Renal injury in liver transplantation

Clinical and mechanistic studies

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To my beloved family

“Now, bring me that horizon”

CAPTAIN JACK SPARROW

ABSTRACT

Background: Renal injury in liver transplantation (LT) is common and presents both as early and late insufficiency. These conditions are interconnected and have substantial impact on the long-term outcome. Multiple factors are involved in its development, often categorized as pre-, intra-, and post-LT risk factors, and comprise both donor and recipient characteristics. Recently, the transplant procedure and liver graft itself have been suggested as important contributors to the immediate acute kidney injury (AKI) post-LT, with hepatic ischemia-reperfusion injury being assigned a causative role for early AKI development. However, the mechanisms by which the liver graft affects remote organs, and by which the renal injury evolves, remain obscure. The current thesis aimed to explore renal injury in LT, from characterization of donor and recipient risk factors, via description of frequency and severity of post-LT renal injury, to in-depth analyses of liver and renal subcellular mechanisms involved in the process. Finally, we explored the role of hypothermic oxygenated machine perfusion (HOPE) of liver grafts in subsequent AKI development.

Methods: Papers I-III originate from data collected during a prospectively conducted study in Gothenburg, comprising of 27 LT patients and their corresponding renal and liver biopsies. Paper IV was a retrospective analysis of the first HOPE treated LTs in our centre, and historic controls. **Paper I** analyzed the frequency of early post-LT renal injury, its evolution and risk factors. Using quantitative proteomics, **Paper II** studied the intraoperative proteomic profiles of liver grafts of patients with and without AKI. **Paper III** explored renal proteome alteration patterns during LT, in patients developing AKI compared to those maintaining pre-LT renal function. **Paper IV** examined the impact of HOPE for older donor livers, on early post-procedure AKI.

Results: Liver transplantation associated with a high risk of substantial decline in measured glomerular filtration rate (mGFR) within days, and AKI within hours of LT, irrespective of pre-LT mGFR, absence of pre-transplant renal pathology, and before introduction of calcineurin inhibitors. Early and late renal injury correlated strongly. Liver graft proteomic profile of LT recipients with AKI differed significantly from that of patients without AKI. Differences were dominated by inflammation and early activation of innate immunity. The same group also presented complex intraoperative renal proteomic alterations, characterized by inflammation, with extracellular matrix-, and mitochondrial modifications. End-ischemic HOPE of liver grafts from older donors, did not reduce the frequency or severity of early post-LT AKI.

Conclusion: Liver transplantation heavily affects post-LT renal function, with substantial consequences for long-term, renal outcome. This thesis uncovered new mechanistic insights into the global proteomic alterations of livers transplanted to recipients developing early AKI. Moreover, our results reveal apparent proteomic renal changes within minutes after liver graft reperfusion. In the absence of overt hemodynamic alterations, we suggest hepatic ischemia-reperfusion injury as the causative event for remote organ, renal injury. HOPE-treatment of high-risk liver grafts of elderly donors did not mitigate neither ischemia-reperfusion injury, nor remote AKI.

Key words: liver transplantation, acute kidney injury, mGFR, hepatic ischemia-reperfusion injury, quantitative proteomics, HOPE

SAMMANFATTNING PÅ SVENSKA

En levertransplantation är ett livräddande ingrepp för patienter med leversjukdom. Under senare decennier har korttidsresultaten förbättrats påtagligt, medan motsvarande trend beträffande långtidsöverlevnaden avstannat. Orsakerna till detta är flera, men en viktig aspekt är nedsatt njurfunktion. Kroniskt försämrad njurfunktion hos organtransplanterade patienter medför ökad dödlighet.[1]

I samband med en levertransplantation kan njurarna skadas av ett stort antal faktorer, vilka delas in i pre-, per-, och postoperativa händelser, beroende på när i förhållande till transplantationsingreppet, de bidrar till skadan. Under uttag och insättning av levern utsätts denna för ischemi och reperfusion (enkelt uttryckt upphävd och återställd blodcirkulation), vilket orsakar ett inflammatoriskt tillstånd med långtgående effekter på såväl levertransplantat som andra avlägsna organ i kroppen. Detta ”fenomen” har under senare år ådragit sig stort intresse. Olika faktorers respektive betydelse, samt när och hur njurfunktionsnedsättning uppkommer, är dock fortfarande oklart. Forskningsfältet är således viktigt för att förbättra livskvaliteten och långtidsresultaten efter levertransplantation.

Syftet med denna avhandling var att studera både övergripande, kliniska och detaljerade molekylära aspekter av njurfunktionen i ett transplantationssammanhang. Vi skapade en prospektiv studie bestående av 27 levertransplanterade patienter i Göteborg, där vi noggrant följde njurfunktionen hos deltagarna och analyserade såväl lever-, som

njurbiopsier, insamlade under transplantationen. Delarbete I-III baserades på detta material, där vi först beskrev njurfunktionsutvecklingen i gruppen som helhet, från acceptans för transplantation och upp till tre år efter operationen. Vi analyserade frekvens och allvarlighetsgrad av akut njursvikt samt dess riskfaktorer. I delarbete II undersökte vi leverbiopsier från operationen med ljusmikroskopi och kvantitativ proteinanalys, med hypotesen att dessa bilder skulle skilja sig åt mellan patienter som drabbades av akut njurskada jämfört med opåverkade patienter. I delarbete III undersökte vi på motsvarande vis njurbiopsier utförda under levertransplantationen. Vår avslutande studie, delarbete IV, prövade huruvida hypoterm syresatt maskinperfusion (HOPE) av levergraft från äldre donatorer kunde påverka ischemi-reperfusionsskadan i levern och därmed minska såväl insjuknandet i, som omfattningen av akut njurskada efter transplantationen.

Sammanfattningsvis konkluderar vi att:

Omfattande njurskada är vanlig och kliniskt uppenbar inom timmar efter levertransplantation, oberoende av tidigare njurfunktion eller inverkan av njurskadlig medicinering. Tidig njursvikt, oavsett allvarlighetsgrad, är starkt associerad med sen och kronisk njurskada.

Proteinuttrycket i levrar, vars mottagare snart utvecklar njursvikt, skiljer sig från det hos patienter med opåverkad njurfunktion. Bilden präglas av inflammation och tidig aktivering av det medfödda immunförsvaret. Detta antyder att den transplanterade levern bidrar till tidig postoperativ njurskada.

Tydliga förändringar av njurarnas proteinmönster sker under levertransplantationen hos patienter som drabbas av tidig njursvikt. Denna bild präglas, likt ovan, av inflammation, modifiering av stödjevävnad och påverkan på celler och signalsubstanser. Begynnande njurskada är således uppenbar redan minuter efter att blodcirkulationen återställts till levertransplantatet.

Varken insjuknandet i, eller allvarlighetsgrad av akut, tidig njurskada tycks påverkas av HOPE-behandling av äldre donatorslevrar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. Norén Å, Åberg F, Mölne J, Bennet W, Friman S, Herlenius G. **Perioperative kidney injury in liver transplantation: a prospective study with renal histology and measured glomerular filtration rates**. Scand J Gastroenterol. 2022 May;57(5):595-602.

II. Norén Å, Oltean M, Friman S, Molinaro A, Mölne J, Sihlbom C, Herlenius G, Thorsell A. **Liver Graft Proteomics Reveals Potential Incipient Mechanisms behind Early Renal Dysfunction after Liver Transplantation.** Int J Mol Sci. 2022 Oct 8;23(19):11929.

III. Norén Å, Boi R, Pullerits R, Mölne J, Friman S, Sihlbom C, Herlenius G, Nyström J, Oltean M**. Proteomic analysis of human kidney biopsies unveils emerging acute kidney injury within minutes after liver graft reperfusion.** Manuscript

IV. Norén Å, Mölne J, Bennet W, Sörensen G, Herlenius G, Lindnér P, Oltean M. **End-ischemic hypothermic oxygenated machine perfusion does not improve renal outcome following liver transplantation from aged donors: A single centre retrospective report.** Submitted

CONTENT

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ABBREVIATIONS

AKI	Acute kidney injury
AKIN	Acute kidney injury network
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BMI	Body mass index
CIT	Cold ischemia time
CKD	Chronic kidney disease
CNI	Calcineurin inhibitor
COD	Cause of death
Cr	Creatinine
CVA	Cerebrovascular accident
CysC	Cystatin C
DAMP	Danger associated molecular pattern
DBD	Donation after brain death
DCD	Donation after circulatory death
DNA	Deoxyribonucleic acid
DRI	Donor risk index
ECD	Extended criteria donor
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FC	Fold change
GFR	Glomerular filtration rate
HIRI	Hepatic ischemia-reperfusion injury
HOPE	Hypothermic oxygenated machine perfusion
HMGB1	High mobility group box 1
IGFBP-7	Insulin-like growth factor binding protein 7
INR	International normalized ratio
IQR	Interquartile range
IRI	Ischemia-reperfusion injury
KDIGO	Kidney Disease Improving Global Outcomes
LT	Liver transplantation
MELD	Model for end-stage liver disease
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NET	Neutrophil extracellular trap
PCA	Principal component analysis
POD	Post-operative day
PRR	Pattern recognition receptor
RIFLE	Risk, injury, failure, loss, end-stage renal disease
ROS	Reactive oxygen species
SAA2	Serum amyloid A2
SD	Standard deviation
TMT	Tandem-mass-tag

INTRODUCTION

Liver transplantation (LT) is the treatment of choice for a wide range of liver diseases, with overall results having improved dramatically, since its broad clinical implementation in the mid-1980's. Advancements in surgical technique, perioperative management, prevention of opportunistic infections, and immunosuppressive therapy have yielded excellent short-term graft and patient survival outcomes, which have become the expected norm. However, long-term results have not followed the same trend. One of the many challenges within this field is to preserve renal function after LT, which has been shown to be an important factor affecting long-term outcomes.[1,2]

From a historical perspective, calcineurin inhibitors (CNIs) have been held responsible for most of the renal impairment after LT, with particular attention on late renal failure. More recently, focus has shifted to early or even immediate post-LT renal damage, and its strong association with chronic kidney disease (CKD).[3] Different mechanisms for renal impairment after LT have been proposed, but a complete and concordant understanding of the problem, as well as targets for intervention, are still deficient and required.

In this thesis we address the acknowledged, albeit incompletely understood, correlation between LT and early renal injury, in a cohort of prospectively enrolled adult LT-patients, scrutinizing demographic, functional, histological, and molecular characteristics. We also examined the effect of hypothermic oxygenated machine perfusion (HOPE) on liver grafts, the proposed mitigated hepatic ischemia-reperfusion injury (HIRI), and whether or not this could be reflected in better preserved renal function after LT.

1.1 GRAFT QUALITY AND RENAL FUNCTION

1.1.1 Assessment of liver graft quality

A thorough evaluation of the liver graft is of paramount importance for the overall outcome of LT. The assessments of a potential donor and its liver are based on a broad consideration of numerous parameters such as donor age, size and medical history, routine blood tests, preoperative imaging modalities, and lastly, the surgeon's judgement during organ procurement. The lack of available organs for LT combined with the raising death rates among patients awaiting a LT, have led to the increasing use of organs from extended criteria donors (ECD). However, although transplant physicians are entirely aware of the significant impact of suboptimal donor characteristics on LT outcome, the quantitative risks associated with combinations of characteristics are unclear.[4] Accordingly, various guidelines and indexes have been proposed, to provide objective information in the decision-making of donor evaluation and organ allocation. One of the first to be introduced, and likely the most frequently used index, was presented by Feng et al. in 2006 and later named the Donor Risk Index (DRI) or Liver Donor Risk Index.[4] Today it comprises of eight donor variables, each assigned a relative risk of graft failure (Table 1). The DRI has proven valuable over the years but has also been criticized for being developed based on a retrospectively assembled material, before the introduction of the MELD (model for end-stage liver disease) concept, in addition to the incorporation of race in the algorithm.[5] In addition, the Discard Risk Index, was created by R Abbas et al., in an attempt to find allocation for even extremely marginal allografts.[6]

TABLE 1. The Donor Risk Index, From Feng et al.[4]

Donor factors	Reference donor
Age (years)	Under 40
Cause of death	Trauma
Race	White
DCD	No
Partial/Split	No
Height (centimeters)	170
Location	Local
Cold ischemia time (hours)	8
Donor Risk Index*	1

*Calculation: Donor risk index = exp[(0.154 if 40≤ age <50) + (0.274 if 50≤ age <60) + (0.424 if 60≤ age <70) + (0.501 if 70 ≤ age) + (0.079 if COD = anoxia) + (0.145 if COD = CVA) + (0.184 if COD = other) + (0.176 if race = African American) + (0.126 if race = other) + (0.411 if DCD) + (0.422 if partial/split) + (0.066 ((170-height)/10)) + (0.105 if regional share) + (0.244 if national share) + (0.010 × cold time)].

COD: Cause of death, CVA: Cerebrovascular accident, DCD: Donation after circulatory death

The terms "extended criteria donors" and "high risk grafts" are not clearly defined, but generally point towards worse than standard quality and imply the risk of inferior post-LT results.[7-9] Most probably the truth is not black or white, but could be better considered a continuum of risks, highlighting the importance of careful selection of ECD and matching recipient, to obtain satisfactory outcomes. During the past decade promising data regarding liver perfusion machines have been reported on two major techniques, a hypothermic, and a normothermic oxygenated perfusion. Encouraging results on viability testing of liver grafts during normothermic machine perfusion have been reportred.[10] Likewise, HOPE after an initial period of cold static preservation has been thoroughly researched, and levels of flavin mononucleotide in the perfusate have been reported as a promising marker of allograft ischemia-reperfusion injury (IRI), and hence post-LT liver function.[11] Most probably more objective methods for viability testing, as well as modification modalities of suboptimal grafts prior to implantation, will be exploited and adopted in routine practice in the future.[12]

1.1.2 Assessment of renal function

Evaluating renal function in patients with liver disease is challenging. The glomerular filtration rate (GFR) is widely accepted as the best overall index of renal function in health and disease, and measured GFR (mGFR) by several methods is considered the "gold standard".[13] However, measurements of GFR are traditionally considered difficult to perform, costly, and inconvenient for repeated testing, and are frequently replaced by estimations of GFR, obtained by assessing the serum levels of endogenous filtration markers, such as creatinine (Cr) and/or cystatin C (CysC).[14,15] Serum Cr, the clinical marker in routine practice, may be influenced by physiologic factors (i.e. gender, age and race, body weight, medications, and diet) and analytical errors, for example laboratory techniques. Furthermore, in a healthy individual, nearly 50-60% of GFR may be lost before a change in sCr is detectable.[16] Cirrhotic patients often have sCr which is affected by reduced hepatic production of creatine, malnutrition, decreased muscle mass, oedema, increased tubular secretion, and may indicate falsely low values.[17,18] As a result, the creatinine-based methods for calculating GFR most often overestimate true GFR in this cohort of patients. CysC, on the other hand, is a non-glycosylated, low molecular weight, basic protein, produced at a constant rate by all nucleated cells. The serum concentration of CysC is less influenced by sex, age, muscle mass, or serum bilirubin than sCr is, and therefore considered mainly influenced by GFR.[19] Different equations, incorporating various demographic variables, in addition to sCr, sCysC, or a combination of both have been proposed, each of which more or less accurate depending on health condition. Therefore, and unfortunately, to date, there is no perfect formula or equation for calculating GFR, that is even near the accuracy of mGFR, especially not during the perturbant circumstances characterizing the peri-transplant period.[20,21]

Renal pathology in liver disease and after LT has been described by others.[22-24] Histological signs of membranous glomerulonephritis, membranoproliferative glomerulonephritis, IgA nephropathy, diabetic lesions, acute tubular necrosis, and other ultrastructural abnormalities are seen in renal biopsies from patients awaiting LT, both with and without previously known or measurable renal impairment. Post-LT renal biopsies show a wide range of pathological abnormalities, of which glomerular disease and expansion of the mesangial matrix are common.[25,26]

1.2 ACUTE KIDNEY INJURY

Acute kidney injury (AKI) is defined by a rapid increase in serum creatinine, decrease in urine output, or both. It is a broad clinical syndrome encompassing various etiologies, ranging from extrarenal pathology (pre-, and/or postrenal obstruction), and non-specific conditions, to more specific, intrinsic, or renal diseases (i.e. interstitial or glomerular nephritis, and acute vasculitic renal diseases). This basic taxonomy has recently been modified to more specific syndromic descriptions such as cardiorenal-, hepatorenal-, nephrotoxic-, and sepsis-associated AKI among others. The rationale behind this is the increasing evidence that each of these syndromes have a unique pathophysiology and treatment.[27] In addition, the risk for AKI is increased by predisposing factors, such as demographic characteristics, genetic burden, and comorbidities. A major clinical challenge in the context of AKI, is that the above conditions often coexist in the same patient or arise as part of other life-threatening conditions (critical illness, sepsis, heart failure, and liver failure). These turbulent circumstances hence tend to compromise the attention drawn to AKI which, even if mild, has fundamental clinical consequences, eventually increasing mortality.[28]

1.2.1 Brief pathophysiology of AKI

Since AKI is a complex clinical syndrome, its pathophysiology varies widely according to the conditions associated with its development, as indicated above. However, regardless of causative circumstances, AKI is believed to be related to a mismatch between oxygen and nutrient delivery to the nephrons, and unmet energy demands, ultimately leading to cellular stress and harm, vascular and tubular injury, and inflammation.[29,30] Briefly, under normal conditions, GFR is determined by the permeability of the glomerular filtration barrier, and by the Starling forces, i.e. the equilibrium between the hydrostatic and colloid osmotic pressures in the glomerular capillaries, and in Bowman's capsule. GFR is also to some extent dependent on renal blood flow. Hence, AKI results under circumstances that decrease GFR through impairment in autoregulation of renal blood flow and filtration. Moreover, when renal compensatory mechanisms are restrained by comorbidities or a decrease in

systemic vascular resistance (for instance the splanchnic vasodilation frequently seen in cirrhotic patients) AKI may arise, predominantly if this condition is paralleled by volume depletion, infection, or other insults.

1.2.2 Definitions of AKI

Changes in sCr and/or urine output form the basis of all diagnostic criteria for AKI. Two similar definitions of AKI, the RIFLE criteria (Risk, Injury, Failure, Loss, and End-stage renal disease) from 2004, later modified into the Acute Kidney Injury Network (AKIN) definition of 2007 have been proposed and validated.[31,32] These definitions were further refined in the 2012 Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guideline for Acute Kidney Injury, which is, at present, the most widely accepted definition of AKI[33] (Table 2). However, all three definitions, in addition to numerous unvalidated descriptions, are used in the literature, making comparisons between studies difficult. Furthermore, the epidemiology of AKI has been hard to describe as a consequence of the different definitions employed.

TABLE 2. AKI, KDIGO definition and stages, From Khwaja et al.[34]

Stage	Serum creatinine	Urine output
1	1.5-1.9 times baseline **or** ≥0.3 mg/dl (≥26.5 µmol/l) increase	<0.5 ml/kg/h for 6-12 hours
2	2.0-2.9 times baseline	<0.5 ml/kg/h for ≥12 hours
3	3.0 times baseline **or** increase in serum creatinine to ≥4.0 mg/dl (≥353.6 µmol/l) or initiation of renal replacement therapy	<0.3ml/kg/h for ≥24 hours or anuria for ≥12 hours

1.2.3 Liver Transplantation and AKI

AKI is a frequent complication after LT, strongly associated with development of CKD.[3] The reported frequencies of post-LT AKI and CKD vary widely, between 12-94% and 10-45% respectively, primarily due to the different definitions in use (see above).[35-37] Occurrence of AKI after LT is associated with a markedly increased 30-day mortality, which is even further augmented if renal replacement therapy is required.[38-40]

The etiology of AKI in the peri-transplant period is thought to be multifactorial and related to pre-transplant aspects (i.e. age, sex, pre-transplant renal function, severity of liver disease), intra-operative events (i.e. hemodynamic instability) and post-transplant factors, such as graft dysfunction, bacterial infections, and drug toxicity.[37,38,41]

Traditionally, CNIs have been blamed for most of the renal injury that arises post-LT, and CNI discontinuation has been shown to improve the severity of already manifest CKD.[42] Immunosuppressive treatment with CNIs is associated with both acute and chronic kidney injury, where the acute nephrotoxicity is driven mainly by reversible vasoconstriction of the afferent arteriole due to an imbalance in vasoconstrictor and vasodilator factors.[43] Chronic CNI exposure, on the other hand, not only induces reversible alterations, but also associates with permanent changes of renal architecture, affecting vessels (arteriolar hyalinosis), tubulo-interstitium (tubular atrophy and interstitial fibrosis), and glomeruli (thickening and fibrosis of Bowman's capsule and focal segmental or global glomerular sclerosis).[44]

In recent times, focus on early renal impairment after LT has shifted to the impact of the LT procedure per se; the effects of extended criteria liver grafts, post-reperfusion syndrome, and especially hepatic ischemia-reperfusion injury. The growing discrepancy between supply and demand of organs for liver transplantation has resulted in an increasing use of liver grafts from ECDs. However, this trend has been paralleled by a higher incidence of early, post-LT AKI, implying that the liver graft itself is an important element in the development of renal dysfunction. Similarly, others have reported a strong relationship between HIRI and peri-operative AKI.[41,45-48]

Interestingly, recent studies indicate that renal metabolism and function are altered already a few hours after liver graft reperfusion, even in the absence of hemodynamic or pharmacologic causes.[49] It is clearly shown that despite the hyperdynamic circulation that normally ensues a LT, and an obvious reduction in renal vascular resistance, measured GFR is profoundly reduced, while renal oxygen consumption is increased.

Despite the accumulating evidence, the potential mechanisms connecting LT to AKI remain unclear. Routine markers of liver damage and graft preservation injury, such as circulating liver enzymes and even microscopic examination of the graft, often fail to discriminate between liver recipients who are going to develop AKI or not. Since treatment options for AKI are few, and consequently rely on supportive and preventive strategies, a better understanding of post-LT AKI is crucial, to improve both early and late outcome.

Figure 1 shows a schematic illustration of the putative pathophysiology behind the AKI to CKD transition.[50]

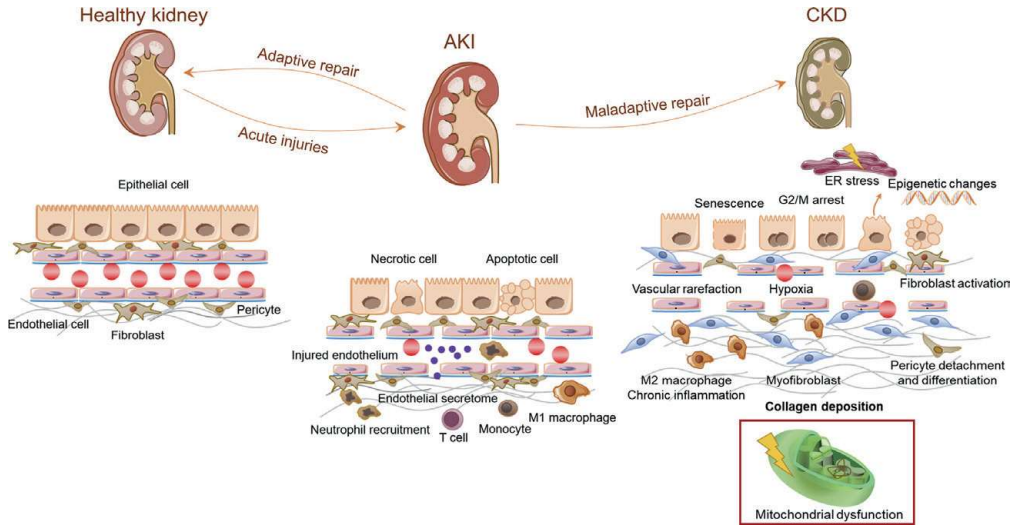


FIGURE 1. Schematic illustration of pathophysiological processes involved in the acute kidney injury (AKI)-chronic kidney disease (CKD) continuum, From Jiang M et al.[50]

ER: Endoplasmic reticulum

1.3 ISCHEMIA-REPERFUSION INJURY

Ischemia-reperfusion injury is a pathological condition characterized by a limitation in blood supply to an organ or tissue, followed by restoration of perfusion and reoxygenation, eventually giving rise to an inflammatory response, similar as seen in sepsis and multi-organ failure.[51] Ischemia-reperfusion injury is encountered in a multitude of clinical settings and contributes to morbidity and mortality in myocardial infarction, ischemic stroke, circulatory shock, vascular and general surgery, and during solid organ transplantation, among other conditions. Hepatic IRI has been extensively studied, both following liver surgery and liver transplantation. Apart from its central role in early allograft function or failure, HIRI has also been found to induce global consequences that affect and harm remote organs, of which the kidneys are particularly vulnerable.[52,53]

1.3.1 Pathophysiology of IRI

The pathophysiology of IRI is initiated by the hypoxic phase, which on a broad cellular level, leads to dysfunction of the electron transport chain in mitochondria, reduced adenosine triphosphate (ATP) production, anaerobic metabolism, and a marked decline in cellular antioxidative substrates. The ensuing energetic failure affects ion channels of cell membranes (including those of intracellular organelles) causing accumulation of sodium, hydrogen and calcium ions, ultimately resulting in hyperosmolarity, cellular swelling, and reduced cellular pH, all of which further impair normal enzyme activity. During reperfusion, the biochemical and molecular changes that occurred during ischemia lead to free radical formation, i.e. reactive oxygen species (ROS), causing oxidative stress that

promotes lipo-peroxidation, membrane injury and DNA damage and eventually resulting in cell death, local inflammatory responses and endothelial dysfunction (further impairing microcirculation and hence contributing to additional hypoxia). Inflammatory cascades and oxidative stress may subsequently induce a cytokine storm, further worsening the initial local and remote cellular injury and death, caused by the initial damage to cellular structures.[54] The mechanisms of HIRI are summarized in Figure 2.

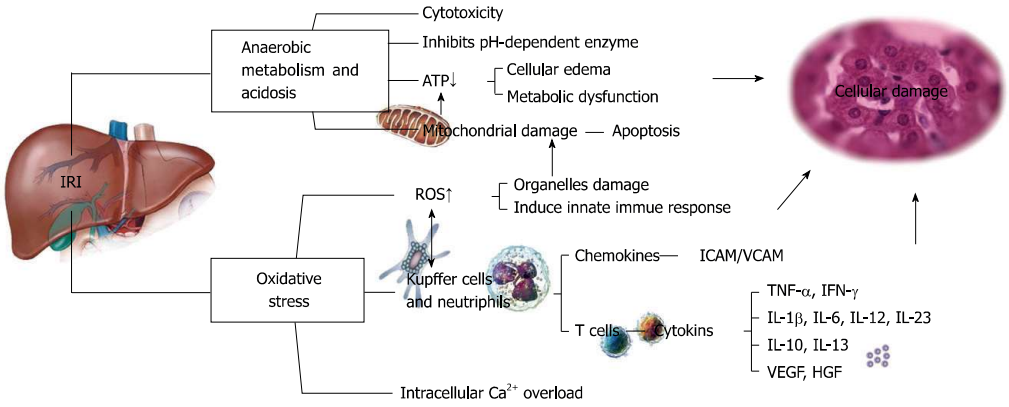


FIGURE 2. Mechanisms of hepatic ischemia-reperfusion injury, From Guan LY et al.[55]

ATP: Adenosine triphosphate; ICAM: Intercellular adhesion molecule; IFN-γ: Interferon-gamma; IL: Interleukin; IRI: Ischemia-reperfusion injury; ROS: Reactive oxygen species; TNF: Tumor necrosis factor; VCAM: Vascular cell adhesion molecule

1.3.2 Immunology of HIRI

Liver IRI activates the innate immune system to orchestrate the complete development of inflammatory hepatocellular injury, universally seen following HIRI. The liver is a unique immunological organ, with the highest content of tissue resident macrophages (Kupffer cells), dendritic cells, and lymphocytes (T cells, NK cells, and B cells), which act together with infiltrating immune cells to respond to and resolve the hazard of IRI.[56] Initial hepatocellular damage, caused by the ischemic insult and subsequent formation of ROS, gives rise to free, extracellular HMGB1 (high mobility group box 1), histone/DNA and ATP, in addition to many more nuclei derived, membrane bound or cytosolic molecules and cellular fragments, all of which can act as danger associated molecular patterns (DAMPs). These DAMPs further operate through different pattern recognition receptors (PRRs), on resident immune cells, other hepatocytes, and liver sinusoidal endothelial cells, to either activate or inhibit liver inflammatory immune responses, depending on graft, and recipient circumstances and factors. Hence, in a more proinflammatory setting, innate and adaptive immune cells are recruited and activated, further increasing the production of ROSs, DAMPs, proinflammatory cytokines and chemokines, as well as neutrophil extracellular traps, known as NETs, while immune regulatory conditions inhibit inflammation.[57] In summary, HIRI activates a complex intercellular signaling network, which transfers inflammatory information to control innate immune cell activation as well as regulate parenchymal cell death. Recent

studies have revealed that DAMPs may trigger not only proinflammatory, but also immune regulatory responses by activating different PRRs or unique intracellular signaling conduits. The one mechanism being the most dominant decides the HIRI outcome. Interestingly there are many publications on how and where to counteract the devastating effects of IRI, but few of these have yet been adopted in clinical practice.[58,59]

1.3.3 Clinical assessment of HIRI

Several clinical tests are routinely used to monitor liver graft function after LT i.e., aspartate aminotransferase (AST), alanine aminotransferase (ALT) bilirubin, and international normalized ratio (INR). More recently, though not a new understanding, the transaminases, have gained much attention as markers of HIRI following LT. However, there is disagreement concerning sensitivity and specificity of these tests, and how or if they describe the event of HIRI. Both AST and ALT are known to increase after LT regardless of significant ischemia-reperfusion injury or not. While some authors present a distinct correlation between post-LT peak serum AST (considered a surrogate marker of HIRI) and succeeding AKI, others highlight an AST threshold at a precise time-point or the kinetics in transaminase recovery post-LT, as evidence of severe HIRI and a potent hazard of ensuing kidney injury.[41,45,46,60,61]

By definition, a biomarker is an indicator of a pathological or physiological state. However, biomarkers are not only crucial in disease diagnosis. They are clearly valuable in other areas of disease management, such as evaluating onset and progression, and predicting the patient´s response and susceptibility to a certain treatment. Although far from clinical practice, advancements in omics techniques have shown promising results, improving the understanding of complex molecular mechanisms on a cellular level. The introduction of clinical proteomics, such as mass spectrometry (MS) based assays, has reshaped the landscape of biomarker identification and validation, allowing the discovery of novel biomarkers at an unprecedented rate and reliability.[62,63] However, up to the present time, no new biomarker or set of biomarkers, have been implemented in routine clinical practice, to predict or even diagnose HIRI. Today, the only established way of verifying the diagnosis of HIRI is the histopathologic evaluation of a liver biopsy often performed using the Suzuki score (Table 3) or modifications thereof.[10,61,64].

TABLE 3. The Suzuki score, From Suzuki et al.[64]

Score	Congestion	Cytoplasmic vacuolization	Parenchymal necrosis
0	No	No	No
1	Minimal	Minimal	Single-cell necrosis
2	Mild	Mild	< 30%
3	Moderate	Moderate	< 60%
4	Severe	Severe	> 60%

AIMS

THE AIMS OF THIS THESIS WERE:

- To describe early and late renal function after LT using mGFR, and to identify risk factors for renal impairment in the intraoperative as well as early post-operative period
- To investigate if liver grafts of patients ultimately developing moderate or severe AKI early after LT, show distinct molecular characteristics compared to the grafts of patients with preserved renal function
- To study if and how the renal proteome is differentially altered in patients presenting early AKI after LT compared to patients without post-LT AKI
- To examine if end-ischemic hypothermic oxygenated machine perfusion of livers from elderly donors impacts on early renal outcome after LT

PATIENTS AND METHODS

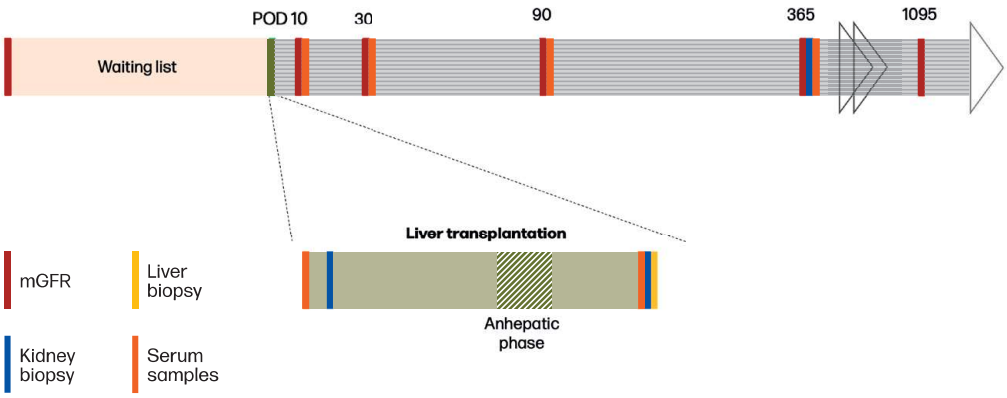


FIGURE 3. Project design "Åsas studie". Patients were followed from waitlisting up to three years post-LT, with repeated mGFRs, intraoperative biopsies and blood sampling. One year post-LT two percutaneous renal biopsies were performed.

mGFR: Measured glomerular filtration rate, POD: Post-operative day

Papers I, **II** and **III** are based on data and samples collected from a prospective, single-centre study, conducted during 2014 and 2015 at Sahlgrenska University Hospital (internally referred to as *"Åsas studie"*). The project design is illustrated in Figure 3.

Paper IV is based on a cohort of patients transplanted with HOPE-treated liver grafts at Sahlgrenska University Hospital, and a group of historic controls. Data were retrieved from the electronic medical records.

Below is a brief description of the patients in each study, followed by an outline of the main methodological aspects of each study. More detailed descriptions of the study design, patient characteristics and methods can also be found in the attached papers and manuscripts.

3.1 PATIENTS

Paper I, II, and III

Th i s single-centre, prospective study was approved by the Regional Ethical Review Board in Gothenburg (Dnr: 598-13) and conducted in accordance with the 2013 Declaration of Helsinki.

Between March 2014 and February 2015, 27 non-consecutive, adult patients, undergoing LT with a donation aft er brain death (DBD) liver graft were enrolled in the study. Exclusion criteria were previous solid organ transplantation, prior need for renal replacement therapy or combined liver and kidney transplantation. Patients were followed from waitlisting for LT, up to 36 months post-LT (Figure 3). Patients participating in the three papers and their distribution between post-LT AKI stages are illustrated in Figure 4.

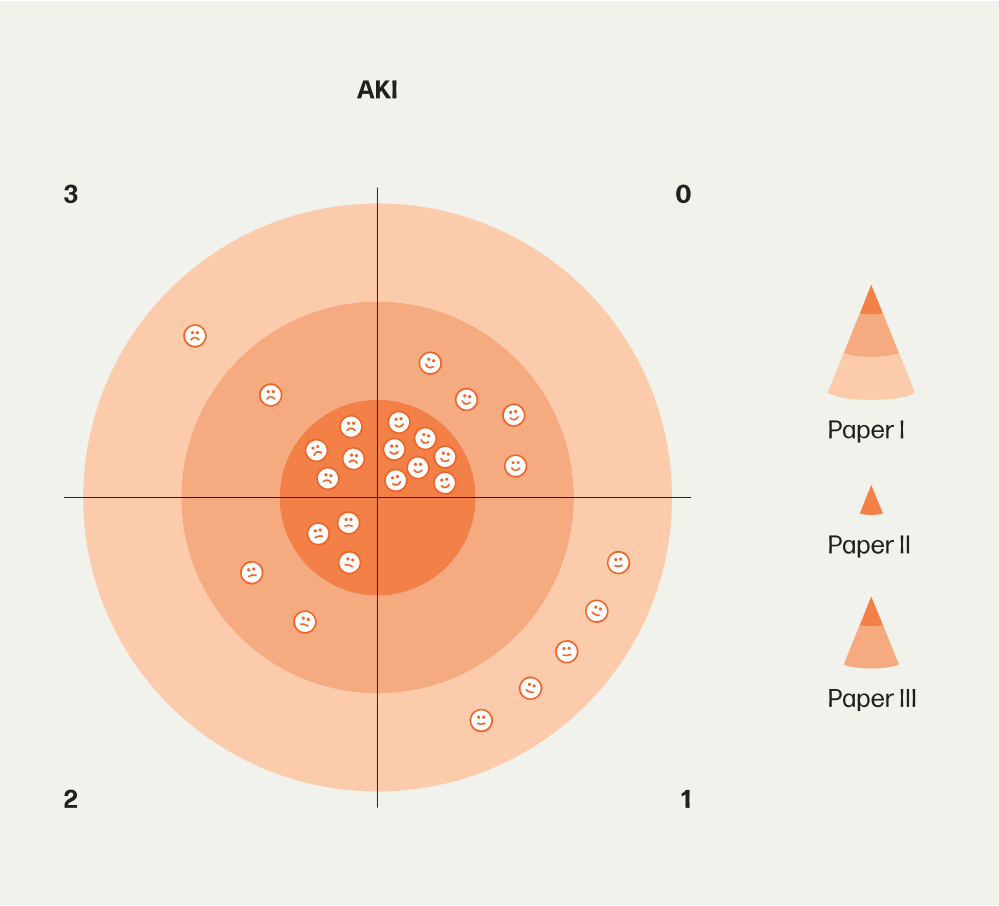


FIGURE 4. Overview of participants in papers I, II and III and their distribution between AKI stages, within 48 hours after liver transplantation. Paper I: n=27, Paper II: n=14, Paper III: n=21

AKI: Acute kidney injury

Paper IV

This was a single-centre, retrospective study of adult patients undergoing primary, single organ liver transplantation between January 1st, 2017 and December 31st, 2022, receiving a liver from a DBD donor, aged 70 years or older. Exclusion criteria were previous solid organ transplants, split grafts, need for preoperative renal replacement therapy, and ABO-incompatible LTs. Data review and collection was approved by the Regional Ethical Review Committee in Gothenburg (Dnr. 048-13). Given the anonymized, retrospective analysis patient consent was waived.

3.2 ORGAN PROCUREMENT AND TRANSPLANTATION

Organ procurement was performed in the standard fashion, using retrograde aortic perfusion with either histidine-tryptophan-ketoglutarate solution, Viaspan-University of Wisconsin solution or Institute Georges Lopez-1 solution, and kept in static cold storage until transplantation (**paper I-III**), with an additional period of end-ischemic hypothermic oxygenated perfusion in **paper IV**.

Anesthesia was induced by propofol and fentanyl or remifentanil and maintained with sevoflurane and either of the opiates used for induction. Transfusions were administered according to the preference of the attending anesthesiologist, and Norepinephrine was given if needed to maintain a mean arterial pressure of >65 mmHg.

LT surgical technique consisted of cavo-caval, side to side anastomosis, and graft reperfusion was initiated after completion of the caval-, and portal vein anastomoses and before performing the arterial and biliary anastomoses. There was no veno-venous bypass or portocaval shunt in studies I-III.

Cold ischemia time (CIT) was defined as duration from the start of cold perfusion in the donor until portal reperfusion in the recipient.

Anhepatic phase was defined as the duration from clamping of the recipient portal vein until reperfusion of the liver graft.

3.3 ASSESSMENT OF RENAL FUNCTION

3.3.1 Renal function, mGFR

Paper I, II, and III

Glomerular filtration rate was measured using ^{51}chromium EDTA or iohexol before acceptance for LT (baseline), at post-operative day (POD) 10, and at 1, 3, and 12 months either at Sahlgrenska University Hospital or at the local hospital. Additionally, 17 patients had GFR measurements at 36 months post-LT.

Paper IV

Measurements of GFR were performed at waitlisting for LT, using either ^{51}chromium EDTA or iohexol.

3.3.2 Renal function, AKI

Paper I-IV

Daily serum Cr levels within the first week were used to evaluate early AKI, in accordance with KDIGO criteria (Table1).

Paper II and III

Patients were considered having renal dysfunction if they presented KDIGO AKI stage 2 and 3 within the first 48h of graft reperfusion. Patients without any evidence of renal dysfunction (AKI stage 0) during the same time frame formed a control group. Patients showing only mild renal dysfunction (AKI stage 1) were excluded from the analyses.

3.3.3 Renal function, CKD

Paper I

Classification of CKD was performed in accordance with the United States National Kidney Foundation Kidney Disease Outcome Quality Initiative (NKF/KDOQI) guidelines.[65] Normal renal function was defined as mGFR >60 ml/min/1.73m^2 (CKD stage 1-2), moderate CKD (stage 3) as 30-59 ml/min/1.73m^2, severe CKD (stage 4) as 15-29 ml/min/1.73m^2, and kidney failure (CKD stage 5) as mGFR below 15 ml/min/1.73m^2 or the need for renal replacement therapy.

3.4 ASSESSMENT OF LIVER FUNCTION

Paper I-IV

Daily liver function tests (AST, ALT, bilirubin, INR) were recorded over the first week as surrogate markers of HIRI and overall liver graft condition and function.

3.5 LIVER AND KIDNEY BIOPSIES

Paper I-IV

During all LTs, a liver graft biopsy was performed using a 14-gauge automated biopsy gun at the end of the transplant procedure. Additionally, four kidney biopsies were obtained from the upper pole of the right kidney, using a 16-gauge automated biopsy gun. Two biopsies at the initial phase of the laparotomy, and two additional biopsies at the end of the transplant procedure, after recirculation of the transplanted liver (paper I-III). Biopsies for histopathologic evaluation were fixed in buffered formalin and processed using standard methods. Biopsies for proteomic analysis were stored in RNA later, at -80°C, until further processing.

3.5.1 Histopathologic evaluation

Paper I-IV

Liver and kidney biopsies were processed using standard routines and stained in accordance with several histochemical and immunohistochemical staining protocols at the Department of Pathology, Sahlgrenska University Hospital. All biopsies were assessed by the same pathologist (JM).

Paper I

Donor liver biopsies were scored for preservation injury, inflammation, fibrosis and macrosteatosis. Macrosteatosis was graded on a scale from 0–3 (0=0%, 1<10%, 2=10–30%, 3>30%) according to percentage of parenchyma.

Only renal biopsies from the beginning of the LT, were evaluated, for estimation of structural renal changes pre-LT. Biopsies were scored using slightly modified Banff criteria[66], for details please refer to the manuscript.

Paper II and IV

Donor liver biopsies were evaluated for preservation and ischemia reperfusion injury, using the Suzuki score (Table 2). Moreover, in paper II immunofluorescence was used to confirm the results of the global proteomics analysis. Slides were examined blindly by the same experienced transplant pathologist and protein overall expression was assessed semi-quantitatively from weak (+) to strong (+++).

Paper III

Sequential, intraoperative kidney biopsies were evaluated according to Banff criteria (same as paper I). Using light microscopy, the following parameters were studied; cytoplasmic vacuolization, loss of microvilli (brush border), tubular dilatation, tubular necrosis, nuclear pyknosis, cellular detachment/luminal cells and scored semi-quantitatively according to Goujon et al.[67] with some modifications, 0: no abnormalities, 1: lesions affecting < 10 % of the kidney sample, 2: lesions affecting 10-50% of samples, 3: lesions affecting > 50% of samples.

3.5.2 Proteomic evaluation

Paper II

Global quantitative proteomics were applied in the analysis of kidney (Paper III) and liver biopsies. In short, proteins were extracted from formalin-fixed paraffin embedded samples, followed by fractionation to remove contaminants and high abundance proteins. Thereafter, proteins were trypsin-digested into peptides, and chemically labelled by tandem-mass-tags (TMTs) with the aim to find differentially expressed proteins between those patients experiencing AKI and those who did not. Labelled samples were combined and multiplexed into one sample prior to nano liquid chromatography mass spectrometry-analysis. The MS^2 fragmentation spectra contained peptide sequence information used for protein identification and the MS^3 spectra were used for relative quantification based on the intensity of the TMTs.

Paper III

Protein expression in paired kidney biopsies was quantified and further analysed, using the same proteomic techniques as presented for the donor liver biopsies above. However, in this case the kidney biopsies had been stored in RNA later, before the analysis, hence the extraction procedure did not involve the paraffin removal step.

3.6 HYPOTHERMIC OXYGENATED MACHINE PERFUSION, HOPE

Paper IV

HOPE has been initiated at our centre in November 2020, for donor livers aged 70 years or older. After an initial period of static cold storage, the liver graft was perfused using VitaSmart™ Machine Perfusion System (Bridge to life Europe Ltd, Wandsworth, London, UK), while recipient hepatectomy was performed. In short, the perfusion system was primed with three litres of Belzer MPS® solution and connected to the graft via the cannulated portal vein. Both ends of the caval vein were left open to allow free outflow of the perfusate. Arterial perfusion was not performed. Continuous perfusion pressure was set to 3 mmHg, and target oxygen concentration was 80-100 kPa in the ~10 °C perfusate. Repeated analyses of oxygen pressure were performed, to verify optimal treatment. After one to two hours of perfusion, the grafts were disconnected and immediately transplanted.

3.7 IMMUNOSUPPRESSION

Paper I-IV

Immunosuppression consisted of induction with intravenous basiliximab (day 0 and POD 4) and intra-operative corticosteroids, and a maintenance protocol, depending on the underlying liver disease. Our standard maintenance protocol consisted of mycophenolate mofetil introduced on day 0 and tacrolimus introduced on POD 3, aiming for a trough level of 5-8 µg/ml during the first three months, 3-5 µg/ml thereafter. Maintenance protocol for primary sclerosing cholangitis and autoimmune hepatitis consisted of oral corticosteroids, in addition to the standard protocol. Serum trough levels of tacrolimus were measured daily during hospital stay.

3.8 STATISTICAL METHODS

Paper I- IV

Continuous, patient related variables were expressed as mean ± standard deviation (SD), median and inter quartile range (IQR) or as absolute and relative frequencies as appropriate, and compared using Mann-Whitney U-test. Correlations were performed using Spearmans correlation method. Categorical variables were compared using Chi Square test or Fisher's exact test. P-values of less than 0.05 were considered statistically

significant. Data were analyzed using IBM SPSS Statistics, Version 25 or GraphPad Prism v. 6 (GraphPad Software, San Diego, CA, USA).

Paper II, and III

For the proteomic analysis, the differential expression analysis was performed using the Perseus software (1.6.15.0) and R. Differentially expressed proteins were identified using a two-sample t-test on log-transformed data. Proteins with a p value < 0.05 and Fold change (FC) $\geq 20\%$ were considered differentially expressed. Principal component analysis (PCA) and heat maps were used as quality control for the samples and clustering of groups.

Paper IV

To control for differing baseline characteristics, propensity score matching was performed on donor- and recipient variables that were considered to impact the outcome. Recipient age and MELD-score, pre-transplant mGFR, donor and recipient BMI and DRI were considered. Group- or TT-matching as well as a Caliper match were performed. An effect size (standardized mean difference) of <0.2 was aimed for, whereas an effect size in the range 0.2-0.5 was considered to have a moderate biasing effect on the outcome analysis.

RESULTS

4.1 PAPER I

Perioperative kidney injury in liver transplantation: a prospective study with renal histology and measured glomerular filtration rates

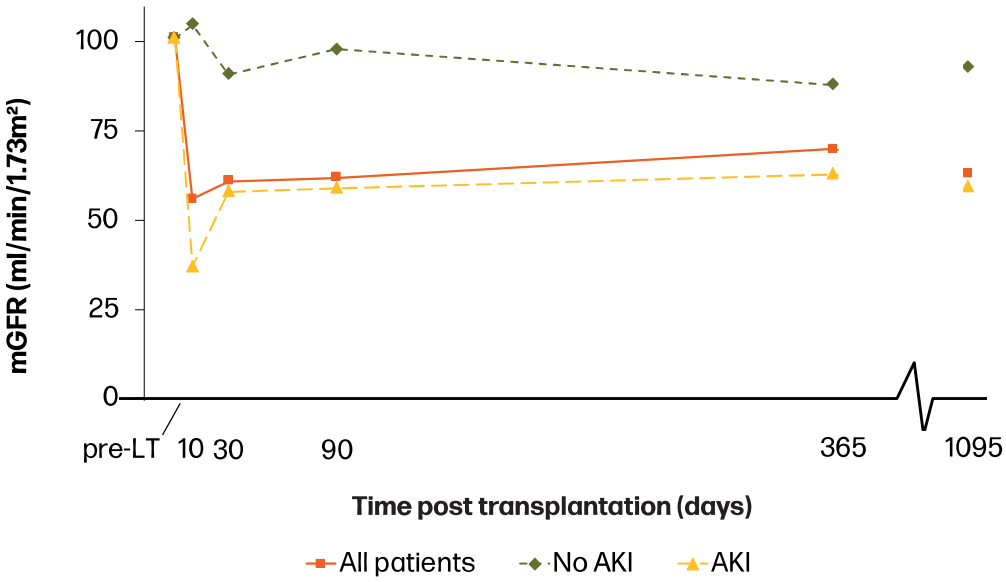
The 27 patients included had a median pre-LT mGFR of 101 (84-108) ml/min/1.73m^2. Median age was 51 (34-58) years, and median MELD score at LT was 12 (18-16). During surgery, no patient experienced post-reperfusion syndrome (defined in accordance with Aggarwal as a decrease in mean arterial pressure >30% below baseline value, lasting for >1 minute, occurring within 5 minutes of graft reperfusion).

Histology

Baseline kidney-, and liver graft biopsies showed only minor histological changes, within the spectrum usually seen in healthy living kidney and liver donors.

Renal function

Measured GFR declined by 45% from pre-LT to POD 10, correlating strongly with mGFR evolution from baseline to 12 months (rs=0.80, p<0.001), and baseline to 36 months (rs=0.82, p<0.001). AKI occurred in 59% of LT recipients within 48 hours of LT, before the introduction of CNIs on POD 3. AKI was strongly associated with mGFR at 12 and 36 months. The evolution of renal function during the study period is illustrated in Figure 5.



	pre-LT	POD10	POD30	POD90	1yr	3yrs
All patients	101 (84-108)	56 (27-94)	61 (44-91)	62 (48-100)	70 (55-89)	63 (43-87)
No AKI	103 (84-113)	102 (35-111)	91 (50-103)	101 (51-104)	89 (63-108)	93 (43-112)
AKI	100 (84-103)	35 (17-56)	57 (27-69)	57 (39-62)	63 (45-71)	59 (39-77)

FIGURE 5. The evolution of mGFR from pre-LT up to three years after LT, in a cohort of 27 adult patients (n = 17 at three years)

Values expressed as median (IQR). Outlined are mGFR evolution for the whole cohort (all patients), patients without acute kidney injury (no AKI) and patients developing acute kidney injury (AKI), within 48 h after LT.

AKI: Acute kidney injury, IQR: Interquartile range, LT: Liver transplantation, mGFR: Measured glomerular filtration rate, POD: Post-operative day, yr: Year

We found donor and recipient BMI, recipient age, MELD score, diagnosis of hepatitis C, and donor cause of death to be potential pre-operative risk factors for early loss of renal function. Bleeding, transfusions, and duration of the anhepatic phase were identified as intraoperative risk factors.

4.2 PAPER II

Liver graft proteomics reveals potential incipient mechanisms behind early renal dysfunction after liver transplantation

The fourteen patients (seven with AKI stage 2/3, and seven without AKI) forming the base of this report did not differ in terms of median age [41 (24-57) vs 49 (28-56) years, p= n.s.] but differed with regard to donor [23 (18-28) vs 29 (24-30) kg/m^2, p=0.04],

and recipient BMI [20 (19-27) vs 29 (21-31) kg/m^2, p=0.02] for the no-AKI and AKI groups, respectively. In addition, median MELD score was significantly higher among AKI patients [8 (7-12) and 15 (12-20), p=0.03].

Liver injury

Liver graft preservation and reperfusion did not initiate a clear disparity in liver function tests, namely AST, ALT and bilirubin, between AKI and no-AKI groups, although the AKI group showed a tendency toward higher values at each time-point. Both groups reached peak liver transaminase values on POD1. Further, histopathologic evaluation of liver biopsies according to the Suzuki criteria revealed no significant differences between the two patient groups.

Proteomic analysis

The global quantitative biopsy analysis, identified a total of 4544 proteins, of which 136 revealed significant differences between the LT patients with and without AKI (FC ≥20%, p <0.05). Moreover, 80 proteins were upregulated and 56 downregulated in patients developing AKI, as clearly illustrated in the heat map (Figure 6A). Most of these proteins are involved in immune and inflammatory responses, host defense and neutrophil degranulation. A more dynamic picture of the proteomic appearance is presented in Figure 6B, showing both the magnitude of fold changes (x axis) and statistical significance (y axis).

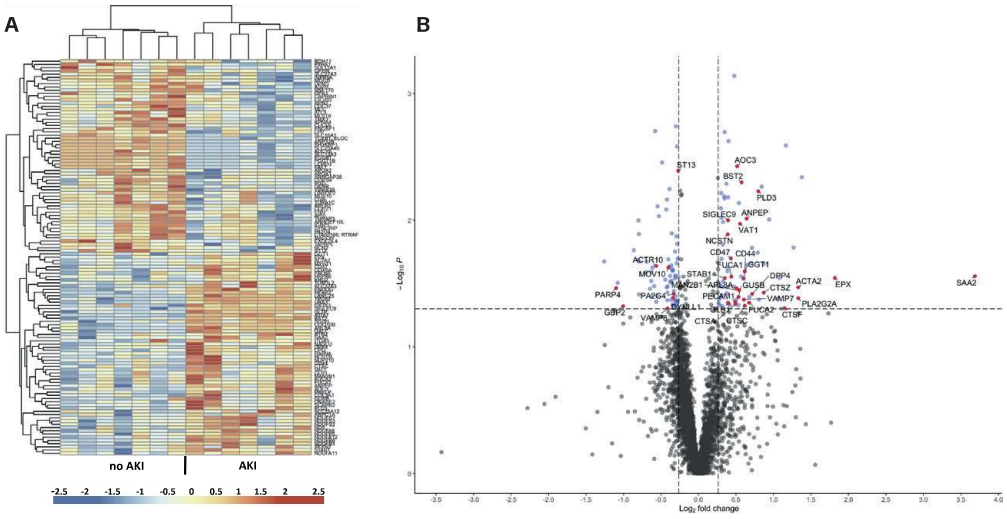


FIGURE 6 A. Heat map view and hierarchical clustering of the proteins showing significant differences between the two groups. The horizontal tree indicates the proteins, and the vertical tree indicates the 14 patients analysed. The colour scheme in the cluster analysis is from blue (low) to red (high), and protein identities are listed on the right. **B.** Volcano plot indicating the proteins showing both the magnitude of fold changes (x axis) and high statistical significance (-log10 of p values, y axis). From Norén et al., paper II.

Cytokines and SAA2

A panel of circulating cytokines was analyzed after 2-4h, and 24h from graft reperfusion, and compared to the preoperative levels as well as with healthy individuals. This analysis did not identify consistent patterns or differences between patients with or without AKI. Patients developing AKI displayed higher IL-6 at 24h compared to healthy individuals.

Serum amyloid A2 (SAA2), a prominent component of the acute-phase response, indicated no differences between groups preoperatively. Similarly, its levels remained unalterd during LT. However, a significant decrease was detected 24 hours after reperfusion, regardless of the presence of AKI.

4.3 PAPER III

Proteomic analysis of human kidney biopsies unveils emerging acute kidney injury within minutes after liver graft reperfusion

Due to concerns regarding duplicate publication and copyright issues of data pending submission, please refer to the manuscript in the last part of the thesis for detailed information.

In brief, 21 patients (ten with AKI 2/3 and eleven without AKI) and their sequential, intraoperative renal biopsies were studied. Donor- and recipient baseline characteristics were similar between groups, except for donor BMI, which was significantly higher in patients developing early AKI compared to those who did not [29 (24-31) vs 23 (19-25) kg/m^2, p=0.02]. Moreover, DRI was higher in the AKI group and causes of donor death differed between groups. However, patients presented with an overall good pre-LT renal function (101 (92-109) ml/min/1.73m^2), with no difference between AKI and no AKI groups.

In sum, there were only minor histopathological differences between the kidney biopsies performed at the beginning of LT and the ones performed after graft reperfusion, with no dissimilarities between groups at the two time-points. Data dependent mode analysis of kidney biopsies identified a total of 8144 proteins, of which 6620 were quantified. At the beginning of LT, proteomic analysis detected 249 differentially regulated proteins (155 upregulated and 94 downregulated, $p<0.05$, FC± 20%) between patients with and without AKI. In post-reperfusion biopsies, the comparative analysis identified 731 differentially regulated proteins (542 up-, and 289 downregulated, $p<0.05$, FC ± 20%). The most upregulated pathways were related to early inflammation, innate immunity, and extracellular matrix alterations. Among the most downregulated pathways, many were traceable to a mitochondrial origin and notably energy metabolism. In addition, there was an intense inflammatory signaling activity in kidneys developing AKI after LT, involving alarmins and DAMPs such as IGFBP-7, HMGB1, caspases and transcription factors.

Regarding circulating alarmins, Interleukin-33 and HMGB1 showed a significant increase after reperfusion compared to baseline measurements, without differences between patients with and without AKI. On the other hand, compared to healthy individuals IGFBP-7 was significantly upregulated in both groups and desreased after reperfusion. Moreover, there were significant differences between AKI and no AKI patients at all three time-points measured. During the short time interval between baseline biopsy and post-reperfusion biopsy, proteins involved in ribosomal processes, immunoglobulin regulation and collagen turnover were particularly regulated in the AKI 2/3 group.

4.4 PAPER IV

End-ischemic hypothermic oxygenated machine perfusion does not improve renal outcome following liver transplantation from aged donors: A single centre retrospective report

Please refer to the manuscript in the last part of the thesis, due to copyright issues, in advance of journal acceptance and publication.

Briefly, after applying inclusion and exclusion criteria, 101 liver transplant recipients, 71 receiving liver grafts preserved using standard, static cold storage, and 30 patients transplanted with a HOPE-treated liver graft were reviewed in this study. All donors were aged 70 years or older, and the two study groups were mostly comparable as regards donor characteristics and recipient baseline and intraoperative descriptions. However, there were differences in recipient BMI [25.5 (23-30) vs 28 (24-34) kg/m^2, p=0.02] and donor age [73 (71-76) in control group vs. 75 (73-77.5) in HOPE group, p=0.015] between groups, and as expected cold ischemia time. Group-matching was performed to include as many control patients as possible to the HOPE-treated group. The best match resulted in 47 control patients to all HOPE patients (n=30). In addition, the Caliper match identified 27 controls to 27 patients in the HOPE cohort.

In sum, there were no differences in terms of frequency or severity of early post-transplant AKI between HOPE and control patients. Serum creatinine increased significantly postoperatively in both groups compared to pre-transplant values. The need for renal replacement therapy was similar between groups. Likewise, there were no significant short-term advantages on immediate graft outcome, as assessed by liver function tests or the occurrence of early allograft dysfunction. Similar findings and no significant differences between static cold storage and HOPE LTs were observed when comparing renal-, and graft-related outcomes in the matched cohorts. Moreover, histopathologic evaluation of transplant biopsies, using the Suzuki score, revealed aggravated HIRI in HOPE-treated grafts compared to those preserved using static cold storage [Suzuki score 5 (5-6) in HOPE treated patients vs 5 (3.5-6) in controls, p=0.038]. The degree of graft steatosis was generally mild, and with no difference between groups.

DISCUSSION

This thesis explores renal impairment in the early period after LT, concerning multiple clinical aspects ranging from pre-transplant risk factors to long-term results, as well as analysing a wide range of histological and molecular changes occurring in liver grafts and kidneys of LT recipients. Commencing with a prospective characterisation of LT recipients, focusing on early renal functional development, we learnt that early AKI is frequent, and heavily impacts on late renal function and LT morbidity. After identifying several peri-transplant risk factors for renal impairment, we went on to examine liver graft quality, on a subcellular and functional level. The proteomic analysis of liver graft biopsies revealed multiple, clear differences between the grafts of patients who would present manifest AKI, compared to those maintaining pre-transplant renal function. The pro-inflammatory shift was evident within two hours of graft reperfusion, and much more distinct than the image given by the histopathologic evaluation. With this in mind, we proceeded by analysing intraoperative, sequential renal biopsies performed during the transplant procedure, exploring differences between the two outcome populations (AKI vs no AKI groups). Again, the proteomic analysis outlined a distinct scenario of early inflammation, extracellular matrix modifications and heavily affected renal energy metabolism, long before any clinical signs of kidney injury were apparent in the AKI group.

Finally, our hypothesis of donor and especially liver graft fuelled impact on recipient early AKI was tested in the fourth study, evaluating whether hypothermic oxygenated perfusion of a liver graft, thought as a method to mitigate HIRI, would abate the development of post-reperfusion renal harm. Interestingly, we found no such tendencies. Nor did we get any indication that HOPE-treatment of older DBD-donors, would present less HIRI than non-treated grafts.

5.1 RENAL INJURY IN LIVER TRANSPLANTATION, ONCE YOU SEE IT, IT'S TOO LATE!

Even though renal injury in liver transplantation is known to be of multifactorial origin and has been thoroughly described over the years,[1,37,38,40,45,47,68,69] the understanding of renal insufficiency early after clinical LT is still incomplete. Renal failure after LT could essentially be described as early and/or late injury. During the last decades, attention has been drawn to the early or *very early* aspects of post-LT renal impairment, where intraoperative events and especially the newly re-perfused liver graft, play key roles.[38,39,41,70] A substantial problem for the study of epidemiology and risk factors for renal injury after LT, is the lack of a uniform definition of AKI. This or rather *these* definitions have changed over the years, as has the entire LT candidate pool, who is constantly expanding, with the acceptance of older and more complicated patients today, compared to historic recipients. The main conclusions from paper I were the profound reduction of mGFR, detected on POD 10, and the high incidence of AKI within 48 hours of graft reperfusion, in a LT-population showing good pre-transplant renal function. Moreover, the introduction of CNI on POD 3, allowed us to disregard CNI-nephrotoxicity as a causative factor. The immediate reduction of renal function correlated strongly with long-term renal outcome. Our overall findings were in line with those of many others.[35,37,38,71] Hilmi et al. reported an incidence of AKI of 52%, within 72 hours of LT, highlighting recipient female sex, recipient weight >100kg, severity of liver disease, and pre-existing diabetes mellitus as important risk factors.[37] Contrasting to our results, they and others describe a higher incidence of pre-transplant CKD stage 4 and 5.[72,73] Furthermore, Hilmi speculated that the prevalence of severe CKD in their cohort would likely be even higher, due to imperfection inherent in the sCr based formula.[37] In line with others we found both donor, and recipient BMI to be risk factors for early AKI after LT. The progression from obesity, to the metabolic syndrome, cardiovascular disease and renal injury is well described in the general population, and steatotic liver grafts carry greater risks of inferior transplant outcome.[74-79] However, we believe that the high baseline mGFR, reinforced by the unremarkable kidney and liver histology, early AKI and repeatedly measured GFRs, added new insight to the complex presentation of a multifactorial event. Although renal function is affected by many pre-, intra-, and post-LT factors, and hence at risk at each phase of the transplant journey, we find it reasonable to highlight the hazard a LT poses on patients in an otherwise satisfactory clinical condition. One methodological concern was the potential changes in mGFR and MELD score between listing and transplantation. We addressed this important note by comparing calculated GFR (MDRD-4) at listing and admission for LT, as well as MELD score at the two time-points, and found no significant differences. Weaknesses of this study are the single-centre setting, and the small size of the cohort limiting our ability to draw advanced conclusions based on clinical parameters. Consequently, multivariate analyses were left out from this paper, hence confounding could not be excluded. Therefore, progressive ideas on mechanisms behind AKI-development, are only speculative, and need to be confirmed.

5.2 IS THE LIVER (PROTEOME) A CRYSTAL BALL FOR AKI?

For obvious reasons a LT could never be better, than the graft we choose to transplant. However, in all honesty, do we actually have all the information needed to assess liver graft quality when the decision is made, or is the mismatch between available organs and constantly increasing waitlist numbers making the decision for us? Hopefully not, but most probably, the criteria we use for evaluating a potential donor today need further refinement.

Ischemia reperfusion injury is a hot topic, not only in the transplantation community. Although the general mechanisms apply for all species and organs, inter-organ differences are not to be neglected. Underlying and predisposing factors differ and are of outmost importance in organ donation and later transplantation. So called ECDs or "marginal donors" and risk factors for HIRI in LT are described by others. Moreover, the relationship between HIRI and peri-operative AKI has also been recognized.[41,47,80] Using samples from the same study cohort as presented above, we wanted to evaluate if subcellular liver graft characteristics differed between patients developing early AKI, compared to those maintaining pre-LT renal function. Hence, we assessed protein expression, using proteomic profiling, of AKI 0 and AKI 2/3 patients, excluding the mild AKI 1 patients from the analyses, in order to reduce confounding. Interestingly, even though the majority of donor variables were similar, and the histopathology of liver biopsies showed no significant differences, the proteomic analysis revealed clear differences in protein expression between groups, even at this very early stage. The protein expression found in "AKI livers" was dominated by inflammation; pathways of innate immunity and neutrophil activity, as well as centred around mitochondrial processes and cell-to-cell interactions. In accordance with others´ analyses of HIRI, we discovered protein patterns consistent with early signs of hepatic IRI, activated endothelial and resident immune cells and neutrophil recruitment and degranulation.[81,82] Vascotto et al. described the proteome of nine LT recipients, analysing intraoperative biopsies and concluding regulation of 36 proteins involved in lipid- and energy metabolism, and redox signalling.[83] Also, in a recent publication by Pulitano et al., they described clear differences in liver graft gene expression, comparing post-reperfusion biopsies between AKI and non-AKI groups.[84] They speculated that intrinsic graft, rather than intraoperative, hemodynamic factors play key roles in the development of AKI early after LT. Similarly, our findings of significantly upregulated levels of hepatic SAA2 and phospholipase A2, only minutes after graft reperfusion made us speculate that this proteomic shift was initiated already in the donor. Upregulation of hepatic SAA2 synthesis during the initial phase of inflammation, is dependent on the coordinated interplay of cytokines and takes hours to complete.[85] Comparisons to other proteomic studies are difficult, firstly since there are not many, and the methodology has improved greatly over the years. Secondly, most research in this field is conducted using laboratory animals, which, by nature, have a different biology than humans, and importantly

also present a more homogenous cohort of individuals. The complexity of this issue was reviewed by López-López et al., who emphasized that <10% of proteins, detected in liver biopsies used for proteomic examination of LT, overlapped between species.[86] In addition, the use of different types of samples, i.e. biopsies, serum, and plasma further complicates comparisons. Strengths of this study are the advanced, state of the art proteomics, used herein and to our knowledge the first proteomic analysis on human, liver graft biopsies performed to answer this research question. On the other hand, the small sample, short observation time between reperfusion and tissue sampling with the latter only being performed at one time-point, add limitations to our conclusions. However, we find it interesting and even likely that this brief period of time, from reperfusion to biopsy performance, reduces the likelihood of de novo protein synthesis and degradation, hence reflecting proteomic differences originating already in the donor or during the period of cold storage.

In conclusion, we found that liver grafts of patients who develop moderate to severe AKI after LT present a distinct proteomic signature, dominated by activation of early innate immunity. We speculate that these proteomic differences were initiated in the donor.

5.3 RENAL PROTEOME DURING LIVER TRANSPLANTATION: EVOLUTION OR INVOLUTION?

On a clinical note, liver transplant recipients often present transiently increased sCr, immediately after the transplant procedure. As previously described, the reason for this is multifactorial in most cases. As a clinician, my impression is that the general opinion on a moderately increased sCr after LT is that the situation will most often return to normal without further interventions, unless dialysis is required. We adjust the dosage of medications and avoid or reduce the administration of nephrotoxic drugs to a minimum, but otherwise we do little. At time of discharge or transferral to the local hospital sCr is frequently within acceptable range and the event of "a temporarily raised sCr" is ancient history.

However, even if mild and transient, AKI is independently associated with morbidity, mortality and long-term adverse events such as CKD and cardiovascular disease.[3,87,88] Since AKI diagnosis is based on two clinical markers that are not renal-specific, identification may be delayed or even missed. Hence, the raised sCr that we calmly observe post-LT, is the sign of already established renal injury. In addition, treatment options are limited, relying on *early* recognition, supportive strategies, and preferably a multidisciplinary approach, in order to minimize progression of renal insufficiency.[89,90] Consequently, preventive interventions and methods to identify AKI without delay are crucial for long-term outcome.

In paper III, we examined the progression of renal subcellular mechanisms during the transplant procedure, comparing patients with AKI 2/3 and AKI 0. In accordance with the above theories that sCr increases only when renal harm is already established, our hypothesis was that structural or mechanistic alterations would be detectable already during the transplant procedure. Interesting findings by Skytte-Larsson et al.[49], clearly show functional changes in terms of a mismatch in renal oxygen delivery and consumption as early as hours after LT. The same group also concluded renal oxygen insufficiency to be the common denominator of AKI associated with sepsis, major cardiac surgery, and cardiopulmonary bypass although the triggering event and resultant renal mechanisms differ.[30] In addition, Jochmans et al. described a distinct increase in sCr, compared to baseline values, as early as six hours after recirculation of the liver graft, in LTs developing AKI.[47] What is more, 19% of patients developing renal injury had reached the threshold for AKI diagnosis within two hours of reperfusion.

Indeed, we found complex, intraoperative, proteomic alterations, implicating emerging renal inflammatory activation and ongoing extracellular matrix (ECM) modifications, during the transplant procedure. There was a clear distinction between AKI 2/3 and AKI 0 patients, demonstrating common features with cellular alterations and signalling pathways seen in emerging AKI and the transition to CKD under circumstances other than a LT.[91-93] With findings from paper II fresh in mind, upregulated levels of liver-derived alarmins and DAMPs, as well as activated, circulating neutrophils and pro-inflammatory cytokines are likely to at least contribute, if not exclusively initiate a renal sterile, inflammatory response of innate immunity. We found serum IGFBP-7 to be of special interest, since this alarmin was found significantly increased before the transplant procedure and decreased uniformly after reperfusion, with significant differences between groups at each time-point measured. Elevated IGFBP-7 is known to induce renal injury, and serum levels are mostly of hepatic origin.[94] Others have shown that IGFBP-7 is associated with liver steatosis, fibrosis, cirrhosis and hence reflecting the pre-transplant circumstances.[95] Moreover, renal matrix metalloproteinases, significantly upregulated in our study, and primarily thought to modulate the ECM, have also been reported as potential regulators of IGFBP-7.[96] Similarly, a recent report indicates that IL-33 is immediately released as an alarmin both after murine, and human liver recirculation and appears to correlate with the severity of liver injury, as well as renal impairment.[97] We found a significant increase of serum IL-33 immediately after graft reperfusion, which returned to normal values within 24h of LT. Consequently, several alarmins are plausible mediators of remote organ ischemia-reperfusion injury acting on renal endothelial cells, tubular epithelial cells, and resident renal immune cells, which in turn are stimulated and amplify the response. Changes in ECM structure and signalling are driven by newly released mediators, such as integrins, cytokines and growth factors.[91,98,99] Additionally, the mismatch between renal oxygen demand and delivery, driven by the incipient inflammation, leads to mitochondrial dysfunction, reduced ATP generation, and a switch towards production of ROS, further promoting ECM degradation and tubular cell injury. We found IGFBP-7 and tissue inhibitors of metalloproteinases (TIMPs) to be upregulated in AKI 2/3 patients

in our study. Both proteins or groups of proteins are known to be expressed and released by renal tubular epithelial cells upon cellular stress, and to induce cell cycle arrest, in order to reduce energy consumption, further DNA damage and renal harm.[100,101] The ensuing period of paucity and potential recovery from an AKI triggering event could thenceforth evolve in either a more healing related direction with cellular division and repair, or a fibrotic, sclerotic and scarring paved pathway leading to later CKD, dependent on surrounding factors such as the presence of IL-33 or pre-existing renal disease.[50,92,102,103]

Limitations of paper III are the restricted number of patients and the lack of information on the accurate tissue compartment, where the reported alterations occurred, which is inherent in the method. Unique to this paper are the sequential renal biopsies performed during LT, to our knowledge not performed in man, as well as the state-of-the-art proteomics method.

In conclusion, kidneys of LT recipients, who will develop early AKI after LT, present complex proteomic alterations within minutes from liver graft reperfusion. These changes involve the innate immune response, ECM and the mitochondria and may represent the first stages of the remote organ injury observed after HIRI, orchestrated by an intense alarmin and cytokine signaling.

5.4 LIVER HOPE: A ROAD WORTH TRAVELLING?

While the risk of post-LT early AKI seems to be influenced by recipient characteristics that are hard to modify (i.e. age, BMI, MELD score), donor risk factors are easier to supervise…or are they? The growing discrepancy between supply and demand for LT has consequently led to the increasing use of higher risk grafts, often referred to as organs from "high-risk donors" or "ECDs". Others have shown the correlation between the use of these organs, the ensuing HIRI, and the increased frequency of AKI.[39,45,104,105]

Over the years, different strategies to reduce the risks associated with these grafts have been explored, from pre-clinical molecular interventions in HIRI signal transduction to changes in organ allocation. Clearly, the majority of these interventions have not gained robust impact, whereas a few have been implemented in clinical practice. Lately, machine perfusion techniques have attracted much attention, with promising theoretical premises of mitigating ischemia-reperfusion injury, reducing post-LT complications and eventually improving transplant outcomes. Ceteris paribus, it would be brilliant if we could treat the liver graft, prior to implantation, to reduce the hazard of serious HIRI in the recipient, and hence modulate the occurrence of AKI. In short, HOPE-treatment helps to improve mitochondrial energetics and function prior to implantation, through mitochondrial metabolic conversion, resulting in reduced accumulation of citric acid cycle metabolites and electron donors, such as succinate and reduced nicotinamide

adenine dinucleotide (NADH) and at the same time preventing reverse electron transfer by mitochondrial complex-1. Consequently HOPE-treated livers are uploaded with sufficient ATP at reperfusion, which enables immediate graft function.[11]

In our centre, we introduced HOPE-treatment for ECD liver grafts, defined as DBD livers from donors aged 70 years or older. In this study, we hypothesized that HOPE therapy would decrease frequency and severity of AKI after LT. Accordingly, we compared the outcome of HOPE and non-HOPE LTs, with respect to the frequency and severity of AKI. In conclusion, and to our surprise we found no significant differences related to either early renal or hepatic outcomes between groups. We truly looked over our methods, since these results were not what we had expected, in the light of recent years tribute to the HOPE method. Nevertheless, even when refining our cohort by propensity score match to homogenize the groups further and minimize confounding, the results remained the same. If anything, there was even a tendency towards a higher incidence of AKI in the HOPE treated cohort, compared to transplants kept in static cold storage. Since our hypothesis was that HOPE as a method would reduce the severity of HIRI and consequently the frequency of early AKI, we also analysed post-reperfusion liver biopsies for Suzuki scoring and graft steatosis. Unexpectedly HOPE-treated livers showed worse Suzuki score while the degree of steatosis was comparable to livers kept in static cold storage. Even though our results differ from several already published reports, there is ongoing controversy whether and how HOPE-treatment for DBD livers truly improves outcome, and if so, which parameters to be considered. The predominant part of publications on end-ischemic HOPE is performed using donation after circulatory death (DCD) grafts, which by default entail a higher risk of post-LT complications, and most probably a more intense IRI. Moreover, there is some evidence that HOPE also offers a valid method for viability testing before implantation.[106,107] On the other hand, the literature on HOPE and DBD is more limited, and the results are inconsistent. Interestingly, Patrono et al. reported a decreased frequency of AKI stage 2/3 and a tendency towards less reperfusion syndrome in their retrospective analysis of HOPE-treated DBD-livers compared to matched controls.[105] The same group later showed somewhat different overall results on post-reperfusion instability and incidence of AKI. Moreover, they reported less need for early post-LT dialysis in the HOPE-, compared to control group.[108] Two randomized controlled trials on HOPE-treated DBD grafts have recently been published, one of which presented, a lesser peak ALT post-LT compared to control patients, interpreted as mitigated HIRI.[109] The other trial, though primarily focusing on the incidence of cumulative Clavien III complications, showed no decreased frequency of need for post-LT dialysis among HOPE patients.[110]

Limitations to this study are the retrospective design and small number of patients, thus far transplanted with HOPE-livers at our centre. In addition, it would have been of greatest interest to compare liver graft biopsies performed *before* HOPE-treatment and static storage to biopsies obtain after reperfusion. One strength of this study is the single criterion for HOPE-treatment being donor age above 70 years, which we consider reduce potential confounding for evaluating treatment effects. In addition, we have

robust measurements of GFR to assess pre-LT renal function. Lastly, our routines and practices have not changed during the study period, hence surgical procedures and medical care remain the same.

Altogether, by 2023, the effects of HOPE-treatment for DBD liver grafts still seem to be unclear. Encouraging results on hypothermic oxygenated perfusion of high-risk DCD and/or ECD organs are by far more convincing. Most probably the fundamental challenge is to sharpen the indication for HOPE-therapy in LT, which requires more consistent definitions of the terms ECD and "high-risk grafts".

5.5 ETHICAL REFLECTIONS ON "ÅSAS STUDIE"

The study was approved by the Regional Ethical Review Board, in Gothenburg (Dnr. 598-13) in December 2013. This approval was later completed with an amendment regarding the analysis of liver graft biopsies routinely obtained during LT (Dnr. 2022-00828-02). Patients were cared for in accordance with the 2013 Declaration of Helsinki.

Performing renal biopsies during LT pose an additional risk of bleeding in patients who are frequently having impaired haemostasis. An adequate exposure of the surgical site, and the duration of time from last biopsy to closure of the abdominal wall, were considered important precautions. Moreover, a renal ultrasound was performed on POD 1, to identify potential biopsy-related complications, such as bleeding, hydronephrosis, or impaired renal circulation.

Ultrasound-guided, percutaneous renal biopsies, also associate with risks of bleeding. Attention was paid to high systolic blood pressure, low haemoglobin, platelet levels, and INR. Moreover, patients were monitored in hospital for two hours after the procedure. In addition, biopsies were performed by experienced radiologists, using as thin needles as would still render enough tissue for the analyses.

The measurement of GFR is sometimes considered troublesome for the patient. In this study mGFR was performed more often than the usual routine. However, as GFR was measured either during the initial hospitalization or scheduled as part of the regular follow-up it has likely not impacted negatively on patients´ day-to-day activities.

The above presented ethical dilemmas of performing kidney biopsies as well as measuring GFR have been thoroughly considered and discussed in our group. In our opinion the advantages that this meticulous assessment and follow-up of renal function present, by far outweigh the downside risk of harm for the patient. Furthermore, the in-depth analyses of biopsy material and multiple mGFRs have been repeatedly explored for different aspects of LT-related renal injury, in three papers…so far.

CONCLUSIONS

Supported by the findings presented in the papers of this thesis, I conclude that:

- Renal injury is frequent, early after LT. The maximum decline in mGFR occurs within days, and AKI within hours of graft reperfusion, irrespective of pre-transplant mGFR, and before the introduction of CNIs. Very early post-LT renal injury has substantial consequences for long-term renal function.
- The proteomic profile of the liver grafts of patients developing early post-reperfusion AKI differ significantly compared to patients maintaining good renal function. Graft proteome is dominated by inflammation, and early signs of innate immunity, suggesting that AKI development is favored by critical graft characteristics.
- Complex proteomic alterations are found in kidneys of LT recipients presenting early post-reperfusion AKI. These changes are dominated by an inflammatory picture, with vast ECM and mitochondrial modifications and are most probably fueled by intensive alarmin signaling. Proteomic changes become distinct, only minutes after graft reperfusion.
- End-ischemic HOPE of liver grafts of patients aged 70 years or older, does not reduce the frequency or severity of early AKI after LT.

- *Renal injury in liver transplantation is a complex issue!* Very early interventions in the LT-procedure are mandatory to improve long-term outcome.

FUTURE PERSPECTIVES

On renal biopsies performed one year after LT

Even though the core of this thesis has been early renal injury after LT, there is a clear correlation between AKI and CKD. Our prospective study ("Åsas studie") also contained one-year renal biopsies, not yet examined. We believe that these biopsies will provide valuable information on the development of renal disease, since to our knowledge, no group has ever performed human, renal protocol biopsies after LT, without any evidence of renal decompensation.

On the comparison of living donor, and baseline LT recipient renal biopsies

Liver diseases may frequently affect the kidneys. In paper I, we described an unremarkable renal histopathology on baseline biopsies obtained before LT, although others have described a range of different findings.[22,23] Comparing renal biopsies of healthy living kidney donors to those of LT recipients, using omic-techniques, would be of great importance to the understanding of renal pathology in liver disease, and hence the point of departure for intraoperative LT renal injury.

On intraoperative hemodynamics and alarmin signaling during LT

In papers I and III, we describe the rapid decline in renal function during the LT procedure, and its effect on renal protein expression. Others have examined hemodynamics and renal metabolism during and after LT. [47,49] We are interested in an intraoperative, functional analysis of these circumstances, linked to timely measurements of potential alarmins and DAMPs from the newly re-perfused liver graft. Hopefully this could help us decipher the hypothesized link between HIRI and AKI.

On organ donors and renal outcome in LT recipients

Hypothesizing that the liver graft significantly impacts renal function after LT, one key piece of knowledge still missing, is when the optimal timing for liver graft optimization would be. Is it possible to foresee early post-LT AKI, already in the donor intensive care unit, even before the potential donor is declared dead? If so, the interventions should start at this time-point to mitigate and prevent risk factors for AKI from progressing and ultimately improving morbidity and longevity for the prospective LT recipient. Although a myriad of studies on donors and donor organ quality have been undertaken, the understanding of how donor factors affect recipient organ systems, others than the ones to be transplanted, is still incomplete.

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I love the sea, and I love adventures.

A PhD has much in common with a journey, and I like to think of mine as a voyage. Beginning as a sailing course, with firm guidance and support, almost not leaving the harbour. Later discovering the archipelago of Bohuslän and Västkusten. After months and years of preparation, I finally dared to realise my dreams, and set out to cross the Atlantic O.

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