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LYSINURIC PROTEIN INTOLERANCE:

A rare disease with several peculiar characteristics

Mari Kärki

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To my mother

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ABSTRACT

Lysinuric protein intolerance (LPI) is a rare, recessively inherited transporter disorder affecting the transport of cationic amino acids (CAAs) lysine, arginine and ornithine in basolateral membrane in intestine and renal tubules. Practically all Finnish LPI patients share the same homozygous variant, c.895-2A > T in the *SLC7A7* gene that encodes CAA transporter y+LAT-1. The loss of function of this transporter leads to reduced intestinal absorption of lysine, arginine and ornithine and their increased excretion in the urine. Renal insufficiency is a common complication and may progress to end-stage renal disease (ESRD), requiring dialysis and eventually kidney transplantation. Severe immunological and pulmonary complications may also occur. In addition, elevated plasma zinc concentrations have been described. The treatment of LPI is based on protein-restricted diet and oral L-citrulline and lysine supplementation. Because renal insufficiency has become increasingly common in patients with LPI, in study I, a cohort of 41 Finnish LPI patients was screened for renal function, oral L-citrulline doses and plasma citrulline concentrations. In study II, the bleeding disorder associated with LPI was investigated using hemostatic and fibrinolytic markers in 15 LPI patients. Lastly, in study III, plasma calprotectin and plasma zinc concentrations of 10 LPI patients were described in detail.

Signs of renal dysfunction were detected in majority of LPI patients, and some patients developed renal insufficiency at an early age. Urine β2-microglobulin was a sensitive early marker of renal involvement. To date, a total of eight Finnish LPI patients had received a kidney transplant. LPI patients show moderate bleeding tendency without spontaneous bleeds. In the present study, defective primary hemostasis, coagulopathy, fibrin abnormality and hyperfibrinolysis were detected in patients with LPI. Plasma calprotectin concentration was extremely high in all LPI patients but plasma zinc concentration was normal or only mildly elevated. There was a positive correlation between plasma zinc and plasma calprotectin concentrations. However, plasma calprotectin did not correlate with markers of renal function. Mechanism of hypercalprotectinemia in LPI remains unknown.

KEYWORDS: lysinuric protein intolerance, renal insufficiency, hemostasis, calprotectin

TURUN YLIOPISTO
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TIIVISTELMÄ

Lysinuurinen proteiini-intoleranssi (LPI) on harvinainen peittyvästi periytyvä kationisten aminohappojen, lysiinin, arginiinin ja ornitiinin, kuljetushäiriö suolen ja munuaistubulusten basolateraalisilla kalvoilla. Kuljettajaproteiinin toimintahäiriön vuoksi lysiinin, arginiinin ja ornitiinin imeytyminen suolesta on alentunutta ja eritys virtsaan lisääntynyttä. Käytännössä katsoen kaikilla suomalaisilla on sama homotsygoottinen variantti, c.895-2A > T, geenissä *SLC7A7*, joka koodaa kationisten aminohappojen kuljettajaproteiinia y+LAT-1:ta. Munuaisten vajaatoiminta on tavallinen LPI:n komplikaatio ja voi edetä dialyysihoitoa ja munuaissiirtoa vaativaan loppuvaiheen munuaisen vajaatoimintaan. Tautiin voi liittyä immunologisia poikkeavuuksia ja keuhkokomplikaatioita. Lisäksi LPI:ssa on raportoitu kohonneita plasman sinkkipitoisuuksia. Hoidossa keskeistä on vähäproteiininen ruokavalio ja L-sitrulliini- ja lysiinilisä. Tutkimuksessa I selvitettiin munuaiskomplikaatioita, L-sitrulliiniannoksia ja plasman sitrulliinipitoisuutta 41 suomalaisen LPI-potilaan ryhmässä. Tutkimuksessa II määritettiin veren hyytymiseen ja toisaalta verihyytymän liukenemiseen eli fibrinolyysiin vaikuttavia tekijöitä 15 LPI-potilaalta. Tutkimuksessa III määritettiin kalprotektiini- ja sinkkipitoisuudet 10 LPI-potilaalta.

Suurella osalla LPI-potilasta oli merkkejä munuaisten vajaatoiminnasta, ja joillekin kehittyi munuaisten vajaatoiminta jo nuorella iällä. Virtsan β2-mikroglobuliinimäärityksellä pystyttiin havaitsemaan munuaisten vajaatoiminta jo varhaisessa vaiheessa. Yhteensä kahdeksan potilasta on saanut munuaissiirteen. LPI potilailla todettiin vuoto-oirekyselyn perusteella kohtalainen vuototaipumus, mutta ei spontaaneja vuotoja. Tutkimuksissa todettiin heikentynyt veren hyytyminen, fibriinin poikkeavuus ja lisääntynyt fibrinolyysi. Kaikilla 10 LPI potilaalla kalprotektiinipitoisuus plasmassa oli erittäin korkea, mutta plasman sinkkipitoisuus oli normaali tai vain lievästi koholla. Kalprotektiinipitoisuus korreloi positiivisesti sinkkipitoisuuden kanssa. Hyperkalprotektinemian mekanismi LPI:ssa on epäselvä.

AVAINSANAT: Lysinuurinen proteiini-intoleranssi, munuaisten vajaatoiminta, hyytymistekijät, kalprotektiini

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Abbreviations

4F2hc	surface antigen 4F2 heavy chain
A10	clot formation after 10 minutes
AAS	atomic absorption spectrometry
ACE	angiotensin-converting enzyme
ACEI	angiotensin-converting enzyme inhibitors
ADP	adenosine-5-diphosphate
ALP	alkaline phosphatase
ALT	alanine aminotransferase
APTT	activated partial thromboplastin time
ARB	angiotensin receptor blocker
BE	base excess
CAA	cationic amino acid
CAT	calibrated automated thrombogram
CFT	clot formation time
CKD	chronic kidney disease
CLT	clot lysis time
CT	clotting time
EPI	epinephrine
ETP	endogenous thrombin potential
ESRD	end-stage renal disease
FDH	Finnish disease heritage
GFR	glomerular filtration rate
GH	growth hormone
HCG-CoA	hydroxymethyl glutaryl coenzyme A
HDL	high-density lipoprotein
HLH	hemophagocytic lymphohistiocytosis
IGF1	insulin-like growth factor-1
IL	interleukin
INF	interferon
LDH	lactate dehydrogenase

LDL	low-density lipoprotein
LPI	lysinuric protein intolerance
LRP1	LDL receptor related-protein 1
MAS	macrophage activation syndrome
MCF	maximal clot firmness
MF	maximum lysis
NH_4	ammonium ion
NO	nitric oxide
OD	optical density
PAP	pulmonary alveolar proteinosis
PAPc	plasmin α2-antiplasmin complex
PFA	platelet function analyzer
PT	prothrombin time
ROTEM	rotational thromboelastometry
SLC7A7	solute carrier, family 7, member 7
TAFI	thrombin activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin complex
TG	thrombin generation
TF	tissue factor
TLR	toll-like receptor
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
TT	thrombin time
uPA	urokinase-type plasminogen activator
vWF	von Willebrand factor
vWF:Ac	von Willebrand factor activity
y+LAT(1/2)	system y+L amino acid transporter, member (1-2)

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals I–III:

I Kärki M, Näntö-Salonen K, Niinikoski H, Tanner LM. Urine beta2-microglobulin is an early marker of renal involvement in LPI. *JIMD Rep,* 2016; 25: 47–55.

II Pitkänen H, Kärki M, Niinikoski H, Tanner LM, Näntö-Salonen K, Pikta M, Kopatz WF, Zuurveld M, Meijers JCM, Brinkman HJM, Lassila R. Abnormal coagulation and enhanced fibrinolysis due to lysinuric protein intolerance associated with bleeds and renal impairment. *Haemophilia,* 2018; 24(5): e312–e321. This original publication has already been included in the thesis book of MD Hanna Pitkänen in year 2018.

III Kärki M, Tanner LM, Lahtinen S, Soukka T, Niinikoski H. Plasma calprotectin is extremely high in patients with lysinuric protein intolerance. *JIMD Rep*, 2023; 64(4): 293–299.

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

1 Introduction

Lysinuric protein intolerance (LPI) is a rare inherited autosomal recessive transport disorder of dibasic cationic amino acids (CAAs) lysine, arginine and ornithine. It was first described in 1965 by Perheentupa and Visakorpi and later established as a disorder of the Finnish disease heritage (FDH). LPI is caused by mutations in the *SLC7A7* gene encoding y+LAT-1 protein, the catalytic light chain subunit of the heteromeric amino acid transporter located at the basolateral membrane of epithelial cells of renal proximal tubules and intestine. The defective CAA transport leads to reduced intestinal absorption of lysine, arginine and ornithine and their increased excretion in the urine, causing depletion of these amino acid in the blood.

Because of lack of lysine, arginine and ornithine, the LPI patients often show failure to thrive in infancy, poor linear growth in childhood, hepatosplenomegaly as well as muscular weakness. They may also have osteoporosis, combined hyperlipidemia, easy bruising and other hematological and immunological defects. Due to defective function of the urea cycle the patients may show poor feeding and even vomiting and hyperammonemia. Renal insufficiency is a common complication and may progress to end-stage renal disease (ESRD). Some patients have suffered from pulmonary alveolar proteinosis (PAP). The treatment of LPI is based on dietary protein restriction, citrulline and lysine supplements and nitrogen scavengers which prevent hyperammonemia and improve protein tolerance.

Increasing number of LPI patients with renal insufficiency have been observed lately. Patients with ESRD may require invasive procedures, i.e. dialysis and kidney transplantation. It has been recognized that LPI patients are prone to bleeds in dental and surgical procedures and deliveries but the pathogenesis behind the bleeding tendency has been unidentified. In addition, elevated plasma zinc concentrations have been described, but the mechanism of this phenomenon is unknown.

The aims of this study were to investigate the prevalence of renal involvement in Finnish LPI patients (study I) by analysing in detail the indicators of renal function, as well as oral L-citrulline doses and plasma citrulline concentrations; to investigate the bleeding disorder associated with LPI and to design perioperative transfusion approaches (study II), and, lastly, to study plasma zinc and plasma calprotectin concentrations in LPI patients (study III).

2 Review of the Literature

2.1 Background

Finnish disease heritage (FHD) is a group of hereditary monogenic disorders caused by founder variants enriched in our population. FHD currently consists of 39 rare diseases (35 original FHD diseases and four candidates) that are more common in Finland than in any other country in the world.[1] Lysinuric protein intolerance (LPI, MIM#222700) also known as hyperdibasic aminoaciduria type 2 or familiar protein intolerance, is the 7th of those 39 diseases, and one of the few affecting amino acid metabolism. LPI hampers cationic amino acid (CAA) transport in the cell membrane. It was first described by Perheentupa and Visakorpi in 1965.[2–4] Today, over 200 patients with LPI have been reported worldwide. The incidence of LPI in Finland is approximately 1:60 000. Thus, in Finland, one child with LPI is born on average once every 1–2 years. Several patients have been reported also from Japan and Southern Italy. [5,6]

2.2 Genetics of LPI

LPI is caused by mutations of the solute carrier family 7 member 7 (*SLC7A7*) gene encoding y+LAT-1 protein, the catalytic light chain subunit of the heteromeric amino acid transporter located at the basolateral membrane of the epithelial cells of the renal proximal tubules and intestine [7–9]. The gene is located on chromosome 14, region q11.2 and the full genomic length is about 46.5 kB [10]. In endoplasmic reticulum, y+LAT-1 together with the heavy chain of surface antigen 4F2 (4F2hc) forms a functional heterodimeric transporter [7,8].

Currently, 69 pathogenic variants of *SLC7A7* have been reported (according to the HGDM® Professional database), including insertions, deletions, missense, nonsense and splice site variants. Almost all Finnish patients share the same homozygous variant, c.895-2A > T (LPI_{Fin}) [11]. The mutation leads to a 10 base pair frameshift deletion in cDNA and formation of a premature stop codon, eliminating the last one-third of the protein [8,9]. No genotype-phenotype correlation for LPI has been established, suggesting that other genetic factors and environment may contribute to the phenotype [12].

2.3 Pathophysiology of LPI

y+LAT-1 is a light chain subunit of the heteromeric amino acid transporter. It has 12 transmembrane regions and its molecular weight is about 40 kDa. Together with 4F2hc it forms a functional heterodimeric CAA transporter. The CAA transporter exists mainly on the basolateral membrane of polarized cells. In LPI patients, the CAA transport defect is localized in the basolateral membrane of the epithelial cells of the renal proximal tubules and intestine (Figure 1), leading to reduced intestinal absorption and renal re-absorption of dibasic cationic amino acids lysine, arginine and ornithine. [4,12–15] The transport defect is also detected in monocytes and alveolar macrophages [16]. However, in fibroblasts, granulocytes and erythrocytes CAA transport is normal. This may be due to compensation of another CAA transporter.[17–20]

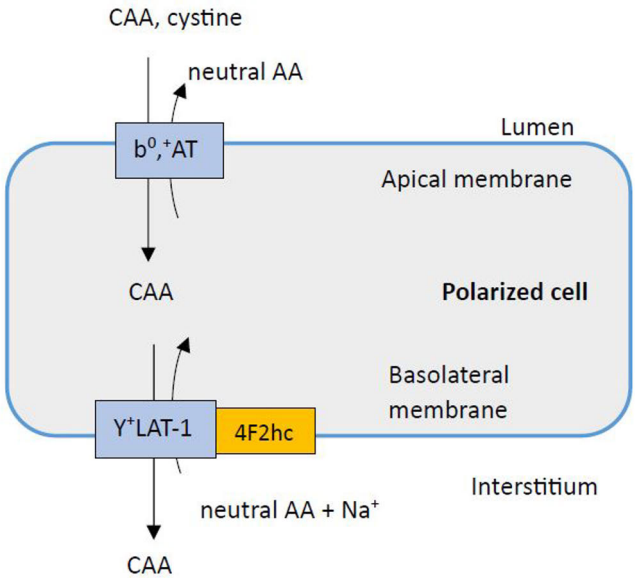

Figure 1. Membranous transport of cationic amino acids (CAAs) in a polarized cell. Transporters are represented as blue rectangles.

Because of the transport defect, plasma concentrations of lysine, arginine and ornithine are low and their excretion in the urine is increased [21]. Interestingly, the intestinal absorption of neutral amino acid citrulline has been shown to be normal, but, after its oral dose, it is excreted in the urine along with arginine and ornithine. This may be because of partial conversion of citrulline to arginine and ornithine in the epithelial cells, leading to increased intracellular concentrations of CAA. This may inhibit the metabolic disposal of citrulline and increase the leakage of the CAA into the tubular lumen. [13,22]

Impairment of intestinal absorption and renal re-absorption of lysine, arginine and ornithine leads to decreased plasma concentration of these CAAs. Arginine and ornithine are needed in the disposal of nitrogen by urea cycle, and, thus, deficiency of these amino acids leads to deficient urea cycle function (Figure 2), decreased tolerance for nitrogen and hyperammonemia after dietary protein loads. [2,21] Lysine, unlike arginine and ornithine, is an essential amino acid for a human being. In mammals, lysine has an important role in several biological processes, including protein synthesis, calcium homeostasis, immune system and growth[23–28]. Lysine is also a precursor of carnitine, which affects lipid metabolism via mitochondrial beta-oxidation[29,30].

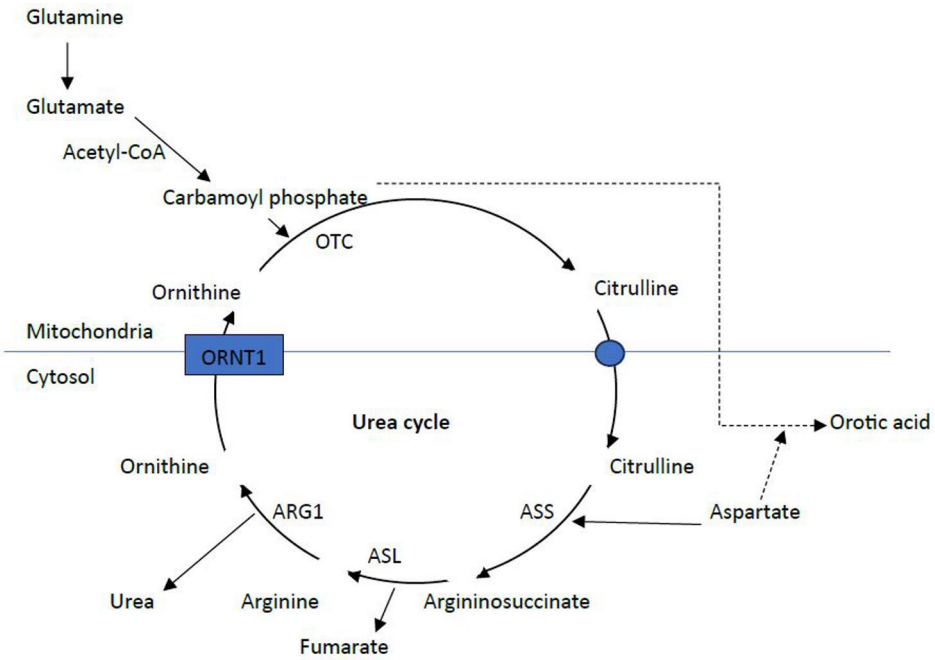

Figure 2. Urea cycle. Transport proteins are in blue. ARG1 = Arginase ASL = argininosuccinic lyase; ASS = argininosuccinic synthetase; ORNT1 = mitochondrial ornithine transporter 1; OTC = ornithine transcarbamylase.

Besides the urea cycle defect, arginine might have a role in the pathophysiology of LPI. Arginine is a precursor substance of endogenous nitric oxide (NO) synthesis. In LPI, the export defect of CAAs has been hypothesized to lead to an increased concentration of arginine in the proximal kidney tubule cells, macrophages and other cells with the transport defect, inducing increased intracellular production of NO. Moreover, synthesis of arginine from citrulline increases arginine production in the kidney, leading to intracellular NO accumulation. Since excess of NO in tubular,

glomerular and mesangial cells is considered to be toxic, this may cause damage and apoptosis in tubular cells and glomeruli. [31–35] It has also been suggested that increased NO synthesis in macrophages and lymphocytes may result in dysfunction of immune cells[31,35]. However, in contrast to this theory, it has been shown that NO levels in LPI macrophages are reduced, suggesting that arginine import and reservoirs are decreased in macrophages. It has been reported, that LPI macrophages present impaired toll-like receptor (TLR) signaling, seen as an overproduction of proinflammatory and anti-inflammatory cytokines.[36] In addition, slightly elevated plasma NO levels have been reported in LPI, correlating with severity of renal insufficiency and implying increased renal production of NO[37]

2.4 Symptoms, signs and clinical findings in LPI

2.4.1 Clinical presentation

Main signs and symptoms of LPI are presented in Figure 3. Most newborns with LPI are asymptomatic during breast-feeding but may develop symptoms when the amount of dietary protein increases at the start of solid foods (from 4–6 months onwards) or if infant formula with higher protein content than in breast milk is used. Feeding with high protein food may lead to hyperammonemia episodes, presenting as nausea, vomiting, diarrhea, even convulsions and coma. Strong, natural aversion to protein-rich food usually develops at approximately one year of age. Later, protein malnutrition leads to growth retardation and failure to thrive. [38–40] Thus, subnormal growth is common in children with LPI. In addition, alterations in growth hormone (GH) / insulin-like growth factor-1 (IGF1) axis have been detected, and two LPI patients with true GH deficiency has been described[41–44]. Delayed skeletal maturation, osteopenia, osteoporosis and decreased collagen synthesis have been observed [45,46]. Hepatosplenomegaly is common and the abdomen may be bulging and muscles weak. Mental development is normal, but psychomotor delay may occur due to previous episodes of severe or prolonged hyperammonemia. [39,40] Pubertal development is normal in adolescence. In addition, hypercholesterolemia, hypertriglyceridemia, hematological and immunological abnormalities including normochromic or hypochromic anemia, thrombocytopenia, leukopenia and deficient B cell function are common findings [38,39,47–49]. Severe long-term complications including renal insufficiency, pulmonary fibrosis and alveolar proteinosis may occur [49–51].

Women with LPI appear to have normal fertility. However, during pregnancy, they are at clearly increased risk for anemia, toxemia, and intrauterine growth retardation, as well as excessive bleeding during delivery. The children of mothers with LPI generally develop normally assuming that serious complications during pregnancy have been avoided.[52]

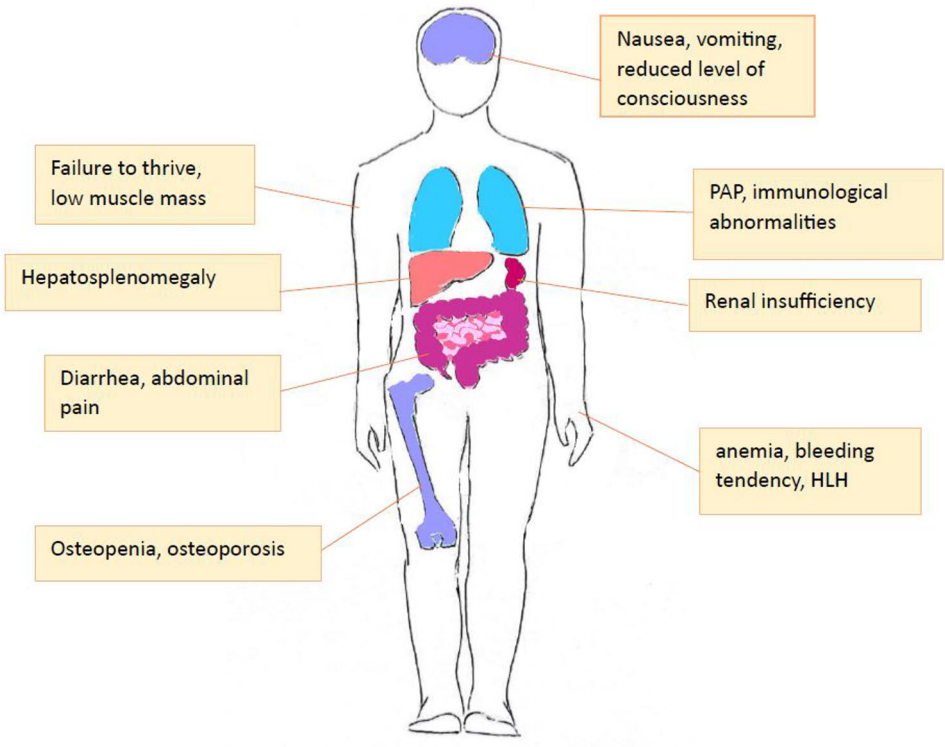

Figure 3. Main signs and symptoms of LPI. HLH = hemophagocytic lymphohistiocytosis; PAP = pulmonary alveolar proteinosis.

2.4.2 Hematological and immunological abnormalities

2.4.2.1 Normal coagulation and fibrinolysis

Primary hemostasis is activated when circulating platelets are exposed to vessel wall components, such as collagen and von Willebrand factor (vFW) immobilized on the subendothelial matrix, in response to tissue trauma. Platelet adhesion and activation leads to platelet aggregation and plug formation. Platelet activation leads to activation of the coagulation cascade components. [53,54]

The classical coagulation cascade is presented in Figure 4. The intrinsic pathway or contact pathway (named as plasma kallikrein-kinin system) is activated when contact is made with certain types of artificial surfaces[55,56]. The extrinsic pathway or tissue factor (TF) pathway is triggered by TF when subendothelial tissue expressing TF is exposed to blood [57]. Both pathways converge on factor X (FX) activation. FXa along with its cofactor factor V (FV), tissue phospholipids, platelet phospholipids and Ca-ions forms the prothrombinase complex, which converts prothrombin to thrombin. Thrombin is the principal enzyme converting fibrinogen to fibrin, which, via polymerization, forms a

hemostatic clot[58]. In addition, thrombin activates factor XIII (FXIII), which stabilizes the clot by crosslinking fibrin polymers covalently[59]. Besides controlling fibrin formation, thrombin has a role in inhibition of fibrinolysis by activating thrombin-activatable fibrinolysis inhibitor (TAFI)[60]. However, the classical coagulation cascade has its limitations, and cell-based model describes coagulation as a time-based process with four steps: initiation, amplification, propagation, and stabilization[61–63] (Figure 5).

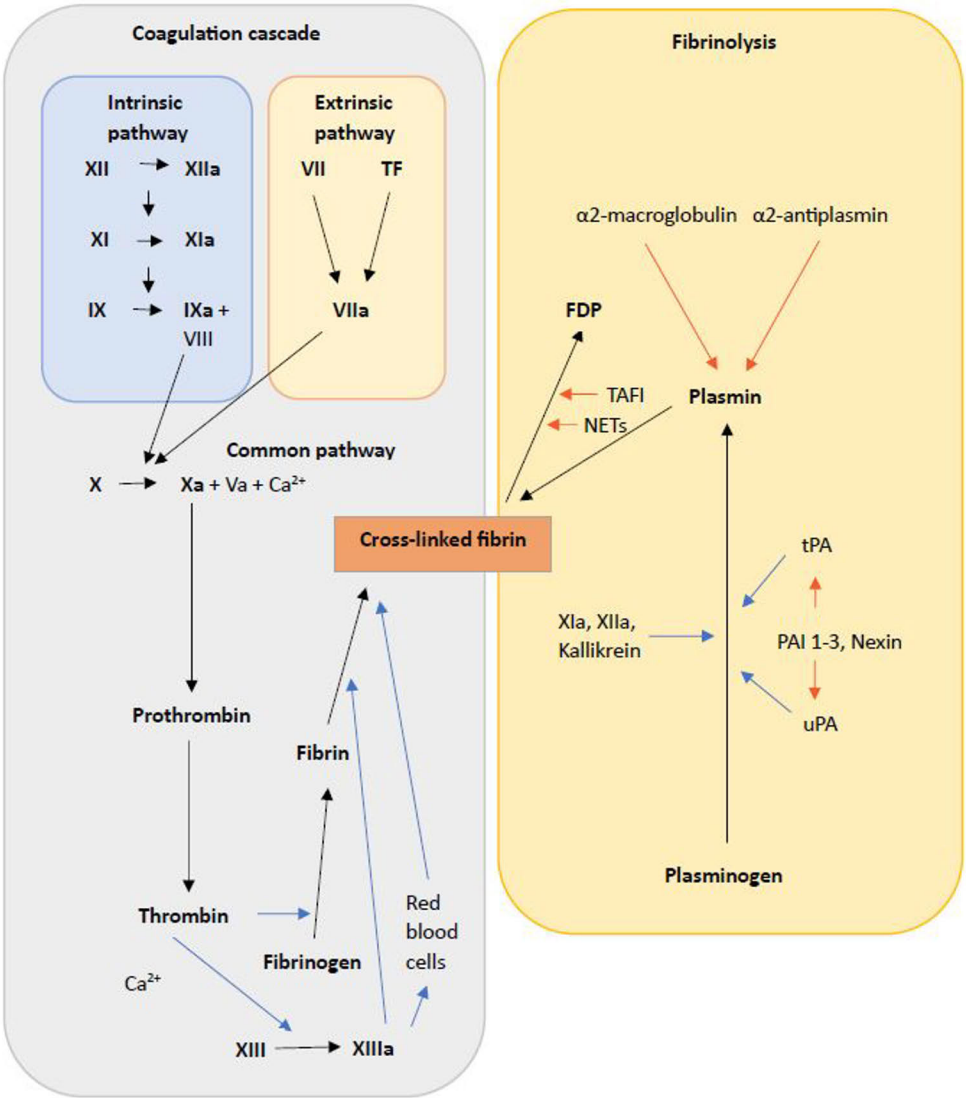

Figure 4. The classical coagulation cascade and fibrinolytic pathway. Activators are presented with blue arrows and inhibitors with red arrows. FDP = fibrin degradation products; NETs = neutrophil extracellular traps; PAI = plasminogen activator inhibitor; TAFI = thrombin activatable fibrinolysis inhibitor; TF = tissue factor; tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator.

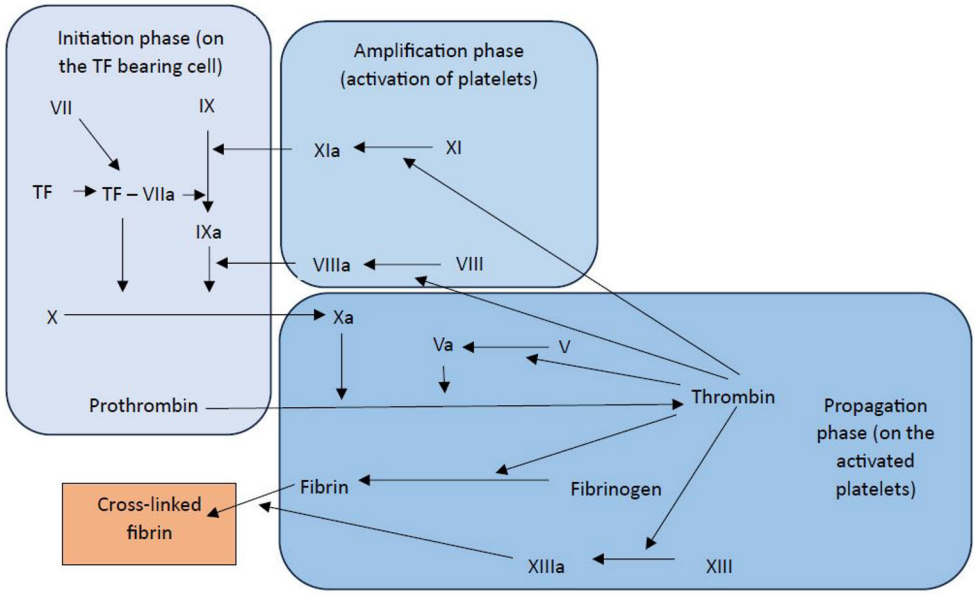

Figure 5. Cell-based model of coagulation (adapted from Hoffman M 2003).

Several variables can affect clot stability, including calcium-ion concentration, local pH, fibrinogen, platelet count and concentration of thrombin, as higher thrombin concentrations generate firmer clots[59]. Fibrinolysis limits excess fibrin formation and enables the removal of thrombi. Fibrin is both a cofactor and substrate for fibrinolytic enzyme plasmin. Like coagulation, fibrinolysis is controlled by several different mechanisms, cofactors, and inhibitors (Figure 4). Initially, plasmin is activated from plasminogen by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Serpins, such as plasminogen activator inhibitor (PAI) -family, nexin and α2-antiplasmin inhibit both tPA and uPA. In addition, α2-macroglobulin is an inhibitor of plasmin, tPA, uPA and kallikrein. [64,65] Within a clot, fibrinolysis can be inhibited by a non-serpin fibrinolysis inhibitor TAFI, which is activated by thrombin-thrombomodulin or plasmin during ongoing fibrinolysis[66]. Moreover, red blood cells have antifibrinolytic effects, since they can be trapped in a fibrin network, forming a clot with a higher lytic resistance[67,68]. Neutrophils also have a role in fibrinolysis, releasing DNA and histones, components of neutrophil extracellular traps (NETs), at the sites of infection and intravascular thrombi and delaying fibrinolysis[65].

2.4.2.2 Hematological and coagulation abnormalities in LPI

Episodes of abnormal mucocutaneous bleedings have been reported in patients with LPI, ranging from minor bleeds associated with dental surgery to fatal postpartum hemorrhage. Mild normochromic or hypochromic anemia, poikilocytosis, thrombocytopenia, leukopenia, mild reticulocytosis, highly elevated lactate dehydrogenase (LDH) activity and serum ferritin levels and decreased haptoglobin levels are common laboratory findings in LPI. Furthermore, low fibrinogen, high D-dimer and elevated levels of thrombin-antithrombin complex (TAT) have been observed. [49,52,69] However, pathogenesis of the abnormal coagulation in LPI is still unclear. Several LPI patients with hemophagocytic lymphohistiocytosis syndrome (HLH) have been described in the literature[70–79]. HLH is characterised by hepatosplenomegaly, hemophagocytosis, cytopenia, high levels of LDH and ferritin, activation of T lymphocytes and macrophages, increased levels of proinflammatory cytokines and multiorgan dysfunction. The condition must be carefully monitored since it can evolve to macrophage activation syndrome (MAS). [35,80,81] Many patients with LPI fulfill the diagnostic criteria for HLH without being acutely ill, suggesting that HLH may also be present as a chronic disease process in LPI instead of an acute episode.

Cytopenias, especially normocytic anemia and thrombopenia, are common. Bone marrow abnormalities, such as erythroblastophagocytosis, has been observed, but it has also been proposed that permanent macrophage activation might be responsible for hematological abnormalities[31,35,70]. In addition, one mechanism behind cytopenias might be increased splenic sequestration due to splenomegaly.[82] Mechanism of splenomegaly is still unclear, but since the white pulp of the spleen consist of T and B lymphocytes, it has been suggested, that defect in y+LAT1 subunit in the lymphocytes could be linked to splenomegaly in LPI.[83] It has also been considered that hepatosplenomegaly in LPI could reflect macrophage activation[31].

2.4.2.3 Immunological abnormalities in LPI

Patients with LPI may also suffer from severe viral and bacterial infections, such as pneumonia, bacterial meningitis, pneumonia, sinusitis, herpes simplex and tuberculosis. Severe Varicella infections have been reported. [49,84] Severe Covid19 infections have not been reported and clinical experience is that Covid19 infections are not especially severe in patients with LPI once appropriate vaccinations have been given (H. Niinikoski, personal communication). In Finnish patients, deficient B cell function with decreased concentration of IgG1-4 subclasses have been detected, leading to impaired humoral immune defense and poor vaccination response. Decreased CD4+ to CD8+ ratio and high CD8+ levels have been reported.[47] In contrast, in non-Finnish patients, CD4+ to CD8+ ratio is high and

levels of IgG, IgA and IgD are elevated. In addition, impaired phagocytic activity of macrophages and natural killer (NK) cells have been observed. [85–87] Several cases of autoimmune diseases have been reported, such as rheumatoid arthritis and systemic lupus erythematosus (SLE) [88–90].

It has been shown that macrophages play a critical role in the pathogenesis of LPI. Immunological defects may result from the impaired TLR signaling in macrophages, leading to an overproduction of proinflammatory and anti-inflammatory cytokines.[36]

2.4.3 Renal involvement

An increasing number of Finnish LPI patients suffer from chronic kidney disease (CKD), which has in several cases progressed to end-stage renal disease (ESRD). The etiology of this common complication is still unknown. Patients have proteinuria, microscopic or macroscopic hematuria, increased levels of serum creatinine and cystatin C and decreased glomerular filtration rate (GFR). Hypertension, hypophosphatemia, decreased bicarbonate levels and base excess (BE) have been described. [51] Renal histological findings are heterogeneous; glomerular and tubular dysfunctions, including glomerular amyloidosis, mild mesangial sclerosis, hyalinous hyperplasia of the arterioles, tubular atrophy, intestinal fibrosis, immune complex mediated (membranous or mesangial) glomerulonephritis and Fanconi syndrome-type tubular dysfunction have been reported [50,51,70,91–95]. However, kidney biopsies are not routinely performed due to bleeding tendency in LPI. Renal insufficiency is often slowly progressive and may lead to dialysis therapy and kidney transplantation [51].

The pathophysiological mechanisms of renal complications in LPI are still unclear, but several explanations have been suggested. High concentrations of cationic amino acids are nephrotoxic in animals, and lysine trapped inside the proximal tubular cells causes direct toxicity and apoptosis [96–98]. In rats, oral lysine supplementation promotes urine protein loss and inhibits albumin reabsorption by the proximal tubular cells [98]. Moreover, delayed renal development, proximal tubular dysfunction and aminoaciduria, as well as growth failure, IGF1 deficiency and delayed liver, lung and skeletal development have been described in *Slc7a7* knockout mouse models[99,100]. In kidneys, intracellular arginine synthesis from citrulline may increase due to oral citrulline supplementation, promoting local production of NO and causing damage and apoptosis in glomerular, mesangial, and tubular cells [33,34,101,102]. Elevated plasma NO levels associating with the stage of renal disease have, indeed, been detected in patients with LPI. [37] However, Kurko et al (2016) showed, that in contrary to the earlier hypothesis, exogenous citrulline did not seem to participate in NO production in CKD, possibly because of decreased

citrulline intake by the injured kidney cells in renal dysfunction [37,103]. Furthermore, combined hyperlipidemia, altered lipid metabolism and long-chain triglycerides seem to be involved in renal disease in LPI patients [37].

2.4.4 Lung involvement

Patients with LPI may develop severe pulmonary complications, including interstitial pneumonia, pulmonary alveolar proteinosis (PAP) and pulmonary fibrosis, which can rapidly lead to acute respiratory insufficiency and become life-threatening [50,88,104,105]. Lung involvement can occur at any age. In LPI, PAP often presents with progressive dyspnea, tachypnea and cough. PAP may also be associated with other organ dysfunctions, leading to a fatal multiple-organ dysfunction syndrome. [49,50,91] In LPI patients with PAP, a large number of cholesterol and cholesterol crystals, dying cells and low levels of surfactant protein D in the airways have been reported. [104] In bronchoalveolar lavage, the number of cells and foamy macrophages are increased. Interstitial lesion can be observed by chest X-rays and chest high-resolution computed tomography (HRCT). Histology may show cholesterol granulomas and alveolar proteinosis. [104,106–108] The treatment consists of high dose systemic corticosteroids and whole-lung lavation [108,109]. In addition, it has been reported that some carefully selected patients might benefit from inhaled granulocyte-macrophage colony-stimulating factor (GM-CSF)[16,110,111]. However, the effectiveness of GM-CSF treatment for secondary PAP in LPI is uncertain, and the prognosis has been poor especially in children with LPI[50,88,104,110]. One patient has received a lung transplant. This patient died 26 months after transplantation with a severe pulmonary infection and recurrent PAP [109]. The mechanism of PAP is still unclear.

2.4.5 Dyslipidemia and liver function

Most of the Finnish LPI patients suffer from combined hyperlipidemia with low levels of high-density lipoprotein (HDL) cholesterol and high levels of triglyceride and total and low-density lipoprotein (LDL) cholesterol. This is evident in many LPI patients already in childhood. Though dietary fat is an important source of energy for LPI patients it does not explain the hypercholesterolemia and hypertriglyceridemia. Dietary treatment alone is seldom effective, but medication (hydroxymethyl glutaryl coenzyme A (HCG-CoA) reductase inhibitors, i.e. statins) has markedly improved lipid values. [48].

Hepatosplenomegaly is a common finding, and liver dysfunction with mildly elevated transaminases might occur, but liver failure is rare [35,112]. Hepatic cirrhosis and cholestasis in hepatocytes have been reported [91,113] However, liver biopsies are

not routinely performed because of the bleeding tendency in LPI. Disturbances in the lipid metabolism have been reported, including altered lipolysis, lipogenesis, and β-oxidation of free fatty acids, demonstrated by the changed expression pattern of lipid-regulating genes, and altered levels of metabolites related to the synthesis and catabolism of lipids. This may lead to hepatic steatosis and further to fibrosis and cholestasis. [37,114]

2.4.6 Hyperzincemia

2.4.6.1 Multiple roles of zinc

Zinc is an essential trace element, occurring as a component at least 300 enzymes and participating in numerous cellular functions [115]. Only 0.1 % of total body zinc is located in the plasma, and most of the plasma zinc is bound to proteins, such as albumin (80–85 %), α2-macroglobulin and S100 proteins [116–119].

Zinc deficiency is associated with immunodeficiency, growth retardation, iron accumulation in tissues, subsequent increase in transferrin receptor levels and ferritin light chain expression [120–123]. It also causes blood clotting disturbances, poor platelet aggregation and increased bleeding time [124,125]. These signs and symptoms are surprisingly similar to those seen in patient with LPI.

Hyperzincemia is a rare entity, and only few patient cases of hyperzincemia have been reported[126–134]. The mechanism of this condition is poorly known. In 2002, Sampson et al reported a new, rare disorder of zinc metabolism, hyperzincemia/ hypercalprotectinemia, currently known as PSTPIP1-associated myeloid-related proteinemia inflammatory syndrome (PAMI-syndrome).[135,136] In literature, recurrent infections, hepatosplenomegaly, anemia, growth failure and neutropenia have been reported in patients with hyperzincemia [135,137], and again, same symptoms are seen in LPI.

2.4.6.2 Dietary zinc intake and hyperzincemia in LPI

Due to protein-restricted diet, patients with LPI are at risk for nutritional deficiencies, including zinc deficiency. Daily dietary intake of zinc in adult LPI patients has been between 8.0 and 8.3 mg per day, rather close to the population reference intake [138], but markedly less than average in the Finnish population (13.6 mg in males and 10.3 mg in females[139]). Thus, less than average plasma zinc values would be anticipated in LPI, but, surprisingly, elevated plasma zinc concentrations (unpublished observation) and serum zinc concentrations[52,140] have been described in patients with LPI. Significance of this phenomenon is unknown.

2.4.6.3 Zinc and calprotectin

Calprotectin (MRP8/14, S100A8/A9, calgranulin A/B), a heterodimer of S100A8 and S100A9, is a calcium- and zinc-binding protein, expressed mainly by neutrophil granulocytes, but also in monocytes and activated macrophages. It also has binding sites for manganese and iron [141–143]. Calprotectin has several biological functions, including antimicrobial, apoptosis-inducing and chemotactic activities [119,143]. Elevated plasma or blood calprotectin concentrations have been detected in several inflammatory conditions, such as rheumatoid arthritis, inflammatory bowel diseases, and cystic fibrosis [144–147]. Calprotectin plays an important role in innate immunity. It chelates essential nutrients zinc and manganese, creating zinc-limited microenvironments, which leads to bacterial metal starvation. [142,148] Furthermore, zinc deficiency has been linked to the increased expression of calprotectin [149,150].

2.5 Treatment of LPI

There is no curative treatment for LPI. The aim of the treatment is to prevent the manifestation of the symptoms. The treatment is based on protein-restricted diet (1–1.5 g/kg/day in childhood; 0.5–1 g/kg/day in adulthood), in order to prevent accumulation of excess nitrogen, and oral L-citrulline supplementation, in order to improve protein tolerance and to prevent hyperammonemia. [113,151,152] Still, most patients suffer from strong aversion to protein-rich foods, and the limited diet may thus lead to deficiencies of vitamins, trace elements and essential amino acids. Therefore, use of a calcium, vitamin D and multivitamin supplements are recommended for all patients.

Citrulline, as a neutral amino acid, uses a different transport route than arginine and ornithine. Citrulline is converted to arginine, and further, to ornithine, thus improving urea cycle function and protein tolerance. [113,151] It is taken at mealtimes and doses (50–100 mg/kg/day) are adjusted to the protein content of the food. The Finnish patients were treated with arginine monohydrochloride during years 1965–1976, and after that with more effective and better tolerated L-citrulline supplementation [151,153]. However, it seems that renal insufficiency has possibly become more common in LPI patients during the L-citrulline therapy, and even children with nephropathy have been observed [51]. Due to the possible role of citrulline in renal complication, only moderate doses on citrulline have been used in the treatment in LPI in Finland, aiming at high normal or only slightly elevated plasma citrulline concentrations.

Since the patients suffer from a deficiency of essential amino acid lysine, L-lysine hydrochloride supplementation (20–30 mg/kg/day) is used to correct lysine deficiency[154], aiming at low-normal range without side effects, e.g. diarrhea. In many patients, sodium benzoate or sodium phenylbutyrate (both up to 250 mg/kg/day or

13 g/m^2/day) or sodium glycerolbutyrate is used to scavenge ammonia in addition to L-citrulline. Some patients also require L-carnitine supplementation [155]. Adult LPI patients with dyslipidemia are treated with HCG-CoA reductase inhibitors ('statins') and elevated blood pressure is treated using antihypertensive medications (angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB)) [48,51].

Subnormal growth is commonly observed in children with LPI, and some patients with poor growth have been treated with growth hormone therapy with a good response [42–44].

3 Aims of the study

Lysinuric protein intolerance is a rare metabolic disease which often leads to various severe clinical complications, the background and pathophysiology of which is still unknown. The aims of the present study were:

1. to investigate the prevalence and course of renal insufficiency in Finnish LPI patients, together with a detailed analysis of markers of renal function, dosage of oral L-citrulline and concentration of plasma citrulline
2. to characterize the bleeding disorder associated with LPI and to design perioperative transfusion protocol
3. to investigate mechanisms of hyperzincemia associated with LPI.

4 Patients and Methods

4.1 Subjects

The follow-up of Finnish LPI patients is unofficially centralized in the department of pediatrics at the Turku University Hospital, Turku, Finland. The majority of Finnish LPI patients are followed-up in Turku, once per year (adults) or even more frequently (infants and children), if needed. A comprehensive laboratory analysis is performed and clinical examinations are done at every visit.

All subjects received routine treatment for LPI. This consisted of a protein restricted diet (1–1.5 g/kg/day in children; 0.5–1 g/kg/day in adults), multivitamin and calcium supplementations, oral L-citrulline treatment and, in many patients, oral L-lysine and ammonia scavenger therapies, and oral carnitine if hypocarnitinemia was observed. Hypercholesterolemia and hypertension were treated according to standard protocols.

A cohort of 41 Finnish patients with LPI followed at Turku University Hospital were included in these studies. Of the 41 patients, 26 were female and 15 were male (Table 1). All these patients were homozygous for LPI_{Fin} variant.

Study I (renal involvement): The study group consisted of all 41 patients (26 females) with LPI followed at Turku University Hospital. The age range in the study group was 3 to 69 years (mean 37.3 years). Six patients were under 18 years of age.

Study II (coagulation): The study group consisted of 15 adult patients (eight females) with LPI followed at Turku University Hospital. The age range in the study group was 23 to 60 years (median 45 years). Eleven of the patients used lysine supplementation. Control plasma was collected from nonmatched volunteer donors in the same age range as patients and with no known illnesses.

Study III (zinc and calprotectin): The study group consisted of 10 adult patients (six females) with LPI followed at Turku University Hospital. The age range in the study group was 27 to 65 years (median 46.5 years). Control plasma was collected from five non-matched adult volunteer donors with no known illnesses.

Table 1. Characteristics of the LPI patients included in each substudy (I-III). F = female; M = male.

Sex	Age at study I	Patient number in study I	Patient number in study II	Patient number in study III	Sex	Age at study I	Patient number in study I	Patient number in study II	Patient number in study III
F	3	1			F	40	22		
M	10	2			M	41	23	14	7
F	10	3			F	43	24	10	4
F	16	4			M	44	25		
F	16	5			M	45	26	11	
F	17	6			F	46	27	15	6
F	18	7			F	46	28	2	8
F	20	8			F	47	29		
F	21	9	4	3	M	48	30	3	
M	22	10	9	9	M	49	31		
F	24	11			F	49	32	12	
F	28	12	7	2	M	50	33	8	
F	29	13			F	53	34		
M	29	14			M	55	35		
M	30	15			F	57	36		
F	32	16			M	58	37	6	5
F	33	17	5		F	58	38		
M	36	18			F	60	39		
M	38	19	13	10	F	62	40		
F	39	20	1	1	M	69	41		
F	39	21							

4.2 Methods

4.2.1 Data collection

Clinical and laboratory data of the patients were obtained from patient records at the Turku University Hospital. In study I, medical records and routinely obtained laboratory test results of 41 Finnish LPI patients from 2007 to 2013 were analyzed retrospectively. The patients received written information about the study and gave written informed consent for coagulation assay studies (study II) and for plasma calprotectin analyses (study III). The studies were approved by the Ethics Committee of University of Turku and Turku University Hospital.

4.2.2 Clinical and laboratory measurements and analyses

Routine follow-up laboratory tests were taken from each patient at their control visits 1–2 times per year. All patients were also clinically examined (including height and weight) annually.

Sitting blood pressure was measured after 15 minutes rest using the average values of three consecutive blood pressure measurements from right arm using an oscillometric noninvasive blood pressure monitor. Size of the cuff was chosen according to the size of the right arm.

All laboratory analyses were performed using standard clinical laboratory methods. Follow-up laboratory tests (as routinely obtained in the follow-up of LPI patients in Turku) were analyzed, including total blood cell count, plasma creatinine, serum cystatin C, urine β2-microglobulin, plasma alanine aminotransferase (ALT), plasma alkaline phosphatase (ALP), plasma ammonium ion (NH_4), plasma calcium, plasma phosphate, total plasma cholesterol, high-density lipoprotein (HDL), triglycerides, plasma prealbumin, total and free serum carnitine, plasma amino acids, and urinary amino acids. Glomerular filtration rate (GFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration Equation (CKD-EPI).

In addition, in study I, GFR was calculated using Cockroft-Gault formula (normal > 90 ml/min), 4-variable Modification of Diet in Renal Disease (MDRD) formula (normal > 60ml/min/1.73 m^2) and CKD-EPI formula (normal > 90 ml/min/1.73 m^2) for adults, and Schwartz formula for patients under 18 years of age.

Urine β2-microglobulin was analyzed in morning spot urine using chemiluminescence detection. Proteinuria was measured from 24 h urine and/or by using urine dipstick test.

In study II, the International Society on Thrombosis and Hemostasis Scientific and Standardization Committee Bleeding Assessment Tool (ISTH/SSC-BAT) questionnaire for scoring the severity of the bleeding tendency was used to evaluate the clinical impact of LPI on hemostasis. Normal values for males were < 4 points and for females < 6 points.

Blood samples were collected in citrate anticoagulant tubes. Platelet poor plasma samples were processed by centrifugation at 2500 rpm for 15 minutes at room temperature and were stored in aliquots at -80°C.

A basic coagulation screen was performed including blood cell count, prothrombin time (PT) (Nycotest PT, Axis-Shield PoC As, Oslo, Norway), activated partial thromboplastin time (APTT) (Actin FSL, Siemens Healthcare Diagnostics, Erlangen, Germany), and thrombin time (TT) (BC Thrombin reagent, Siemens Healthcare Diagnostics, Erlangen, Germany). PT determines the abnormalities in the extrinsic pathway and APTT in the intrinsic pathway. Coagulation factors, such as von Willebrand factor activity (vWF-Ac) (INNOVANCE VWF Ac., Siemens Healthcare Diagnostics, Erlangen, Germany), FXIII:C activity (Berichrom

chromogenic FXIII, Siemens Healthcare Diagnostics, Erlangen, Germany) and fibrinogen (modified Clauss method, Multifibren U, Siemens Healthcare Diagnostics, Erlangen, Germany) were also measured. Fibrinolysis was assessed by measuring D-dimer (immunoturbidometric Tina-quant D-Dimer, Roche Diagnostics, Mannheim, Germany), plasminogen and α2-antiplasmin (Diagnostica Stago S.A.S. Asnières sur Seine, France), plasmin α2-antiplasmin complex (PAPc) (DRG Diagnostica, Marburg, Germany) and TAFI activity (PEFA kit, Pentapharm, Basel, Switzerland).

Primary hemostasis was studied with epinephrine (EPI) or adenosine-5-diphosphate (ADP) and recorded as closure times of collagen membrane cartridges in the Platelet Function Analyzer (PFA-100, Siemens Healthcare Diagnostics, Erlangen, Germany).

Thromboelastometric variables were studied with a rotational thromboelastometry (ROTEM) device (TEM International GmbH Munich, Germany) at 37°C in citrated whole blood according to the manufacturer's instructions. INTEM, EXTEM and FIBTEM tests were used. ROTEM tracings were recorded up to 60 minutes to exclude late fibrinolysis. INTEM is activated by phospholipids and ellagic acid and mimics intrinsic pathway. EXTEM uses tissue factor (TF) as an activator, exploring extrinsic pathway. In INTEM and EXTEM, clot firmness is influenced by platelets and fibrinogen. FIBTEM is a modification of EXTEM and reflects the isolated effect of fibrinogen under cytochalasin D induced platelet inhibition. Clotting time (CT), clot formation time (CFT), clot formation after 10 min (A10), maximum clot firmness (MCF) and maximum lysis (ML) were recorded.

Thrombin generation (TG) was measured *in vivo* by the prothrombin fragments 1 + 2 assay (Enzygnost monoclonal, Siemens Healthcare Diagnostics, Erlangen, Germany) and *in vitro* by the Calibrated Automated Thrombogram (CAT) (Labscan, Thermo Fisher, Helsinki, Finland) assay using the Thrombinoscope software (Thrombinoscope, Maastricht, The Netherlands) and the validated reagents (Diagonistica Stago, Asnières sur Seine Cedex, France) according to the manufacturer's instructions. Lag time (time to initiation of thrombin formation), endogenous thrombin potential (ETP; the area under the curve; nmol thrombin x min), and peak (maximum thrombin concentration) were measured.

Fibrin generation and clot lysis were determined by optical density (OD) of clotting plasma, triggered with TF and in the presence of tissue plasminogen activator (tPA) to induce fibrinolysis (TF, phospholipids, tPA and $CaCl_2$ at final concentrations of 0.5 pmol/l, 4 μmol/l 50 ng/ml, and 15 mmol/l). Carboxypeptidase inhibitor (Sigma Aldrich St. Louis, MO, USA) was included in parallel incubations to inhibit activated TAFI. OD_{max} was derived directly from OD tracing measured at 405 nm, and clotting time (CT) and clot lysis time (CLT) were derived from the first

derivative of the OD tracing. CT was defined as the time elapsed from the reagent addition to maximum fibrin generation, and CLT as the time between maximum fibrin generation and the maximum rate of clot lysis.

In study III, plasma zinc concentration was measured by photometry and plasma copper concentration by spectrophotometry. Blood manganese, 24-hour urine zinc and urine copper levels were measured by inductively coupled plasma mass spectrometry (Synlab/MVZ Labor Dr- Limbach & Kollegen, Heidelberg, Germany). Blood samples for plasma calprotectin measurements were collected in EDTA tubes and they were centrifuged at 3000 rpm for 10 min. Plasma samples were stored in aliquots at 75°C until use. Plasma calprotectin was measured with enzyme linked immunosorbent assay (Calprotectin ELISA [ALP]) from the CALPROLAB™ (Lysaker, Norway) according to the instructions of the manufacturer. Absorbance was measured at 405 nm using an ELISA plate reader (Hidex Sense). The standard curve was based on measuring six standards in the range 0–500 ng/ml.

4.2.3 Statistical analyses

Statistical analysis of the data was made using IBS SPSS Statistics 22 software (study I ja II) and IBS SPSS Statistics 27 software (study III). The Shapiro-Wilk normality test was performed. Groups were compared using the Mann-Whitney test. Correlations were calculated with the Spearman correlation coefficient, also using linear regression models where pertinent. *P*-values < 0.05 were considered significant.

The studies were approved by the Ethics Committee of University of Turku and Turku University Hospital.

5 Results

5.1 Urine β2-microglobulin as a marker of renal involvement in LPI

Characteristics of 41 patients with LPI are presented in Table 2. During this follow-up, majority of the patients (70 %) developed renal dysfunction (i.e. proteinuria, decreased GFR, hypophosphatemia and/or decreased bicarbonate concentration). Two of them were under 18 years of age. Twenty of 36 patients (56 %) had proteinuria (urine protein > 0.1 g (24 h) or urine albumin > 30 mg (24 h)) and 19 of them (53 %) had hematuria (positive urine dipstick test). Plasma creatinine concentration was increased in 19 (48 %) of 40 patients and serum cystatin C was elevated in 24 (62 %) of 39 patients. Urine β2-microglobulin was measured from 31 patients and was elevated in 28 (90 %) of them. GFR was calculated using 4-variable MDRD formula, Cockcroft-Gault formula and CKD-EPI formula, showing decreased GFR in 18 (44 % of the whole cohort), 28 (68 %) and 25 (61 %) patients. CKD-EPI formula seemed to be the most reliable in LPI patients, who typically have low muscle mass. There was a correlation between GFR and urine β2-microglobulin (r = -0.69, p < 0.001), and, in some LPI patients, a rapid elevation of urine β2-microglobulin preceded decrease of GFR. Base excess (BE) was decreased in 21 of 26 patients (81 %). Twelve patients had oral supplementation of bicarbonate, phosphate or both. Nineteen patients (46 %) had hypertension, and 16 of them were treated with antihypertensive drugs.

The amount of L-citrulline supplementation depended on individual protein intake. Therefore, daily doses varied widely, from 28 to 229 mg/kg. Mean adult dose was 82.9 mg/kg (SD ±27.4), while children had higher mean dose 122.7 mg/kg (SD ±61.9). There were no differences in either weigh-based L-citrulline doses or plasma citrulline concentrations between the patients with normal and decreased renal function (Table 3).

Table 2. A summary of the follow-up data of the 41 patients with LPI at Turku university hospital. ESRD = end-stage renal disease. (Modified from Table 1 in original publication I.)

					Estimated GFR					
Sex	Age at study	Proteinuria	Hematuria	Hyperten-sion	4v-MDRD*	Cockroft -Gault	CKD -EPI	Elevated serum cystatin C	Elevated urine β2-microglobulin	ESRD
F	3				162					
M	10				289				+	
F	10				205				+	
F	16		+		104				+	
F	16				109				+	
F	17				112			+	+	
F	18	+			75			+	+	
F	20				111	116	126		+	
F	21	+	+		31	32	34	+	+	
M	22	+	+		46	43	47	+		
F	24	+	+		60	74	69	+	+	
F	28				80	79	93		+	
F	29	+		+	79	64	93			+**
M	29	+	+	+	32	38	35	+	+	+**
M	30				79	109	90		+	
F	32			+	84	159	99	+	+	
F	33	+	+		46	51	52	+	+	
M	36		+	+	99	81	111			
M	38		+	+	77	99	87		+	
F	39		+		82	102	96		+	
F	39	+	+		8	9	8	+	+	+
F	40				72	61	83	+	+	
M	41	+	+	+	59	85	66	+	+	
F	43				29	32	32	+		
M	44	+	+	+	122	116	115			
M	45	+	+	+	20	25	21	+	+	
F	46	+	+	+	71	68	81		+	
F	46	+	+	+	45	44	50	+	+	
F	47	+	+		36	48	39	+	+	
M	48			+	32	50	34	+	+	
M	49	+	+		79	71	78		+	
F	49	+		+	59	57	66	+	+	+**
M	50	+		+	66	76	73	+	+	
F	53	+		+	68	52	76	+		
M	55	+	+	+	11	11	11	+	+	+
F	57	+	+		38	53	41	+	+	
M	58			+	87	69	94			
F	58	+		+	41	34	44	+	+	
F	60	+	+	+	7	8	7	+		+**
F	62	+	+	+	74	61	80	+	+	+**
M	69	+	+	+	55	61	56		+	

*<18 years old with Schwarz formula

**patient had received a kidney transplant

Table 3. Weight-based oral L-citrulline doses in LPI patients with or without nephropathy. (Table 5 from original publication I.)

	Normal GFR		Decreased GFR**		
	N (female)	Mean ± SD	N (female)	Mean ± SD	*p*-value***
Age	8 (4)	36 ± 12	20 (12)	42 ± 13	0.055
GFR	8 (4)	103 ± 13	20 (20)	57 ± 20	< 0.001
Weight-based citrulline dose (mg/kg)	8 (4)	82.4 ± 21.9	19 (11)	79.5 ± 29.2	0.619
Fasting plasma citrulline (µmol/l)	4 (2)	64.8 ± 23.0	15 (10)	80.3 ± 20.1	0.362

*GFR was calculated using CKD-EPI formula
**Patients with ESRD were not included
****P*-value by using non-parametric tests: independent samples

At the time of this study, in 2013, a total of seven patients (18 %) had progressed to end-stage renal disease (ESRD), two of them already in their early twenties. Two of them were treated with peritoneal dialysis, and a total of five patients had received a kidney transplant (mean age 39.4 years, range 20 to 50 years; Table 4) (To date, 8 Finnish LPI patients have received a kidney transplant). Three patients had experienced episodes of graft rejection and one patient lost her transplant. Moreover, anemia and recurrent or chronic infections have remained a problem after the kidney transplantations. In addition, four of the patients had proteinuria since operation and GFR remained normal in only one patient.

During this follow-up, two patients died. One patient with ESRD died eight years after the kidney transplantation at the age of 60. She lost her transplant three years after the transplantation and was then treated with peritoneal dialysis. One patient with renal dysfunction died at the age of 16 years.

Table 4. A summary of the data of the five LPI patients with a renal transplant. CsA = cyclosporin; MPA = mycophenolic acid; TAC = tacrolimus. (Modified from Table 6 in original publication I.)

	Age			Complications after the transplantation				Immunosuppressive drugs	
Sex	At study	At start of dialysis	At trans-plantation	Infections	Anemia	Graft rejection	Graft loss	After the transplantation	At study
F	29	20	24	+	+	+		CsA	TAC, MPA
M	29	20	20	+	+	+		MPA, TAC	CsA
F	49	45	46	+	+			CsA, MPA	CsA, MPA
F	60	51	52	+	+	+	+	CsA, MPA	-
F	62	53	55	+	+			CsA, MPA	CsA, MPA

5.2 Coagulation and fibrinolysis in LPI

The study group consisted of 15 adult patients (eight females) with LPI. The age range was 23 to 60 years (median 45 years). Eleven patients (73 %) had elevated plasma creatinine concentration and ten patients had elevated serum cystatin C. Twelve patients had decreased GFR. Urine beta2-microglobulin was elevated in 14 (93 %) patients. Plasma ALT levels were moderately elevated in three patients and NH_4 was elevated in four patients (up to 62 μmol/l). Prealbumin was in the normal range in all patients. Plasma lysine was decreased in four patients, while urine lysine was measured from 12 patients and was elevated in all of them. BE was decreased in ten patients. Ten patients (67 %) had elevated total cholesterol, 12 patients had combined hyperlipidemia and only five patients had normal HDL. Ten patients had cholesterol lowering medication, and only one patient had normal cholesterol levels without medication.

Median bleeding score was 4 points (range 0–9 points). Six of 15 patients had elevated bleeding score, and three of those six were female (Table 5). Two female patients had experienced severe postpartum hemorrhage. One of these patients also had bleeding during a splenectomy due to abdominal trauma and kidney transplant surgery. Two female patients had normal labor and puerperium. One male patient bled after a splenectomy due to spontaneous cyst rupture. A total of seven patients reported bleeding complications due to invasive procedures, and only one male patient underwent minor surgery without bleeds. One male patient had a spontaneous gastrointestinal bleed, otherwise no spontaneous bleeds were reported. Mucocutaneous bleedings most commonly occurred from minor wounds, oral cavity, after tooth extraction, after surgery or major trauma, during menstruation and postpartum.

Table 5. ISTH/SSC-BAT bleeding score in 15 LPI patients. (Modified from Table 1 in original publication II.)

Patient number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sex	F	F	M	F	F	M	F	M	M	F	M	F	M	M	F
Age	40	48	49	23	34	60	29	52	23	45	46	51	40	43	48
Epistaxis	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Cutaneuous bleeding	1	1	0	1	0	3	1	0	0	0	1	1	0	1	0
Bleeding from minor wound	0	0	0	1	0	0	0	0	0	2	0	1	0	0	0
Oral cavity bleeding	1	0	0	1	1	0	0	0	1	1	1	0	1	1	0
Bleeding after tooth/teeth extraction	3	0	-1	0	2	0	0	0	-1	3	3	-1	3	0	0
Gastrointestinal bleeding	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Bleeding after surgery or major trauma	0	0	0	0	0	0	0	4	0	0	0	2	0	0	0
Muscle hematomas (spontaneous)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hemarthrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anemia	+	+		+	+		+	+		+		+		+	+
Iron substitution	+	+		+	+		+	+		+		+		+	+
Family history of bleeds	+	+				+		+							+
Menorrhagia	0	0		2	2		0			2		2			3
Postpartum hemorrhage	3	-1		0	0		0			0		3			0
Cumulative	8	0	-1	5	5	0	1	4	0	9	6	8	4	2	3

Median hemoglobin in females was 123 g/l (range 109–139 g/l) and in males 131 g/l (range 106–147 g/l) (Table 6). Six patients had mild thrombocytopenia (median 154, range 95–309 10^9/l), but it was below 100 only in one patient. White blood cell counts were in the normal range. Plasma ferritin levels were high in all patients (median 1846 µg/l, range 599–9069 µg/l). None of the patients had diagnosis of hemophagocytic lymphohistiocytosis syndrome (HLH), but eleven of 15 patients met three of its eight diagnostic criteria (including splenomegaly, hypertriglyceridemia, hypofibrinogenemia, high ferritin).

PT, APTT, TT and vWF:Ac were all in the normal range. In contrast, median FXIII activity was decreased and was within normal range only in four patients. D-dimer was very high in all patients (median 32 mg/l, range 12–109 mg/l; normal reference value < 0.5 mg/l). Four patients had decreased fibrinogen level, without prolonged TT.

Eleven patients had prolonged PFA closure time in the EPI cartridge. ADP closure time was measured from eleven patients and was prolonged in all of them. There was a correlation between platelet count and EPI closure time (r = -0.55, p = 0.03). However, the platelet count did not correlate with ADP closure time or vWF:Ac levels.

Table 6. Hematological and fibrinolytic variables; blood cell count, platelet function and fibrinolysis markers. (Modified from Table 3 in original publication II.)

	Median (range)	**Reference range**	
Hemoglobin	F: 123 (109–137) M: 131 (106–147)	F: 117–255 M: 134–167	g/l
Erythrocytes	F: 4.3 (3.5–4.6) M: 5.0 (3.5–5.4)	F: 3.9–5.2 M: 4.2–5.7	10^{12}/l
Leukocytes	5.3 (3.5–10.6)	3.4–8.2	10^9/l
Platelets	154 (95–309)	150–360	10^9/l
Ferritin	F: 995 (678–9069) M: 2075 (599–5000)	F: 5–100 M: 10–220	µg/l
Platelet function			
PFA-EPI	179 (102–300)	82–150	s
PFA-ADP	115 (102–162)	62–100	s
Coagulation markers			
vWF:Ac	110 (47–211)	44–183	%
PT	103 (77–160)	70–130	%
APTT	25 (20–29)	23–33	s
TT	22 (19–30)	17–25	s
Fibrinogen	2.5 (1.0–4.3)	1.7–4.0	g/l
FXIII	58 (27–141)	76–156	%
Prothrombin fragment 1 + 2	> 1200 (961–>1200)	53–217	pmol/l
Fibrinolysis			
Plasminogen	120 (91–155)	80–120	%
α2-antiplasmin	88 (50–105)	80–120	%
Plasmin-antiplasmin complex	> 2000	47–562	µg/l
D-dimer	32 (12–109)	< 0.5	mg/l
TAFI	72 (48–121)	64–125	%

In vitro thrombin generation (TG) in CAT was impaired in patients, as seen by decreased ETP (median 754 nmol x min, range 444–1266 nmol x min) compared to healthy controls (median 1165 nmol x min, range 542–1688 nmol x min) ($p = 0.02$). (Figure 6) However, lag time and peak TG did not differ from controls. In contrast, circulating prothrombin fragment 1 + 2 levels were increased (median > 1200 pmol/l, range 961– > 1200 pmol/l), indicating markedly elevated *in vivo* TG.

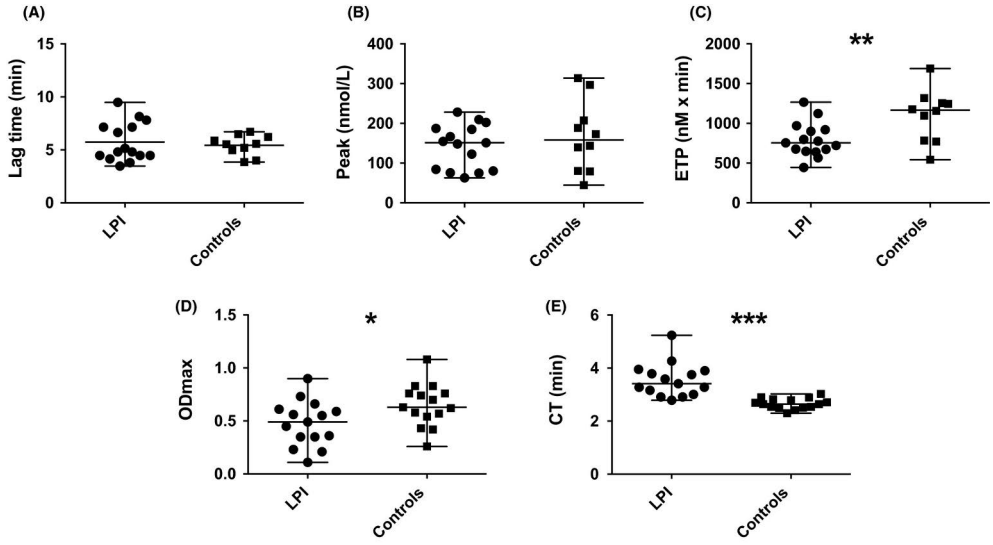

Figure 6. Thrombin generation and fibrin clot formation in LPI, compared to healthy controls. (A) median lag time in Calibrated Automated Thrombogram (CAT), (B) CAT median peak thrombin generation, (C) Endogenous thrombin potential (ETP) in CAT, (D) Fibrin clot formatition traced by OD_{max}, and (E) OD_{max} derivative clotting time (CT). The lines represent median and range. * $P = 0.03$, ** $P = 0.02$, *** $P < 0.001$. (Figure 6 from original publication II.)

In ROTEM (Table 7), seven patients had decreased FIBTEM MCF. FIBTEM MCF recognized decreased fibrinogen levels, as expected. Besides low fibrinogen, low FXIII:C correlated with FIBTEM MCF ($r = 0.63$, $p = 0.01$). INTEM CT was in normal range. INTEM CFT was prolonged in six patients and four patients showed reduced INTEM MCF. Seven patients showed prolonged EXTEM CT and CFT. EXTEM MCF was reduced in five patients. INTEM CFT, A10 and MCF and all EXTEM variables correlated with fibrinogen levels, but not with platelet count. Fibrin clot formation traced by OD_{max} was reduced and OD_{max} derivative CT was prolonged (median 3.41 min, range 2.79–5.24 min) compared to healthy controls ($p = 0.03$) (Figure 6).

Table 7. ROTEM variables in LPI. CFT = clot formation time; CT = clotting time; MCF = maximum clot firmness; ML = maximum lysis. (Modified from Table 4 in original publication II.)

Variable		Median (range)	Reference range	
EXTEM	CT	72 (61–213)	38–79	s
	CFT	141 (88–425)	34–159	s
	MCF	52 (35–73)	50–72	mm
	ML	1 (0–14)	0–15	%
INTEM	CT	178 (149–214)	100–240	s
	CFT	109 (59–702)	30–110	s
	MCF	55 (25–73)	50–72	mm
	ML	0 (0–14)	0–15	%
FIBTEM	MCF	9 (3–20)	9–25	mm

Median plasminogen levels were elevated (median 120 %, range 91–155 %), and PAPc levels were extremely high (> 2000 µg/l). α2-antiplasmin levels were lower compared to healthy controls ($p < 0.001$), leading to a higher plasminogen/α2-antiplasmin ratio (median 1.50, range 1.10–2.02), indicating increased fibrinolysis in LPI. (Table 6) TAFI was slightly decreased in five patients. OD derivative CLT was shorter in patients than in healthy controls ($p < 0.01$).

Renal function markers (GFR, serum cystatin C) correlated with urine lysine levels ($r = 0.63$, $p = 0.03$ and $r = -0.64$, $p = 0.03$). Loss of renal function correlated with diminished fibrin formation and markers of fibrinolysis. Creatinine, GFR, serum cystatin C, BE and bicarbonate all correlated with decreased fibrin formation (OD_{max}) and α2-antiplasmin levels and elevated D-dimer levels (Table 8).

Table 8. Renal insufficiency, coagulation and fibrinolysis: correlations between renal function markers, fibrin formation (OD_{max}) and enhanced fibrinolysis (D-dimer, α2-antiplasmin). (Table 9 from original publication II.)

		Creatinine	GFR	Cystatin C	BE	Bicarbonate
ODmax	*r* =	-0.87	0.84	-0.82	0.64	0.70
	P =	<0.001	<0.001	<0.001	0.02	0.01
D-dimer	*r* =	0.61	-0.56	0.55	-0.72	0.70
	P =	0.02	0.03	0.04	<0.01	0.01
α2-antiplasmin	*r* =	-0.69	0.62	0.63	0.75	0.73
	P =	<0.01	0.04	0.04	<0.01	<0.01

5.3 Plasma zinc, other trace elements and calprotectin in LPI

The study group consisted of 10 adult patients (six females) with LPI. The age range was 27 to 65 years (median 46.5 years). Plasma zinc concentration was elevated only in two patients (median 14.9 µmol/l, range 9–25.3 µmol/l), when measured by photometry. Previously, in 2007-2012, plasma zinc was measured by atomic absorption spectrometry (AAS), and it was elevated in most of the patients (Table 9). Only one of six patients had elevated urinary zinc concentration (24h urine). In the present study, plasma copper levels were within normal range, but urine copper was slightly elevated in three of six patients. Blood manganese concentrations were elevated in three patients (median 10.2 µg/l, range 1.6 – 22.6 µg/l). Plasma iron concentrations were low or normal (median 11.5 µmol/l, range 8–22 µmol/l). Plasma calcium concentration was normal in all patients. Seven patients had increased plasma creatinine and serum cystatin C concentrations, and GFR was decreased in all patients (median 50 ml/min/1.73m^2, range 17–88 ml/min/1.73m^2). Urine beta2-microglobulin concentration was measured from eight patients and was elevated in seven of them. (Table 10)

Table 9. Plasma zinc concentration in LPI patients in 2005–2012 at the time of the study in 2020-2021 and in 2023. (Modified from Table 2 in original publication III.)

Patient number	Plasma zinc (µmol/l)*								Study III	
	2005	2006	2007	2008	2009	2010	2011	2012	2020–2021	2023
1	14.2			25.8		35.4		16.0	15.3	23.0
2		18.6				25.8			14.1	25.2
3		98.5	65.4		53.0	69.8		20.2	18.2	54.6
4	11.4					10.3			12.0	
5	28.4		40.5	38.5					14.7	34.9
6			33.7	31.8	27.8	31.1		28.7	14.4	
7			62.4			50.5			15.1	61.2
8			52.5	68.4	56.1	25.9	26.5		25.3	55.7
9									17.8	43.3
10			34.3	32.6	31.1	36.9			12.5	32.9

*Referenge range was 10-20 µmol/l in 2005-2012 and 9–18 µmol/l in 2020–2021, and 9–22 µmol/ in 2023.

Method/Laboratory: 2005-2011 AAS/Tykslab; 2012 AAS/Yhtyneet Medix laboratoriot Oy, 2020-2021 Photometry/Synlab/MVZ Labor Dr. Limbach & Kollegen; 2023 AAS/Vita Laboratoriot Oy/Labor Dr. Kramer & Kollegen.

Table 10. Clinical and laboratory data of 10 adult patients with LPI in study III. (Modified from Table 1 in original publication III.)

	Median (range)	Reference range	
Age	46.5 (27–65)		
Sex (M/F)	4/6		
Plasma creatinine	128.5 (76–308)	W: 50–90	µmol/l
		M: 60–100	
Serum cystatin C	1.46 (0.72–2.31)	0.62–1.11	mg/l
Urine beta2-microglobulin	45.2 (0.07–98.9)	0–0.3	mg/l
GFR (CKD-EPI)	50 (17–88)		ml/min/1,73m2
plasma ALT	28.5 (15–83)	<35	U/ml
plasma ALP	84 (38–173)	35–105	U/l
plasma NH_4	27 (20–66)	<50	µmol/l
plasma prealbumin	0.28 (0.23–0.38)	0.2–0.4	g/l
Total plasma cholesterol	5.3 (3.7–7.8)	<5	mmol/l
Plasma LDL	2.9(1.7–4.5)	<3	mmol/l
Plasma HDL	1.16 (0.73–1.66)	>1.2	mmol/l
Plasma triglycerides	2.85 (1.4–6.2)	0.45–2.6	mmol/l
Plasma lysine	118 (98–139)	114–289	µmol/l
Urine lysine	578 (242–855)	2–63	µmol/mmol Krea
Plasma calcium	2.31 (2.19–2.45)	2.15–2.51	mmol/l
Plasma zinc	14.9 (12–25.3)	9–18	µmol/l
Plasma copper	16.5 (10.4–25.1)	10.7–26.6	µmol/l
Blood manganese	10.2 (1.6–22.6)	6.0–11	µg/l
Plasma iron	11.5 (8–20)	9.0–34	µmol/l
Urine zinc (24 h)	4.2 (1.4–20)	2.3–12	µmol/24h
Urine copper (24 h)	0.98 (0.25–1.61)	<0.94	µmol/24h
Plasma calprotectin	622338 (18817–1063291)	Controls: 608 (291–1695)	µg/l

Calprotectin was measured using an enzyme-linked immunosorbent assay (ELISA). It was extremely high in all patients (median 622 338 µg/l, range 18 817 – 1 063 291 µg/l). There was a correlation between plasma zinc concentration and plasma calprotectin concentration ($r = 0.806$, $p = 0.005$; Figure 7). However, there was no correlation between renal function markers (serum cystatin C, creatinine, GRF, urine beta2-microglobulin) and plasma calprotectin levels. ALT was moderately elevated in two patients (median 28.5 U/ml, range 15–83 U/ml) and ALP was slightly elevated in four patients (median 84 U/l, range 38–173 U/l). Both ALT

and ALP correlated with plasma calprotectin concentration (r = 0.760, p = 0.011, and r = 0.721, p = 0.019). Prealbumin concentration was normal in all patients.

Six out of the ten patients had hypertriglyceridemia and three patients had elevated LDL cholesterol concentration. However, serum lipoprotein (a) concentration was elevated only in two patients (median 64 mg/l, range < 25–956 mg/l; unpublished data).

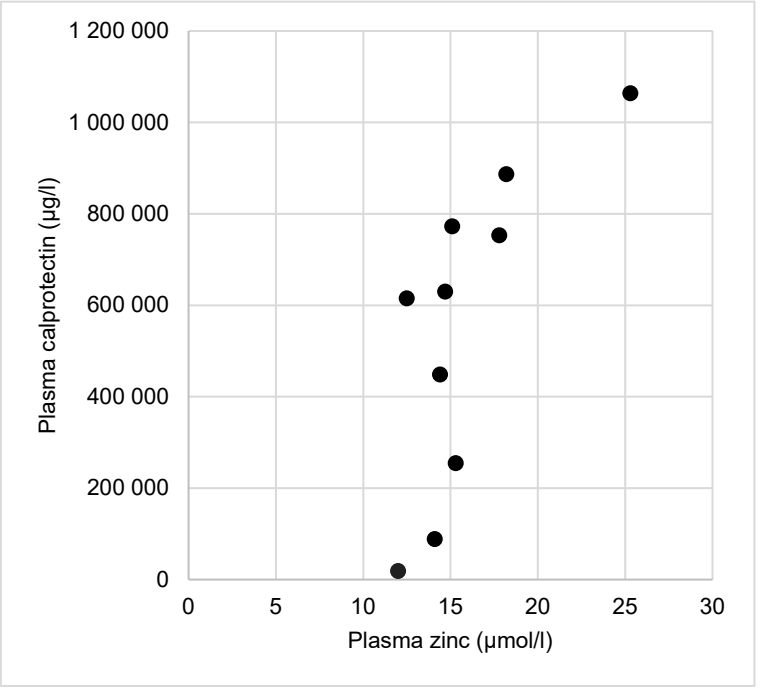

Figure 7. Correlation of plasma calprotectin with plasma zinc in 10 LPI patients. P = 0.005. (Modified from Figure 1 in original publication III.)

6 Discussion

LPI is a rare metabolic disease, influencing many organ systems and leading to various severe clinical complications. In this study, some new data about the renal involvement of LPI was obtained. Signs of renal dysfunction were detected in majority of LPI patients, and some patients developed renal insufficiency already at an early age. Urine β2-microglobulin was a sensitive early marker of renal involvement (study I). To date, a total of eight (19 %) Finnish LPI patients have received a kidney transplant.

This study aimed at determining the pathophysiology behind the bleeding tendency in LPI. LPI patients showed moderate mucocutaneous bleeding tendency without spontaneous bleeds. Impaired primary hemostasis, altered thrombin generation, fibrin abnormality and enhanced fibrinolysis were detected. Moreover, hyperfibrinolysis correlated with renal insufficiency (study II).

In addition, one of the aims of this study was to investigate the mechanism of hyperzincemia in LPI (study III). Plasma calprotectin concentration was extremely high in all LPI patients but, surprisingly, plasma zinc concentration was normal or only mildly elevated. There was a positive correlation between plasma zinc and plasma calprotectin concentrations. However, plasma calprotectin did not correlate with markers of renal function. Mechanism of hypercalprotectinemia in LPI remains unknown.

6.1 Renal insufficiency in LPI

6.1.1 Urine β2-microglobulin as a marker of renal dysfunction

Plasma creatinine is a relatively unsensitive marker of renal function in LPI patients due to the reduced muscle mass and protein malnutrition. Therefore, serum cystatin C is a more reliable marker of renal function in LPI. A low-molecular-weight protein β2-microglobulin is a component of the major histocompatibility class I molecule (MHC I) and is presented in all nucleated cells [156]. It is eliminated by glomerular filtration, and, thus, serum β2-microglobulin has been considered as a potential

marker of renal dysfunction. Nor muscle mass, body weight or gender affects its concentration. It has been shown that serum beta2-microglobulin increases earlier than serum creatinine. [157] In addition, the production of β2-microglobulin can be increased in several diseases, such as rheumatoid arthritis, Sjögren's syndrome, systemic lupus erythematosus and hematological and solid malignancies[158]. β2-microglobulin is almost completely reabsorbed in proximal tubular cells, and, thus, tubular dysfunction leads to an increased urinary excretion of β2-microglobulin[159,160]. In this study (study I), urine beta2-microglobulin was elevated in 90% of the patients, and in some patients, it started to elevate before any changes in GFR were detected and plasma creatinine and serum cystatin C were still within the reference range. It seems that beta2-microglobulin is currently the most sensitive early marker of renal disease in patients with LPI.

6.1.2 The role of L-citrulline therapy in the renal impairment

The role of L-citrulline therapy in the renal complications in LPI has been hypothesized. Large amounts of citrulline increase the intracellular synthesis of arginine, which may cause damage and apoptosis in tubular, glomerular and mesangial cells via increased production of nitric oxide [31–35]. Furthermore, high concentrations of cationic amino acids are nephrotoxic in animal [96]. However, in the present study, no differences in weight-based L-citrulline doses and plasma citrulline concentrations were observed between patients with normal and decreased renal function. One of the male patients with ESRD neglected citrulline therapy for several years but his renal function still decreased rapidly. However, due to the possible role of citrulline in renal insufficiency in LPI, L-citrulline doses had been slightly reduced during the last decade, and mean weight-based dose is now 80-90 mg/kg/day.

6.1.3 ESRD and kidney transplantations

Renal insufficiency is a common and progressive complication in LPI. In Finland, almost every adult LPI patient has impaired renal function, despite regular follow-up and careful treatment. Pathogenesis of this life-threatening complication is still poorly understood although several explanations have been suggested, as described before. It may even be a part of natural progression of LPI. In some cases, CKD has led to renal failure at an early age, and several patients have received a kidney transplant. Thus, signs of renal involvement should be monitored regularly and risk factors (hypertension, hyperlipidemia) should be treated carefully.

One case report of overall improvement in LPI symptoms after kidney transplantation has been published. After receiving a kidney transplant with functioning y+LAT1 transporter, renal losses of CAAs diminished. The female

patient's protein tolerance improved, her amino acid profile normalized, and she could even stop taking citrulline.[161] However, in our data, urine lysine, arginine and ornithine levels have been normal only in one patient after transplantation. Dietary intake protein has been unchanged after transplantation.

6.2 Bleeding tendency, impaired hemostasis and hyperfibrinolysis in LPI

Spontaneous bleeds are rare in patients with LPI. However, in invasive procedures, there is a risk for bleeding complications. Mucocutaneous bleeds triggered with invasive interventions and postpartum bleeds are typical. Since renal insufficiency has become more frequent and patients develop ESRD requiring invasive procedures (dialysis, kidney transplantation), it is important to identify pathogenesis of the abnormal coagulation associated with LPI.

6.2.1 Impaired hemostasis and hyperfibrinolysis

In this study, we discovered impaired primary hemostasis in patients with LPI. *In vivo*, thrombin formation and fibrinolysis activation were strongly elevated, but *in vitro*, thrombin generation was significantly reduced. Renal insufficiency associated with elevated lysine loss in urine, and markers of fibrinolysis correlated with the loss of renal function. In general, impaired hemostasis, hypercoagulability and altered fibrinolysis have been reported to associate with renal dysfunction[162–165]. However, in LPI, D-dimer levels were approximately 20 times higher as reported for patients with CKD in general. Fibrinogen level was normal or low in patients with LPI, while, in general, in CKD fibrinogen is elevated. [165,166]

Previously, it has been suggested that in LPI disseminated intravascular coagulation is caused be reduced NO production [69]. Elevated D-dimer, prothrombin fragment 1 + 2 levels and PAPc levels may support this suggestion. However, there was no sign of significant coagulation factor consumption. In ROTEM, altered EXTEM and INTEM variables correlated with fibrinogen but not with platelet count, suggesting that in LPI decreased fibrin formation contributes to impaired clot formation. *In vitro*, ETP was reduced and CT prolonged. Plasminogen/α2-antiplasmin ratio was elevated, which might lead to enhanced fibrinolysis, as evidenced by high D-dimer and PAPc levels and shortened CLT. It seems that impaired primary hemostasis, minor consumption of coagulation factors with enhanced fibrinolysis might explain the bleeding tendency in LPI. In addition, patients with liver cirrhosis have been described with a resembling clinical condition [167]. It is possible, that high prothrombin fragments 1 + 2, PAPc and D-dimer may reflect impaired hepatic clearance, due to liver involvement in LPI. The mentioned

molecule complexes are breakdown products of protease-inhibitor complexes with a short half-life [167]. Moreover, dyslipidemia further overloads liver. Since the hepatic clearance of both lipids and prothrombin fragments 1 + 2, PAPc and D-dimer is mediated by the LDL receptor related-protein 1 (LRP1) receptor [168], their prolonged clearance in LPI might be caused by exhaustion of LRP1 receptor.

We discovered that severity of renal insufficiency in LPI correlated with hyperfibrinolysis. Moreover, elevated plasminogen/α2-antiplasmin ratio may lead to plasminemia, which is known to be harmful to kidneys in animals [169]. In addition, decreased fibrin generation and clot formation, resulted by low or normal levels of fibrinogen and decreased FXIII activity, associated with markers of renal function.

6.2.2 Lysine and tranexamic acid in fibrinolysis

Lysine has an important role in fibrin formation and fibrinolysis. Both tissue plasminogen activator (tPA) and plasminogen have fibrin binding sites which often contain lysine binding sites, binding to internal and C-terminal lysine residues. C-terminal lysine residues, generated by plasmin, have an important role for a positive feedback mechanism for the stimulation of fibrinolysis [65].

The role of tranexamic acid, a synthetic analogue of lysine, in LPI is unknown. In general, tranexamic acid inhibits fibrinolysis by binding the lysine-binding sites on the plasminogen, preventing the binding of plasminogen to fibrin, and reducing the activation of plasminogen to plasmin.[170] Tranexamic acid can also be profibrinolytic; an increase in mortality has been reported if tranexamic acid was given more than three hours after injury[171]. However, the role of tranexamic acid in managing hyperfibrinolysis in LPI could be worth studying.

6.2.3 Lysine, calcium and coagulation

Lysine is also an important modulator of calcium homeostasis, and it affects calcium transport in both intestine and kidney[172]. Calcium is a key cofactor in the coagulation cascade, and hypocalcemia is associated with coagulopathy in trauma patients[173,174]. Due to dietary restrictions, patients with LPI are at risk for calcium deficiency, and calcium supplementation is recommended for all patients. However, plasma calcium levels have been in normal range in LPI patients.

6.2.4 Bleeding tendency and perioperative care in LPI

Patients with LPI are prone to bleeds in invasive procedures, such as kidney transplantations, and deliveries [52]. Therefore, perioperative coagulation screening should be performed carefully. In addition to routine screening, platelet count and

function, fibrinogen, FXIII and D-dimer should be measured in LPI patients. We recommend testing primary hemostasis with PFA-100 or PFA-200 (a newer platelet function analyzer). ROTEM was able to identify the patients with decreased fibrinogen and FXIII levels, and it may be useful in emergency situations and operative settings, guiding transfusion therapy in LPI patients. Platelets should be given preoperatively, and fibrinogen and FXIII concentrates should be given if necessary.

D-dimer is very high in LPI and therefore it is unusable for thrombosis diagnosis in patients with LPI. Venous ultrasound or/and computerized tomography may be needed to diagnose thrombosis.

6.3 Hypercalprotectinemia in LPI

Plasma calprotectin concentration was extremely high in all the studied 10 Finnish LPI patients, being nearly 1000 times higher compared to healthy controls. In general, plasma calprotectin levels observed in different inflammatory diseases have been much lower than in the present study. On the other hand, in patients with PAMI syndrome, plasma calprotectin can be even 500–12 000 times the normal levels [135,136].

6.3.1 Calprotectin in renal diseases

Increased serum and plasma calprotectin levels have been reported in patients with renal disease. In 2010, Malícková et al reported significantly elevated plasma calprotectin levels in patients with ESRD (mean 24 380 6 μg/l) [175], whereas in patients with acute antineutrophil cytoplasm antibody (ANCA) -associated vasculitis (AAV), serum calprotectin levels have varied from 5000–40 000 μg/l [176]. Moreover, a correlation between calprotectin levels and severity of disease in different types of glomerulonephritis has been reported [177]. It has been suggested that, besides being a disease biomarker, calprotectin may have a more direct role in pathogenesis of vasculitis and glomerulonephritis[178]. In glomeruli, infiltrating macrophages produce calprotectin and its subunits. Once released, calprotectin interacts with several receptors, including TLR4, activating productions of proinflammatory cytokines (e.g. interleukin 6 (IL-6), tumor necrosis factor α (TNF-α)) and stimulating autoreactive IL-17 producing T cells associated with vasculitis and glomerulonephritis.[179–181] Moreover, calprotectin has an ability to bind to the endothelium via heparin sulfate moieties and carboxylated glycans, resulting in release of leucocyte chemoattractants (such as CXCL1) and inducing endothelial cell injury, necrosis and apoptosis[182–185]. In renal biopsies of patients with AAV, a glomerular infiltration of calprotectin in active crescents as well as areas of focal

necrosis have been found [176]. In this study, we measured extremely high plasma calprotectin concentrations in LPI patients, and it is possible that calprotectin might have a role in the pathogenesis of renal disease in LPI.

6.3.2 The role of macrophages in the pathogenesis of LPI

It is recognized that macrophages have a key role in the development of severe complications in LPI. LPI macrophages display an impaired phagocytosis and TLR signaling, and downregulation in type I interferons (IFNs) and overexpression of pro-inflammatory and anti-inflammatory cytokines has been detected in LPI macrophages[36,87,186]. Elevated plasma concentrations of inflammatory chemokines CXCL8, CXCL9 and CXCL10 have been detected in patients with LPI, and two of these chemoattractants correlated with loss of renal function[36]. In addition, it has been showed that genes related to chemotaxis and inflammatory responses are upregulated in LPI[114]. Moreover, y+LAT1 protein directly modulates inflammatory phenotype of macrophages and regulates the innate immune responses[186].

Calprotectin, expressed mostly in neutrophils, monocytes and early differentiated macrophages, is an endogenous ligand of TLR4[119,187]. Calprotectin has intrinsic cytotoxic and proinflammatory properties, and it has been associated with several inflammatory diseases, infections and malignancies.[187,188] Moreover, it has been reported that plasma calprotectin levels correlate with the severity of Covid19 infection and with comorbidities[189]. Hypercalprotectinemia in LPI is a novel finding, supporting the theory of a systemic inflammatory state in LPI.

6.3.3 Hyperzincemia

Hyperzincemia is a rare condition. Generally, slightly elevated plasma zinc concentration is attributed to sample contamination, hemolysis or prolonged storage before separation of plasma[190,191]. In addition, hyperzincemia can be caused by zinc supplementation and parenteral solutions with excessive zinc content, but in such cases marked hyperzincuria is detected[128,192]. In PAMI syndrome, plasma/serum zinc levels are clearly elevated and plasma calprotectin is extremely high. This rare disease is caused by specific deleterious variants in the gene encoding PSTPIP1, which is a regulatory phosphatase protein modulating T-cell and phagocyte activation, cytoskeletal organization and IL-1 production. The pathophysiological mechanisms of this disease are not completely understood, but deleterious variants in the *PSTPIP1* are suggested to lead to and increased binding to the pyrin inflammasome, causing inflammasome activation and IL-1beta overproduction.[136,193] However, patients with PAMI syndrome do not have symptoms of zinc intoxication, and hyperzincemia is suggested to represent a

pseudo-elevation, since functional zinc stores are depleted due to calprotectin's high zinc-binding affinity[137,194].

Elevated plasma and serum zinc concentrations have been described in patients with LPI[52,140]. Surprisingly, in the current study, plasma zinc levels, measured by photometry, were normal or only mildly elevated. At the time of the current study, zinc analyses were performed in different laboratory with a different measurement method, and this might have influenced the results. Also, one possible explanation for this is that some of the zinc may be bound to calprotectin so tightly that it is not detected by the photometric method, although it is detected by AAS. However, after this study (III) was published, we measured plasma zinc by AAS, and with this method, plasma zinc concentrations were remarkably elevated (Table 11). Furthermore, patients with extremely high plasma calprotectin concentrations might suffer from zinc deficiency since calprotectin has a high binding capacity for zinc. In addition, due to dietary restrictions, LPI patients are at risk for nutritional deficiencies, and zinc deficiency is described to increase calprotectin levels[149,150].

6.4 Diagnostic challenges in LPI

LPI is a complex multisystem disease with wide spectrum of symptoms, varying from nearly normal growth to severe multi-organ disease. Majority of the patients with LPI develop strong aversion to dietary protein at an early age and, thus, classical symptoms of LPI can be absent or minimal. LPI lacking disease-specific manifestations can mimic various diseases, and it may be difficult to differentiate LPI from other conditions. Posey et al (2014) described a 5-year-old boy with idiopathic osteoporosis, lacking typical symptoms of LPI. Whole exome sequencing was performed and the patient was diagnosed with LPI. [195] Lokuhewage et al (2023) reported a 2.5-year-old girl with LPI, presenting with pancytopenia and splenomegalia, mimicking acute leukemia, without any other classical features of LPI.[83] Hanafusa et al (2023) reported a case of 47-year-old woman who was initially diagnosed with renal tubular acidosis/Fanconi syndrome and rickets, and afterwards, with LPI performing comprehensive genetic analysis[95]. IJzermans et al (2023) reported a 52-year-old woman with LPI, initially diagnosed with fibrillary glomerulonephritis and hemochromatosis [161]. In Finland, a 55-year-old woman with ESRD of previously unknown etiology was diagnosed with LPI during pretransplant evaluation (unpublished observation). One female patient had a postpartum psychotic episode, probably due to hyperammonemia, and she was subsequently diagnosed with LPI (unpublished observation). In addition, one case of LPI associated with moyamoya vasculopathy has been reported[196].

These cases show that inherited metabolic diseases, including LPI, should be considered in the differential diagnosis of renal tubular diseases, or when patients

present with clinical features that do not fit accurately to a known clinical entity. In addition, use of exome/genome-wide testing as a first-tier diagnostic approach has led to LPI diagnosis in cases with highly atypical phenotype.

6.5 Strengths and limitations of the study

In Finland, the follow-up of LPI patients is, although unofficially, centralized in Turku University Hospital. The patients included in this study represent the largest LPI patient cohort in the world. Large-scale coagulation tests performed in LPI patients provided new information about coagulopathy in LPI, and plasma calprotectin concentration measurements were performed for the first time in LPI patients. Moreover, routine follow-up of all LPI patients in Turku included, apart from clinical investigation, also regular laboratory analysis of several metabolic parameters.

This study also carries some limitations. As in all rare (metabolic) diseases, the small number of patients hampered the use of statistics. Statistically significant results are seldom obtained in clinical studies with a low number of human subjects, and clinically meaningful differences and findings may therefore have been missed. A matched control group without renal insufficiency was not included in the study design; such a group of LPI patients is in real life impossible to find. In studies II and III, LPI patients were compared with healthy individuals only.

In study II, one laboratory-related limitation was the absence of the second step of centrifugation during sample collection, which is recommended for the CAT assay.

In study III, plasma zinc levels differed from earlier measurements. However, at the time of the current study, zinc analyses were performed in different laboratory with a different measurement method, and this might have influenced the results.

6.6 Future considerations

Mechanisms behind the complications of LPI are still poorly understood. The phenotype varies from mild symptoms and nearly normal growth to severe multi-organ disease. Current treatment largely prevents the acute manifestations of the disease, but it has shown to be poor in preventing long-term complications. Renal insufficiency is a common complication in LPI, and many patients have developed ESRD, requiring dialysis and kidney transplantation. The exact mechanisms of renal impairment are still unknown. The possible role of the current treatment protocols in the development of severe complications is still unclear. Renal insufficiency may even be a part of natural progression of LPI. In some cases, CKD had led to renal failure at an early age. Thus, signs of renal involvement should be monitored

regularly and risk factors, such as hypertension and dyslipidemia, should be treated carefully.

Since most of the LPI patients develop strong aversion to dietary protein at an early age, classical symptoms of LPI can be absent. Thus, in several cases, LPI has been detected only in adulthood. On the other hand, the phenotype may be very variable and resemble other clinical entities, therefore leading to diagnostic delay. Use of exome/genome-wide testing as a first-tier diagnostic approach has led to diagnosis even in cases with highly atypical phenotype, concurrently broadening our understanding of this multifaceted disease.

In future, positron emission tomography (PET) could be an interesting non-invasive method to investigate metabolic changes in LPI and to determine whether hepatic clearance is impaired in LPI. A total body PET imaging provides an opportunity to study multiple organs simultaneously, which could be particularly valuable when metabolic diseases with multi-organ involvement are studied.

Calprotectin is an important inflammatory biomarker, and it seems to be useful for monitoring inflammatory activity in several diseases. Extremely high plasma calprotectin in LPI is a novel finding. It might suggest previously unknown molecular mechanisms behind the pathogenesis of complications. Hypercalprotectinemia supports the theory of a systemic inflammatory state in LPI, but further studies are required to confirm the relevance of this finding. Moreover, it is possible that there is a link between abnormal coagulation and hypercalprotectinemia in LPI. Immunohistochemistry of renal, liver and lung biopsies could help to detect possible calprotectin overexpression in these tissues, but one must remember that invasive procedures can be highly risky for LPI patients and thus biopsies are not routinely performed.

In addition, patients with LPI might actually suffer from hypozincemia due to calprotectin's high zinc binding capacity. Zinc has numerous important roles, and zinc deficiency is known to result in immune dysfunction and impaired hemostasis. The role of zinc supplementation in LPI would be worth studying.

7 Summary/Conclusions

LPI is a complex multisystem disease. It influences many organ systems and the human metabolism. It has been shown that despite the treatment, patients are in risk for severe complications which may manifest even decades after diagnosis. Mechanisms behind renal insufficiency, pulmonary, immunological, and hematological complications are still poorly understood.

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

I Renal insufficiency is a common complication in Finnish LPI patients. Urine beta2-microglobulin is currently the most sensitive early marker of renal complication, and it should be monitored regularly in LPI patients. Currently, a total of eight (19 %) Finnish LPI patients have received a kidney transplant. The prognosis after renal transplantation has been satisfactory. In this study, there was no significant correlation between weight-based L-citrulline dose and renal function. However, due to the possible role of citrulline in the development of renal disease, we have slightly reduced L-citrulline doses and monitored plasma citrulline concentrations regularly. More investigation on this subject is clearly needed.

II Impaired hemostasis and minor consumption of coagulation factors together with enhanced fibrinolysis may explain the bleeding tendency in LPI. High levels of F1 + 2, D-dimer and PAPc could reflect impaired hepatic clearance, but not consumption of coagulation factors as previously suggested. The severity of renal insufficiency correlated with hyperfibrinolysis. Spontaneous bleeds are rare in patients with LPI, but they are prone to bleed in invasive procedures and deliveries. Perioperative coagulation screening should include total cell count, platelet function, fibrinogen, FXIII, D-dimer and evaluation of primary hemostasis with PFA-100 or PFA-200. Platelet level should be corrected preoperatively, likewise fibrinogen and FXIII should be adjusted with concentrates. ROTEM may be a useful tool in emergency situations, guiding transfusion therapy. D-dimer is very high in LPI and therefore it is unusable for thrombosis diagnosis in patients with LPI.

III We observed extremely high plasma calprotectin concentration in all 10 studied LPI patients. This novel clinical finding is a new addition to the peculiarities observed in LPI. It might suggest previously unknown mechanisms behind pathogenesis of LPI. However, further studies are needed to confirm the relevance of this finding and its significance in the course of LPI as well its predictive value for the onset of renal, coagulation or systemic complications.

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