet *et al.* 2014, Sahota *et al.* 2014).

2.3 Innate immunity

2.3.1 General aspects

The mammalian immune system is composed of an innate and adaptive immunity. Innate immunity is an evolutionarily ancient part of the immune system, and it provides the first line of defence, consisting of physical barriers (e.g. the skin, endothelial cell layer in the respiratory and gastrointestinal tracts), mononuclear phagocytes (e.g. monocytes and macrophages), dendritic cells (DCs), granulocytes (neutrophils, eosinophils and basophils), mast cells, NK cells, platelets and humoral factors, including the complement system, acute phase proteins, inflammasomes and cytokines. The advantage of the innate immune system is its ability to rapidly respond to invading microbes, but its downside is a lack of specificity and memory. (Janeway and Medzhitov 2002, Li *et al.* 2007, Medzhitov 2007, Stokes and Granger 2012.) The characteristics and differences of the two parts of the immune system are described in Table 5.

When pathogens escape the physical barriers of the body and cause an infection, the innate immune system initiates an acute inflammation response which is characterised by four components: inducers, sensors, mediators and effectors. First, the infection is recognised through the binding of the pathogens (inducers) by the receptors (sensors) of the tissue-resident macrophages and mast cells. The activation of the cells results in the secretion of inflammatory mediators, including proinflammatory cytokines and chemokines, histamine and lipid mediators such as prostaglandins and leukotrienes. These mediators induce neutrophil (effectors) migration to the infection site which is accompanied by vasodilation and increased permeability of vascular endothelial cells, thus allowing neutrophil extravasation and simultaneous protein-rich plasma fluid exuding into the tissue. Subsequently, in the tissue, the neutrophils destroy the pathogens by a

respiratory burst releasing reactive oxygen and nitrogen species (ROS and RNS) and proteases from their granules. At this point, the inflammation becomes apparent as heat, swelling, redness, pain and loss of function in the affected tissue. After the pathogen clearance, the inflammatory state is switched into a resolution phase by anti-inflammatory lipoxins and TGF-β suppressing further neutrophil recruitment. Consequently, tissue repair and healing are promoted by phagocytic macrophages ingesting apoptotic neutrophils and debris. However, when an inflammatory response by the innate immune machinery is unable to fully clear infectious agents in a short time, the innate immune cells induce a specific adaptive immune response by recruiting other immune cells, such as T and B lymphocytes, to the site of infection. (Serhan 2007, Medzhitov 2008, Ashley *et al.* 2012.)

Table 5. Properties and differences in innate and adaptive immunities.

	Innate/non-specific	Adaptive/acquired
Anatomical components	Skin, respiratory tract, gastrointestinal tract	Bone marrow, thymus, mucosal-associated lymphoid tissue, lymph node
Cells	Monocytes, macrophages, dendritic cells, natural killer cells, neutrophils, mast cells, eosinophils, basophils	T and B lymphocytes
Proteins	Cytokines, complements, collectins and lysozymes	Immunoglobulins
Receptors	Pattern recognition receptors (encoded in a germline)	Antigen-specific receptors (rearranged during development, somatic recombination)
Distribution of receptors	Non-clonal	Clonal
Targets of recognition	Conserved molecular patterns (LPS, LTA, glycans)	Details of molecular structure (proteins, peptides, carbohydrates)
Specificity	Non-specific activity	Specific (molecular) activity
Onset of response	Immediate (hours)	Delayed (days)
Memory	No	Yes
Self-discrimination	Yes, but indiscriminate tissue damage can occur	Yes, but it is imperfect (autoimmunity)

LPS = lipopolysaccharide, LTA = lipoteichoic acid
Modified from Janeway and Medzhitov 2002, Li *et al.* 2007.

2.3.2 Cytokines

Cytokines are small proteins secreted and responded to by most cells, especially different immune cells such as macrophages, B cells, T cells and mast cells, but also by nonimmune cells like epithelial cells, endothelial cells (Vernier *et al.* 1996) and fibroblasts (Imatani *et al.* 2001). The term 'cytokine' refers to a molecule made by one cell to act on another; in fact, cytokines are actually growth factors and hormones of the immune and hematopoietic systems (Ozaki and Leonard 2002, Dinarello 2007). In addition to communication that cytokines mediate between the neighbouring cells (paracrine signalling), they also act on the cells that release them (autocrine signalling) or on quite distant cells (endocrine signalling), by binding to specific cell surface receptors (Simón and Polan 1994). Different cell types may secrete the same cytokine, which may act on different cell types and exhibit several functions (pleiotropy). Similarly, many different cytokines may share overlapping activities (redundancy). Cytokines can also act synergistically or antagonistically. (Ozaki and Leonard 2002, Zhang and An 2007.)

Cytokines include interleukins, chemokines, interferons, colony stimulating factors and tumour necrosis factors. Some cytokines are primarily lymphocyte growth factors, whereas others polarise the immune response to antigens. The latter can be divided into proinflammatory and anti-inflammatory cytokines based upon whether they induce or suppress inflammation; however, some cytokines have both pro- and anti-inflammatory properties. Proinflammatory cytokines are produced predominantly by activated macrophages, and they include IL-1β, IL-6 and TNF-α. The anti-inflammatory process is mediated by cytokines such as IL-1RA, IL-4, IL-10, IL-11 and IL-13. (Dinarello 2007, Zhang and An 2007.) More than 40 cytokines are defined as interleukins (cytokines made by one leukocyte and acting on others), and their induction and secretion profiles in $CD4^+$ Th cells have been used to divide these cells into distinct subclasses (Akdis *et al.* 2011). Chemokines are a group of cytokines that induce chemotaxis in order to activate the migration of leukocytes to the site of inflammation. Most of the chemokines belong to either the CC or CXC subfamily, including, for example, MIP-1α (monocyte chemoattractant protein 1 alpha, CCL3) or IL-8 (CXCL8), respectively. (Zhang and An 2007.) Two types of interferons have been depicted: type I IFNs (IFN-α and IFN-β) against viral attacks secreted mainly by plasmacytoid DCs (pDCs) (Gilliet *et al.* 2008), and type II IFN (IFN-γ) produced by IL-12- and IL-18-induced NK cells, DCs, macrophages and T cells to mediate a wide range of immune functions (Schroder *et al.* 2004).

After the clearing of an infection, the cytokine genes are shut down and the cytokine release and cell activation ceases. However, immune responses may fail to be turned off, leading to a condition of chronically activated cells resulting in a cytokine storm, which represents systemic inflammation, haemodynamic instability, multiple organ dysfunction and potentially death. The cytokine storm may derive from excessive proinflammatory stimuli including superantigens triggering nonspecific but massive activation of T-cells, toll-like receptor ligands, allergens or proinflammatory cytokines themselves; humoral or cellular anti-inflammatory regulation may also be affected. Several cytokine storm syndromes exist, HLH and MAS being good examples (described in the chapter 2.2.5.2). (Canna and Behrens 2012.)

2.3.3 Macrophages

Macrophages are the key cells in innate immune responses as they are the main pathogen-recognising cells initiating inflammation, mediators of phagocytosis and antigen presenters, and they also initiate specific T cell responses. Macrophages mature from the circulating monocytes migrating into tissues in the steady state to maintain homeostasis or in response to infection. They are found in almost every tissue, such as the AMs in the lungs, Kupffer cells in the liver, Langerhans cells in the skin, osteoclasts in the bone, histiocytes in the connective tissue, microglia in the CNS (Gordon 2003, Mosser and Edwards 2008, Laskin 2009) and macrophages in the adipose tissue (Suganami and Ogawa 2010), where they are specialised in different functions.

Macrophages can be divided into classically activated M1 and alternatively activated M2 macrophages. Resting macrophages are activated into M1 as a response to microbial stimuli and Th1 cytokines IFN-γ or TNF-α (Flesch *et al.* 1995, Skeen *et al.* 1996), and they secrete high

amounts of proinflammatory mediators, such as IL-1, IL-6, IL-12, IL-23, TNF-α, ROS and RNS that are required to kill pathogens (Martin and Dorf 1990, Flesch *et al.* 1995, Forman and Torres 2001, Verreck *et al.* 2004, Martinez and Gordon 2014). Consequently, the IL-12 secretion induces Th1 cell responses (Mahon *et al.* 1996), but IL-1, IL-6 and TGF-β are essential for the differentiation of the Th17 cells (Acosta-Rodriguez *et al.* 2007, Manel *et al.* 2008), and IL-23 is further needed to induce the pathogenic Th17 cells detected in autoimmune reactions (Lee *et al.* 2012). In contrast, M2 polarization is a more complex phenomenon than that of M1, thus the division of the M2 class into the three subclasses, M2a, M2b and M2c, has been suggested (Mantovani *et al.* 2004). IL-4- and IL-13-induced M2a macrophages mediate allergic and antiparasite responses by parasite encapsulation. M2b polarization by immune complex-ligation or LPS is characterised by an increase in IL-10 secretion, phagocytosis and Th2 differentiation. M2cs, induced by IL-10 and TGF-β, are deactivating macrophages as after inflammation, they mediate resolving by deactivating respiratory burst, downregulating the M1 macrophages and pro-inflammatory cytokines, and phagocytosing apoptotic neutrophils. As a generalization, the classical macrophage activation promotes inflammation and Th1 responses, and the Th2-associated M2-directed activation protects the host by preventing excessive inflammation and promoting tissue repair and remodelling. (Gordon 2003, Mantovani *et al.* 2004, Laskin 2009, Biswas and Mantovani 2010, Gordon and Martinez 2010, Martinez and Gordon 2014.) In addition, activation of iNOS for NO synthesis (killing) and arginase for polyamine (cell growth and proliferation) and proline (wound healing) production (Figure 1) are prominent in further dividing macrophages into the M1 and M2 classes, respectively, at least in murine macrophages (Munder *et al.* 1998, Martinez *et al.* 2009). The human peripheral blood monocytes can also be differentiated into the M1 and M2 macrophages with GM-CSF and M-CSF, respectively (Verreck *et al.* 2006).

2.3.4 Toll-like receptors (TLRs)

Macrophages and other innate immune system cells sense microbes through pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) (Akira *et al.* 2006, West *et al.* 2006). PAMPs are conserved and important constituents of microbes, and they are expressed broadly in different pathogens but not in the host cells, which makes possible the discrimination between self and non-self (Akira *et al.* 2006, West *et al.* 2006, Medzhitov 2007, Kawai and Akira 2009). PRRs are expressed constitutively in the given type of host cells. They are germline-encoded, non-clonal and independent of immunologic memory, and they have a broad specificity enabling them to bind into a large number of molecules. (Akira *et al.* 2006, West *et al.* 2006, Medzhitov 2007.) Pathogen recognition at the cell surface or lysosomal and endosomal membranes is mediated by toll-like receptors (TLRs), which were initially identified in *Drosophila melanogaster* (Lemaitre *et al.* 1996, Beutler 2009). In contrast, cytosolic detection for intracellular PAMPs is mediated by retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Akira *et al.* 2006, Kawai and Akira 2009).

In humans, TLRs are expressed in various immune cells, mainly in macrophages, DCs and neutrophils, but also at some level in B and T cells, and even in non-immune cells such as fibroblasts

and epithelial cells. TLRs are membrane glycoproteins comprising extracellular, membranous and intracellular domains. Their extracellular N-terminal domain contains leucine-rich repeat (LRR) motifs in varying numbers and a cytoplasmic C-terminal signalling domain, which is homologous to that of the interleukin 1 receptor (IL-1R). (Akira *et al.* 2006, West *et al.* 2006.) Thus far, 13 TLR family members in mammals have been identified: TLR1-9 are conserved between humans and mice, TLR10 is functional only in humans, and TLR11-13 have been lost in the human genome (Kawai and Akira 2009). TLRs are specialised in recognising bacterial, viral, fungal and protozoan PAMP structures (Akira *et al.* 2006, West *et al.* 2006). In addition, endogenous ligands, released during tissue damage (Miyake 2007), and synthetic ligands (Fasciano and Li 2006) for TLRs have been detected and created, respectively. TLRs can mainly be divided into two groups by their ligand specificity and subcellular location: TLR1, TLR2, TLR4 and TLR6 mainly recognise bacterial membrane lipids and are expressed on the cell surface, but TLR3, TLR7, TLR8 and TLR9 identify microbial nucleic acids and remain in the membranes of intracellular compartments such as endosomes and lysosomes, into which their ligands are internalised. In addition, TLR5 resides in the cell membrane and recognises bacterial flagellin. Concerning the human TLRs, cell surface-localised TLR10 has for long been a mystery. (Akira *et al.* 2006, Kawai and Akira 2010, De Nardo 2015, Jiménez-Dalmaroni *et al.* 2015, Pandey *et al.* 2015.) However, it has been lately demonstrated that TLR10 recognises pathogens of both bacterial (Regan *et al.* 2013) and viral (Lee *et al.* 2014) origin. The ten known TLRs and their associated PAMPs are presented in Table 6.

Table 6. The ten known human TLRs and examples of their associated ligands.

TLR	TLR adapter	TLR location	Ligand examples	References
TLR2/TLR1	MyD88	Cell surface	triacyl lipopeptides, Pam_3CSK_4 (synthetic)	(Rock *et al.* 1998, Takeuchi *et al.* 2002, Jin *et al.* 2007)
TLR2/TLR6	MyD88	Cell surface	diacyl lipopeptides, LTA, Zymosan	(Rock *et al.* 1998, Takeuchi *et al.* 1999, Ozinsky *et al.* 2000, Takeuchi *et al.* 2001)
TLR3	TRIF	Endolysosome	dsRNA (viral), poly(I:C) (synthetic)	(Rock *et al.* 1998, Alexopoulou *et al.* 2001)
TLR4	MyD88/TRIF	Cell surface/ endosome	LPS (bacterial), HSP 60/70 and fibrinogen (endogenous)	(Medzhitov *et al.* 1997, Poltorak *et al.* 1998)
TLR5	MyD88	Cell surface	Flagellins (bacterial)	(Rock *et al.* 1998, Hayashi *et al.* 2001)
TLR7	MyD88	Endolysosome	ssRNA (viral), IAQ (synthetic)	(Du *et al.* 2000, Hemmi *et al.* 2002, Diebold *et al.* 2004, Lund *et al.* 2004)
TLR8	MyD88	Endolysosome	ssRNA (viral), IAQ (synthetic)	(Du *et al.* 2000, Heil *et al.* 2004)
TLR9	MyD88	Endolysosome	CpG DNA (bacterial/viral)	(Du *et al.* 2000, Hemmi *et al.* 2000, Lund *et al.* 2003, Krug *et al.* 2004)
TLR10/ TLR2?	MyD88?	Cell surface	bacterial and viral	(Chuang and Ulevitch 2001, Guan *et al.* 2010, Regan *et al.* 2013, Lee *et al.* 2014)

Pam_3CSK_4, Pam_3(tripalmitoylated)CysSerLys$_4$; LTA, lipoteichoic acid; dsRNA, double-stranded RNA; poly(I:C), polyinosinic-polycytidylic acid; LPS, lipopolysaccharide; HSP, heat shock protein; ssRNA, single-stranded RNA; IAQ, imidazoquinolines; CpG, cytidine-phosphate-guanosine

The confronting and binding of PAMPs by TLRs results in TLR dimerisation and conformational changes needed for the adaptor binding to induce the appropriate signalling cascades (Jin *et al.* 2007, Jin and Lee 2008, Liu *et al.* 2008, Yoon *et al.* 2012). Signalling through TLRs is either MyD88 (myeloid differentiation factor 88)-dependent (Muzio *et al.* 1997, Wesche *et al.* 1997) or MyD88-independent/TRIF [Toll/IL-1R (TIR) domain-containing adapter inducing IFN-β]-dependent (Kawai *et al.* 1999, Kawai *et al.* 2001, Yamamoto *et al.* 2002, Oshiumi *et al.* 2003), based on the signalling routes and immune responses mediated by TLRs. However, heterodimers of different TLRs, such as TLR4/TLR5, may engage both adaptors (MyD88 or TRIF) for additional or varied responses (West *et al.* 2006). Subsequently, TLR pathway activation by PAMP binding leads to immune responses involving the production and secretion of inflammatory cytokines and interferons in order to initiate pathogen clearance. TLR2/1, TLR4 and TLR9 signalling routes, which were studied in this thesis, will be discussed in more detail below, and are described in a simplified manner in Figure 3.

TLR2/1 signalling

TLR2, which is expressed in, for example, macrophages and DCs, recognises a wide range of microbial products due to its ability to form heterodimers with either TLR1 or TLR6. The TLR2/1 dimer binds bacterial triacyl lipopeptides (LP), including those from mycobacteria and Gram-negative bacteria such as meningococci (Takeuchi *et al.* 2002), whereas the TLR2/6 complex recognises diacylated lipoproteins and peptidoglycans from mycoplasma and Gram-positive bacteria (Takeuchi *et al.* 2001). (Akira *et al.* 2006, West *et al.* 2006, Kawai and Akira 2010.) One of the PAMP molecules used to efficiently activate the TLR2/1 pathway is Pam_3CSK_4, which is a synthetic triacylated LP mimicking the acylated amino terminus of bacterial LPs (Jin *et al.* 2007). Stimulation with LPs causes the TLR2/1 heterodimer to associate with co-receptor CD14 (Triantafilou *et al.* 2006). An activation of TLR2/1 leads to the recruitment of MyD88 to the receptor complex with the aid of a sorting adaptor, TIRAP (TIR domain-containing adaptor protein)/Mal (MyD88-adaptor-like) (Fitzgerald *et al.* 2001, Horng *et al.* 2001), and to the subsequent interaction of MyD88 with the IRAK proteins (IL-1 receptor-associated kinase) (Cao *et al.* 1996a, Muzio *et al.* 1997, Wesche *et al.* 1997) (Kawai and Akira 2010). The activation of the IRAK proteins results in their association with TRAF6 (tumour necrosis factor receptor-associated factor-6) (Cao *et al.* 1996b) and the induction of TAK1 (TGF-β-activated kinase 1) (Sato *et al.* 2005) (Kawai and Akira 2010). TAK1 further activates mitogen-activated protein kinases (MAPKs), consequently leading to the induction of transcription factors such as IRF5 (Takaoka *et al.* 2005), AP-1 (Karin *et al.* 1997, O'Neill and Greene 1998) and NF-κB (Cao *et al.* 1996b, Wesche *et al.* 1997, Hayden and Ghosh 2004). This activation results in the expression of genes encoding proinflammatory cytokines including IL-1β, IL-6, IL-12p40 and TNF-α (Kawai and Akira 2010).

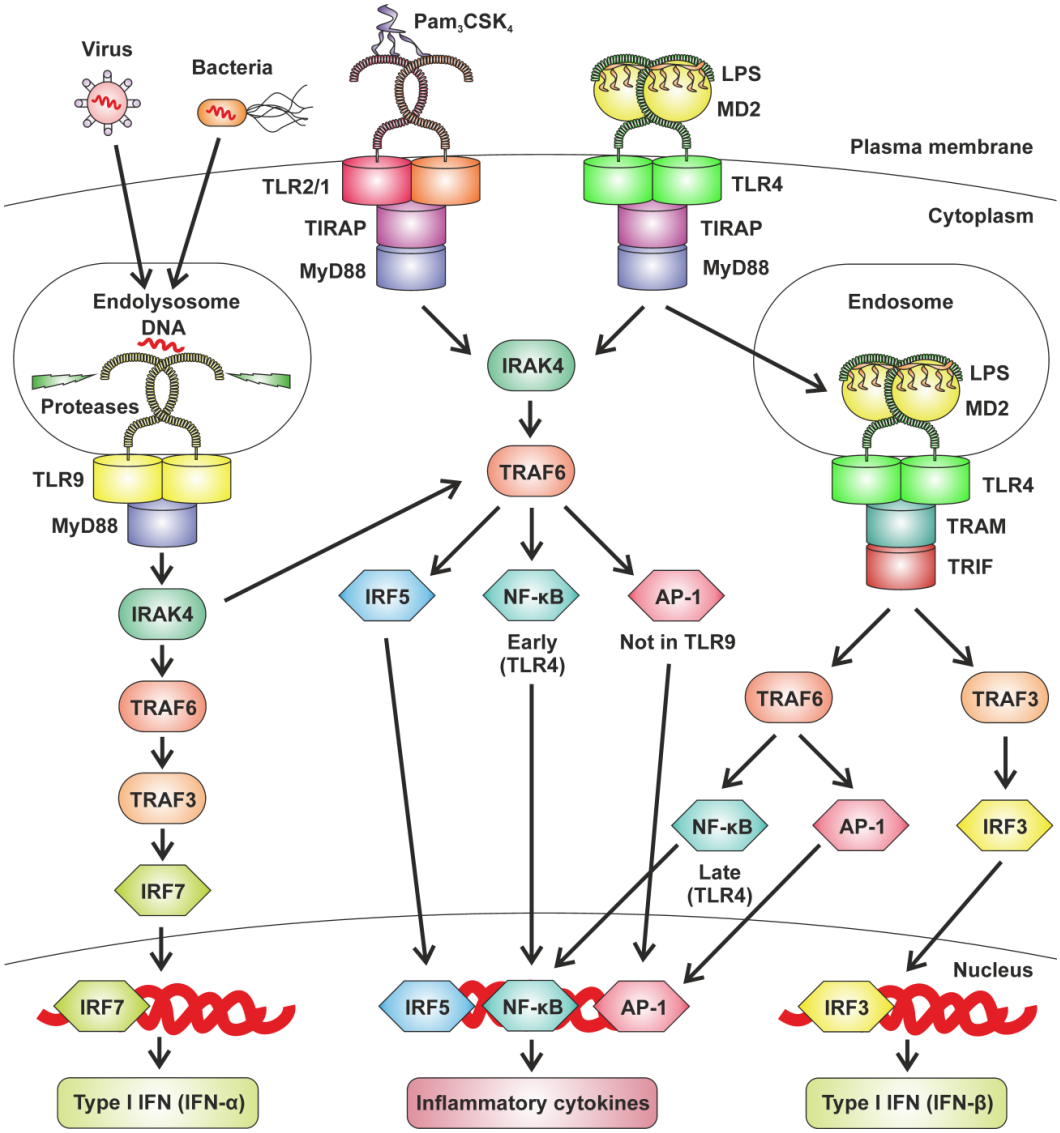

Figure 3. TRL2/1, TLR4 and TLR9 signalling pathways. Modified from Kawai and Akira 2010, Jiménez-Dalmaroni *et al.* 2016, Pandey *et al.* 2015.

TLR4 signalling

A mammalian homologue of the *Drosophila* Toll receptor, subsequently named TLR4, was the first mammalian TLR to be identified (Medzhitov *et al.* 1997). TLR4 recognises LPS of the outer membrane of Gram-negative bacteria (Poltorak *et al.* 1998) with the aid of additional membrane-linked and soluble molecules. First, LPS aggregates are extracted from bacteria by a LPS binding protein (LBP) (Schumann *et al.* 1990, Park and Lee 2013) that transports LPS to the co-receptor CD14 (Wright *et al.* 1990, Tobias *et al.* 1995, Park and Lee 2013) found either in a soluble form (Frey *et al.* 1992) or linked to the cell surface (Lee *et al.* 1993). Second, CD14 splits LPS aggregates into monomers and presents them to the TLR4–MD-2 (myeloid differentiation protein 2) complex (Shimazu *et al.* 1999, Schromm *et al.* 2001, Park *et al.* 2009, Park and Lee 2013). The binding of LPS to the receptor complex is followed by the recruitment of MyD88 (MyD88-dependent pathway) into the TLR4 complex with the aid of TIRAP. This results in an early-phase induction of IRF5, MAPKs/AP-1 and NF-κB and, further, in the secretion of inflammatory cytokines. (Kawai and Akira 2010.) Subsequently, the TLR4-MD-2-LPS complex is internalised into the endosome where it triggers a second, TRIF-dependent, signalling cascade (Kagan *et al.* 2008, Tanimura *et al.* 2008). First, TRIF is recruited into the TLR4 complex with the assistance of the TRAM (TRIF-related adapter molecule) protein (Fitzgerald *et al.* 2003b, Oshiumi *et al.* 2003, Yamamoto *et al.* 2003a, Yamamoto *et al.* 2003b). The signalling goes either through TRAF6 or TRADD/Pellino/1-RIP1 to TAK1, MAPKs/AP-1 and late-phase NF-κB activation for the induction of inflammatory cytokine production (Kawai and Akira 2010). In addition, the TRIF-dependent pathway leads to the activation of TRAF3, instead of TRAF6, in order to activate TBK1 (TANK-binding kinase 1) and, subsequently, the IRF3 (interferon regulatory factor 3) transcription factor to induce the secretion of IFN-β (Fitzgerald *et al.* 2003a, Sato *et al.* 2003, Gohda *et al.* 2004, Hoebe and Beutler 2006, Häcker *et al.* 2006). However, for the production of inflammatory cytokines, the activation of both MyD88- and TRIF-dependent pathways is required in order to trigger the early- and late-phase inductions of NF-κB (Kawai and Akira 2010).

TLR9 signalling

TLR9 recognises bacterial and viral DNA that contains unmethylated 2'-deoxy CpG (cytidine-phosphate-guanosine) DNA motifs (Takeshita *et al.* 2001, West *et al.* 2006). The mammalian CpG motifs are highly methylated and they occur at a low frequency, thus preventing confusion of self and non-self (West *et al.* 2006). Synthetic CpG oligodeoxynucleotides (ODNs) are also strong TLR9 pathway inducers in pDCs, macrophages and B cells (Kawai and Akira 2010). After ligand internalisation, TLR9 translocates from the endoplasmic reticulum into the endosome where it is proteolytically cleaved before binding into DNA motifs (Ahmad-Nejad *et al.* 2002, Latz *et al.* 2004, Nishiya and DeFranco 2004). The TLR9 signalling mainly follows the MyD88 pathway; however, the route is divided based upon the pathogen origin of the ligand. By activating TRAF6, bacterial DNA induces the activation of the transcription factors NF-κB and IRF5 and, consequently, the production of inflammatory cytokines. (Akira *et al.* 2006, West *et al.* 2006, Kawai and Akira 2010.) The viral DNA-induced TLR9 response requires association with TRAF3 in addition to TRAF6, in order to activate the IRF7 transcription factor and induce the production of type I IFNs, most importantly IFN-α (Kawai *et al.* 2004, Hoebe and Beutler 2006, Kawai and Akira 2010). Secreted type I IFNs

enhance, by a positive feedback loop, the expression of the *IRF7* gene and further the induction of type I IFNs (Honda *et al.* 2006). pDCs are the main viral recognising cells and the main producers of IFN-α through TLR9 signalling (Lund *et al.* 2003, Akira *et al.* 2006, Gilliet *et al.* 2008), and IRF7 has been demonstrated to be expressed constitutively in these cells (Izaguirre *et al.* 2003, Kerkmann *et al.* 2003). However, constitutive IRF7 expression and viral-induced IFN-α production are also detected in monocytes, although to a lesser degree than in pDCs (Izaguirre *et al.* 2003).

2.4 'Omics' technologies in molecular studies

The term 'omics' describes technologies aiming at universal identification of variations in DNA (genomics), RNA (transcriptomics), proteins (proteomics), metabolites (metabolomics) and lipids (lipidomics) in a specific biological sample (Horgan and Kenny 2011). The 'omics' technologies have a broad range of applications from examining gene and protein expression at the single-cell level to discovering new disease-causing mutations in specific tissues, and further to the monitoring of systemic changes in the metabolome in different biofluids. Using these technologies in order to integrate transcriptomic, proteomic and metabolic information is the ultimate goal of systems biology, which aims at comprehending the complex network of all cellular processes and pathways (Chuang *et al.* 2010).

2.4.1 Transcriptomics – The RNA microarray versus RNA sequencing

Transcriptomics is a study of the complete set of RNA transcripts (transcriptome) produced by the whole genome in cells or tissues of interest using high-throughput methods, such as microarrays and RNA sequencing. A comparison of transcriptomes enables the identification of gene expression level changes either in a single cell (Stegle *et al.* 2015) or in distinct cell populations in various disease stages (e.g. cancer, immune diseases) (Rhodes and Chinnaiyan 2005) or in response to different treatments (e.g. medicines, cytokines) (Himes *et al.* 2014). Although each individual has the same genome in every cell, the gene expression varies in different cell types and is influenced by daily (Whitney *et al.* 2003) and seasonal (De Boever *et al.* 2014) fluctuations, as well as developmental stages in different tissues (Francesconi and Lehner 2014). In addition, age and gender affect the differences in gene expression patterns (Whitney *et al.* 2003). Gene expression level changes may reveal novel biomarker genes whose over- or underexpression could implicate the onset of a condition or the severity of a disease stage (Butte 2002). The whole-blood cells provide easily accessible and cheap sample material for gene expression studies. The disadvantage of using whole-blood is that information from individual blood cell types cannot be obtained. However, it has been demonstrated that the whole-blood peripheral cells express approximately 80% of the genes in the human genome, and that the whole-blood cells and at least nine other human tissues share 80% of their gene expression (Liew *et al.* 2006). Therefore, as the transcriptome of the whole-blood cells provides a near-comprehensive expression profile of an organism, it can be used to detect biomarkers of several human traits and diseases, especially when specific tissue samples are impossible to obtain. However, analysis of specific tissue samples are still needed in many cases as the whole-blood gene expression may not correlate with the levels of circulating

molecules originated from other tissues (Haring *et al.* 2015). In addition to biomarkers, transcriptomic analyses also provide tools for drug discovery by finding new drug targets (Butte 2002).

The traditional way of detecting gene expression is by using DNA microarrays, also known as DNA chips, which allow the measurement of the expression of a large number of genes of specific interest (e.g. immune chips) or the whole genome. Two basic types of microarrays are available for gene expression profiling: the cDNA microarray and oligonucleotide microarray. In the cDNA microarray, PCR amplicons are first printed or spotted at specified sites on glass slides as probes. Next, the target RNA from two samples is converted into single-stranded cDNA in the presence of nucleotides labelled with a fluorescent dye, either cyanine 3 or cyanine 5. After mixing, sample cDNAs are hybridised onto the array, resulting in competitive binding of differentially labelled cDNAs to the probes. Fluorescence scanning of the array produces relative signal intensities and mRNA ratios between the two studied samples. In contrast, oligonucleotide microarray technology (Affymetrix GeneChip as an example) exploits unique database-derived short oligonucleotides that are spotted or *in situ*-synthesised onto arrays. Sample RNAs are converted into double-stranded cDNA and, subsequently, *in vitro*-transcribed to cRNA into which biotin-labelled nucleotides are incorporated. Then, each cRNA sample is hybridised onto a separate probe array, and the target binding is detected by staining with a fluorescent dye coupled to streptavidin. The signal intensities detected from the arrays are used to calculate relative mRNA amounts and, further, to compare mRNA levels between arrays with different samples. (Schulze and Downward 2001, Miller and Tang 2009.)

Different platforms for the transcriptomics studies have been harnessed by different companies. In addition to the solid surfaces, such as quartz wafers used in Affymetrix GeneChips and glass slides by Agilent, Illumina Sentrix BeadChip provides arrays covered by beads with ~ 700 000 probes attached to each bead. With GeneChip and BeadChips it is only possible to measure one sample per array because of their limitation with one label, while the Agilent platform allows two-colour, cyanine 3 and cyanine 5, hybridisations. (Miller and Tang 2009, Slonim and Yanai 2009.) Recently, it has become possible to measure hundreds of thousands of coding and non-coding transcript variants simultaneously, produced by alternative splicing and even separate exons (http://www.affymetrix.com).

The disadvantage of the microarrays is that only transcripts with *a priori* information can be studied. A relatively new technique based on the next-generation deep-sequencing is RNA sequencing (RNA-Seq), which provides a comprehensive view of a transcriptome by directly sequencing all RNA molecules and, therefore, allowing the detection of totally new, previously unidentified transcripts. The measure of expression in RNA-Seq is the number (depth or coverage) of times a nucleotide is read and mapped to a particular reference transcript, or assembled *de novo* without a reference sequence. RNA-Seq is a sensitive method, detecting transcripts expressed at a low level and showing only little background noise, whereas hybridisation-based approaches are susceptible to a high rate of false-positives when identifying transcripts with low expression levels. (Wang *et al.* 2009, Flintoft 2010, Malone and Oliver 2011, Sims *et al.* 2014, S. Zhao *et al.* 2014.) There is a wide range of different techniques available in RNA-Seq as different companies offer their own specific methods. After library preparation, the actual sequencing is performed using 'sequencing by synthesis' technology; for example, by pyrosequencing using the 454 GS FLX Titanium system (Roche), bridge amplification using the HiSeq system (Illumina) and detecting a change in the pH

using the Ion personal genome machine (Ion Torrent), or sequencing by oligo ligation detection using the SOLiD system (Applied Biosystems/Thermo Fisher Scientific). In addition, the third generation sequencing techniques based on real-time detection have been developed. These include a direct observation of an enzymatic reaction using the single-molecule real-time method (Pacific Bioscience) and the disruption in an electric current through a biopore channel using the Nanopore system (Oxford Nanopore). (Liu *et al.* 2012.)

In addition to traditional gene expression analysis, RNA-Seq enables the identification of alternatively spliced transcripts, allele specific expression, post-transcriptional modifications, gene fusions, new mutations and SNPs (single nucleotide polymorphisms) and can present new information on the positions of promoters, exons, and 5' and 3' ends (Wang *et al.* 2009, Malone and Oliver 2011, Sims *et al.* 2014). One of the growing fields in RNA research is the study of regulatory non-coding RNAs (ncRNA), such as small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), small interfering RNAs (siRNAs), microRNAs (miRNAs), long interspersed ncRNAs, promoter-associated RNAs, terminator-associated RNAs, transcription start site-associated RNAs, transcription-initiation RNAs and many more (Jacquier 2009). The direct target approach with RNA-Seq also enables the usage of new model organisms whose genomes have not yet been sequenced (Wang *et al.* 2009, Malone and Oliver 2011). By using RNA-Seq, the study of 'personalised medicine', especially based on pharmacogenomics, may revolutionise the study of medicine and pharmaceutics in the future and bring vast improvements in the treatment of patients with severe conditions. However, the downside of RNA-Seq is that it requires extensive expertise in computational methods; therefore, the data analysis and storage may present challenges in applying the technology in a clinical setting (S. Zhao *et al.* 2014).

2.4.2 Metabolomics and lipidomics – the liquid and gas chromatography (LC and GC) and mass spectrometry (MS) techniques

Metabolomics is a study of the whole metabolite profile (metabolome) in a specific biological sample. The metabolome represents a collection of thousands or even tens of thousands of low-molecular-weight molecules (e.g amino acids, nucleotides, sugars, organic acids, polyphenols, alkaloids and vitamins) produced during cellular processes in a single cell, tissue, organ or organism (Kaddurah-Daouk *et al.* 2008, Zhang *et al.* 2012). Although lipidomics can be classified under the field of metabolomics, it is now thought to be a distinct discipline due to the functional specificity of lipids when compared to other metabolites. The most commonly used samples in metabolomics and lipidomics studies are derived from the plasma and urine, which can be reached non-invasively and, thus, are easy to obtain. A number of other fluids such as cerebrospinal fluid, bile, seminal fluid, amniotic fluid, synovial fluid, gut aspirate and saliva have also been studied and, in addition, intact tissue samples can be used for biomarker detection (Gowda *et al.* 2008). Metabolomics is a historically old "technology" as human urine was analysed as early as 6 000 years ago (Armstrong 2007), and ancient Chinese doctors used ants for the detection of diabetes from the urine with high glucose levels (van der Greef and Smilde 2005). In the Middle Ages, colours and sedimentation of the urine were categorised with urine charts in order to recognise medical conditions of metabolic origin (Armstrong 2007). However, the term 'metabolic profile' was not introduced until 1971,

when Horning and others (Horning and Horning 1971, Gates and Sweeley 1978) demonstrated compounds in the human urine by the gas chromatography-mass spectrometry (GC-MS) technique. In 2007, the Human Metabolome Project (HMP), aiming to identify and quantify all detectable metabolites (>1 μM) in different human body fluids, was completed and The Human Metabolome Database (HMDB) containing the associated data was established. The database has since been regularly updated and transformed into a more public-deposition model. (Wishart *et al.* 2007.)

A wide variety of methods has been developed to separate, identify, characterise and quantify metabolites, but no single analytical platform is capable of capturing all metabolic information in a sample (Zhang *et al.* 2012). However, when combining platforms, it is possible to gain more metabolic data than using only one technique. Of these different platform combinations, GC-MS, liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) are commonly used. The first part of the device is for separating isolated metabolites, mainly in the gas or liquid phase by either GC or LC, respectively, or using electric field by CE. However, metabolites that are not volatile must be derivatised chemically before entering GC (M. Li *et al.* 2014). Adding a GC or LC part to the MS instrument enables the identification and quantification of metabolites by both compound retention time in the chromatography column and molecular mass spectrum derived from MS. (Rochfort 2005, Kaddurah-Daouk *et al.* 2008, Patti *et al.* 2012, Maher *et al.* 2015, Redman *et al.* 2015.) MS is both a sensitive and specific technique, and also allows the detection of the presence of molecules that are yet unidentified. When entering the MS part of the device, compounds are first ionised in the ionisation source of MS, after which they travel through the mass analyser where they separate and finally arrive at different parts of the detector according to their mass/charge (m/z) ratio. After the ions have come into contact with the detector, useable signals are generated, recorded and displayed as a mass spectrum by a computer showing their relative abundance based on the m/z ratio of the ions. (Ho *et al.* 2003, Rochfort 2005, Kaddurah-Daouk *et al.* 2008, Patti *et al.* 2012, Maher *et al.* 2015.)

Different ionisation techniques, including matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI), are used depending on the sample phase. In addition, various mass analysis and separation principles may be utilised in MS, such as quadrupoles (Q), which separate ions based on their stability within a quadrupolar field, the quadrupole ion trap (QIT), which utilises a three-dimensional field to trap ions, orbitrap, which exploits electrodes that trap ions in an orbital motion, time-of-flight (TOF), which separates ions in time and the recently invented distance-of-flight (DOF), that sorts ions in space. MS can also be exploited as a tandem or hybrid in the form of, for example, triple quadrupoles (QqQ) or quadrupole time-of-flight (Q-TOF) MS. (Hill *et al.* 1990, Maher *et al.* 2015.) A tandem MS is characterised by multiple rounds of mass analysis and fragmentation of ionised molecules whose m/z ratio is measured and then used for structural identification (Patti *et al.* 2012).

Metabolomics can either be targeted or non-targeted profiling, depending upon the study question. In targeted metabolomics, a list of specified metabolites focusing on a few related pathways of interest, such as drug metabolism or enzyme activities, is measured. Standard compounds for the metabolites of interest are first used to set up selected and optimised reaction monitoring methods and to generate standard curves against which the metabolites in the study samples are quantified. The platform often used in the targeted metabolite profiling is QqQ-MS, owing to its

sensitivity and specificity. Untargeted metabolomics, for which QTOF-MS is commonly used, is global profiling as it aims at simultaneously measuring as many metabolites as possible. In non-targeted metabolomics, bioinformatics software is used for performing the retention time alignment and identifying differing peaks between samples. Metabolite identification is carried out by searching for the m/z values of peaks of interest in metabolite databases and comparing the retention time and MS/MS data of a standard compound to that of a research sample. (Patti *et al.* 2012.) Widely used public metabolite spectral databases for the peak identification are HMDB (Wishart *et al.* 2007), Golm database (Kopka *et al.* 2005) and METLIN database (Smith *et al.* 2005) but, in addition, in-house libraries may be generated. Many metabolites may however remain unidentified, in which case a *de novo* characterisation with traditional methods is required (Patti *et al.* 2012). Further information, such as the biochemical pathways in which the metabolites participate, can be determined by using the HMDB (http://www.hmdb.ca/), KEGG (http://www.genome.jp/kegg/) and PubChem (http://pubchem.ncbi.nlm.nih.gov/) database searches.

Lipidomics aims at the full analysis of lipid species and their cellular pathways and networks through quantifying complete lipid profiles (Brügger 2014, M. Li *et al.* 2014). The estimated number of different lipid species varies from 10 000 to 100 000 (Wenk 2010), but lipids are mainly divided into the following eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (Sud *et al.* 2007). In cellular systems, lipid metabolism is crucial in energy homeostasis, membrane structure, dynamics and signalling (Wenk 2010, Köfeler *et al.* 2012). Lipidomics is still a relatively new 'omics' discipline, although it has been growing rapidly during the last few years (Wenk 2010, Brügger 2014). In lipidomics, similar methods and instruments are utilised as in metabolomics; however, lipids must be first extracted from the samples in order to remove small molecules that could disturb the analysis. This is commonly performed using the liquid-liquid extraction method exploiting chloroform/methanol as a solvent. (Brügger 2014, M. Li *et al.* 2014.) The largest amount of information on the lipidome is gained by the use of MS in connection with, for example, CE, GC, high-performance LC, or its improved version, ultrahigh-performance LC (UHPLC). ESI and MALDI are used particularly in the ionisation of lipids before their entering MS, but many new methods have been developed. (M. Li *et al.* 2014.) The global lipidomics data analysis follows the procedure used in the metabolomics data analysis (Wenk 2010, Köfeler *et al.* 2012). Lipid identification can be carried out and structures detected with the help of in-house libraries or databases such as the LIPID MAPS structure database (LMSD) (Sud *et al.* 2007).

Probably the most significant application of metabolomics and lipidomics is quantifying differences between a disease state and the normal condition, in order to understand disease mechanisms, and to identify new diagnostic markers (Kaddurah-Daouk *et al.* 2008). To date, metabolic and lipid fingerprints have been reported for several diseases, such as Alzheimer's disease, Huntingtons's disease, Parkinson's disease, schizophrenia, type 2 diabetes, Crohn's disease, cardiovascular diseases, hypertension, hyperlipidemia and different cancers (Kaddurah-Daouk *et al.* 2008, M. Li *et al.* 2014). In addition to identifying already known molecules, metabolomics and lipidomics also aim to discover totally new metabolites and lipids, and to recognise the biochemical pathways that they have a role in.

3 AIMS OF THE STUDY

The aetiology of severe LPI complications, especially those of immunological, renal and pulmonary nature, or that of combined hyperlipidemia, is not solved, and no correlation between the genotype and phenotype has yet been established in LPI. Therefore, the objective of this study was to examine 1) molecular factors contributing to the various symptoms, 2) innate immune responses in macrophages exposed to pathogens, and 3) the systemic metabolic and lipid profiles of the Finnish LPI patients by using basic and high-throughput molecular techniques in order to provide new hypotheses and models for the pathophysiology of LPI.

The specific aims of the present study were:

1. To unveil the effect of the LPI_{Fin}-mutation on the genome-wide trancriptome pattern in whole-blood cells (I)

2. To analyse the amino acid transporter gene expression level changes in whole-blood cells, peripheral blood mononuclear cells (PBMCs), monocyte-derived macrophages (MDMs) and reticulocytes in LPI (I-II and unpublished results)

3. To examine the immune- and red cell-related gene expression patterns in whole-blood cells, PBMCs and reticulocytes in LPI (I and unpublished results)

4. To explore the innate immune responses and TLR signalling in pathogen-stimulated MDMs in LPI (II)

5. To define systemic amino acid, metabolite and lipid profiles in LPI, particularly in the patients with chronic kidney disease (III)

4 SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Patients and controls (I-III and unpublished results)

A total of 36 Finnish LPI patients were included in this study. The controls were healthy Finnish volunteer children and adults, age- and sex-matched to the patients. However, a variable number of subjects were included in each sub-study. The sexes and ages of the patients and controls from whom the samples were collected are described in Table 7, and the detailed descriptions of the individual patients are presented in Table 8. All the patients' samples were collected during their clinical follow-up visits at the Department of Pediatrics in Turku University Hospital and University of Turku. The samples of the patients and controls were collected between 9 am and noon and the samples were non-fasting. All samples were stored at -80 °C for a period from under a year to 8 years at maximum. Informed consent was obtained from all the patients or their parents. The investigation corresponds to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of the Hospital District of Southwest Finland.

Table 7. Sex, age and description of samples of the LPI patients and controls included in this study.

	Whole blood		MDMs[a]	PBMCs[b]/ Reticulocytes
	Whole-blood cell RNA	Plasma cytokines/NO/ metabolites	RNA medium cytokines/NO	RNA
Patients (n)[c]	36	26	23	11
Sex				
female (n)	24	17	15	7
male (n)	12	9	8	4
Age at study (years)				
mean	31.0	38.0	37.5	37.4
range	0.9-61.0	12.0-65.0	12.0-65.0	14.0-57.0
Controls (n)	10	19[d]	15[d]	11
Sex				
female (n)	5	12	10	7
male (n)	5	7	5	4
Age at study (years)				
mean	30.0	40.1	39.3	40.0
range	9.0-48.0	12.0-65.0	12.0-65.0	23.0-56.0

[a] Monocyte-derived macrophages
[b] Peripheral blood mononuclear cells
[c] Number
[d] The same controls were chosen for the peripheral whole-blood plasma and MDM studies with the exception of four controls from whom MDM samples were not available.

Table 8. The description of the individual patients included in each substudy.

Patient	Sex	Age at diagnosis (y)[a]	Whole-blood cells		Plasma/MDMs[b]		PBMCs[c]/Reticulocytes	
			Patient ID in I	Age at study (y)	Patient ID in III	Age at study (y)	Patient ID	Age at study (y)
1	F	1.0	**P1**	6.9	P18	12.5	P1	14.6
2	F	4.9	**P2**	10.7			P2	18.4
3	F	3.5	**P3***	14.8	P9*	19.7		
4	F	3.0	**P4**	18.8	P11	23.7		
5	M	20.6	**P5**	20.6	P2	25.7		
6	M	5.4	**P6***	31.8	P12*	37.1		
7	F	3.0	**P7**	36.4	P4	41.4	P7	44.4
8	F	0.2	**P8**	37.1				
9	M	7.0	**P9***	39.3			P9*	46.6
10	M	0.3	**P10**	39.6			P10	47.0
11	M	2.2	**P11**	41.6			P11	48.8
12	F	12.0	**P12**	44.8	P19	49.7		
13	M	30.6	**P13***	46.6	P22*	51.6	P13*	53.5
14	F	1.3	P14	7.2	P20	12.1	P14	14.1
15	F	1.8	P15	8.1				
16	F	1.2	P16*	8.3				
17	F	1.4	P17*	11.6	P23*	17.3		
18	F	3.6	P18*	21.5				
19	F	8.7	P19*	23.8	P8	27.7	P19	30.2
20	M	0.2	P20	26.7	P26	32.5		
21	M	29.6	P21*	30.0	P16	34.5		
22	F	5.0	P22*	29.5	P24*	35.2		
23	F	0.8	P23*	30.1	P5	34.6	P23	37.6
24	F	2.8	P24	31.3	P25*	37.1		
25	F	1.5	P25*	34.4	(P14)*	39.4		
26	M	1.5	P26	35.9	P21	41.0		
27	M	0.2	P27*	37.8	(P13)*	40.9		
28	F	3.0	P28*	38.6	(P15)*	43.7		
29	F	10	P29*	40.0	P6*	45.0		
30	F	0.2	P30*	48.2	P3*	53.2		
31	F	12.0	P31	48.5	P1*	53.6	P31*	56.6
32	M	10.0	P32	49.2	P10	54.0		
33	F	14.0	P33*	51.3				
34	F	15.0	P34*	52.9	P17*	58.4		
35	M	25.0	P35	60.9	P7*	65.3		
36	F	0.25	P36 (*IFI27*)	0.9				

The 13 patients in bold were included in the microarray study. From the three patients in parentheses, only the plasma samples were obtained. The patients with asterisks (*) suffer from chronic kidney disease.

[a] years

[b] monocyte-derived macrophages

[c] peripheral blood mononuclear cells

F = female, M = male

More detailed patient descriptions with symptoms, medications and supplements are presented in online Supplementary Table 1A and B in I and in Table 1 in III.

4.1.2 Laboratory analyses (I, III)

All the patients in this study display the LPI_{Fin} mutation in the *SLC7A7* gene. Clinical laboratory variable analyses utilised in this study [arginine, citrulline, glutamine, lysine, creatinine, haemoglobin, ferritin, thrombocytes, leukocytes, albumin, alkaline phosphatase (ALP), alanine transaminase (ALT), ammonium (NH_4), carnitine, total, LDL and HDL cholesterols and TGs from the patients' plasma or serum samples (Table 1 in I, and Table 2 in III)] were performed using routine laboratory methods. Reference value limits were obtained from the Turku University Hospital Central Laboratory.

4.1.3 Determination of estimated glomerular filtration rate (eGFR) and chronic kidney disease (CKD) stages (II-III)

In order to evaluate the kidney glomerular function in 26 patients included in sub-studies II and III, eGFR values were calculated with the CKD-EPI formula. The eGFR values of the children under 18 years were calculated using the Bedside Schwartz formula. The eGFR values are represented as mL/min/1.73 m^2. By using pre-defined cutoff values, the patients were divided into five different CKD stages (Table 1 in III).

4.2 Sample collection

4.2.1 Peripheral whole-blood cells (I and unpublished results)

The peripheral whole-blood samples for the whole-blood cell gene expression studies were collected into the PAXgene Blood collection tubes (PreAnalytix, Hombrechtikon, Switzerland) and stored at -80 °C. The whole-blood samples for the lymphocyte flow cytometric assays were collected into BD Vacutainer® K_3 EDTA blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

4.2.2 Peripheral blood mononuclear cells (PBMCs) (II and unpublished results)

For the MDM study (II), peripheral whole-blood samples were collected in lithium heparin tubes (Vacuette, Kremsmünster, Austria). First, PBMCs were extracted after centrifugation with Ficoll-Paque™ PLUS (GE Healthcare Bio-Science AB, Uppsala, Sweden), after which 1.5 million cells per well were suspended in 24-well plates (Falcon Multiwell™ Primaria™ 24 Well, Becton, Dickinson and Company) in the RPMI-1640 medium with a GlutaMAX supplement (Invitrogen, Life Technologies, Carlsbad, CA, USA).

For the PBMC study (unpublished), the peripheral whole-blood samples were collected using BD Vacutainer® CPT sodium citrate tubes (Becton, Dickinson and Company). PBMCs were extracted after centrifugation and stored in RNA Protect Cell Reagent (Qiagen, Hilden, Germany) at -80 °C.

4.2.3 Reticulocytes (unpublished results)

The peripheral whole-blood samples were collected using BD Vacutainer® CPT sodium citrate tubes (Becton, Dickinson and Company). Erythrocytes and reticulocytes were extracted following a centrifugation, and stored in RNA Protect Cell Reagent (Qiagen) at -80 °C.

4.2.4 Plasma (II-III)

Plasma samples were extracted from the peripheral whole-blood samples collected in lithium heparin tubes (Vacuette) by centrifugation and stored at -80 °C.

4.3 Lymphocyte flow cytometric analysis (I)

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, UK) according to the manufacturer's instructions. The stained samples were incubated overnight at +4 °C and run with the BD FACScan flow cytometry analyser. The percentages of the lymphocyte subpopulations were compared to the reference values in use in two Finnish university hospitals (Helsinki and Turku).

4.4 Cell culture (II)

4.4.1 Monocyte-derived macrophage (MDM) differentiation

The PBMCs were cultured for six days at 37 °C in 5.1% CO_2 in the macrophage SFM-medium (Invitrogen) supplemented with 10 ng/ml GM-CSF (ImmunoTools, Friesoythe, Germany) and 100 U/ml penicillin-streptomycin in order to differentiate monocytes into macrophages. As an exception to the above, on the last day of culturing, the cells were left without GM-CSF for two hours before the experiments.

4.4.2 MDM PAMP stimulations

Differentiated macrophages were stimulated separately with three PAMPs: Pam_3CSK_4 (synthetic bacterial lipoprotein; 1 µg/ml), LPS (lipopolysaccharide; 0.5 µg/ml) and ODN 2216 CpG DNA (CpG oligonucleotide type A; 3µM) (all three from InvivoGen, San Diego, CA, USA), to activate the TLR2/1, TLR4 and TLR9 signalling pathways, respectively. First, the non-stimulated (0 h) cell culture medium and cell samples were harvested. Second, the PAMP-stimulated cell culture medium and cell samples were collected 4 h and 24 h after stimulation. In total, samples were collected from 23 patients and 15 controls at the time points mentioned above with three PAMP stimulations, with the exception of those samples for which the number of cells available was insufficient: for the patients, there were 22 samples of the 24-h Pam_3CSK_4 stimulations, 19 samples of the 4-h and 24-h LPS stimulations and 20 samples of the 4-h and 24-h CpG DNA stimulations, whilst for

the controls 14 samples of the 24-h LPS stimulations were achieved. The medium and cell samples were stored at -80 °C; however, the cell samples were first suspended in RNA Protect Cell Reagent (Qiagen).

4.5 Gene expression studies

4.5.1 RNA extraction (I-II and unpublished results)

The total RNA was extracted from the peripheral whole-blood cells (I) using the PAXgene Blood RNA kit (PreAnalytix) according to the manufacturer's instructions, although instead of using the elution buffer, RNase-free water was used for RNA elution. The total RNA was extracted from the MDM (II), PBMC (unpublished) and reticulocyte (unpublished) samples using the NucleoSpin RNA XS, NucleoSpin RNA Midi and NucleoSpin RNA Blood Midi kits (Macherey-Nagel, Düren, Germany), respectively, according to the manufacturer's instructions, with the exception of the lysis step in the reticulocyte samples, which was excluded. The eluted RNA from the PBMC samples was concentrated using the NucleoSpin RNA Clean-up XS kit (Macherey-Nagel). The concentration and purity of the RNA was measured with the NanoDrop spectrophotometer (NanoDrop technologies, Wilmington, DE, USA).

4.5.2 Genome-wide RNA microarray (I)

5 µg of the total RNA of each patient and of the pooled control samples was amplified using the RiboAmp® OA1 Round RNA Amplification Kit (Arcturus, Sunnyvale, CA, USA) according to the manufacturer's instructions. cDNAs were *in vitro*-transcribed into cRNA using the Illumina RNA Amplification Kit (Ambion, Huntingdon, UK). During the reaction, the cRNA was labelled with biotine 11 dUTP (PerkinElmer, Wellesley, MA, USA). The concentration of the cRNA samples was measured using the NanoDrop spectrophotometer (NanoDrop Technologies). After amplification, the labelled samples were hybridised on a Sentrix® HumanRef-8 Expression BeadChip Array (Illumina, San Diego, CA, USA) according to the instructions for Illumina® BeadStation 500X Revision D. Hybridisation was detected using 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).

4.5.3 Quantitative real-time PCR (qRT-PCR) (I-II and unpublished results)

The expression level changes of genes in the whole-blood cell, PBMC, MDM and reticulocyte mRNA samples were further studied using quantitative real-time PCR. First, the mRNA was reverse-transcribed into cDNA using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The gene-specific primers (Biomers, Ulm, Germany) (Appendix Table 1) were designed with the Beacon Designer 4 program (PREMIER Biosoft International, Palo Alto, CA, USA). Amplification of the template cDNA was performed with the SYBR Green method (IQ™ SYBR® Green Supermix, Bio-Rad and Maxima™ SYBR Green/Fluorescein qPCR

Master Mix, Thermo Fisher Scientific, Waltham, MA, USA) using iCycler® IQ™5 (Bio-Rad). As exceptions to the above, the expression levels of *IRF7*, *IFNB1*, *NOS2* and *SLC7A2* were analysed with a TaqMan® protocol using KAPA PROBE FAST qPCR Master Mix (ABI Prism™, Boston, MA, USA) and the 7900HT Fast Real-Time PCR System (Applied Biosystems). All the measurements were carried out in duplicate in two independent runs. The relative gene quantifications (ΔΔCt values) were calculated using *GNB2L1* (guanine nucleotide binding protein, beta-peptide 2-like 1) as an internal reference gene. In addition, *TRAP1* (TNF receptor-associated protein 1) was used in the whole-blood cell studies. The gene expression levels were expressed as $2^{-\Delta\Delta Ct}$ $\log_2$ fold change values relative to the average of control samples.

4.6 Cytokine secretion measurements (II)

Cytokine levels were measured in the MDM culture medium and plasma samples. The plasma samples were first diluted twofold with the assay diluent of the kit used (Invitrogen, Carlsbad, CA, USA). Single-well measurements were carried out using the Human Cytokine 25-Plex Panel (Invitrogen) [including following cytokines: GM-CSF, IFN-α, IFN-γ, IL-1RA, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (Eotaxin) and TNF-α] according to the manufacturer's instructions, using a Luminex apparatus (Luminex 100TM IS V2.3 Luminex Corporation, Austin, Texas, USA). IFN-β was measured with the ProcartaPlex Human Basic kit and Human IFN-beta Simplex kit (eBioscience, Affymetrix, Vienna, Austria) according to the manufacturer's instructions. The results were analysed with Bio-Plex Manager™ Software 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

4.7 NO level measurements (II-III)

The total concentrations of NO were measured in the plasma and unstimulated MDM culture medium samples. The plasma and medium samples were first diluted tenfold and twofold, respectively, with the reaction buffer of the kit used (Enzo Life Sciences, Lausen, Switzerland) and then ultra-filtrated for 15 minutes through a 10K MWCO filter (Amicon Ultra-0.5 Centrifugal Filter Devices, Millipore, Billerica, MA, USA). NO measurements were made using the Nitric Oxide (total) detection kit (Enzo Life Sciences) according to the manufacturer's instructions. Optical densities were measured with the Wallac Victor^2 1420 Multilabel Counter (Wallac, PerkinElmer, Turku, Finland) at 570 nm.

4.8 Targeted amino acid, and global metabolome and lipidome analyses (III)

The targeted amino acid, global polar metabolomics and global lipidomics analyses were performed using the HPLC-QqQ-MS/MS, GC×GC-TOFMS and UPLC-Q-TOFMS techniques, respectively. The detailed methods are described in III. A total of 42 amino acids were analysed and quantified with the Cliquid® software (Ab Sciex, MA, USA). Amino acids (argininosuccinic acid,

anserine, carnosine, cystathionine, hydroxylysine, homocysteine, phosphoethanolamine and phosphoserine) with no measurable concentrations in the study samples were removed from the data. For the metabolome data, the Golm online database was used for functional group prediction of the unknown metabolites, and, subsequently, the biochemical pathways of the identified metabolites were determined by the KEGG and PubChem database searches. The identifications of lipids were based on the internal lipid library.

4.9 Statistical analyses (I-III and unpublished results)

The statistical analyses are described in detail in I, II and III. However, in short, the scanned microarray raw data (I) was first normalised, and then the signal log ratios (SLR) of the LPI and average control signals were computed. After the initial filtering, the preserved genes were further filtered using a $\log_2$ fold change limit of ± 0.8 and a P-value limit of < 0.05 for the results of the *t*-test. The microarray data were deposited in the EMBL-EBI microarray database (accession number E-TABM-572; https://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-572/). The changes in the gene expression levels and routine laboratory values were tested using the *t*-test (I-III and unpublished results). The expression changes of the amino acid transporter genes were tested for correlation (Pearson's correlation) (I). The changes in the cytokine data were tested using the Cochran-Mantel-Haenszel (CMH) method (II). The P-values obtained from the MDM gene expression and cytokine data were Bonferroni-corrected (II). P-values of < 0.05 were regarded as significant (I-III and unpublished results). The changes in the NO (II-III), amino acid, metabolite and lipid levels (III) were tested using the Mann-Whitney *U* test. The P-value limit of < 0.05 was Bonferroni-corrected (amino acids) or the minimum false discovery rate (FDR) (metabolites and lipids) was estimated as the maximum q values (III). The amino acids with P < 0.0015 and metabolites and lipids with q < 0.05 were regarded as significant (III). The Spearman's rank correlation coefficients were computed between the selected variables (II-III). P-values derived from the pairwise correlations were corrected by the Benjamini-Hochberg procedure (III). P-values of < 0.05 were regarded as significant (II, III). The *post-hoc* analyses of the eGFR-correlated metabolites were performed with the Kruskall-Wallis test, and the P-value limit of < 0.05 was Bonferroni-corrected (III).

The statistical analyses were performed using the SPSS software (IBM SPSS Statistics 11.0.1 or 22, Armonk, NY, USA), SAS®version 9.3 (SAS Institute, Cary, NC, USA) and the packages for the R software (Team 2011).

5 RESULTS AND DISCUSSION

5.1 The whole-blood genome-wide transcriptome patterns in LPI (I)

5.1.1 Microarray data analysis – Gene ontology (GO) classification

In this study, the transcriptomes of a cohort of 13 Finnish LPI patients were analysed for the first time, using microarrays. The object of this study was to examine the effect of the Finnish *SLC7A7* mutation on the genome-wide gene expression profiles in the whole-blood cells when compared to the healthy age- and sex-matched controls. Although all the Finnish patients share the same LPI_{Fin} mutation, it was thought that there could be some alterations between the patients to be observed in the transcription levels of various genes. Accordingly, it was hoped that this study would reveal any genotype-phenotype correlation expected to be detected in the patients with a wide range of symptoms. Therefore, the patients with 'classic' symptoms were chosen, some also suffering from more severe complications such as CKD and PAP.

The microarray analysis revealed that, by using a $\log_2$ fold change limit of ± 0.8 and a P value limit of < 0.05, expression changes were found in a total of 935 transcripts, representing 926 individual genes of which 487 were upregulated and 439 downregulated. Those genes with an altered expression were seen to contribute to a variety of basic cellular functions. In order to make the most of the vast transcriptome data, the genes were categorised into different gene ontology (GO) classes based on the biological processes that they represent. The GO analysis revealed that the genes over-expressed in the LPI patients were related to, for example, immune and inflammatory responses, chemotaxis, apoptosis, and cell shape and cell size control (Figure 4). Among the under-expressed genes, the affected biological processes were development, regulation of transcription, proteolysis and peptidolysis (Figure 4). Many of these altered processes directly associate with the aetiology and suggested pathophysiology of LPI. For example, an increased expression level of genes related to immunological processes clearly indicates immunological deficiencies in the patients. The decreased expression pattern of genes pertaining to skeletal and muscle development, and lipid catabolism and metabolism may be a marker of ongoing osteoporosis and muscle hypotonia, and combined hyperlipidemia, respectively, consistently detected in LPI.

It is well recognised that epigenetic factors, including nutrients, influence gene expression profiles (Cousins 1999, Choi and Friso 2010). Since LPI patients are on a permanent low-protein diet, it is to be expected that the changes in their transcriptomes, especially in genes regulating basic cellular functions, are partly due to the altered nutritional homeostasis. It should be also noted that the expression of some genes is controlled by hormones; therefore, their expression is highly susceptible to changes during the day (Butte 2002). The hierarchical clustering (data not shown) of the patients by differentially expressed transcripts did not reveal any distinct connection between the severity of symptoms and gene expression changes. That is, those patients with a more severe clinical picture, including CKD and PAP or haematological and immunological deficiencies, showed no association based on their gene expression status. Hence, no transcript-phenotype correlation could be presented based on this study. Therefore, the symptoms of different severity suffered by LPI patients could arise from a complex combination of

not only gene expression but also proteome and metabolome regulation; that is, the combination of different parts in systems biology.

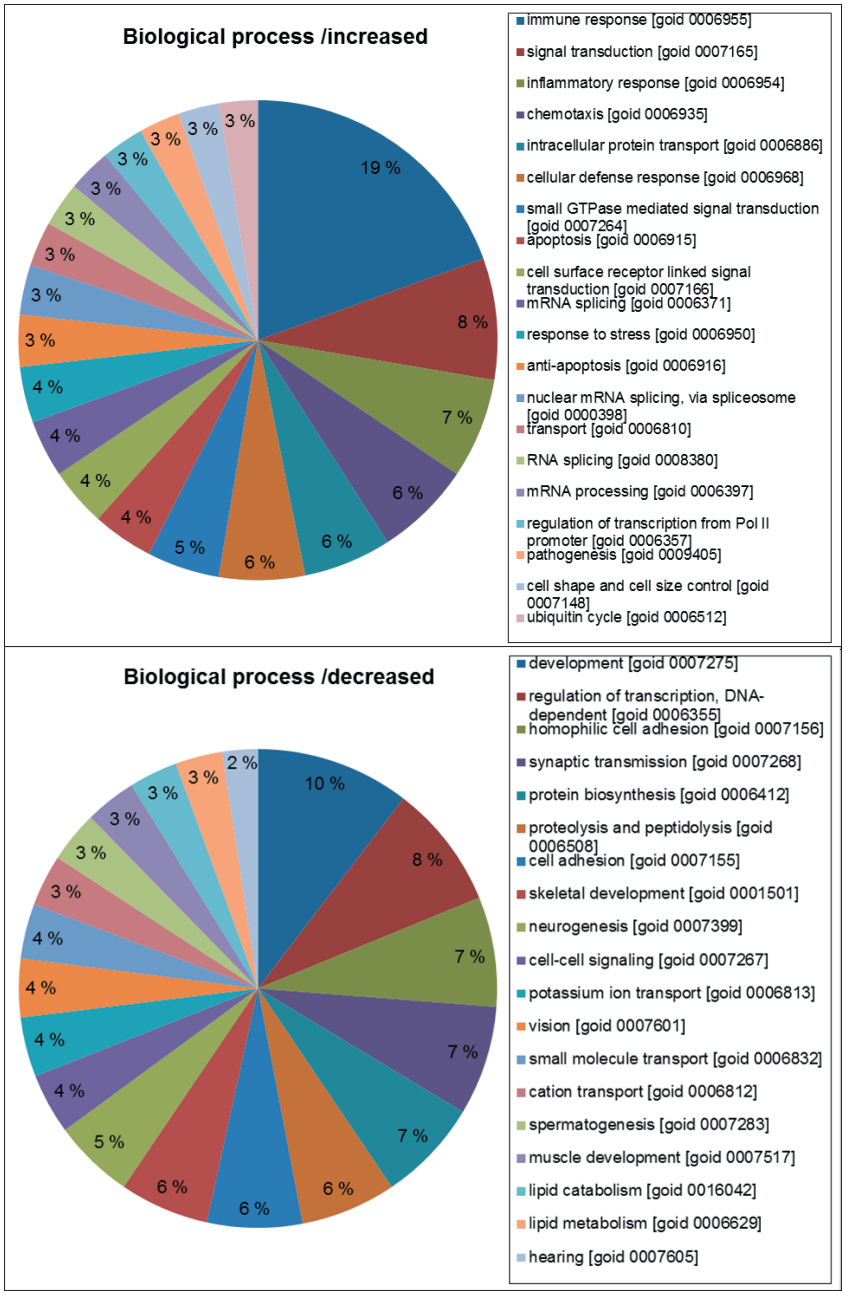

Figure 4. The most enriched gene ontology classes in the LPI patients. The up and downregulated genes in the LPI whole-blood cells were categorised into different gene ontology (GO) classes based on the biological processes that they contribute to.

The involvement of other genes in the pathogenesis of the LPI mouse was demonstrated earlier by Sperandeo and others (Sperandeo *et al.* 2007), who discovered hundreds of genes with altered expression patterns in the hepatic and small intestinal samples of the *Slc7a7*$^{-/-}$ mice. The genes with an altered expression level were mainly related to transport but they also associated with metabolism, apoptosis and proteolysis. The IGF genes were highly represented in the mouse transcriptome as *Igfbp1* was downregulated 2.6-fold in the foetal liver and upregulated 71.1-fold in the adult liver. It is known that IGFBP1 has an inhibitory role on IGF-stimulated growth and differentiation (Verhaeghe *et al.* 2001). Therefore, the upregulation of *IGFBP1* 1.9-fold in this study may in part be a contributing factor in the growth failure and osteoporosis seen in the patients. As the murine study was performed on the liver and intestine, the main target organs of LPI, it provided important information on the LPI pathophysiology. However, as organ biopsies are difficult to obtain in humans, particularly in LPI patients with a bleeding diathesis, and it is known that immune cells along with the intestine, kidney and liver express *SLC7A7*, the peripheral whole-blood cells were a perfect choice for the transcriptomic analysis as they provide valuable information of gene expression changes at the systemic level. In addition, this study proved that the whole blood can be a useful study material, reflecting conditions from other tissues as shown in the results in the next chapter. To support this, it has already been demonstrated that the whole-blood peripheral cells express approximately 80% of the genes in the human genome, and that the whole-blood cells and many other human tissues share most of their gene expression (Liew *et al.* 2006).

5.1.2 Microarray data analysis – DAVID and Ingenuity pathway (IPA) analyses

The functional annotation analysis with the DAVID bioinformatics tool was carried out on those genes with significantly altered expression levels. The analysis revealed several affected biological processes highly enriched in LPI, including immune response, chemotaxis, inflammatory response, erythrocyte differentiation and processes related to cell death and apoptosis (Table 2 in I). These results clearly indicate that different immunological processes and cellular destruction are prevailing states in LPI at the systemic level.

The Ingenuity pathway analysis (IPA) revealed that a significant ($P < 0.01$) number of genes had an altered expression pattern in canonical pathways related to antiviral immune response ('interferon signalling'), cellular growth and differentiation ('ERK/MAPK signalling') and liver and renal functions, as seen in Table 3 in I. The altered gene expression levels in the 'interferon signalling' may reflect the weakened antiviral responses causing severe viral infections in LPI. Changes were also detected in the expression of genes belonging to the 'hepatic fibrosis/hepatic stellate cell activation', 'hepatic cholestasis', 'PXR/RXR activation' and 'PPARα/RXRα activation' pathways, which implies changes in the cellular functions of the liver and kidney. It seems that hepatic and renal alterations may, at least partly, also have an immunological background based on the genes involved in these pathways. Interestingly, the IPA analysis also uncovered a significant involvement of the differentially expressed genes in 'renal necrosis/cell death (apoptosis of kidney cell lines)' pathway which may be directly linked to the CKD observed in LPI.

5.2 Plasma amino acids and amino acid transporter gene expression patterns in different cell types in LPI (I-III and unpublished results)

No large-scale amino acid analysis had been made before in LPI in a case-control setting. We therefore carried out an LC-MS-based targeted amino acid analysis on the plasma samples of the LPI patients. The amino acid profiling revealed that the levels of ornithine, arginine, lysine, tryptophan, tyrosine, leucine, methionine, valine and phenylalanine were significantly ($P < 0.0015$) decreased in the LPI patient plasma samples when compared to the healthy control individuals (Table 3 in III). In contrast, the levels of homocitrulline, citrulline, beta-aminoisobutyric acid (BAIBA), glutamic acid, glycine, aspartic acid, proline and serine were significantly increased in the LPI patients. Overall, the analysis demonstrated that, of the proteinogenic amino acids, the levels of essential amino acids were decreased whilst those of non-essential ones were increased in LPI. This type of amino acid profile is to be expected as the patients are on a permanent low-protein diet leading to the amino acid malnutrition for which the body apparently attempts to compensate by an accelerated *de novo* synthesis of dispensable amino acids. However, the level of non-essential tyrosine remained low since its biosynthesis is dependent upon essential phenylalanine. In addition, the levels of non-essential arginine, essential lysine and non-proteinogenic ornithine were decreased directly due to the transport defect of the mutated y^+LAT1. The pairwise correlation analysis (Figure 2 in III) further revealed that, as expected, amino acids with similar features, such as branched-chain (leucine and valine) and aromatic (phenylalanine, tryptophan and tyrosine) amino acids, correlated considerably. Surprisingly, despite the fact that the patients receive a citrulline supplement, the amount of exogenous citrulline did not correlate with the plasma citrulline levels.

Due to the disturbance of the amino acid balance in the LPI plasma, changes concerning amino acid transport were to be expected. Consistently, the whole-blood microarray data uncovered several genes related to transport in general, but especially to amino acid transport. In total, 16 upregulated genes involved in cellular transport were detected, 10 of which (*SLC4A1*, *SLC2A1*, *SLC1A5*, *SLC7A1*, *SLC5A2*, *SLC21A3*, *SLC21A8*, *SLC7A5*, *SLC13A2* and *SLC36A3*) were genes of different SLC family members. Seven transporter genes, including six SLC genes (*SLC7A7*, *SLC15A4*, *SLC4A7*, *SLC38A2*, *SLC40A1* and *SLC22A4*), were downregulated. Of the SLC7 amino acid transporter family, there were three members with altered expression: *SLC7A1*, *SLC7A5* and *SLC7A7*. Thus, the amino acid transporter genes, especially those of the SLC7 family, were of particular interest. Although the defect in the y^+LAT1 transporter disrupting the CAA transport in the blood cells has been observed so far only in monocytes and macrophages (Barilli *et al.* 2010, Barilli *et al.* 2012), it is expected that the defect impacts on other cells of the immune system as well. Therefore, it was scrutinised whether the amino acid transporter genes in the blood cells respond to the CAA transport defect and a reduced level of CAAs and essential amino acids in the plasma. The changes in the expression levels of the amino acid transporter genes, *SLC1A5*, *SLC7A1*, *SLC7A2*, *SLC7A5*, *SLC7A6*, *SLC7A7* and *SLC3A2* encoding ASCT2, CAT1, CAT2, LAT1, y^+LAT2, y^+LAT1 and 4F2hc, respectively, were therefore chosen to be studied in the whole-blood cells, PBMCs, MDMs and reticulocytes with qRT-PCR. The results of the gene expression changes in these cell types are shown in Table 9, Table 4 in I, Supplementary Tables II-IV in II and Figure 1A-C in II.

Table 9. The qRT-PCR results of the amino acid transporter genes in four cell types in the LPI patients compared to the controls.

Gene symbol	Whole-blood cells		PBMCs[b]		MDMs[c]		Reticulocytes	
	log_2 FC[a]	P	log_2 FC[a]	P	log_2 FC[a]	P	log_2 FC[a]	P
SLC1A5	1.86	< 0.001	0.00	NS	-0.14	NS	1.10	< 0.05
SLC7A1	0.73	< 0.001	-0.12	NS	-0.01	NS	0.08	NS
SLC7A2	ND		ND		ND		ND	
SCL7A5	1.79	< 0.001	0.48	NS	-0.52	NS	1.20	< 0.01
SLC7A6	-0.79	< 0.05	-0.43	< 0.01	-0.21	NS	-0.01	NS
SLC7A7	-3.05	< 0.001	-2.81	7.83×10^{-11}	-4.72	7.20×10^{-29}	-1.46	< 0.05
SLC3A2	0.04	NS	0.18	NS	-0.17	NS	0.27	NS

[a] FC = fold change
[b] peripheral blood mononuclear cells
[c] monocyte-derived macrophages
NS = not significant, ND = not detectable

The *SLC7A7* gene was heavily downregulated in the patients in all studied cell samples, but to the largest extent in MDMs. This may be due to the nonsense-mediated mRNA decay eliminating truncated and defective products caused by mutations leading to premature translation-termination codons (Brogna and Wen 2009). However, some transcripts may escape the process and produce faulty protein products. This is the case with the LPI_{Fin} mutation, which disrupts the carboxyl terminus of the nascent protein and produces a putatively truncated protein. These undestroyed aberrant mutant proteins may be deleterious to the viability of a cell, as suggested by the upregulated apoptosis pathways currently detected in LPI. The mRNA decay is further supported by the observation of the downregulation of *SLC7A7* even in the LPI reticulocytes in which the system y^+L transport is unaltered, mediated either by y^+LAT2 or some other, yet unidentified transporter (Smith *et al.* 1988, Boyd *et al.* 2000).

It has been suggested that the y^+LAT2 transporter could compensate for the transport defect of y^+LAT1 in LPI. In cultured fibroblasts, the system y^+L transport was thought to be intially mediated by y^+LAT2 or by the combination of y^+LAT2 and y^+LAT1; in the latter case, the absence of y^+LAT1 in LPI would be compensated for by y^+LAT2 (Dall'Asta *et al.* 2000). Barilli and others (Barilli *et al.* 2010) actually showed that *SLC7A6* was expressed at a high level in cultured fibroblasts of one Italian patient, suggesting a compensatory effect of y^+LAT2 on y^+LAT1. The same phenomenon has been proposed to take place in the cultured lymphoblasts in which the *SLC7A6* gene was upregulated in Japanese patients (Shoji *et al.* 2002). However, the current results from the four cell types in the Finnish patients indicate something quite different. The expression level of *SLC7A6*, as an average of all the studied patients, was unchanged in MDMs and reticulocytes, but, curiously, decreased in the whole-blood cells and PBMCs. However, in the study of Barilli and coworkers (Barilli *et al.* 2010), *SLC7A6* expression was very low in monocytes, even lower in the patient cells. The above-mentioned results and the current study propose that in the blood cells y^+LAT2 cannot compensate for the CAA transport defect caused by mutated y^+LAT1, unlike in cultured fibroblasts and lymphoblasts. However, it appears that another CAA transporter, CAT1, may try to augment CAA transport in the whole-blood cells, since the expression of its gene *SLC7A1* was

increased in those cells. Given that the expression of *SLC7A1* remained unchanged in other studied cells types, it may imply that only polymorphonuclear cells are the targets of increased CAT1-mediated CAA transport in LPI. Surprisingly, the *SLC7A2* expression was totally absent from all studied cell samples in both the controls and patients, indicating that in the studied cells other transporters than CAT2 dominate CAA transport. These results are supported by the studies of Barilli and others (Barilli *et al.* 2010, Barilli *et al.* 2012) who demonstrated that the y^+ type transport is low in LPI monocytes and macrophages.

As NAA transporter genes *SLC1A5* and *SLC7A5* were upregulated in the whole-blood cells and reticulocytes, but not in PBMCs and MDMs, it seems that in addition to reticulocytes their increased expression is derived from the polymorphonuclear cells. Curiously, the amino acid transporters encoded by these two genes are closely related to the mTOR system needed for normal protein synthesis and cell growth (Nicklin *et al.* 2009). However, a deficiency of essential amino acids, especially that of leucine, may lead to the inactivation of the mTOR system and induce autophagy for the breakdown of cellular components in order to secure energy supply (Nicklin *et al.* 2009). In LPI, starvation of essential amino acids is a permanent and consistent state as revealed by this study. However, it seems that the body attempts to inhibit autophagy in LPI by upregulating ASCT2 and LAT1 to increase glutamine uptake and, subsequently, elevate leucine intake, respectively, which are necessary for the mTOR activation.

5.3 Immune system dysfunction in LPI

5.3.1 Lymphocyte subpopulations (I)

Since leukopaenia is associated with LPI, the patients' lymphocyte subpopulations, including T lymphocytes ($CD3^+$), B lymphocytes ($CD19^+$), helper/inducer T cells ($CD4^+$), suppressor/cytotoxic T cells ($CD8^+$), NK cells ($CD16^+/56^+$) and activated T cells, were measured, and the $CD4^+/CD8^+$ ratio was calculated. The T lymphocyte and $CD4^+$ T cell population values varied from slightly subnormal to normal, whereas the B cell percentages varied from slightly decreased to slightly elevated. The NK cell and $CD8^+$ T cell populations were normal, apart from one patient with a slightly increased $CD8^+$ cell population; therefore, a decreased ratio of $CD4^+/CD8^+$ cells was observed. This differs from an earlier observation of a mainly decreased $CD4^+$ to $CD8^+$ ratio in the Finnish patients (Lukkarinen *et al.* 1999). On the whole, the proportions of the lymphocyte subpopulation were fairly low: they were either fractionally subnormal or just within the normal limits. In contrast to this, the proportions of activated $CD3^+$ HLA-DR^+ T cells were notably reduced in almost all patients studied. This may be the factor contributing to severe pathogen infections in the LPI patients, as HLA DR^+ cells are important in exogenous antigen presentation to $CD4^+$ helper T-lymphocytes (Rea *et al.* 1999).

5.3.2 TLR-induced gene expression and cytokine secretion by PAMP-stimulated MDMs (II)

It has been suggested that a prolonged inflammation state and impaired macrophage functions may play a role in life-threatening conditions such as PAP and CKD in LPI (Sebastio *et al.* 2011,

Ogier de Baulny *et al.* 2012). LPI patients suffer also from immunological defects leading to severe *Varicella* and bacterial infections. It is known that the *Varicella-zoster* virus induce both the TLR9 (Yu *et al.* 2011) and TLR2 (Wang *et al.* 2005) pathways in human PBMCs and MDMs, respectively. Thus, the aim of this substudy was to establish whether the CAA transport defect in LPI has an effect on the TLR signalling in classically-activated macrophages. It was decided to study the signalling of the PAMP-stimulated TLR2/1, TLR4 and TLR9 pathways at 4- and 24-hour time points in order to examine both immediate and late TLR responses. The total of 26 cytokines and 35 genes (Appendix Table 1), in addition to the seven amino acid transporter genes presented in the chapter 5.2, were studied from the unstimulated and PAMP-stimulated MDMs.

5.3.2.1 Amino acid transporter gene expression patterns

As shown in the chapter 5.2, the only differentially expressed amino acid transporter gene in unstimulated MDMs was *SLC7A7*. However, when MDMs were stimulated with PAMPs for 24 h, expression level changes were observed in several amino acid transporter genes. First, it was shown that the LPI MDMs manifested massive and significant downregulation of *SLC7A7* in all studied TLR stimulations and time points (Figure 1A-C in II and Supplementary Tables II-IV in II). However, the activation of the TLR9 pathway for 24 h significantly increased *SLC7A7* expression in both controls and LPI patients (Figure 1C in II and Supplementary Table IV in II). As it is known that CAA transport, especially that of arginine, is orchestrated mainly by y^+LAT1 and not by *SLC7A1*-encoded CAT1 in human macrophages (Rotoli *et al.* 2004, Rotoli *et al.* 2007, Barilli *et al.* 2011), these results suggest that CAA transport through y^+LAT1 may be particularly necessary for the macrophage activation needed to clear viral pathogens. However, in LPI, the increase in the expression of mutated *SLC7A7* is futile as defective y^+LAT1 does not reach the plasma membrane (Mykkänen *et al.* 2000). In addition, it was seen that none of the other studied CAA transporters showed any compensatory effect for *SLC7A7*. On the contrary, both NAA and CAA tranporter genes were downregulated as a result of the PAMP stimulations. *SLC7A5* was downregulated significantly in the patients at the 4-h time point after the TLR4 pathway induction and at the 24-h time point when the TLR2/1 and TLR9 were induced (Figure 4A and C in II and Supplementary Tables II-IV in II). *SLC7A5* is known to be upregulated during classical macrophage activation (Martinez *et al.* 2006) and in macrophages confronting pathogens (Nau *et al.* 2002), contrary to what was observed in the LPI macrophages. *SLC7A1* showed significant downregulation at the 24-h time point in the LPI MDMs when TLR2/1 and TLR9 were induced (Supplementary Tables II and IV in II). Similarly, *SLC7A6* was significantly downregulated in the patients at the 4-h time point after the TLR2/1 pathway activation and at the 24-h time point after the induction of TLR2/1 and TLR4 (Supplementary Tables II-III in II). Therefore, it seems that y^+LAT2 cannot provide the necessary compensatory aid for y^+LAT1 in macrophages attacked by pathogens. The total absence of the expression of CAT2-encoding *SLC7A2* in both LPI and control MDMs further supports the importance of y^+LAT1 in human macrophages.

SLC3A2, encoding 4F2hc (CD98hc), was downregulated in the LPI MDMs after 24-h TLR4 and TLR9 stimulations. 4F2hc is vital for the well-being of cells as it participates in many major cellular functions, such as proliferation, differentiation, adhesion and fusion (Devés and Boyd 2000). Since the LPI_{Fin}-mutated y^+LAT1/CD98hc complex does not reach the plasma membrane, it may cause a

dominant-negative effect on the CD98-mediated integrin signalling (Feral *et al.* 2005) and immune functions such as fusion, antigen-presentation and phagocytic activity of macrophages (Tsumura *et al.* 2012), clonal expansion of T and B cells (Cantor *et al.* 2009, Cantor *et al.* 2011, Cantor and Ginsberg 2012) and antibody responses (Cantor *et al.* 2009).

Extracellular arginine is essential in NO and polyamine production (Shin *et al.* 2011), which are further needed for pathogen clearance and normal cell growth, respectively. Arginine is also needed in macrophage-mediated tumour cell cytotoxicity (Evoy *et al.* 1998). It is also known that a dietary lysine deficiency impairs immune responses, limits the synthesis of proteins, including cytokines, and the proliferation of lymphocytes, and that an oral lysine supplementation weakens the *Herpes simplex* virus infections (Li *et al.* 2007). As the CAA influx in monocytes and macrophages is mediated by y^+LAT1 (Rotoli *et al.* 2004, Rotoli *et al.* 2007, Barilli *et al.* 2011) and other CAA transporters do not seem to compensate for the transport defect, it seems that in the LPI MDMs the intracellular level of CAAs may be crucially reduced, which could in turn have a deleterious impact on macrophage function in innate immunity.

5.3.2.2 The TLR2/1 and TLR4 signalling pathways

The cytokine measurements in the MDM medium revealed that a 4-hour stimulation of the TLR2/1 pathway with Pam_3CSK_4 led to significantly increased levels of IL-1RA ($P = 0.0018$), IL-12 ($P = 0.0342$) and TNF-α ($P = 0.0030$) (Figure 5A) in the patients' MDMs compared to those of the controls. In addition, the TLR2/1 pathway induction was shown to lead to the activation or repression of a total of 20 genes (control MDM results in Supplementary Table II in II). Statistically significant expression level changes in the LPI patients were found in nine of the genes studied when compared to the controls. *TICAM1* (TRIF), *STAT4*, *IL12B*, *TNF* and IFN-γ receptor genes *IFNGR1* and *IFNGR2* were upregulated in the LPI samples after a 4-h TLR2/1 pathway stimulation. In contrast, downregulation after a 4-h stimulation was seen in *IRF3*. At the 24-h time point, *TLR1* was upregulated and *TLR9* showed downregulation in the patients versus controls. The TLR2/1 pathway gene expression results are shown in Supplementary Table II in II and Figure 1A in II.

A 24-hour stimulation of the TLR4 pathway with LPS resulted in significantly increased levels of IL-1RA ($P = 0.0138$) and IL-12 ($P = 0.0471$) in the patients' MDM medium compared to that of the controls (Figure 5B). At the gene expression level, the TLR4 pathway induction led to the activation or repression of four genes (control MDM results in Supplementary Table III). Of these four genes, *TLR9* was activated in both study groups after a 4-h stimulation, but significant downregulation at the 4-h time point was detected in the LPI patients compared to the controls. *IFNGR1* was repressed after the 4-h stimulation only in the controls, thus significant upregulation at the 4-h time point was detected in the patients versus the controls. In addition, *TLR4*, *IFNB1* and *IFNGR2* were significantly upregulated and *STAT4* was significantly downregulated at the 4-h time point in the patients compared to the controls. The TLR4 pathway gene expression results are shown in Supplementary Table III in II.

It seems that the TLR2/1 pathway-induced cytokine secretion directly followed the upregulation of the *TLR1*, *TNF* and IL-12p40-encoding *IL-12B* genes. However, the expression level of IL-12 p35-

encoding *IL12A* was very low, and it showed no activation whatsoever when the TLR2/1 pathway was activated. The activation of *STAT4*, which encodes the IL-12-induced transcription factor, after TLR2/1 induction shows that in addition to the NK and Th1 cells, *STAT4* is also expressed in macrophages, both in unstimulated and PAMP-stimulated states. Frucht and others (Frucht *et al.* 2000) have demonstrated earlier that the STAT4 mRNA and protein are expressed in activated, but not in unstimulated, monocytes and rheumatoid synovial macrophages, respectively. In another study, monocytes and MDMs expressed *STAT4* only at a very low level (Lehtonen *et al.* 2005); however, these results were obtained by Northern blotting, which is not as sensitive a method as qRT-PCR for mRNA detection. Since IL-12 is known to activate the *STAT4* gene, and MDMs in this study both produced IL-12, and expressed *STAT4* and IL-12 receptor genes *IL12RB1* and *IL12RB2*, an autocrine induction would be a possible explanation for the IL-12-induced immune responses in LPI. Overactivation of the TLR4 pathway in LPI is, along with the cytokine result, suggested by the increased *TLR4* expression. However, the IL-12 genes were not activated at all after the TLR4 pathway induction when measured at the 4 h or 24 h time points, neither in the controls nor patients, in spite of the increased IL-12 secretion from the MDMs. It may be that the autocrine induction could have turned off the IL-12 gene activation since *STAT4* was downregulated in the patients' TLR4-stimulated MDMs. Curiously, *IL1RN* encoding IL-1RA showed no upregulation in the patients neither after the TLR2/1 nor TLR4 pathway induction in spite of the elevated cytokine level. It was observed that the genes *IFNGR1* and *IFNGR2* encoding a receptor for IFN-γ, one of the key cytokines in innate immunity, were upregulated in the patients after the TLR2/1 and TLR4 pathway activations, which suggests that LPI patients may be more receptive to IFN-γ induction after a bacterial encounter.

The observation of the LPI MDMs secreting increased levels of IL-12 and TNF-α indicates that the patients develop a stronger inflammatory response than controls when their macrophages confront pathogens. IL-12 and TNF-α are proinflammatory cytokines induced by the MyD88-activated NF-kB pathway and mainly produced by macrophages (Komastu *et al.* 1998, Wajant *et al.* 2003, Kawai and Akira 2011). TNF-α is released in large amounts upon induction by bacterial products, and it is also an important mediator of many autoimmune diseases (Wajant *et al.* 2003, Clark 2007). Upregulation of IL-12 in the serum has also been associated with different disease states, such as SLE (Tokano *et al.* 1999) and other autoimmune conditions, due to its role in NK and Th1 cell activation (Kobayashi *et al.* 1989, Hsieh *et al.* 1993, Trinchieri 1995, Trinchieri 2003). However, it seems that in LPI, there may be an attempt to compensate for inflammation by an overproduction of IL-1RA, an anti-inflammatory agent that prevents IL-1 from binding to its receptor and mediating inflammation and subsequent tissue damage (Arend *et al.* 1998, Arend 2002). An elevated level of IL-1RA in the blood has been detected in patients with infections, acute or chronic inflammation, SLE, rheumatoid arthritis, lung diseases, chronic renal failure (Arend *et al.* 1998, Arend 2002) and metabolic diseases (Perrier *et al.* 2006). Curiously, the increased secretion of TNF-α and IL-1RA can also be seen in the serum of those LPI patients suffering from HLH/MAS (Duval *et al.* 1999). In conclusion, the TLR2/1 and TLR4-induced increased levels of TNF-α, IL-12 and IL-1RA by the LPI patients' MDMs suggest that the patients' response to bacterial infection is excessive and that LPI macrophages may sustain an inflammatory state in their residence tissue by an overproduction of cytokines.

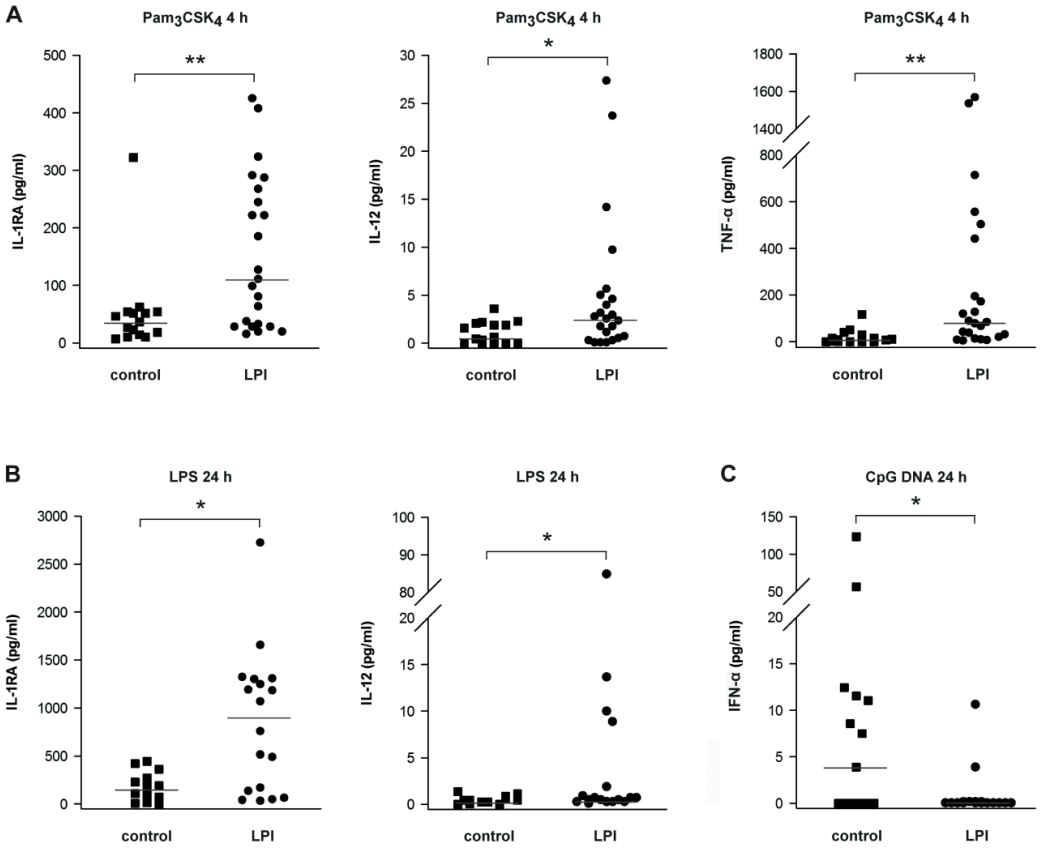

Figure 5. Significantly altered cytokine secretion by LPI macrophages stimulated with TLR2/1, TLR4 and TLR9 agonists. Peripheral whole-blood monocyte-derived LPI and control macrophages were stimulated with Pam_3CSK_4 (A), LPS (B) and CpG DNA (C) to activate the TLR2/1, TLR4 and TLR9 pathways, respectively. Cell culture medium samples were collected after 0 h (unstimulated), 4 h and 24 h of TLR stimulation. In total, the levels of 26 cytokines were measured. The lines represent the median. Cytokines with $P < 0.05$ are shown. *$P < 0.05$, **$P < 0.01$. (Figure 2 from original publication II.)

5.3.2.3 The TLR9 signalling pathway

A 24-h CpG DNA stimulation of the TLR9 pathway led to significantly increased IFN-α levels in the control MDMs ($P = 0.0327$) when compared to the patients (Figure 5C), although the IFN-α level remained relatively low in both the controls and patients. Another type I IFN, IFN-β, was also secreted at a low level by MDMs when stimulated with CpG DNA, more prominently in the controls [median 3.05 (IQR 2.75-4.90) pg/ml] than in the patients [median 2.75 (IQR 2.36-2.95) pg/ml]. However, the difference in the secretion levels of IFN-β between the controls and patients was not statistically significant. It may be that a longer stimulation time than 24 h is required for higher expression levels of IFN-α and IFN-β in MDMs. In addition, the induction of TLR9 in MDMs resulted in the activation or repression of sixteen genes (control MDM results in Supplementary Table IV in II). Overall, the LPI patients had statistically significant expression level changes in seven of the genes studied when compared to the controls. *IRF3* and *IFNB1,* encoding IFN-β, were

downregulated in the patients at the 4-h time point compared to the controls. At the 24-h time point, *TLR9*, *IRF7*, *SOCS1* (suppression of cytokine signalling 1), *IL12A* and *IL12RB1* were downregulated in the patients compared to the controls. The TLR9 pathway gene expression results are shown in Supplementary Table IV in II and Figure 1C in II.

The TLR9 pathway is important in both viral and bacterial defence through the production of type I IFNs and inflammatory cytokines, respectively (Akira *et al.* 2006, West *et al.* 2006). It is known that the induction of type I IFN through TLR9 occurs mainly in pDCs, the main viral pathogen-recognising cells (Lund *et al.* 2003, Akira *et al.* 2006, Gilliet *et al.* 2008), and that the IRF7 protein, which is essential in IFN-α production, is more strongly expressed in pDCs than in monocytes (Izaguirre *et al.* 2003). Nonetheless, the results demonstrated that TLR9 stimulation also activates IFN-α production in MDMs and that *IRF7*, although expressed at a low level, is activated to a greater degree after the TLR9 stimulation in MDMs. Interestingly, *IRF3*, the protein product of which is needed for IFN-β production in the TRIF-mediated pathway, was activated after the TLR9 induction. Initially, TRIF was thought to contribute only to TLR3 and TLR4 signalling (West *et al.* 2006), but it has been demonstrated that the TLR9 pathway can also be activated through TRIF and IRF3 (Volpi *et al.* 2013). Since the activation of *TICAM1*, the gene that encodes TRIF, was not observed, it is possible that some TLR-independent pathway activates IRF3. To support this, it has been shown that cytosolic DNA is capable of inducing IRF3 independently of TLR (Stetson and Medzhitov 2006). In addition, the downregulation of *SOCS1* after TLR9 pathway induction is quite surprising since the SOCS1 protein is needed for the inhibition of excessive cytokine production and signalling. However, in macrophages, SOCS1 is also known to be induced by TLR stimulation and to negatively regulate the TLR signalling through inhibiting autocrine type I IFN signalling by blocking IFN-α/β receptor-induced pathways (Baetz *et al.* 2004, Gingras *et al.* 2004). Therefore, it may be that the downregulation of *SOCS1* in LPI is the system's response during viral infection to boost type I IFN mediated signalling. Although the exact signalling mechanisms resulting in the type I IFN production are not covered in this study, downregulation of *TLR9*, *IRF7*, *IRF3*, *IFNB1*, *SOCS1* and decreased secretion of cytokines IFN-α and IFN-β in the patients compared to the controls after TLR9 stimulation clearly indicate that, in LPI, the response to viral recognition is reduced, particularly after the 24-h exposure, which may explain the severe outcome of viral infections seen in LPI.

5.3.3 NO levels in MDM medium and plasma (II-III)

NO has been suggested to be the key 'villain' in the pathogenesis of LPI. It has been hypothesised that LPI patients may have elevated NO production due to the increased levels of arginine trapped in the cells with disabled y^+LAT1, further boosted by citrulline supplementation, and that this increased toxic NO tampers with crucial cell functions (Sebastio *et al.* 2011, Ogier de Baulny *et al.* 2012). Therefore, the levels of NO produced by MDMs and circulating in the blood were measured in the patients' and controls' unstimulated MDM culture medium and plasma samples, respectively. Contrary to the earlier theory, the NO levels produced by MDMs were actually significantly decreased in the LPI patients compared to the controls (Figure 6). This suggests that, in contrast to the earlier hypothesis, intracellular arginine reservoirs in the LPI macrophages may actually be diminished, not increased, due to the reduced influx of arginine by defective y^+LAT1 as proposed

already in the chapter 5.3.2.1. In addition, the expression level of *NOS2*, encoding the iNOS enzyme that produces NO from arginine in macrophages, was studied in the unstimulated and PAMP-stimulated MDMs. Surprisingly, the expression of *NOS2* was not detected either in the LPI or in the control MDMs. In contrast, the Italian group (Mannucci *et al.* 2005) detected a high NO_2^- level but still a low iNOS level in LPI fibroblasts. Nevertheless, it has been demonstrated that the arginine transport is normal in LPI fibroblasts due to the compensating transport of y^+LAT2 (Dall'Asta *et al.* 2000).

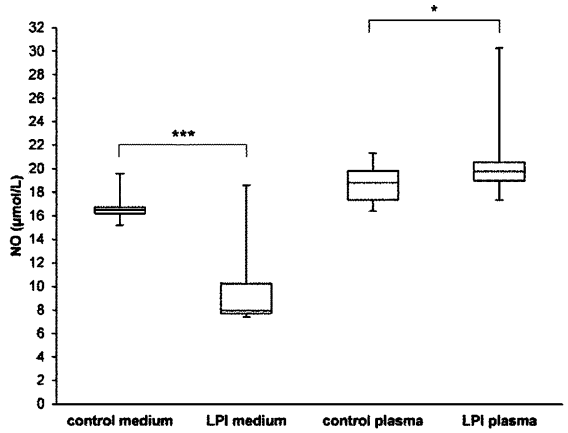

Figure 6. MDM medium and plasma NO levels of the LPI patients and controls. The NO levels were measured in unstimulated peripheral whole-blood monocyte-derived macrophage culture medium samples and plasma samples of the LPI patients and controls. The data are represented as median and quartiles ± maximum/minimum values. The statistical significance was determined using the Mann–Whitney *U* test. *P < 0.05, ***P < 0.001. Modified from Figure 4 in II and Figure 1 in III.

There is considerable controversy concerning the NO production and iNOS expression in macrophages in the current literature. Most of the studies of NO have been carried out with murine macrophages (Kakuda *et al.* 1999, Nicholson *et al.* 2001, Yeramian *et al.* 2006b), which differ from their human counterparts (Venketaraman *et al.* 2003). In human macrophages, NO and iNOS expression have been scarcely detectable in healthy subjects. However, iNOS and NO have been observed in those patients with infections and inflammatory diseases (MacMicking *et al.* 1997, Thomas and Mattila 2014). The current result supports the idea that human macrophages do not express *NOS2*, but still produce NO, possibly by using only a minute amount of iNOS indetectable at mRNA level. NO production is known to depend on extracellular arginine in murine macrophages (Granger *et al.* 1990, Assreuy and Moncada 1992, Bogle *et al.* 1992, Baydoun *et al.* 1993) and human endothelial cells (Shin *et al.* 2011). However, it has been suggested that human monocytes may also produce NO from the *de novo*-synthetisised arginine from glutamine converted to citrulline in the absence of extracellular arginine (Murphy and Newsholme 1998). Therefore, it may be that in the LPI macrophages, some level of an endogenous synthesis of arginine takes place to facilitate the NO production, but in insufficient quantities to maintain the normal level of NO. It is interesting that NO has been shown to reduce NF-κB activation and cytokine production

in human stimulated MDMs and AMs (Fiorucci *et al.* 2000, Thomassen and Kavuru 2001). However, NO may also interact with oxidants to form toxic compounds which may reduce the availability of NO for blocking inflammatory cytokine production (Thomassen and Kavuru 2001). Consequently, the decreased NO could partly explain the increased cytokine production in the stimulated LPI macrophages.

In contrast to the MDM medium, the plasma levels of NO were slightly, but significantly ($P = 0.02$) elevated in the patient [median 19.75 (IQR 18.98-20.56) µmol/L] samples compared to the control [median 18.81 (IQR 17.35-19.80) µmol/L] samples (Figure 6). The increased plasma levels of NO indicate intensified circulation of NO which may have an impact both at the cellular and systemic levels. However, it is difficult to deduce from which tissue or cells the NO originates from. Kamada and others (Kamada *et al.* 2001) have shown decreased levels of NO in the LPI plasma, hypothesising that it may be derived from endothelial cells suffering from reduced arginine levels. In this study, it was observed that the LPI plasma NO concentrations correlated inversely with the eGFR ($r = -0.40$, $P < 0.05$), implying that the increased NO level associates with reduced kidney glomerular function, and that NO in the plasma may, indeed, be derived from the kidney. This could be a direct consequence of an increased arginine level in the kidney tubule cells due to the CAA export defect and may be further induced by citrulline supplementation (Morris 2007). The participation of exogenous citrulline in the elevated NO production or CKD was not, however, detected. An explanation for that may be that arginine production is known to be decreased in CKD as a result of a reduced citrulline uptake in the kidney (Tizianello *et al.* 1980). Curiously, both an excess and a deficit of NO have been detected in different disease states in the kidney. However, the toxicity of NO is supported by studies in which increased NO reacting with oxygen and nitrogen has been demonstrated to cause postischemic renal failure and immune-mediated kidney disorders such as lupus nephritis, renal fibrosis and glomerulonephritis (Kone 1997, Peters *et al.* 1999, H. Peters *et al.* 2003), also detected in LPI.

5.3.4 Plasma cytokine levels (II)

Since systemic inflammation may be one of the explanatory factors of the LPI aetiology, concentration levels of 26 different cytokines were analysed in the plasma samples of the patients and controls. The measurements revealed that the secretion levels of chemokines CXCL8 (IL-8) ($P = 0.0462$), CXCL9 (MIG) ($P < 0.0001$) and CXCL10 (IP-10) ($P = 0.0375$) were significantly increased in the patients compared to the controls (Figure 7). It is known that CXCL8 induces chemotaxis of neutrophils and other granulocytes to the site of infection (Baggiolini and Clark-Lewis 1992) and that activated T cells and other leukocytes are attracted to the site of inflammation by CXCL9 and CXCL10 (Taub *et al.* 1993, Liao *et al.* 1995, Qin *et al.* 1998). This has also been observed in other disease conditions: IL-8, for example, occurs at high levels in the plasma of infants with respiratory syncytial virus bronchiolitis (Hull *et al.* 2000), and CXCL9 and CXCL10 levels are inceased in the plasma and serum in rheumatoid arthritis, multiple sclerosis (Patel *et al.* 2001, Lee *et al.* 2009) and SLE (Lit *et al.* 2006, Kong *et al.* 2009), in which infiltrating leukocytes attracted by CXCL9 and CXCL10 play an important role in tissue injury. It has been proposed that LPI macrophages and lymphocytes may sustain an inflammatory state induced by NO in the kidney cells with defective CAA transport (Sebastio *et al.* 2011, Ogier de Baulny *et al.*

2012). Therefore, the association of these leukocyte-attracting chemokines with eGFR was tested. The concentrations of CXCL9 (r = -0.59, P = 0.0016) and CXCL10 (r = -0.43, P = 0.0301) had a significant negative correlation with eGFR, indicating that the LPI patiens with reduced glomerular function have particularly elevated plasma chemokine levels. Consequently, these results support the theory that the LPI patients may indeed have a systemic inflammatory state, which, in those patients with renal dysfunction, further causes leukocyte attraction to the injured kidney to perpetuate the inflammation.

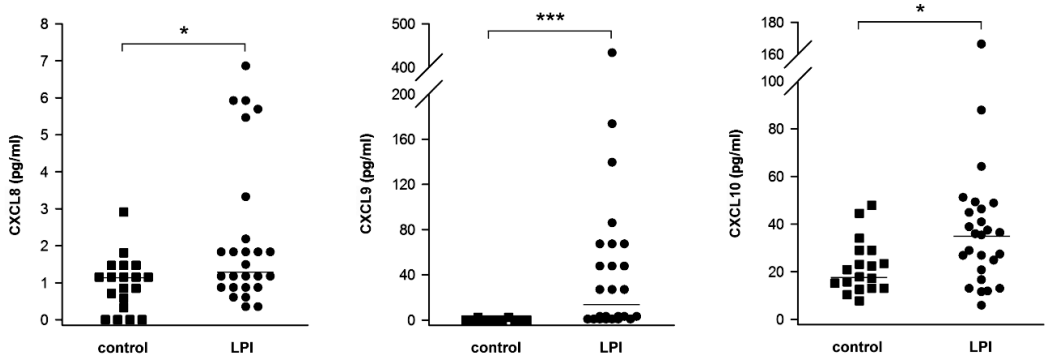

Figure 7. Plasma chemokine levels of the LPI patients and controls. The levels of 26 cytokines were measured in the peripheral whole-blood plasma samples of LPI patients and controls. The lines represent the median. The cytokines with P < 0.05 are shown. *P < 0.05, ***P < 0.001. (Figure 3 from original publication II.)

5.3.5 Expression level changes of immune-related genes in the whole-blood cells and PBMCs (I and unpublished results)

Based on the microarray and MDM results, the immune-related genes of interest and those with highly changed expression levels were further studied using qRT-PCR in the peripheral whole-blood cells and PBMCs. The results revealed that *IFI27* [interferon (IFN)-α-inducible protein 27] was the fourth most upregulated gene in the LPI patients' whole-blood cell samples. The qRT-PCR validation confirmed that, on average, the expression level of *IFI27* was upregulated 24-fold in the patients compared to the controls (Table 10). Furthermore, in seven patients, the *IFI27* expression level was as much as 1 000 times higher compared to the controls (Figure 8); three of those patients had experienced serious PAP after which two had died. However, in PBMCs, the average patient expression level was considerably lower, only 14-fold compared to the controls (Table 10). The expression differences between the whole-blood cells and PBMCs may be partly explained by the fact that the PBMC results were obtained only from 11 patients (Figure 8).

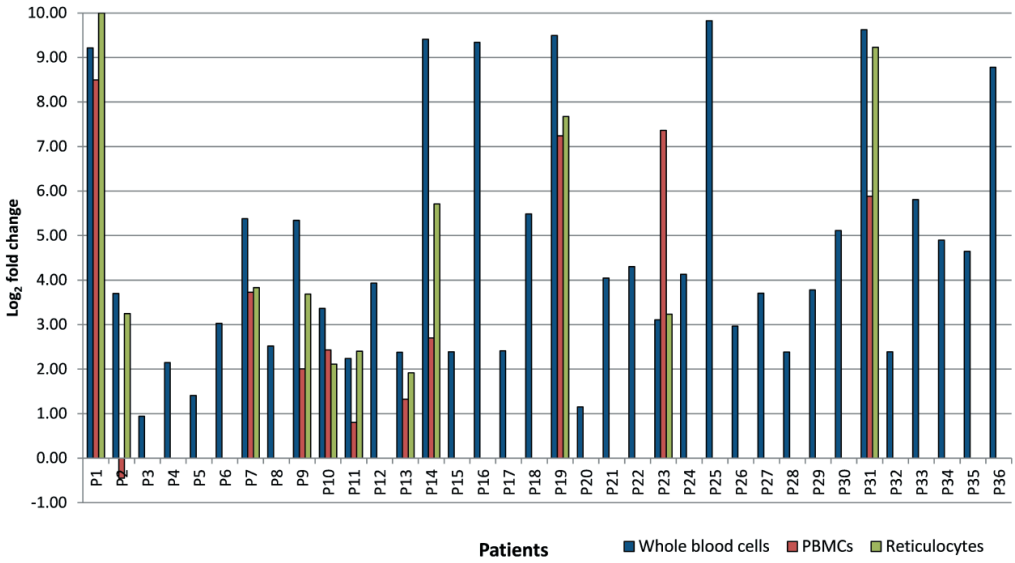

Figure 8. The *IFI27* expression level changes in the three cell types in the LPI patients compared to the controls. The expression level changes were measured in the whole-blood cells, and PBMC and reticulocyte samples of 36 and 11 patients, respectively, using qRT-PCR. PBMC = peripheral blood mononuclear cell

IFI27 belongs to the group of IFN-stimulated genes contributing to the innate immune responses of IFNs (Cheriyath *et al.* 2011, Malhotra *et al.* 2011). *IFI27* has been found to be upregulated in different viral and autoimmune states in the whole-blood cells and PBMCs, respectively (Ishii *et al.* 2005, Fjaerli *et al.* 2006, Ioannidis *et al.* 2012). It has also been shown to suppress viral proliferation when over-expressed in cells (Itsui *et al.* 2009). Interestingly, in the control and LPI MDMs, *IFI27* expression was strongly induced after a 24-h CpG DNA stimulation (Figure 9 and Supplementary Table IV in II) supporting the idea that *IFI27* has a role in the innate immune response against viral pathogens.

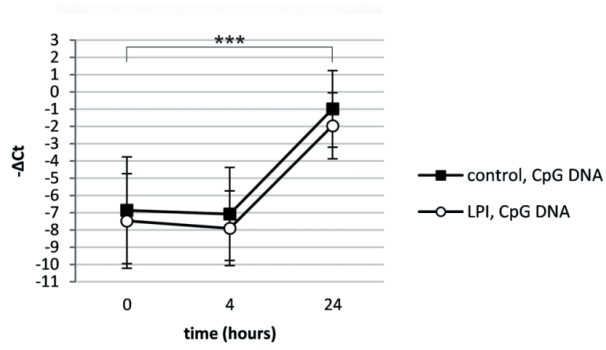

Figure 9. Gene expression analysis of *IFI27* in the TLR9 pathway-induced macrophages. *IFI27* expression was studied in the CpG DNA-stimulated monocyte-derived macrophages (MDMs) of the LPI patients and controls using qRT-PCR. The results are shown as −ΔCt values relative to the expression of the reference gene, *GNB2L1*. The data are represented as the mean ± SD. The statistical significance was determined using the *t*-test. The P-value was Bonferroni-corrected according to the three time points. ***P < 0.001.

NAMPT (*PBEF1*, pre-B-cell colony enhancing factor 1) was the fourth most downregulated gene in the microarray study. *NAMPT* codes for a protein, PBEF1, promoting growth and differentiation of B cell precursors (Samal *et al.* 1994) and plays an important role in innate immunity (Müller *et al.* 1999). It is upregulated in neutrophils and monocytes in response to different cytokines and microbial stimuli in order to inhibit the apoptosis of activated neutrophils (Jia *et al.* 2004), and its expression is known to increase in systemic inflammation states (Luk *et al.* 2008). Surprisingly, in the LPI patients, *NAMPT* was upregulated in the PBMCs but downregulated in the whole-blood cells (Table 10). In PBMCs, it might be responding to the prevailing inflammatory state and the deficiencies of the patients' B-cell functions by increasing its expression. The downregulation in the whole-blood cells, on the other hand, may enhance the apoptosis of neutrophils, which is particularly important during a resolution phase after an inflammatory state.

In the microarray analysis, the four most downregulated cytokine genes, *IL-1B*, *CXCL8* (IL-8), *CXCR2* (IL-8RB) and *IL-18RAP,* were also validated by qRT-PCR. The genes for IL-1B, which mediates inflammation and tissue damage (Arend 2002), and CXCL8, which induces chemotaxis of neutrophils and other granulocytes to the site of infection (Baggiolini and Clark-Lewis 1992), showed upregulation in the PBMCs and downregulation in the whole-blood cells (Table 10). The increased expression level of *CXCL8* in the PBMCs is consistent with the cytokine result of the elevated level of CXCL8 in the plasma in LPI presented in the chapter 5.3.4, indicating that increased CXCL8 secretion may originate from mononuclear cells. The genes encoding CXCR2, a receptor for CXCL8, and IL-18RAP, an accessory protein of the IL-18 receptor (Born *et al.* 1998) that helps IL-18, an activator of NK and T cells (Okamura *et al.* 1995), to bind IL-18R1, were downregulated in the whole-blood cells and PBMCs (Table 10). These results clearly indicate that the LPI patients' cytokine regulation is, in addition to a cytokine secretion level, weakened at the gene expression level in the whole-blood cells and PBMCs.

Table 10. The qRT-PCR results of the immune-related genes in the LPI patients compared to the controls.

Gene symbol	Whole-blood cells		PBMCs[b]	
	log_2 FC[a]	P	log_2 FC[a]	P
IL1RN	NA		0.77	< 0.05
IL1B	-1.36	< 0.001	1.79	< 0.01
CXCL8	-1.58	< 0.001	3.74	6.56×10^{-5}
CXCR2	-1.32	< 0.001	-0.93	< 0.001
IL12B	NA		-0.30	NS
IL18RAP	-1.98	1.17×10^{-6}	-1.38	7.94×10^{-5}
TNF	NA		0.19	NS
NAMPT	-2.09	6.29×10^{-6}	1.56	< 0.001
IFI27	4.58	5.41×10^{-6}	3.78	< 0.001
LAMP2	NA		0.43	< 0.05

[a] FC = fold change
[b] peripheral blood mononuclear cells
NA = not available, NS = not significant

In addition, the expression levels of *IL1RN, IL12B, TNF* and *LAMP2* were analysed in the PBMC samples. The expression of *IL1RN* and *LAMP2* was upregulated (Table 10), which is in contrast to the results from MDMs, where their levels were unchanged in the patients. However, as the secretion of IL-1RA by the stimulated MDMs was increased, it may be that IL-1RA production could also be increased in other mononuclear cells in the patients.

5.4 Reticulocyte-specific gene expression level changes in LPI (I and unpublished results)

Among the genes whose expression levels were increased in the patients in the whole-blood cells studied with microarrays were genes related to different haematological processes, such as erythropoiesis and haem synthesis, and also genes encoding erythrocyte membrane proteins, transporters, enzymes and blood group antigens. Some of the genes of interest and those with the highest expression level changes between the patients and controls were further validated by qRT-PCR in the whole-blood cell and reticulocyte samples. The results are shown in Table 11.

Table 11. The qRT-PCR results of the reticulocyte-specific genes in the LPI patients compared to the controls.

Gene symbol	Whole-blood cells		Reticulocytes	
	$\log_2$ FC[a]	P	$\log_2$ FC[a]	P
ALAS2	1.75	3.58×10^{-5}	1.12	< 0.01
BLVRB	2.80	7.94×10^{-11}	0.69	< 0.05
BSG	2.29	7.44×10^{-9}	1.09	< 0.01
CAI	4.26	6.29×10^{-10}	2.46	1.36×10^{-6}
EPB42	2.76	4.78×10^{-9}	1.67	3.24×10^{-5}
ERAF	4.16	2.60×10^{-12}	2.99	3.41×10^{-7}
FECH	1.72	1.32×10^{-5}	0.04	NS
HBQ1	2.34	5.03×10^{-7}	0.73	< 0.05
HEMGN	0.90	< 0.05	-1.10	< 0.01
HRI	1.11	< 0.001	0.96	< 0.05
MSCP	1.48	9.39×10^{-5}	0.81	< 0.05
SELENBP	2.77	1.20×10^{-7}	1.26	< 0.001
SLC2A1	2.12	1.16×10^{-8}	2.80	1.48×10^{-6}
SLC4A1	2.19	4.03×10^{-7}	1.64	< 0.01
IFI27	4.58	5.41×10^{-6}	4.82	5.94×10^{-5}

[a] FC = fold change
NS = not significant

ERAF (erythroid associated factor), which encodes ASHP (alpha-haemoglobin stabilizing protein), was the most upregulated gene in the transcriptome data. This gene is expressed during erythropoiesis and regulated by GATA1 (globin transcription factor 1) (Kihm *et al.* 2002), a transcription factor inducing the expression of many erythropoiesis-related genes (Welch *et al.* 2004). GATA1 was also upregulated in the LPI patients' whole-blood cells (microarray $\log_2$ FC 0.84). ASHP forms a stable complex with free alpha-globin but not with beta-globin or haemoglobin A in a cell, and

thus prevents the precipitation of any alpha-globin in excess of beta-globin (Kihm *et al.* 2002). The expression of *ERAF* is known to increase as a result of the expression of the alpha-globin gene (dos Santos *et al.* 2004); therefore, it seems that there may be a high alpha-globin/beta-globin ratio in the LPI erythrocytes.

CA1 encodes carbonic anhydrase 1 (CA1), which is the most abundant non-haemoglobical protein in the erythrocytes (Sly and Hu 1995). CA1 catalyses the hydration of carbon dioxide into bicarbonates and hydrogen ions (Sly and Hu 1995); the bicarbonates are further transported outside the cell by an anion exchanger, erythrocyte membrane protein band 3 (Jay 1996), encoded by *SLC4A1* which also has an increased expression level in LPI. CA1 contains 85% of the total zinc concentration in the erythrocytes. Curiously, the erythrocyte zinc concentration as well as the expression of CA1 are known to increase in patients with CKD (Mafra and Cozzolino 2004). CKD (Mafra and Cozzolino 2004) and the upregulation of CA1 (Mondrup and Anker 1976) are also often associated with anaemia. In LPI, despite the inappropriately high ferritin levels, the serum iron levels are normal. However, the serum concentration of zinc is markedly increased in the patients. It has been demonstrated that an iron supplement administered to CKD patients decreases the level of zinc in erythrocytes but increases its level in the serum (Mafra and Cozzolino 2004). Further, it seems that an intracellular iron transport may be enhanced in LPI as *MSCP*, encoding mitoferrin, which transports iron into the mitochondrial matrix (Shaw *et al.* 2006), was upregulated in the patients. In conclusion, it appears that the increased plasma zinc levels in LPI may be due to the enhanced haemolytic anaemia exceeding the incorporation of zinc into the newly-formed CA1 during reticulocytosis. Increased haemolytic anaemia is also supported by the elevated level of the *BLVRB* gene, which codes for biliverdin reductase which turns biliverdin, the product of haem breakdown, to bilirubin (Kapitulnik and Maines 2009). The expression of this gene in the LPI whole-blood cells may possibly originate from monocytes, as macrophages are mainly responsible for haemolysis.

In LPI, the upregulation of three genes encoding the enzymes of haem synthesis were detected: *ALAS2* (aminolevulinic acid synthase), *PBGD* (porphobilinogen deaminase) (microarray $\log_2$ FC 0.98), and *FECH* (ferrochelatase). Another important upregulated gene related to the haem regulation is *HRI* which encondes the haem-regulated initiation factor 2-alfa kinase expressed particularly in reticulocytes. It inhibits the excessive translation of alpha and beta globins by inactivating translation factor eIF-2alpha when the concentration of haem is decreased against globins. (Chen and London 1995, Han *et al.* 2001.)

The most abundant reticulocyte-specific group of genes upregulated in the patients was that of genes encoding erythrocyte membrane proteins that form the structure of the cell cytoskeleton (Tse and Lux 1999, Birkenmeier and Barker 2004). The following genes were the most upregulated in the microarray analysis ($\log_2$ FC value in parenthesis): *EPB42* (erythrocyte membrane protein band 4.2/palladin) (1.96), *ANK1* (ankyrin 1) (1.64), *SPTB* (beta-spectrin) (1.61), *EPB49* (erythrocyte membrane protein band 4.9) (1.41), *SLC4A1* (erythrocyte membrane protein band 3) (1.41), *MPP1* (membrane protein p55) (1.39), *GYPC* (glycophorin C) (1.38), *TPM3* (tropomyosin 3) (0.84) and *TMOD1* (tropomodulin 1) (0.84). However, the expression levels of the genes coding for two other important membrane proteins, actin and alpha-spectrin, were not altered according to the microarray data. In the study by Whitney and others (Whitney *et al.* 2003), it was seen that in the

whole-blood cells the expression pattern of a cluster of reticulocyte-specific genes correlated significantly with red cell distribution width (RDW) measuring the variability in the red blood cell size. This gene cluster includes many of the genes whose expression was also revealed to be altered in this study, for example *EPB42*, *SLC4A1*, *ANK1, TMOD* and *ALAS2*.

Upregulated consistent reticulocyte gene expression may, to some degree, be due to the increased reticylocytosis in response to haemolytic anaemia. However, as the expression level of *IFI27* was seen to differ considerably between the patients (Figure 8) it may prove that, at least at some level, the upregulation of the reticulocyte genes may derive from the changes at the single cell level. For example, the altered expression pattern of the above-mentioned genes coding for membrane proteins may either cause or reflect the abnormal morphology of the patients' erythrocytes.

5.5 Metabolic imbalance in LPI

5.5.1 Altered metabolite pattern in LPI (III)

The global polar plasma metabolite composition in the LPI patients was analysed using a GC-MS-based technique. In total, significantly ($q < 0.05$) changed levels were detected in 146 metabolites, 58 of which were fully identified (Table 4 in III). Of these 58, 36 had increased levels and 22 decreased levels in the LPI patients compared to the controls. The biochemical pathway analysis revealed that these metabolites participate in, for example, sugar metabolism (ascorbate and aldarate metabolism, galactose metabolism and starch and sucrose metabolism), energy metabolism (TCA cycle), amino acid metabolism (phenylalanine, tyrosine and tryptophan metabolism, valine, leucine and isoleucine metabolism, alanine, aspartate and glutamate metabolism, and glycine, serine and threonine metabolism) and fatty acid and lipid metabolism (fatty acid biosynthesis/β-oxidation, alpha linolenic and linoleic acid metabolism, and glycerolipid/glycerophospholipid metabolism).

In order to cluster both the LPI patients and controls according to the levels of the 58 changed metabolites, and also to cluster the metabolites, a heatmap with dendrograms was computed. The resulting hierarchical clustering demonstrated that the patient and control samples formed two separate clusters, which were further divided into smaller clusters (Figure 3 in III). The cluster of decreased metabolites in the LPI patients roughly consisted of two subclusters including sugar derivatives and amino acids (Figure 3 in III). The cluster of increased metabolites in the patients was divided into three subclusters (Figure 3 in III). The first subcluster was formed by myo-inositol, 2,5-furandicarboxylic acid (FDCA), 4-hydroxyphenylacetic acid (HPA), threonic acid, 2,4-dihydroxybutanoic acid, 3,4-dihydroxybutanoic acid, galactaric acid, fucose, galacturonic acid, glucopyranose derivative 1 and 2-deoxy-erythro-pentonic acid. The second subcluster consisted of increased amino acids, and the third subcluster contained saturated fatty acids: palmitic acid, stearic acid, lauric acid and myristic acid and unsaturated omega fatty acids: oleic acid, linoleic acid, linolenic acid, 11-eicosenoic acid and 9-tetradecenoic acid. Although it was seen that the highest essential fatty acid levels were concentrated in one particular patient subgroup, no connection between the upregulation of fatty acids and the clinical picture of these patients could be drawn.

In order to better understand the associations of metabolites, pairwise correlations of metabolites, NO, clinical laboratory variables, statin medication and supplementations were performed.

One of the most interesting findings was that α-KG and malic acid correlating with one another (r = 0.60), and aspartic acid and glutamic acid, also correlating with each other (r = 0.53), all had increased levels in LPI. These four metabolites are tightly linked to cellular energy production and urea cycle function. However, in LPI, urea cycle function is impaired due to the shortage of its intermediates arginine and ornithine, leading to a high systemic ammonia level after dietary protein loads. A high ammonia level in the brain is known to inhibit α-KG-dehydrogenase (α-KGDH), an enzyme that is needed in the TCA cycle to convert α-KG into succinyl CoA. This may further disturb the TCA cycle and cause α-KG to accumulate in the plasma, as seen in hyperammonaemic patients during hepatic coma (Ott *et al.* 2005). In LPI, the highly increased levels of both α-KG and glutamic acid may be markers of augmented ammonia clearance in the brain as elevated α-KG is known to result in the accelerated formation of glutamate and further glutamine from ammonia (Ott *et al.* 2005). Ott and others (Ott *et al.* 2005) have also suggested that since ATP production may be affected by an impaired TCA cycle, glycolysis must be enhanced, which may be seen in LPI as the reduced levels of glucopyranose (der2) and two fructose derivatives, indicating that their consumption is enhanced. To support this, the following correlations between α-KG and fructose derivative 2 (r = -0.45), glucopyranose derivative 2 and fructose derivative 2 (r = 0.53), and fructose derivatives 1 and 2 (r = 0.67) were observed. α-KG, malate, aspartate and glutamate are also each a part of the glycolysis-linked malate-aspartate shuttle needed for transporting NADH into the mitochondria for ATP production. However, if α-KGDH is inhibited, aspartate can provide malate for the TCA cycle, thus decreasing its availability for the malate-aspartate shuttle and affecting the NADH levels. High ammonia conditions may lead to a compensatory mechanism for energy production in the TCA cycle by the breakdown of amino acids valine and isoleucine, and leucine and isoleucine for the synthesis of the TCA cycle intermediates succinyl-CoA and acetyl-CoA, respectively. However, in LPI, the levels of these branched-chain amino acids (BCAAs) are initially reduced due to the protein malnutrition, as are the levels of 2-oxoisovaleric acid and 4-methyl-2-oxovaleric acid, the direct breakdown products of valine and leucine, respectively. The following correlations of the BCAAs and their catabolites were detected: valine correlated with 2-oxoisovaleric acid (r = 0.71), 4-methyl-2-oxovaleric acid (r = 0.66), leucine (r = 0.75) and isoleucine (r = 0.57); leucine and isoleucine correlated with each other (r = 0.85) and with 4-methyl-2-oxovaleric acid (r = 0.70 and r = 0.63, respectively); and leucine and 2-oxoisovaleric acid (r = 0.41) were seen to correlate. This supports their associations in this common process. In LPI, the elevated ammonia level also leads to increased orotic acid excretion in the urine (Rajantie 1981). However, it is possible to inhibit orotic aciduria to some extent by citrulline supplementation (Rajantie 1981). In the current study, exogenous citrulline correlated inversely with uridine (r = -0.53), a subsequent intermediate in the pyrimidine pathway following orotic acid; therefore, it likely decreases the elevated level of uridine in LPI. There may be further evidence for the impaired TCA cycle in LPI as it seems that the exploitation of glycerol by oxidation in the TCA cycle (Bortz *et al.* 1972) may be decreased, suggested by the elevated level of glycerol and the decreased levels of its oxidised products, highly correlating glyceric acid and tartronic acid (r = 0.87) (Gil *et al.* 2011). The above-mentioned pairwise correlations are presented in Figure 4 in III.

Clues suggesting prevailing oxidative stress were provided by this study as the increased levels of pyroglutamic acid, glutamic acid, cysteine and glycine, all the metabolites of the γ-glutamyl cycle needed for the GSH synthesis (Meister and Tate 1976), were observed in the LPI patients. Oxidative stress in general involves excessive ROS production and their decreased clearance by antioxidants, such as GSH (Valko *et al.* 2007). In addition, it was shown that pyroglutamic acid correlated with s-methylcysteine (r = 0.50) (Figure 4 in III), also increased in LPI, which in several studies has been observed to have antioxidant effects (Wassef *et al.* 2007), and to induce GSH levels in the kidney (Yin *et al.* 2007) and the GSH peroxidase activity in the plasma (Huang *et al.* 2004). According to these results, it may be hypothesised that the consumption of antioxidants and their increased need in LPI is accelerated in response to increased oxidative stress.

5.5.2 Lipid metabolism in LPI based on the lipidome, metabolome and transcriptome analyses (I, III)

The LC-MS-based global lipidome analysis revealed a total of 447 lipids with significantly ($q < 0.05$) changed levels in the LPI patients, and of these 244 could be identified. Of the identified lipids, 198 had increased and 46 decreased levels in the LPI patients compared to the controls. Further clustering of the identified lipids grouped the lipidome data into eight lipid clusters (LC1-LC8) (Table 6 in III). As expected, the lipid cluster division mainly followed the functional and structural lipid groups. LC1 consisted of ceramides, phosphatidylcholines (PCs), lysoPCs, phosphatidylethanoamines (PEs) and sphingomyelins (SMs). LC3 contained mainly PCs with polyunsatured fatty acids. The rest of the clusters (LC2 and LC4-LC8) included TGs containing monounsaturated, polyunsatured and saturated fatty acids. LC6 and LC8 consisted mainly of long-chain TGs, and LC7 included short-chain TGs. Except for LC3, which was significantly decreased, all the lipid clusters were significantly increased in the patients. To further scrutinise the data, the associations of the eight lipid clusters, NO, laboratory findings, statin medication and supplementations were tested using pairwise correlations (Figure 5 in III). It was seen that the routine laboratory TGs correlated positively with LC1 (r = 0.64) and all lipid clusters containing TGs, except for LC5. These lipid clusters also correlated highly with one another. As expected, a positive correlation was seen between LC1 and LC3 containing PCs (r = 0.63) with increased and reduced levels, respectively.

The metabolome analysis revealed that fatty acids were elevated in the LPI plasma and correlated positively with glycerol, the levels of which were increased in the patients, and negatively with glycerol-3-phosphate (G3P) with decreased levels in the patients. It has been observed that combined hyperlipidemia with high TG and cholesterol levels detected in LPI is not explained merely by dietary fat consumption, and it manifests even with statin medication (Tanner *et al.* 2010). Based on the results of the three 'omics' used in this study, it can be proposed that the LPI patients may suffer from serious lipid overload and hepatic steatosis, which was detected in some liver biopsies decades ago but not examined in LPI since then. It is known that elevated TGs and low HDL levels, also seen in LPI, are detected in the plasma of hepatic steatosis patients (Targher *et al.* 2005). It seems that in LPI, elevated TG, fatty acid and glycerol plasma levels may indicate an increased synthesis and release of TGs from the liver in very low-density lipoproteins (VLDLs). This may be a consequence of accelerated lipolysis and release of free fatty acids from the adipose tissue and their subsequent conversion along with glycerol to TGs in the liver. The uptake of fatty acids into the liver from the

circulation and lipogenesis are induced by nuclear receptor PXR (pregnane X receptor) acting as a transcription factor (Ihunnah *et al.* 2011). Interestingly, the whole-blood transcriptome study revealed that the genes activating PXR have an altered, mainly upregulated, expression pattern in LPI, indicating that lipogenesis could be accelerated in LPI. Another important transcription factor in the lipid metabolism is PPARα (peroxisome proliferator-activated receptor alpha), regulated by free fatty acids, which induces production of enzymes needed in fatty acid β-oxidation (Nguyen *et al.* 2008). The transcriptome analysis suggests that β-oxidation of free fatty acids and, therefore, energy combustion could be defective in LPI, as the genes activating PPARα were mainly downregulated in the patients. Reduced fatty acid oxidation further intensifies the TG synthesis and induces hepatic steatosis, ultimately leading to the apoptosis of hepatocytes (Feldstein *et al.* 2003, Reddy and Rao 2006). Dying hepatocytes release TGs, which, along with the unmetabolised fatty acids, leads to lipotoxicity and further steatohepatitis. As the liver is injured by toxic lipids, it is also susceptible to secondary assaults by ROS, gut-derived endotoxins and cytokines leading to oxidative stress and inflammation which further results in the activation of stellate cells and hepatic fibrosis (Reddy and Rao 2006, Del Ben *et al.* 2014). The genes included in the hepatic fibrosis/hepatic stellate cell activation pathway were shown to have an altered expression pattern in the patients, which supports the occurrence of hepatic fibrosis in LPI.

Furthermore, increased plasma levels of ceramides, lysoPCs and SMs detected in LPI are all known to associate with hepatic steatosis (Natarajan *et al.* 2006, J. F. Li *et al.* 2014, Xia *et al.* 2015). In addition, decreased levels of PCs, the main phospholipid components of all lipoprotein classes (Cole *et al.* 2012), were detected in the patients. This is consistent with the observed reduced synthesis of PCs in the liver (Natarajan *et al.* 2006, Puri *et al.* 2007) and a deficiency of dietary methionine (Rinella and Green 2004, Corbin and Zeisel 2012), the PC synthesis intermediate also reduced in LPI, in the hepatic steatosis patients. As it has been shown that the plasma PC levels may directly reflect the hepatic PC synthesis (Pynn *et al.* 2011), reduced levels of certain PCs in the LPI plasma may be a marker of hepatic steatosis.

Intriguingly, it appears that exogenous citrulline may have a beneficial role in inducing lipolysis in LPI as citrulline supplementation correlated inversely with G3P (r = -0.46), routine laboratory TGs (r = -0.51), and LC1 (r = -0.39), LC4 (r = -0.45) and LC8 (r = -0.44) containing ceramides, lysoPCs, PCs, PEs, SMs and TGs, and positively with glycerol (r = 0.39) and ethanolamine (r = 0.51), a component of PEs.

In conclusion, these data suggest that lipid metabolism in the liver, normally balanced between fatty acid and TG synthesis by lipogenesis (energy intake) and degradation by lipolysis and further fatty acid β-oxidation (energy combustion), is disrupted in LPI. This is supported by an altered expression pattern of lipid-regulating genes, altered levels of metabolites directly related to the synthesis and catabolism of lipids and hepatosplenomegaly, often observed in hepatic steatosis.

5.5.3 Genes, metabolites and lipids associating with CKD in LPI (I, III)

A careful follow-up has revelaled that over half of the Finnish LPI patients suffer from renal insufficiency as indicated by high plasma creatinine and cystatin C levels, and high urine β2-microglobulin levels. In order to assess the kidney function of the cohort of 26 patients included in the substudies II an III, the eGFR values were calculated. By using predefined cut-off values, the patients were divided into five different CKD stages (Table 1 in III). One patient was classified as a stage 5 CKD patient suffering from an end-stage kidney failure. Three patients had stage 4 CKD with severely reduced kidney function, and four patients were classified as stage 3 CKD patients suffering from moderately reduced kidney function. Six patients had stage 2 CKD with mildly reduced kidney function. Of these, patient 17 had undergone a kidney transplant. Normal kidney function (CKD1) was observed in 12 patients. The hierarchical clustering grouped the patients with CKD into two clusters: The first consisted of stage 3-5 CKD patients and one CKD2 patient, and the second included the rest of stage 2 CKD patients and one CKD3 patient (Figure 3 in III). Interestingly, it was seen that the patients with the most severely reduced kidney function (CKD3-5) had the highest concentration levels of myo-inositol, FDCA, HPA, threonic acid, 2,4-dihydroxybutanoic acid, 3,4-dihydroxybutanoic acid, galactaric acid, fucose, galacturonic acid, glucopyranose derivative 1 and 2-deoxy-erythro-pentonic acid (highlighted with a red rectangle in Figure 3 in III). As it became clear that over half of the patients included in this study suffered from CKD of different stages and that the patients with CKD shared common metabolites, one of the foci in the amino acid, metabolome and lipidome analyses was to define more closely those markers associating with CKD. Therefore, the correlation of eGFR with the concentration levels of significantly changed amino acids, metabolites and lipid clusters was tested (Figure 4 in III). Metabolites with the most significant correlations ($P < 0.001$) with eGFR were myo-inositol ($r = -0.93$), galactaric acid ($r = -0.67$), threonic acid ($r = -0.67$), HPA ($r = -0.80$), FDCA ($r = -0.72$) and indole-3-acetic acid (IAA) ($r = -0.73$). Of the amino acids, homocitrulline ($r = -0.70$) and BAIBA ($r = -0.77$) correlated most significantly ($P < 0.001$) with eGFR. Figure 10 shows an example of FDCA and threonic acid correlating with eGFR in the current LPI patient cohort. In order to discover whether the metabolites were uniquely elevated in the patients with CKD, a *post-hoc* analysis of the above-mentioned eight metabolites was performed between the patients with and without CKD, and controls. The analysis confirmed that all the metabolites but homocitrulline had significantly elevated levels in the patients with CKD compared to those without (Table 5 in III).

Of the eight eGFR-correlating metabolites in this study, myo-inositol is the most known to be associated with renal dysfunctions, such as uremia (Bultitude and Newham 1975, Vanholder *et al.* 2003), membranous nephropathy (Gao *et al.* 2012) and CKD (Holub 1986, Zhao 2013). Therefore, it was unsurprising to discover that it also related to LPI-associated CKD. As the metabolite data already indicated that oxidative stress may be a phenomenon associated with LPI, excessive ROS production is also often connected to CKD (Cachofeiro *et al.* 2008, Massy *et al.* 2009). It is interesting that threonic acid, which has been observed in addition to myo-inositol in some kidney diseases (Gao *et al.* 2012, Shah *et al.* 2013), is an oxidised degradation product of ascorbic acid (Isbell and Frush 1979, Englard and Seifter 1986), a well-known antioxidant (Massy *et al.* 2009). As it is known that increased oxidised forms of antioxidants in the plasma are good indirect markers of oxidative stress (Massy *et al.* 2009), it may be that in the LPI patients with CKD, elevated

threonic acid indicates ongoing oxidative stress. Therefore, it would be meaningful to measure the concentrations of plasma ascorbic acid in LPI, as its decreased level in the plasma has been observed in CKD (Takahashi *et al.* 2011).

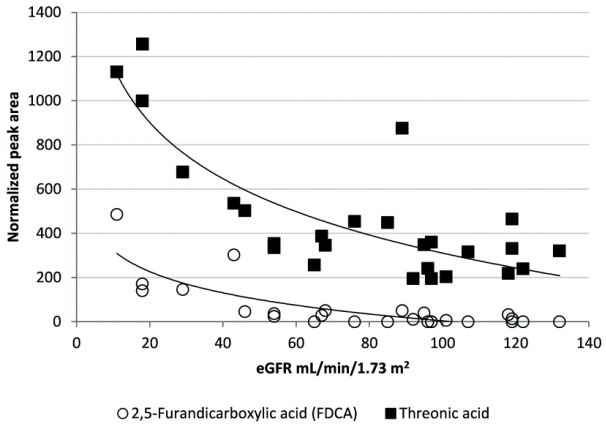

Figure 10. Correlations between estimated glomerular filtration rate (eGFR) and two metabolites increased in the LPI patients. Logarithmic regression lines between the 26 patient samples are shown.

The HPA and IAA levels were also increased in the patients with CKD. These metabolites are processed from dietary amino acids by intestinal bacteria. An increased level of HPA, the breakdown product of phenylalanine and tyrosine (van der Heiden *et al.* 1971, Curtius *et al.* 1976, Chalmers *et al.* 1979), has been detected in the urine in, for example, the bacterial overgrowth syndrome (Chalmers *et al.* 1979) and membranous nephropathy (Gao *et al.* 2012). IAA, the catabolite of tryptophan (Weissbach *et al.* 1959, Chung *et al.* 1975), is a uremic solute (Ludwig *et al.* 1968, Vanholder *et al.* 2003, Yavuz *et al.* 2005) known to induce inflammation and endothelial dysfunction in CKD (Gondouin *et al.* 2013, Dou *et al.* 2015). Surprisingly, when compared to the controls, IAA levels were decreased in the patients without CKD, suggesting that the reduced availability of essential amino acid tryptophan due to protein malnutrition could be a general limiting factor decreasing IAA production by the gut bacteria in LPI. In the patients with CKD, however, the highly increased levels of both HPA and IAA, the latter almost at the same level as in the controls, may be the result of their impaired renal clearance, but also, perhaps, by the altered activity of intestinal bacteria.

Another metabolite of gut bacteria metabolism found to be increased in LPI-associated CKD was FDCA. There is scarcely any information available on this metabolite; however, it is known to be a normal constituent of human urine (Mrochek and Rainey 1972, Pettersen and Jellum 1972) and plasma (Lewkowski 2001), although it was barely detected in the control and non-CKD patient samples of this study. It is not completely clear how FDCA is metabolised in humans; it is most possibly derived, by the activity of gut bacteria, from glucuronidated furan derivative 5-hydroxymethylfurfural (M. Zhao *et al.* 2014) produced from sugars by strong heating during food preparation (Pettersen and Jellum 1972, Perez Locas and Yaylayan 2004). Galacturonic acid, which also correlated with eGFR in LPI (r = -0.57, P = 0.003), is the main component of fruit-derived

pectin fermented by human intestinal bacteria to produce galacturonic acid and further galactaric acid and FDCA (Endress and Mattes 2012). Interestingly, galactaric acid was also one of the eight elevated metabolites most significantly correlated with eGFR. In contrast to this result, its decreased level has been associated with diabetic nephropathy patients by Hirayama and others (Hirayama *et al.* 2012). It remains to be seen whether galactaric acid and FDCA, with high levels in LPI CKD patients, could possibly be used as novel biomarkers for CKD.

These results suggest that in addition to the impaired renal clearance of metabolites in LPI, the patients may also suffer from an altered intestinal biochemical environment and alterations in the activity of the gut bacteria. This may not be surprising as LPI patients experience protein malnutrition and an amino acid imbalance, and it is indeed proven that dietary modifications and medicinal interventions can cause altered microbial intestinal environment and result in changes in metabolite composition in the plasma and urine (Vaziri 2012, Griffin *et al.* 2015). In addition, malnutrition and altered gut microbe composition have been detected in advanced CKD, which may lead to the formation of pro-oxidant and proinflammatory byproducts contributing to uremic toxicity, inflammation and cardiovascular complications (Stenvinkel *et al.* 1999, Vaziri 2012). Protein energy malnutrition is also connected to oxidative stress and inflammation (Stenvinkel *et al.* 1999, Jain *et al.* 2013); as a result, it is evident that these phenomena and CKD are all linked tightly together.

Several amino acids correlated with the reduced kidney function, BAIBA most significantly. Its level is increased in the serum in uremia influencing the development of uremic toxemia (Gejyo *et al.* 1977, Kraus and Kraus 2001, Jaisson *et al.* 2012). Quite recently, it was reported that BAIBA induces white fat browning (Roberts *et al.* 2014), and its plasma concentration was demonstrated to increase with exercise, whereas a low plasma level associated with metabolic risk factors such as high total cholesterol, TGs and body mass index (Roberts *et al.* 2014). Thefore, in the LPI patients, especially those with CKD, the increased level of BAIBA may be the system's attempt to protect itself from metabolic perturbations.

It is known that cardiovascular disease and endothelial dysfunction (Stenvinkel *et al.* 1999, Go *et al.* 2004, Cachofeiro *et al.* 2008) with high VLDL, LDL and low HDL cholesterol levels (Attman *et al.* 1993, Prinsen *et al.* 2003, Batista *et al.* 2004, Kwan *et al.* 2007) as well as intracellular lipid overload (Lee 2011) are commonly associated with CKD. Yet, in the patient cohort included in this study, the routine laboratory lipid values did not differ significantly between the patients with and without CKD (Table 2 in III), and they did not correlate with eGFR either (Figure 5 in III). However, it was seen that the lipid clusters LC6 (r = -0.54) and LC8 (r = -0.60) containing elevated levels of long-chain TGs in the patients correlated inversely with eGFR ($P < 0.01$). This is in accordance with the results of Druml and others (Druml *et al.* 1992), who have demonstrated that during acute renal failure the elimination of long-chain TGs is considerably decreased. This suggests that different lipid classes should be monitored more carefully in LPI in the future.

It has been proposed that lipid alterations in CKD could be a consequence of several factors, including lipoprotein oxidation, impaired catabolism of lipoporoteins and their elevated TG content due to the decreased lipoprotein and hepatic lipase functions (Batista *et al.* 2004, Kwan *et al.* 2007, Vaziri 2009). Further, it is suggested that in CKD, the uptake of TG-rich VLDLs by glomerular

cells is increased, and that the increased accumulation of fatty acids in podocytes leads to apoptosis and glomerulosclerosis (Lee 2011). As apoptosis is known to promote the loss of renal epithelial cells (Sanz *et al.* 2008), it may be initially induced by oxidative stress (Kannan and Jain 2000). In LPI, both of these phenomena could plausibly occur in CKD, as indicated by the increased oxidation of ascorbic acid and increased expression level of genes related to stress response and pathogenesis, and most interestingly, by the altered expression pattern of genes related to the apoptosis of kidney cell lines. In addition, the changed expression profile of genes activating nuclear receptors PXR/RXR and PPARα/RXRα, expressed in the kidney in addition to the liver, may have a role in renal cholesterol clearance and detoxification, and oxidative stress, inflammation, fatty acid β-oxidation and lipotoxicity, respectively (Tovar-Palacio *et al.* 2012).

Statin medication correlated inversely with eGFR (r = -0.46) and positively with urine proteins (r = 0.57), which could be due to the fact that statin medication-requiring hyperlipidemia is detected widely in CKD. However, of the studied lipids, only LC6 and LC8 were shown to associate with CKD in LPI, and statins were observed to correlate positively with LC8 (r = 0.48), indicating that the patients with high levels of long-chain TGs are also those who receive statins for hyperlipidemia. What is surprising is that statins do not seem to ameliorate hyperlipidemia in the current patient cohort. Nevertheless, this may be due to the fact that at the time the samples were collected, not all the patients with combined hyperlipidemia were medicated, whereas some patients without altered lipid values received statins.

5.6 Study strengths and limitations

The patients included in this study represent one of the largest and well-characterised LPI patient cohorts in the world. The global metabolome and lipidome analyses performed for the first time in LPI patients provided large-scale systemic information about the alterations in metabolic and lipid pathways. As for metabolomics and lipidomics, the transcriptomics study was performed for the first time on LPI patients, and it revealed, in addition to the obvious blood cell-related gene expression changes, altered levels of transcripts reflecting those of the actual LPI target tissues, such as the kidney and liver. However, if it is necessary to know from which cell types the mRNA expressions are derived from, specific cell samples are warranted.

Studies on LPI monocytes, MDMs and AMs remain few, concerning mainly the transport activies of the system y^+L and y^+ transporters and the reduced phagocytosis properties of MDMs (Barilli *et al.* 2010, Barilli *et al.* 2012). The current results of the TLR signalling alterations and NO production have provided valuable information about the LPI macrophages confronting pathogens, both viral and bacterial, and also arginine metabolism, respectively. MDMs used in this study are easy to obtain; however, as the pathogenesis of LPI is suggested to be partly caused by defective macrophages perpetuating inflammation in the target tissues, it should be recognised that MDMs are not tissue macrophages but derived from differentiated cultured monocytes. Therefore, it is expected that MDMs do not directly represent tissue macrophages. It is known that *in vitro*-derived macrophages can generate different responses from macrophages obtained *in vivo* in humans

(Thomas and Mattila 2014). For example, iNOS or arginase activity in macrophages has been identified by some groups but not by others. In part, this may be due to the differences between MDMs and tissue macrophages used in these studies, but also some groups use the detection of enzyme protein rather than enzyme activity as an evidence of enzyme expression. Therefore, although the presence of the iNOS protein or the *NOS2* mRNA, as in our study, is not detectable, it may be that only a minuscule amount of a protein is actually needed for the iNOS activity. (Thomas and Mattila 2014.)

It is known that LPS is a strong inducer of the TLR4-mediated immune responses; however, in this study, responses to the LPS stimulation remained relatively low. It has been demonstrated that human *in vitro*-derived macrophages show variability in their responses to LPS between genetically diverse individuals (Thomas and Mattila 2014). In general, human macrophages are not as responsive to LPS as mouse macrophages, possibly due to the lower environmental exposure of humans to LPS. Further, it is known that human macrophages take a longer time to respond to the stimulatory factors *in vitro* than mouse macrophages, and it is thought that time-points in experiments using human MDMs may actually have been too short to detect the response. (Thomas and Mattila 2014.) This may also be true in this study, in which only low levels of type I IFNs and IL-12 after CpG DNA and LPS stimuli, respectively, were detected. Therefore, it seems that a longer PAMP exposure time than 24 h may be required for some cytokines. In addition, as it has been shown that IL-23 instead of IL-12 is the most important cytokine mediating inflammation by macrophages (Verreck *et al.* 2004), the levels of IL-23 should also be measured in LPI.

One important point should be taken into account when interpreting the current MDM results: the MDMs were cultured in a perfectly normal nutritional situation, including all essential amino acids. Therefore, this study indicates how LPI MDMs with a CAA transport defect and supposedly a deficiency of intracellular CAAs mediate responses to microbial infections in an otherwise normal nutritional state. It would be tempting to scrutinise MDMs in a condition deficient of CAAs and essential amino acids, thus mimicking the situation in the systemic environment of LPI. It should also be noted that one can only speculate on the actual cellular concentration of arginine in LPI monocytes or macrophages in culture conditions and *in vivo* due to a low plasma level of arginine and its transport defect until it is actually measured. To better understand this, the influx and efflux transport activities of CAAs should be examined in MDMs and *in vivo* macrophages. In addition, in order to fully cover the TLR signalling pathways in LPI macrophages, one must not only rely on gene expression and cytokine secretion changes, but also scrutinise the molecular mechanisms at the protein expression and activation levels.

6 SUMMARY AND CONCLUSIONS

6.1 Summary of the main results

Since the description of LPI in 1965, considerable research has been conducted in order to clarify the pathophysiology of LPI. Recently, it has been shown that despite proper treatment, patients may develop severe complications that may manifest even decades after diagnosis. The exact mechanisms behind renal, pulmonary, hepatic, immunological and haematological complications in addition to consistent combined hyperlipidia remain unknown. These complications may be life-threatening as they can be manifested simultaneously and therefore result in a multiorgan failure. The aim of this study was to scrutinise the effect of the CAA transport defect on the systemic gene expression and metabolite levels and also macrophage responses in a large and well-examined patient cohort, and therefore to explore the cellular processes with which the LPI_{Fin} mutation tampers.

The main results of the current study may be summarised as follows:

- The whole-blood transcriptomics study revealed altered levels of transcripts participating in immune responses, apoptosis and pathways related to hepatic and renal lipid metabolism, hepatic fibrosis and cholestasis and also apoptosis or necrosis of kidney cells. The transcriptome data was expected to reveal unique gene expression patterns between the patients with symptoms of different severity. Unfortunately, unambigious transcript-phenotype correlations could not be detected.
- The targeted amino acid analysis showed that in the LPI plasma the levels of CAAs and essential amino acids were decreased and non-essential ones were increased. The LPI gene *SLC7A7* was highly downregulated in all studied cell types, but its upregulation was seen after viral induction in MDMs. *SLC7A6* did not compensate for the reduced *SLC7A7* expression in any of the cells studied. *SLC1A5*, *SLC7A5* and *SLC7A1* were upregulated in the LPI whole-blood cells. The PAMP-stimulated *SLC7A1, SLC7A5*, *SLC7A6* and *SLC3A2* were downregulated in the LPI MDMs.
- High upregulation of erythrocyte-related genes encoding enzymes, blood group antigens, transporters and proteins participating in erythrocyte membrane structure, erythropoiesis and haem synthesis may partly explain anaemia and the morphological changes of erythrocytes in LPI.
- In the whole blood, high upregulation of *IFI27* and considerably reduced levels of activated antigen presenting $CD3^+$ $HLA\text{-}DR^+$ T cells were seen in the patients.
- In LPI MDMs, activation of the TLR2/1 pathway led to the increased expression of the *TLR1*, *TNF*, *IL12B, STAT4* and IFN-γ receptor genes and the elevated secretion of IL-12, TNF-α and IL-1RA. Stimulation of the TLR4 pathway led to the upregulation of *TLR4*, *IFNB1* and *IFNGR2* and the increased production of IL-12 and IL-1RA. The TLR9 pathway activation resulted in the downregulation of *TLR9*, *IRF7*, *IRF3, IFNB1* and *SOCS1* and the reduced secretion of IFN-α and IFN-β. NO levels were decreased in the LPI MDM medium.

- Plasma levels of chemoattractant CXCL8 (IL-8), CXCL9 (MIG) and CXCL10 (IP-10) were increased in the LPI patients. Further, elevated levels of CXCL9 and CXCL10 correlated with the reduced glomerular function.
- Slightly increased NO levels in the LPI patients' plasma showed inverse correlation with eGFR, suggesting that arginine trapped inside the kidney tubule cells may accelerate NO production in the kidney. Citrulline supplementation did not correlate with the increased NO plasma levels nor associate with the CKD stages.
- The global metabolomics study revealed changes in the plasma metabolites participating in the sugar, amino acid, fatty acid and TCA cycle metabolisms, for example. The levels of eight metabolites (myo-inositol, threonic acid, FDCA, galactaric acid, HPA, IAA, BAIBA and homocitrulline) correlated to a considerable degree with the reduced glomerular function in CKD.
- The global lipidomics analysis showed dysregulation of TGs, PCs, lysoPCs, PEs, SMs and ceramides. Long-chain TGs correlated with the reduced glomerular function.

6.2 New hypotheses on the LPI pathophysiology

As a result of this thesis, novel genes, cytokines and metabolites participating in different biochemical pathways associated with LPI were detected. Based on these results, the following hypotheses summarised below and in Figure 11 can be put forward concerning the pathophysiology of LPI.

- Protein malnutrion and amino acid deficiency in LPI results in a reduced plasma level of the essential amino acids and an increased level of the non-essential ones by either an enhanced *de novo* amino acid synthesis or protein breakdown. As the plasma pool is short of essential amino acids, especially leucine, the mTOR system may be inactivated and autophagy induced to aid cellular survival. It is possible, however, that the system attempts to prevent this by the upregulation of the ASCT2 and LAT1 transporters for glutamine and large branched-chain and aromatic NAAs, respectively. Increased ammonia levels after dietary protein loads may affect the TCA cycle and malate-asparate shuttle and further enhance glycolysis. Overall, it may be that the body attempts to increase energy production in LPI.
- Immunological defects in LPI, including severe viral and bacterial infections, may result from the impaired TLR signalling in macrophages. The disruption in the TLR pathways is seen as an overproduction of proinflammatory and anti-inflammatory cytokines, and this indicates that in LPI the response to bacterial infection is inappropriately increased and that, subsequently, the anti-inflammatory response is accelerated. Therefore, during bacterial infection, macrophages may perpetuate inflammation in their current residence tissue. The reduced secretion of IFN-α by the LPI macrophages confronting viral DNA suggests an impaired response to viruses which may directly contribute to the severity of viral infections, such as *Varicella*, seen in some LPI patients. Further, the NO needed for the pathogen destruction is reduced in macrophages, suggesting, along with the TLR result, that, in contrast

to the prevailing hypothesis, intracellular arginine and lysine reservoirs may actually be decreased in LPI macrophages due to an influx, not efflux, defect by faulty y^+LAT1 and further reduced plasma levels of CAAs.

- Ongoing systemic inflammation and oxidative stress may be constant conditions in LPI as suggested by increased chemoattractant chemokine and antioxidant glutathione synthesis metabolite levels in the plasma. In the patients with CKD in particular, leukocytes may be attracted to the injured kidney to maintain the inflammation, and antioxidant defence could be enhanced by the oxidation of ascorbic acid.
- Urea cycle dysfunction, hepatosplenomegaly and combined hyperlipidemia are the main liver-associated defects consistently detected in LPI. There is severe lipid overload, along with the possibly increased uptake of fatty acids by hepatocytes and lipogenesis, and reduced lipolysis and fatty acid β-oxidation by an altered activation of nuclear receptors. This may lead to hepatic steatosis, lipotoxicity, steatohepatitis, apoptosis of hepatocytes, oxidative stress, inflammation, and further to hepatic fibrosis and cholestasis. Overall, as it is known that muscles utilise carbohydrates as their principle energy source, it may be that the ultimate driving force for the lipid overload in LPI could be the low carbohydrate consumption by the minute muscle tissue and the eventual conversion of the excess carbohydrates into fat. Therefore, hepatic steatosis may be a constant condition associating with hepatosplenomegaly and, further, with HLH in LPI.
- The defective CAA export causing an increased level of arginine in the kidney tubule cells, the main site for arginine synthesis, seems to enhance NO production and impair the kidney function, especially that of the glomerulus, and may further decrease the level of circulating arginine, depleting it from other cells. The toxicity of NO, inflammation, oxidative stress and the altered expression of genes related to renal apoptosis or necrosis could lead to the apoptosis of kidney cells. Further, apoptosis and glomerular injury may result from the uptake of TGs by glomerular cells and an increased accumulation of fatty acids in podocytes. Long-chain TGs in particular seem to be involved in CKD in LPI. An altered activation of lipid-regulating nuclear receptors may result in reduced renal cholesterol clearance and fatty acid β-oxidation and, subsequently, increased lipotoxicity. This may further cause tubulopathy and glomerulonephritis which lead to reduced GFR and the accumulation of uremic toxins. Metabolites of intestinal bacterial sugar and amino acid metabolism are also increased, indicating that protein malnutrition may cause changes in the gut microbe environment. In contrast to earlier suggestions, citrulline supplementation increasing arginine synthesis does not seem to be a causative agent in CKD or to increase NO levels, which may be due to the decreased citrulline uptake by the kidney cells in CKD generally. Therefore, as CKD seems to be a relatively recent complication in LPI, it may actually be a result of the more careful treatment and improved protein tolerance of the patients leading to a longer life expectancy than earlier and allowing CKD to develop over time.
- The aetiology of PAP may be caused by similar processes to those of CKD and hepatic steatosis. Lipid and cholesterol accumulation has already been seen in LPI AMs, and systemic inflammation and oxidative stress may further induce the processes leading to this severe lung disease.

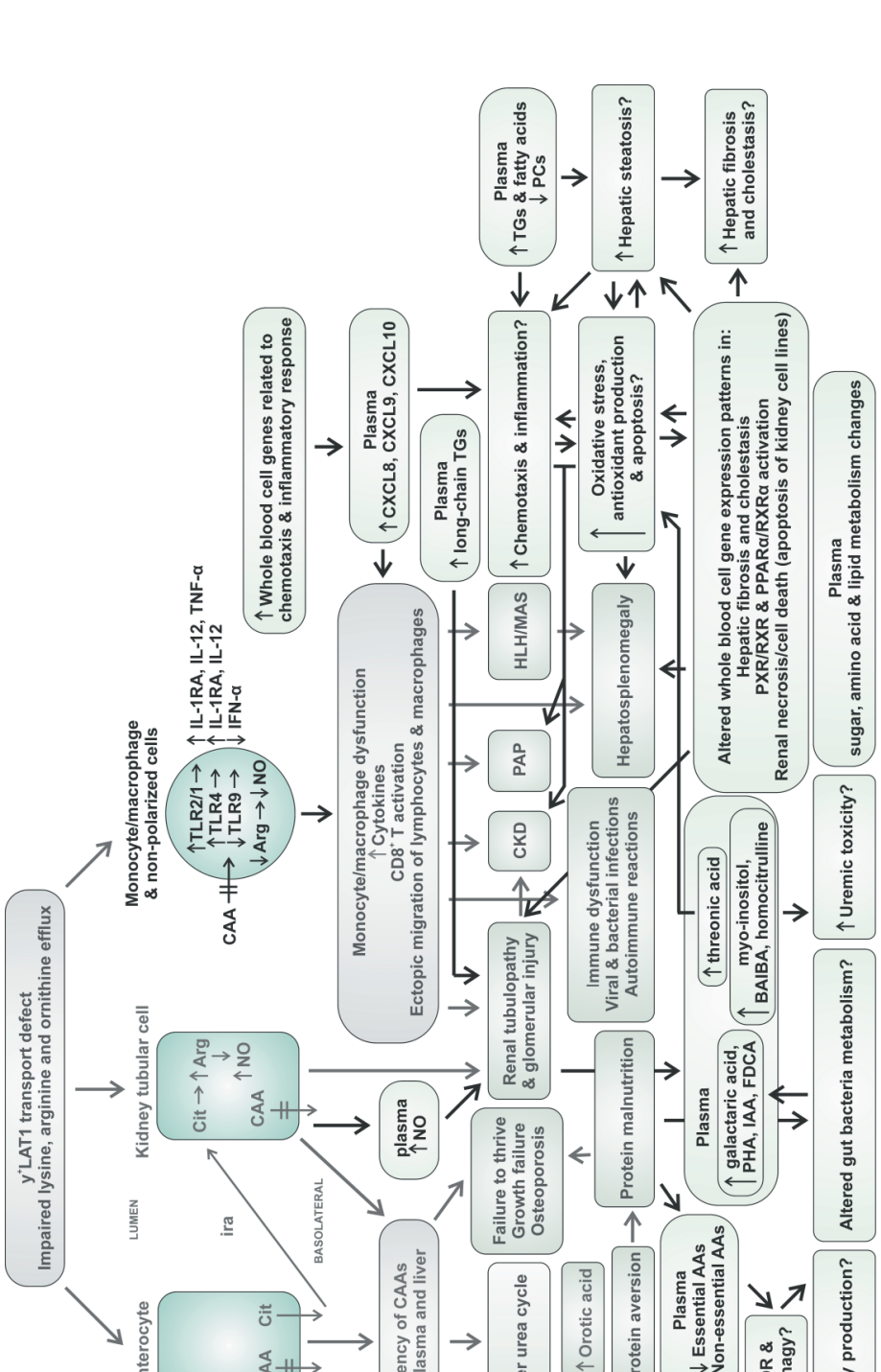

Figure 11. The revision of the model and pathways in the pathophysiology of LPI based on the results of the thesis. CAA, cationic amino acid; Cit, citrulline; ira, intestinal-renal axis; Arg, arginine; NO, nitric oxide; NH_3, ammonia; CKD, chronic kidney disease; PAP, pulmonary alveolar proteinosis; HLH, haemophagocytic lymphohistiocytosis; MAS, macrophage activation syndrome; TG, triacylglycerol; PC, phosphatidylcholine; PHA, 4-hydroxyphenylacetic acid; IAA, indole-3-acetic acid; FDCA, 2,5-furandicarboxylic acid.

6.3 Future aspects and challenges

This study has provided a considerable quantity of new information aiding in elucidating the molecular mechanisms and pathways behind severe complications in LPI. It may be possible that in the future the prognosis of kidney function in the CKD patients could be predicted by measuring the plasma marker metabolites. In addition, *IFI27* would be the most promising whole-blood gene marker for predicting alveolar and renal complications. Since oxidative stress seems to be associated with LPI, especially in those patients with CKD, it would be rational to measure oxidative stress markers (e.g. GSH) and to study whether the patients suffer from a shortage of ascorbic acid.

Although the whole blood is a highly useful, easy to obtain and noninvasive sample material for molecular studies offering a wide range of information about different complications, research on specific cells and tissues, such as the kidney and liver, is still warranted in order to fully understand the pathophysiology behind the disease. Histological examinations of the kidney and liver have been performed previously in LPI, but gene expression, cytokine, metabolite and lipid levels should also be scrutinised in these tissues. Further, the urinary global metabolome could offer more knowledge of the kidney function in LPI. As this study suggests that protein malnutrion may lead to an altered intestinal microbe metabolism, it would be interesting to examine the intestinal microbial composition of the patients. Amino acids are at the centre of LPI research; therefore, the characteristics of the amino acid transport in LPI macrophages *in vitro* and *in vivo* should be scrutinised in order to clarify whether the CAA transport defect is influx- or efflux-directed, thus causing a deficit or excess of intracellular arginine and lysine reservoirs, respectively. In addition, possible mTOR inactivation and induction of autophagy in LPI due to the low plasma NAA levels should be studied carefully. Finally, this study has revealed new molecular mechanisms and pathways contributing to the life-thereatening complications in LPI; however, more fine-tuned molecular studies are needed to resolve the complex clinical picture of LPI.

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APPENDIX

Appendix Table 1. The primer sequences for qRT-PCR used in the study.

Primer name	The primer sequence 5' → 3'	Cell type studied			
		MDM	PBMC	Whole-blood cells	Reticu-locyte
SLC1A5 Forward	CCTCATCTACTTCCTCTTCACC	x	x	x	x
SLC1A5 Reverse	GCCACGCCATTATTCTCCTC	x	x	x	x
SLC7A1 Forward	CCGAGAGCAAGACCAAGC	x	x	x	x
SLC7A1 Reverse	AAGCCTATCAGCATCCACAC	x	x	x	x
SCL7A5 Forward	CGTGGACTTCGGGAACTATC	x	x	x	x
SLC7A5 Reverse	GAGCCTGGAGGATGTGAACA	x	x	x	x
SLC7A6 Forward	CAGATGTCCTTAGCAGTGATGC	x	x	x	x
SLC7A6 Reverse	ACCTTGATGAAGCAAAGATGGAT	x	x	x	x
SLC7A7 Forward	TTGTGGCTGCTTCTAGGCTTTTC	x	x	x	x
SLC7A7 Reverse	CACTGGTGTGAACCGCTCAAC	x	x	x	x
SLC3A2 Forward	ATCAAGGTGGCGGAAGAC	x	x	x	x
SLC3A2 Reverse	AGAAGAGCAGCAGCAGTG	x	x	x	x
TLR1 Reverse	CACACATTTGATATTAGATAGTTCC	x			
TLR2 Forward	TGGATGGTGTGGGTCTTGG	x			
TLR2 Reverse	AGGTCACTGTTGCTAATGTAGG	x			
TLR4 Forward	TGGAAGTTGAACGAATGGAATG	x			
TLR4 Reverse	AGATACTACAAGCACACTGAGG	x			
TLR9 Forward	CCTGGAGTATCTGCTGTTGTC	x			
TLR9 Reverse	AGGTGGCTGAAGGTATCGG	x			
MYD88 Forward	CCCAGCGACATCCAGTTTG	x			
MYD88 Reverse	AGAGACAACCACCACCATCC	x			
TICAM1 Forward	TGGAGGAAGGAACAGGACAC	x			
TICAM1 Reverse	CTGGAGGTAGGCTGAGTAGG	x			
TRAF6 Forward	GACACTCAATTACAGCCTTCAC	x			
TRAF6 Reverse	AGCACCACATCTCTCATTTCC	x			
SOCS1 Forward	GTAGGATGGTAGCACACAAC	x			
SOCS1 Reverse	GAGGAAGAGGAGGAAGGTTC	x			
NFKB1 Forward	AATCATCCACCTTCATTCTCAAC	x			
NFKB1 Reverse	AATCCTCCACCACATCTTCC	x			
NFKB2 Forward	ACCGACAGACAACCTCACC	x			
NFKB2 Reverse	CCTCAGCAGCCTCACTCC	x			
IRF1 Forward	AAGACCAGAGCAGGAACAAG	x			
IRF1 Reverse	GTCCATCAGAGAAGGTATCAGG	x			
IRF3 Forward	GACGCTCACCACGCTATG	x			
IRF3 Reverse	GCAGGTCCACAGTATTCTCC	x			
IRF7 Forward (Taqman)	AGCTGTGCTGGCGAGAAG	x			
IRF7 Reverse (Taqman)	CATGTGTGTGTGCCAGGAA	x			
STAT1 Forward	CGACAGTATGATGAACACAGTATAG	x			
STAT1 Reverse	GAAGGAACAGAGTAGCAGGAG	x			
STAT4 Forward	CCAATGTCAGTCAGTTACCTAATG	x			
STAT4 Reverse	GCTCATCACCTCCAGTAGTTG	x			

Primer name	The primer sequence 5' → 3'	Cell type studied			
		MDM	PBMC	Whole-blood cells	Reticu-locyte
ATF3 Forward	GCGACGAGAAAGAAATAAGATTG	x			
ATF3 Reverse	GCCTTCAGTTCAGCATTCAC	x			
TBX21 Forward	AACGGATGAAGGACTGAGAAG	x			
TBX21 Reverse	TAGTTAGGGCAGAGGATGGG	x			
HMGB1 Forward	AAGAAGACCTGAGAATGTATCCC	x			
HMGB1 Reverse	GTTTCCTGAGCAGTCCATATTTAG	x			
IL1RN Forward	GCCTGCCTGTTCCCATTC	x	x		
IL1RN Reverse	TGTTGTGACGCCTTCTGAG	x	x		
IL1RAP Forward	AACTACAGCACAGCCCATTC	x			
IL1RAP Reverse	ACCACAGCACATCTTTCTCC	x			
IL1R2 Forward	CCTGGAAGATGCTGGCTATTAC	x			
IL1R2 Reverse	GAAACACCTTACACGGGATTG	x			
IL12A Forward	ATGAGGAAACTTTGATAGGATGTG	x			
IL12A Reverse	CAGAGGTATCATGTGGATGTAATAG	x			
IL12B Forward	CAGAGCAGTGAGGTCTTAGG	x	x		
IL12B Reverse	AAGCAGCAGGAGCGAATG	x	x		
IL12RB1 Forward	CCTGCGGTGTTGCCTTAG	x			
IL12RB1 Reverse	ACTTCTCTGTCTGGTTCCTG	x			
IL12RB2 Forward	ATACGGAGTTCTATACCAGAGTTG	x			
IL12RB2 Reverse	AAGGCTTCACAGTCACATCG	x			
CXCL10 Forward	GGTGAGAAGAGATGTCTGAATC	x			
CXCL10 Reverse	TAGGGAAGTGATGGGAGAGG	x			
TNF Forward	GCGGTGCTTGTTCCTCAG	x	x		
TNF Reverse	GCTACAGGCTTGTCACTCG	x	x		
IFNG Forward	GCAGGTCATTCAGATGTAGC	x			
IFNG Reverse	TGTCTTCCTTGATGGTCTCC	x			
IFNGR1 Forward	CAAGTCCTTGATCTCTGTGGTAAG	x			
IFNGR1 Reverse	GTTCTTCTGTATGTTCCACTTTTCC	x			
IFNGR2 Forward	TCGGGCATTTAAGCAACATATC	x			
IFNGR2 Reverse	CAGGACCAGGAAGAAACAGG	x			
LAMP2 Forward	TGATACTTGTCTGCTGGCTAC	x	x		
LAMP2 Reverse	ATACTTAATGGTGCTGCTATTGAG	x	x		
NLRP3 Forward	TGAGCATTCTGAGCCTGTG	x			
NLRP3 Reverse	CCTGTCTTGGTAGAGTGTCC	x			
IFI27 Forward	GTCCTCCATAGCAGCCAAG	x	x	x	x
IFI27 Reverse	TAGAACCTCGCAATGACAGC	x	x	x	x
NAMPT Forward	CCGACTCCTACAAGGTTACTCAC		x	x	
NAMPT Reverse	GTAGACATCTTTGGCTTCCTGG		x	x	
IL1B Forward	GGCTTATTACAGTGGCAATGAGG		x	x	
IL1B Reverse	GTAGTGGTGGTCGGAGATTCG		x	x	
CXCL8 Forward	GACATACTCCAAACCTTTCCACCC		x	x	
CXCL8 Reverse	CTCAGCCCTCTTCAAAAACTTCTCC		x	x	
CXCR2 Forward	GCTGTCGTCCTCATCTTCC		x	x	
CXCR2 Reverse	CAGAATCTCGGTGGCATCC		x	x	
IL18RAP Forward	GAGTATTCCGCATCACATAAGC		x	x	
IL18RAP Reverse	CCATTCTTGTACCAGGTTACC		x	x	

Primer name	The primer sequence 5' → 3'	Cell type studied			
		MDM	PBMC	Whole-blood cells	Reticu-locyte
ALAS2 Forward	GGAGCGTGATGGAATTATGC				x
ALAS2 Reverse	CAGAGAAGTGGTAAAGATGAAGC				x
BLVRB Forward	GCGTGCCGAGACTCTGAG				x
BLVRB Reverse	TCACTTCGTAACCTGCTTGC				x
BSG Forward	GAAGTCGTCAGAACACATCAAC				x
BSG Reverse	GCCTTGTCCTCAGAGTCAG				x
CAI Forward	CAATTAAAACCAAGGGCAAACGAGC				x
CAI Reverse	CATTTGATAGAAGGCTGCGGAATTG				x
EPB42 Forward	CCATTTGTAGACCACACCTTG				x
EPB42 Reverse	GAACGGAATCTGTAGCTCCTC				x
ERAF Forward	CAGCAGGTCTTCAATGATCCTCTCG				x
ERAF Reverse	GCCTTGTCTCGCTCTTGGG				x
FECH Forward	TCAACCGCAGAAGAGGAAG				x
FECH Reverse	GTCCAAGAAGAGTCTCAGAAGG				x
HBQ1 Forward	GCGTCGCTGGACAAGTTC				x
HBQ1 Reverse	GGGAGAGGCTTTACTCAAACAC				x
HEMGN Forward	GAACCATTCTCCAGAAGTCATTG				x
HEMGN Reverse	TGTTCTCTGCTGCTTGCG				x
HRI Forward	GAAGAGAACACCAACACATACG				x
HRI Reverse	AGCAGGACCACACCCAAGCTG				x
MSCP Forward	AGCAGAAGTGGTGAAGCAG				x
MSCP Reverse	ATAGGTGATGAAGTGGATGGAC				x
SELENBP1 Forward	TCGCATCTATGTGGTGGAC				x
SELENBP1 Reverse	GGCTGGTGTGGAGAAAGG				x
SLC2A1 Forward	GCTTCCTGCTCATCAACCG				x
SLC2A1 Reverse	TCATCTGCCGACTCTCTTCC				x
SLC4A1 Forward	GATACCTACACCCAGAAACTCTC				x
SLC4A1 Reverse	GAATATGAGGATGAAGACCAGCAG				x
GNB2L1 Forward	CAAATACACTGTCCAGGATGAGAG	x	x	x	x
GNB2L1 Reverse	GCTTGCAGTTAGCCAGGTTC	x	x	x	x
GNB2L1 Forward (Taqman)	CCTAACCGCTACTGGCTGTG	x			
GNB2L1 Reverse (Taqman)	CTACAATGATCTTTCCCTCTAAATCC	x			
TRAP1 Forward	CTGCACCTTCGTGAGTTTGA			x	x
TRAP1 Reverse	GACCCCAGCACATTTCTCAT			x	x