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NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN IN KIDNEY AND LIVER TRANSPLANTATION

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Academic dissertation

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If at first an idea isn't absurd then there is no hope for it
– Albert Einstein

To Tobias and August

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LIST OF ORIGINAL PUBLICATIONS

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3. **Hollmén M**, Kyllönen L, Merenmies J, Salmela K. Serum neutrophil gelatinase-associated lipocalin and recovery of graft function after kidney transplantation. BMC Nephrol 2014; 15: 123.
4. Åberg F, Lempinen M, **Hollmén M**, Nordin A, Mäkisalo H, Isoniemi H. Neutrophil gelatinase-associated lipocalin associated with irreversibility of pre-liver transplant kidney dysfunction. Clin Transplant 2014: 28 (8): 869-876.

LIST OF ABBREVIATIONS

ADH	Anti diuretic hormone
AKI	Acute kidney injury
CADI	Chronic allograft damage index
CAN	Chronic allograft nephropathy
CNI	Calcineurin inhibitor
CKD	Chronic kidney disease
DCD	Donation after cardiac death
DGF	Delayed graft function
ECD	Expanded criteria donor
EGF	Early graft function
ELISA	Enzyme-linked immunosorbent assay
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
HLA	Human leucocyte antigen
ICU	Intensive care unit
INR	International normalized ratio
IRI	Ischemia/reperfusion injury
KDIGO	Kidney disease: improving global outcome
MARS	Molecular adsorbent recirculating system
MELD	Model for end-stage liver disease
NGAL	Neutrophil gelatinase-associated lipocalin
OPTN	Organ Procurement and Transplantation Network
POC	Point-of-care
RIFLE	Risk, injury, failure, loss, end-stage criteria
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SD	Standard deviation
UNOS	United Network for Organ Sharing

ABSTRACT

Background

Expanding the criteria for deceased organ donors increases the risk of delayed graft function (DGF). DGF, especially when long lasting, complicates kidney transplant outcome. Liver transplant recipients are at an increased risk for kidney injury both before and after transplantation and renal dysfunction strongly associates with morbidity and mortality. Identifying kidney injury early is crucial in achieving favorable outcome after transplantation. However, there are currently no reliable methods for predicting kidney damage in transplant patients.

Neutrophil gelatinase-associated lipocalin (NGAL) is a novel marker for acute kidney injury (AKI). The aim of the study was thus to test whether donor and recipient urine and serum NGAL could predict DGF, prolonged DGF lasting >14 days, or quality of kidney function in non-DGF transplantations, and whether plasma NGAL obtained prior to liver transplantation could predict prolonged kidney injury after liver transplantation.

Methods

Ninety-nine consecutive, deceased, heart-beating donors and their 176 adult kidney recipients were prospectively included. Serum and urine samples were collected from the donors before organ retrieval. From the recipients, serum and urine samples were collected at arrival to the transplant unit and in the mornings of days 1 and 14 after transplantation. Serum NGAL was analyzed using the enzyme-linked immunosorbent assay (ELISA) and point-of-care (POC) methods. Urine NGAL was analyzed using the ARCHITECT® method. For the liver study, all adult deceased donor liver transplant recipients (n=203) at our center during the years 2005 to 2010 were included and pretransplant plasma NGAL was analyzed using the POC method.

Results

DGF was seen in 70/176 patients. The duration of DGF was prolonged (>14 days) in 26 cases. Transplants with prolonged DGF had worse 1-year graft survival (73%) compared to transplants with shorter DGF (100%, p=0.001).

The mean donor sNGAL was 218ng/mL and uNGAL 18ng/mL. Donor sNGAL and uNGAL concentrations correlated directly with donor plasma creatinine and indirectly with estimated glomerular filtration rate (eGFR). Donor NGAL did not predict DGF as such. High (>mean) donor uNGAL concentration associated with prolonged DGF and worse 1-year graft survival (90.3%) compared to the low uNGAL group (97.4%, p=0.048). In a multivariate analysis, uNGAL and expanded criteria donor status emerged as independent risk factors for prolonged DGF.

Recipient uNGAL was significantly higher in patients with DGF compared to recipients with early graft function (EGF) at all measured time points. Day-1 uNGAL predicted DGF and prolonged DGF with an AUC of 0.75. Day-1 uNGAL also predicted DGF in cases which seemed to have early function: in 15/112 cases with day-1 urine output >1L (AUC 0.70) and in 19/86 cases with >50µmol/L decrease in creatinine on day 1 (AUC 0.74). In a multivariate analysis, day-1 uNGAL emerged as an independent predictor of DGF.

Recipient sNGAL was significantly higher in patients with DGF compared to patients with EGF at all measured time points. Day-1 sNGAL predicted DGF with an AUC of 0.85 and prolonged DGF with an AUC of 0.83. In a multivariate analysis, day-1 sNGAL emerged as an independent predictor of DGF.

In the liver transplant recipients, pretransplant pNGAL modestly correlated with creatinine and cystatin C levels eGFR. There were no significant differences in the mean pNGAL concentrations between patients with or without posttransplant AKI or a need for posttransplant renal replacement therapy. The mean pNGAL was significantly higher among liver transplant patients with eGFR <60mL/min at three months after transplantation compared to those with eGFR >60 mL/min ($p=0.001$).

Conclusions

Donor uNGAL measurements give additional value in the evaluation of donor kidney function and suitability for kidney donation. Recipient day-1 uNGAL predicts DGF when it is not clinically expected early on and prolonged DGF, which leads to poor graft survival. Day-1 sNGAL predicts clinically significant DGF. Measuring recipient sNGAL and uNGAL on the morning following kidney transplantation is therefore useful in individualizing patient care. Pretransplant pNGAL associates with reduced kidney function at three months after transplantation and helps in optimizing and individualizing patient care.

1. INTRODUCTION

Kidney transplantation is the best available treatment for end-stage renal disease (ESRD). It significantly improves patients' quality of life and has been reported to markedly decrease the risk of death compared to patients on maintenance dialysis (1). At Helsinki university hospital 5-year kidney graft survival is more than 80%. As the prognosis for patients on maintenance dialysis has improved, the number of patients needing a kidney transplant has increased, and the gap between supply and demand has widened. Consequently, the donor criteria have been expanded which has led to an increase in delayed graft function (DGF) after transplantation.

Liver transplantation is the standard and only curative treatment for patients with liver failure. The prognosis after liver transplantation has improved significantly over the past decades: 75–85% of recipients have a functioning graft at five years after transplantation (2,3). This improvement results from advances in immunosuppression, organ preservation, surgical techniques and pretransplant treatment of patients with liver failure (4). Despite these improvements, liver transplant recipients are at an increased risk for kidney injury both before and after transplantation and renal dysfunction strongly associates with morbidity and mortality (5–7).

Both DGF and renal failure associated with liver failure are forms of acute kidney injury (AKI). AKI is a general term describing acute renal impairment of various, multifactorial origins and to varying degrees. Its pathogenesis is poorly understood, diagnostic methods inadequate, and treatment options limited. AKI inflicts a considerable health and economic burden (8–10). At present, DGF and AKI associated with liver disease cannot be predicted, diagnosed early, or treated. The exact molecular mechanisms also remain unknown. Being able to diagnose these conditions early would aid the development of new treatments.

Neutrophil gelatinase-associated lipocalin (NGAL) is a new, noninvasive marker of AKI (11–21). NGAL has been proposed as a marker of DGF, as an association with NGAL and DGF has been found in studies where urine or serum NGAL had been measured very soon after transplantation (22–38). The role of NGAL in predicting AKI after liver transplantation has also been proposed (39–50).

The aim of this study was thus to examine the role of NGAL in DGF in a large prospective study including both deceased kidney donors and recipients and to study whether NGAL measured from liver transplant recipients before transplantation predicts prolonged kidney injury after liver transplantation.

2. REVIEW OF THE LITERATURE

Kidney transplantation is the only curative treatment for end-stage renal disease (ESRD). The results of kidney transplantation have significantly improved over recent years. The outcome after transplantation depends on recipient factors, donor issues and events related to transplantation. However, the increasing age and cardiovascular morbidity of both recipients and donors, DGF, lack of efficient treatment for chronic allograft nephropathy (CAN), and adverse effects of immunosuppressive drugs such as cancer, diabetes, and infection have a negative effect on long-term results (51–53).

2.1 Recipient factors affecting kidney graft survival

The prevalence of patients on maintenance dialysis has grown far more rapidly than the growth rate of the general population due to the increasing age, morbidity—particularly diabetes—and longer life expectancy for patients with ESRD (54, 55). Thus, recipients have become older and more commonly have multiple chronic disorders influencing the long-term results after kidney transplantation.

Age at the time of transplantation has a clear impact on graft survival; an older age leads to inferior long-term outcomes (56). Cardiovascular disease is an independent risk factor for impaired graft survival and death (57–59). Time spent on maintenance dialysis also directly correlates with poorer long-term survival (59). An advantageous factor regarding graft and patient survival is human leukocyte antigen (HLA) matching (52, 60). The presence of HLA antibodies, and especially donor specific antibodies, is associated with the occurrence of acute rejection (AR) and decreased graft survival after transplantation (61, 62). Primary kidney disease, CAN and a recurrence of the primary kidney disease in the transplanted graft are important causes of kidney graft loss (52, 63). However, the most significant cause for graft loss is the death of the recipient (64, 65).

2.2 Donor factors affecting kidney graft survival

There is a constant disparity between the need for and the availability of kidneys for transplantation; according to the Organ Procurement and Transplantation Network (OPTN) database, every year the number of patients added to the waiting list for kidney transplantation increases whereas the number of performed transplantations remains stable or even declines (66). For example, between 2000 and 2009 in the US alone 1,155,897 patients were added to the kidney waiting list, while only 125,968 (10.9%) received a transplant (67). The majority were deceased donor transplantations (68%) and every fourth kidney came from an expanded criteria donor (ECD) (67). Hence, there is a substantial need to increase the living and deceased donor pool. In Finland, the majority of donors are deceased donors, with only about 2–5% being living donors.

Living donor kidneys are uniformly superior to deceased donor kidneys due to, for example, the possibility of a precise pretransplant evaluation of the donor and the donor kidney, lack of trauma causing brain death and the destructive effects of brain death, and very short CIT

(52). The limiting factor is finding a suitable and willing donor. The paired-donor exchange program is one example of how the living donor pool can be increased. It enables living donor kidney transplantation for those without a compatible kidney donor of their own; kidneys can be exchanged simultaneously between two to three, or even more, pairs (68). Additionally, de-sensitizing protocols enable transplantation across blood group and from an HLA-incompatible donor (69,70).

The selection criteria for deceased donors have also been widened, meaning that many older donors with comorbidities who would have previously been rejected are now accepted for transplantation. In the short term, this seems to result in a negative effect on the onset of graft function after transplantation. However, in historical data the improvement in recipient care seems to, at least partly, override this negative effect regarding 1-year graft survival (71).

There are many definitions for ECDs, but the most commonly used classifies ECDs as brain-dead donors older than 60 or older than 50 with more than one additional risk factor (hypertension, cerebrovascular accident as cause of death or plasma creatinine 1.5mg/dL/>132μmol/L) (72). Additionally, there are programs for accepting kidneys from donors after cardiac death (DCD), further adding to the potential donor pool. Not surprisingly, this has led to new problems, as the quality of donor kidneys has a clear impact on long-term outcome (53, 72–74). The use of ECD kidneys is associated with reduced recipient and graft survival and increased incidence of DGF compared to standard criteria donor (SCD) kidneys (72, 75, 76). Interestingly, although the use of DCD kidneys is associated with increased occurrence of DGF and primary nonfunction, it does not seem to affect long-term results (77). However, not all ECDs are alike; some have very well-functioning kidneys without any histological changes in biopsies. Kidneys from very old deceased donors (>75 years) did not increase the risk of DGF or primary nonfunction if the donor had been healthy with no comorbidities such as cardiovascular disease (78). In addition, many non-donor related factors, such as cold ischemia time (CIT) and cold preservation, with or without machine pump perfusion, affect the results (79). Various algorithms have been developed to evaluate ECD donors but they also include non-donor related variables such as CIT and HLA match (80–83). In practice, these are not helpful for a clinician trying to decide whether to accept or reject a donor. Hence, there is a substantial need for new tools for assessing donor kidney quality.

SCD kidneys rarely have abnormalities in their kidney biopsies, but when ECD kidneys are used the issue of preexisting abnormalities becomes more relevant (53). A preimplantation donor kidney biopsy is thus helpful in deciding whether to transplant or discard an ECD kidney. Donor biopsies also provide important baseline information from the kidney that subsequent biopsies can be compared to (83, 84). Most donor biopsies show at most mild unspecific changes, most commonly global sclerosis, arterial sclerosis, tubular atrophy and interstitial fibrosis (71, 84). The data on whether histological changes correlate with graft survival is controversial, yet some studies have found that the severity of histological changes correlates with graft survival, vascular disease and fibrosis (53, 75, 81, 83, 84).

There is no uniform prevailing practice concerning the use and allocation of ECD kidneys, as they are of somewhat inferior quality. A recent study found that transplanting ECD kidneys instead of SCD kidneys to patients <60 years old significantly increased mortality (85). Generally,

age matching (old to old) is used and has been suggested to attenuate the effect (65, 85, 86). Age matching leads to worse outcome compared to transplantation from SCD in older patients, but overall the results are significantly better compared to a long waiting time on maintenance dialysis (74, 85). The United Network of Organ Sharing (UNOS) Kidney Transplantation Committee has suggested a new allocation policy for ECD kidneys based on donor quality. Hopefully, this will help in increasing the donor pool further and optimize the use of ECD kidneys.

2.3 Transplantation related factors affecting kidney graft survival

Ischemia/reperfusion injury (IRI), AKI, DGF, immunosuppression and CAN all have a major impact on long-term graft survival.

2.3.1 Ischemia/reperfusion injury

All transplanted kidneys are subject to cold ischemia, warm ischemia and reperfusion resulting in direct cell death and increased inflammatory response with ensuing cell death and tissue damage (Figure 1). Ischemia causes hypoxia and the accumulation of reactive oxygen species (ROS). Reperfusion presents the transplanted kidney to the hosts' immune system, activating the innate and adaptive defense mechanisms, which target the graft in multiple ways (87). Reperfusion additionally triggers a pathologic vasoconstriction, which is further augmented by increased endothelin levels (88, 89).

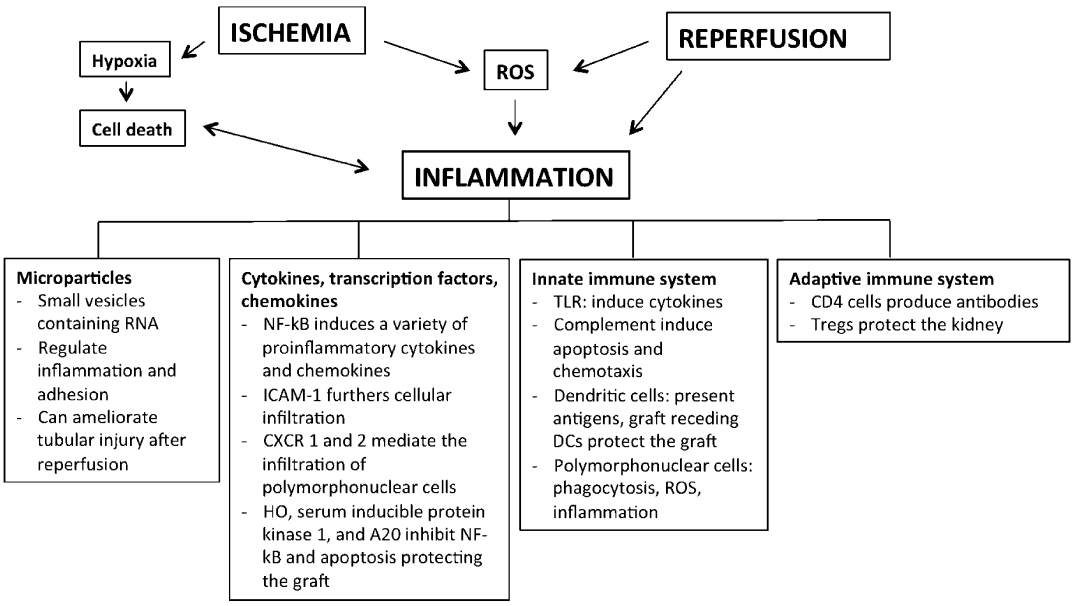

Figure 1. The molecular effects of ischemia/reperfusion injury.
ROS=reactive oxygen species, ICAM=intercellular adhesion molecule, CXCR=CXC chemokine receptor, HO=heme oxygenase, TLR=toll like receptor, DC=dendritic cell, Treg=T regulatory cell, NF-kB=nuclear factor kappa-light-chain-enhancer of activated B cells.

IRI mostly targets endothelial cells in the glomerulus and epithelial cells in the tubule (90). IRI predisposes the graft to tubular cell necrosis and fibrosis, and increases immunogenity, thus causing AKI, DGF, rejection and CAN (91,92). However, it is not known how the dose of IRI is related to the degree of damage. T regulatory cells, graft derived dendritic cells and microparticles play a role in protecting the graft from IRI (93).

2.3.2 Acute kidney injury

In practice, all transplanted kidneys show changes comparable with changes seen in AKI. The degree of injury varies from clinically undetectable to DGF and primary nonfunction. AKI is a multifactorial condition leading to various degrees of renal failure with poorly understood pathogenesis and inadequate diagnostic tools to detect it early enough/in real time. AKI is associated with morbidity, mortality, prolonged hospitalization and increased costs (9, 10, 94).

Today, assessing kidney function is based on measuring serum/plasma creatinine levels and urine output. Various classifications can be used to assess and categorize AKI, but the most widely acknowledged and used are the RIFLE (Risk, Injury, Failure, Loss, Endstage) criteria (95), Acute Kidney Injury Network (AKIN) criteria (96), and KDIGO Clinical Practice Guideline for Acute Kidney Injury (94). There are no applicable classifications for AKI in kidney transplantation as the recipients already have ESRD. Creatinine is a poor marker for AKI as it needs to be measured in a steady state, it reacts slowly to changes in kidney function and many non-renal factors affect its concentration (97–99). The hunt for a good kidney injury marker has been ongoing for decades, but so far nothing has undermined the position of creatinine.

Fluid resuscitation is the most beneficial and the only widely accepted treatment to prevent and attenuate AKI (100, 101). Dialysis treatment is of additional value in the presence of hyperkalemia, fluid overload or metabolic acidosis. Of other treatments, intravenous sodium bicarbonate, levosimendan, N-acetyl cysteine administration before radiocontrast and natriuretic peptides in major surgery have all been shown to have somewhat promising results in alleviating AKI (102–106). However, in general, therapies are lacking success, as the diagnosis is usually delayed and treatment cannot be targeted because the mechanisms behind AKI are still poorly understood.

2.3.3 Delayed graft function

According to the Organ Procurement and Transplantation network database (http://optn.transplant.hrsa.gov) the incidence of DGF is approximately 24.3%. However, the reported incidences of DGF vary from 4% when using living donor kidneys to more than 50% for deceased donor kidneys (107–109). DGF rarely complicates living donor transplantations. However, in deceased donor transplantation DGF affects a significant number of transplantations: one-third at our center (110). The rate of DGF is constantly rising as more ECDs are accepted for transplantation (74, 111, 112).

DGF is a multifactorial condition in which many donor, recipient and transplantation related factors play a role. The precise mechanism behind DGF is still somewhat obscure but the key factors are donor brain death and the subsequent catecholamine and cytokine storm, IRI and the activation of innate and adaptive immune responses in the recipient (80, 113). There are currently no means to predict DGF.

There are several definitions in the literature for DGF (108, 109). This lack of a uniform definition complicates the comparison of different studies, hinders diagnosis and impedes the development of potential treatment.

The most popular definition for DGF is the need for dialysis during the first week after kidney transplantation. Between 1984 and 2007 this definition was used to define DGF in 69% of reviewed studies (108, 109). It is simple and easy to use. However, it falsely includes patients needing one dialysis, for example due to high potassium levels or fluid overload, and excludes patients with good diuresis without need for dialysis, but with slowly decreasing creatinine which does not require dialysis during the first week. At our center, DGF has been defined using the Halloran et al. (114) definition: urine output <1L/24 hours for >2 days, or plasma creatinine concentration >500μmol/L throughout the first week, or >1 dialysis session needed during the first week.

DGF increases the need for dialysis and post-transplant biopsies, increases the risk of AR and masks it, extends the duration of the post-transplantation hospital stay, increases the risk of CAN and causes considerable economic burden (110, 112, 115–118). Additionally, DGF has adverse long-term effects and is associated with increased mortality (113, 119). Not all studies have found an association between DGF and graft survival and there is no general agreement on the ultimate effect of short DGF (lasting less than one week) on graft survival. However, there is strong evidence concerning the damaging effect of prolonged DGF on graft survival (112, 120, 121).

Risk factors for DGF are related to the donor (ECD, DCD, old age, female gender or obesity), transplantation (long warm and cold ischemia time or absence of machine perfusion), and recipient (obesity, diabetes, mode of and time on maintenance dialysis before transplantation, male gender or immune sensitization) (72,122–124). Thus, including these risk factors in a nomogram can help in risk assessment (80, 81).

DGF cannot be treated. However, all patients with DGF should be biopsied to exclude AR and acute tubular necrosis (125). Ultrasound scanning should be performed to exclude hematomas, urinary tract obstruction or problems in the vessels of the graft. Other treatment during DGF consists of dialysis, sustaining hemodynamic stability and avoiding nephrotoxins (125).

2.3.4 Immunosuppression

Immunosuppression has a major role in preventing rejection and improving short-term outcomes. Different immunomodulating strategies have also enabled transplantation for highly sensitized recipients (126). Short-term outcomes after kidney transplantation were dramatically improved in the 1980s when calcineurin inhibitors (CNIs) were included in the immunosuppression (51, 127). However, immunosuppression has many side effects, such as direct nephrotoxicity to the graft, increased susceptibility to infections, and increased risk for malignancy, diabetes and cardiovascular disease (52). CNI nephrotoxicity can lead to progressive and irreversible fibrosis, sclerosis and deterioration in renal function (128). CNIs have a narrow therapeutic window and express notable variability in adsorption, distribution, metabolism and elimination (129). Therefore, the use of these drugs is demanding and the through levels need

frequent monitoring. Consequently, various CNI-free or CNI-sparing therapies have emerged, which have not improved long-term outcomes (130–132). There is a consensus that CNIs contribute to CAN development but it is unclear how big this impact is (127, 133).

KDIGO guidelines suggest that immunosuppression after kidney transplantation should consist of triple therapy: CNI (cyclosporine A or tacrolimus), antiproliferative agent (mycophenolate mofetil), and steroids with or without induction therapy with induction agents, preferably interleukin 2 receptor antagonists (IL-2R) (125).

2.4 Alleviating ischemia/reperfusion injury

Timely diagnosis and management of post-transplant complications is essential for graft survival. Alleviating IRI might help in preventing DGF.

2.4.1 Donor treatment

Brain death induces extensive metabolic changes, multiple organ dysfunction and widespread injury in the donor (134, 135). It generates systemic inflammation that by the time of organ recovery will have lasted several hours (136, 137). Additionally, there are alterations in endocrine function, vascular regulation and coagulopathy, which further increase the inflammation and hemodynamic instability (134, 138, 139). Limiting the time between brain death and organ procurement to less than 24 hours decreases the risk of DGF (140, 141). Early identification and treatment of brain death related complications, such as diabetes insipidus and pulmonary edema, additionally improve the outcome of the transplanted organ in the recipient (142).

2.4.1.1 Fluids

It is beneficial to maintain a mean arteriolar pressure >70mmHg in all donors by fluid resuscitation (143). Hydroxyethyl starch use has been found to associate with early graft dysfunction and DGF in the kidney recipient (144, 145) in addition to its other negative effects. Using a large volume (>1250ml) of other colloids, for example albumin, has been found to associate with reduced risk for DGF (146). Hypernatremia in the donor commonly results from disturbed fluid balance and induces osmolality and swelling, and aggravates IRI (147, 148). Hypernatremia >150 mmol/L has been found to associate with graft dysfunction and loss after transplantation (147, 148).

2.4.1.2 Hormonal resuscitation

The brain death induced catecholamine storm incapacitates the donors' endocrine function, resulting in a considerable decrease in hormone levels including cortisol, thyroxine, insulin and vasopressin (149). Treating donors with intravenous steroids alleviates the effect of systemic inflammation (150). In kidney recipients this was associated with better 1-year graft survival and in liver recipients with amelioration of IRI and decreased incidence of AR (151, 152). However, not all studies have found these associations (153,154).

Low levels of triiodothyronine and thyroxine have a major role in donor anaerobic metabolism and depletion of glycogen. The etiology is unclear but permanent damage to the hypothalamus is suggested as a possible cause. Treatment with triiodothyronine and thyroxin has been suggested

to result in a reversal from anaerobic to aerobic metabolism and stabilization and improvement of cardiac function (155,156). However, a recent meta-analysis found that treating donors with triiodothyronine did not have any beneficial hemodynamic effects (149).

Additional use of desmopressin or vasopressin in diabetes insipidus has been found to be beneficial in some studies (143). Nevertheless, the recent meta-analysis did not find any advantageous effects of desmopressin administration and kidney function or outcome after transplantation (149). In the case of patients without diabetes insipidus, treatment with arginine vasopressin was found in one study to reduce the need for inotropes (157).

Brain dead donors are usually hyperglycemic as brain death increases gluconeogenesis and insulin resistance and decreases insulin secretion (149, 158). Hyperglycemia and high variability in blood glucose levels associate with decreased graft function after transplantation (159, 160). Therefore, aggressive management of hyperglycemia in the donor is recommended (UNOS guideline).

2.4.1.3 Vasopressors

Dopamine is a neurotransmitter that has central and peripheral actions. Peripheral dopamine mediates blood flow, glomerular filtration rate, sodium excretion and catecholamine release. Hence, it is used as a vasopressor in acute situations and donor treatment. It also induces heme oxygenase-1, which catabolizes unstable iron and antagonizes free radical generation (87). One large multicenter study found that treating the donor with low dose dopamine vs. no dopamine treatment improved early kidney graft function and decreased the incidence of DGF and prolonged graft survival, but did not affect patient survival (161, 162).

Donor epinephrine or adrenaline infusion associates with an increased risk for DGF (146). However, vasopressor support is imperative if hemodynamic status requires it (141, 143).

2.4.1.4 Anticoagulation

Brain death causes significant disturbances in blood coagulation. Deceased organ donors are usually treated with anticoagulants to prevent thrombosis. It has been suggested that the prevalence of microscopic thrombosis is underestimated (163). In a pig model of kidney transplantation, it was shown that treating the donor with a thrombin inhibitor melagatran just before perfusion with University of Wisconsin solution might improve graft survival (163, 164). Additionally, timely diagnosis and treatment of disseminated intravascular coagulopathy and other dysfunctions is needed (143).

2.4.2 Organ preservation

Preserving the kidney in cold buffer fluid for the time of transportation helps to protect the kidney by decreasing metabolism, and reducing osmotic injury/edema and acidosis (87, 165). The UW solution is one of the most commonly used buffers. It contains adenosine to improve aerobic metabolism and allopurinol to inhibit xanthine oxidase.

Machine perfusion has been suggested to reduce the risk of DGF and improve 1-year graft survival after transplantation of both SCD and ECD kidneys (166). However, this was not found

in recent meta-analysis (167). Using extracorporeal membrane oxygenation in DCD has shown promising results in deducting DGF (168)

2.4.3 Recipient treatment

Many different strategies such as ischemic preconditioning; stem cell therapies; molecules to prevent inflammation, endothelial injury, and vasoconstriction; and complement regulators have been studied in experimental models to limit IRI and especially the reperfusion injury in the recipient. Only a few have been studied in humans, with very minor results. Adequate intraoperative fluid treatment is one of the rare effective means to reduce the risk of DGF (87, 169–174).

2.4.3.1 Immunosuppression

Reperfusion activates the hosts' innate immune system: macrophages and neutrophils migrate into the graft and the complement cascade is activated, further activating the adaptive immune system, resulting in the formation of ROS and direct cell lysis (87, 173, 174). Targeting the recipients' immune system before transplantation with induction therapy, for example using anti-CD25, anti-CD52, anti-CD3 or antithymocyte immunoglobulins, may decrease the incidence of DGF (87). Initiating CNIs before transplantation or in the early postoperative period does not seem to increase the risk of DGF or delay graft recovery, despite previous beliefs (175).

Many molecules have anti-inflammatory actions. Administrating these molecules to alleviate the effects of the activated immune system has been proposed to have a role in preventing DGF. However, small studies on erythropoietin, P-selectin and human annexin V homodimer have been performed without significant results (87).

2.4.3.2 Vasodilatation

Reperfusion causes vasoconstriction and inflammation further increases it by damaging the endothelium. Additionally, CNIs cause vasoconstriction. Calcium channel blockers, endothelin receptor antagonists and adenosine antagonists have all been proposed to be useful in preventing DGF (87). Calcium channel blockers somewhat decrease the incidence of DGF and result in significantly better long-term kidney function that is independent of blood pressure lowering effects (89, 176, 177). The other drugs have so far only been used in experimental models of transplantation.

2.5 Long-term kidney graft function

Kidney function at 1-year after transplantation is an independent predictor of long-term outcome and correlates with graft failure and death with a functioning graft (125, 178). This is probably mostly due to increased cardiovascular disease, as in kidney transplant patients graft dysfunction is an independent risk factor for cardiovascular disease (179). All kinds of proteinuria (micro- or macro-proteinuria) occurring at any level or time after transplantation have a negative impact on graft survival (180). In summary, 1-year kidney function is an important prognostic factor for long-term kidney function.

2.6 Acute kidney injury after liver transplantation

Kidney injury is an important and major complication in patients with advanced liver disease; approximately one in five patients hospitalized with decompensated cirrhosis will develop renal failure (181, 182). In most cases this is caused by prerenal kidney injury resulting from infection, hypovolemia or hemorrhage and in 70% of cases it can be treated by volume expansion (182). Hepatorenal syndrome is a liver-disease related kidney injury resulting from complex changes in vasodilatation and vasoconstriction in systemic and renal circulation. It affects 18% of patients with advanced cirrhosis at one year and 39% at five years (183). Prognosis of hepatorenal syndrome is poor: at best median survival time is six to seven months (184).

Liver transplant recipients are at significant risk for pre- and posttransplant AKI. The reported prevalence varies widely, from 8% to 78%, with 8% to 17% of patients needing renal replacement therapy (RRT) (185–187). The etiology of kidney injury is multifactorial, resulting from acute and chronic liver failure and factors related to transplant surgery and medication. The predominant risk factors for AKI are hepatorenal syndrome, perioperative hypotension, large volume transfusion, extended cross clamping time, use of CNI, nephrotoxic antibiotics and contrast media, infection, hypertension, diabetes and pre-existing chronic kidney disease (CKD) (186–189). Pre-existing CKD and pre- and posttransplant AKI associate with poorer outcomes after liver transplantation. Hence, a combined kidney–liver transplantation and individually designed immunosuppression would lead to better results among these patients (190). However, nowadays it is not possible before liver transplantation to identify patients who are going to develop posttransplant AKI or which patients on pretransplant RRT would benefit from a combined kidney–liver transplantation.

Liver transplant patients are also at increased risk for developing CKD after liver transplantation mainly due to CNI therapy, hypertension, diabetes and chronic hepatitis C infection (191).

At present, the assessment of renal function is based on measuring urine output, serum/plasma creatinine and cystatin C levels, or using RIFLE, AKIN or the KDIGO Guideline for AKI classifications. As previously stated, creatinine is not a good marker of kidney injury in an acute unstable setting. Better markers for identifying patients at risk of AKI, and especially irreversible AKI resulting in permanent need for RRT, are needed.

2.7 Measuring kidney function

2.7.1 Creatinine

Creatine is broken down to phosphocreatinine in skeletal muscle and consequently non-enzymatically to creatinine in the liver. In most people, muscle mass turnover is relatively constant and thus serum/plasma creatinine is relatively constant. There are several factors directly affecting serum creatinine levels (age, gender, diet, muscle mass, drugs, race, fluid status and exercise) or affecting the analysis method (hyperglycemia, bilirubin, cimetidine and cephalosporines) (192, 193). Creatinine is freely filtered by the glomerulus and actively secreted by the tubules. The rate of secretion depends on blood creatinine concentration. Additionally,

creatinine secretion accounts for only 10% to 40% of creatinine clearance that can mask decline in GFR (194).

When the GFR declines, the creatinine concentration in the blood rises. However, serum creatinine is insensitive to GFR changes and serum creatinine only becomes abnormal after 50% of GFR is lost. This is due to increased extrarenal metabolism and secretion of creatinine by the tubules. Additionally, it takes up to 24 hours to reach a steady state (192, 195).

Creatinine is especially poor in assessing kidney function in patients with end-stage liver disease. The serum creatinine concentration in these patients tends to be lower than expected, since the synthesis of creatinine is reduced in the liver, they usually have reduced muscle mass, they exercise less than healthy individuals and their intake of protein is usually reduced. Additionally, patients with liver disease often have edema, further diluting the creatinine concentration in the blood (196).

2.7.2 Glomerular filtration rate (GFR)

The normal GFR is >90mL/min. GFR varies greatly between individuals due to age, body size, gender and race, but it is mostly constant within each individual. However, age-related decline in GFR is 0.75-1 mL/min per year after mid-adulthood (between 30 and 40 years of age).

GFR cannot be measured directly; thus, a surrogate marker is needed. An ideal marker for GFR measurement would be produced at a constant rate/exogenous, freely filtered by the glomerulus, without tubular reabsorption or secretion, or extrarenal metabolism. Additionally, there should be available an accurate inexpensive assay. Inulin clearance is the gold standard for measuring GFR, but it is too difficult and expensive for clinical work. In fact, measuring clearance for any substance is laborious, slow and inaccurate, as 24 hours of urine collection is needed. Hence, in the clinic the assessment of GFR relies on the estimation of creatinine clearance using mathematical equations, which try to overcome some of the limitations related to urine collection (197). All of the equations are susceptible to the limits in use of creatinine as a marker of kidney injury. Additionally, they have been developed for use in a stable CKD population and they have not been validated and should not be used in an acute setting, although they commonly are.

The Cockroft-Gault, modification of diet in renal disease (MDRD), and CKD-EPI equations are used in patients over 18 years. In pediatric patients, GFR is estimated using the Schwarzt equation (198–201). The Cockroft-Gault equation was the first widely used estimation for GFR:

> GFR = [(140-age) x weight]/72 x serum creatinine. In females the result is multiplied by 0.85.

This estimation is inaccurate, as it uses weight as an index of muscle mass. Additionally, it overestimates GFR at low values in patients with CKD.

The MDRD equation is considered more accurate than the Cockroft-Gault equation as it normalizes the results to body surface area (202):

GFR = 170 x serum creatinine$^{-0.999}$ x age$^{-0.176}$ x 0.762 for females and x 1.18 if the patient is black.

This equation may underestimate GFR in CKD stage 1 (if kidney function is normal but there is other evidence of kidney disease) and overestimate it in CKD stages 4 and 5 (if GFR <30 ml/min/1.73m^2). The MDRD equation has been validated in adults with impaired kidney function (GFR<60ml/min).

The CKD-EPI equation is the newest equation for estimating GFR. It is considered more accurate than the MDRD equation as it has less bias in CKD stage 1. The National Kidney Foundation and KDIGO recommend using the CKD-EPI in estimating GFR (203).

GFR = 141 x $\min(cr/\kappa,1)^{\alpha}$ x $\max(cr/\kappa,1)^{-1.209}$ x 0.993^{age}
Cr=serum creatinine, min=minimum serum creatinine / κ or 1
max=maximum serum creatinine / κ or 1, α= -0.329 in females and –0.411 in males
κ= 0.7 in females and 0.9 in males

The Schwartz formula is used to estimate GFR in children:

GFR = (k x height)/serum creatinine.
The coefficient (k) is: 0.33 for low birth weight infant <1 year, 0.45 for normal birth weight infant <1 year, 0.55 for child or adolescent girl, 0.70 for adolescent boy.

2.7.3 Urine output

Urine output is an approximate measure of kidney function. Many drugs, fluid status, and other circumstances such as surgery affect urine output. In AKI, urine output can be decreased, normal or increased depending on tubular injury and tubular urine concentration capacity (204).

2.7.4 Urea

Urea is a small by-product of protein metabolism that is freely filtered by the glomerulus but also significantly reabsorbed. The rate of renal clearance of urea is not constant and varies, for example due to fluid status. Urea concentration in blood increases with a decrease in GFR. However, nutritional status, protein intake, fluid volume, steroid administration, fever, trauma, liver disease and gastrointestinal bleeding affect its concentration (205). The blood urea nitrogen to creatinine ratio has been suggested to discriminate between prerenal azotemia and acute kidney injury, but recent findings do not support this (206).

2.7.5 Cystatin C

Cystatin C is a 13kDa protein produced in all nucleated cells at a relatively constant rate. It is not bound to plasma proteins and thus is freely filtrated through the glomerulus and reabsorbed in the proximal tubulus. It is not secreted into urine, so its clearance cannot be determined

(207). The appearance of cystatin C in the urine is thus related to kidney injury and cystatin C concentration correlates with GFR (208). However, there are some shortcomings concerning the use of cystatin C in the diagnosis of kidney injury: albuminuria may inhibit reabsorption of cystatin C by its receptor megalin and hence increase its concentration in urine (209, 210). Serum cystatin C concentration is affected by hypertriglyceridemia, diabetes, inflammation, hyperthyroidism and steroid use (207, 211, 212).

Overall, cystatin C is not the perfect biomarker for kidney injury but, as Shlipak et al. concluded in a recent meta-analysis, measuring serum cystatin C alone or in combination with creatinine adds value when evaluating the association between GFR and the risk of ESRD or death (213). Therefore, measuring serum cystatin C has been included in the KDIGO CKD guidelines. In a recent study of a panel of plasma and urine biomarkers, plasma cystatin C was found to moderately predict AKI (214).

2.8 Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is a 22–25kDa iron carrier protein. NGAL was first identified in human neutrophils bound with matrix metalloproteinase-9 (215, 216). Later, an iron binding siderophore, enterochelin, was also identified as a ligand for NGAL (217, 218).

NGAL is a part of the lipocalin protein family. The lipocalins are secreted or cytosolic proteins that bind and transport several low molecular weight proteins such as retinoids, prostaglandins, iron, fatty acids, steroids and arachidonic acid (217, 219, 220). The ligand-binding pocket of NGAL is much larger and lined with more polar and positively charged amino acid residues than in other lipocalins.

NGAL binds siderophores, a diverse group of small iron binding chemicals produced in bacteria, fungi, and plants (218, 221). NGAL associated with siderophore and iron (=holo-NGAL) enters the cell via an endocytic multiprotein megalin-cupulin receptor. Another transmembrane receptor, 24p3R, has also been proposed to transport NGAL (222, 223). After entering the cell, NGAL traffics to endosomes and releases iron, resulting in the regulation of iron responsive genes coding, for example, ferritin and transferrin receptors (223, 224). By increasing intracellular iron uptake, NGAL plays a part in mesenchymal-epithelial transformation (225). NGAL without a siderophore-iron complex (apo-NGAL) can scavenge iron, for example from bacterial cells, and exit via the endosomal-recycling pathway (223) resulting in cell apoptosis, inhibition of bacterial growth and erythropoiesis (217, 223, 226).

NGAL also acts as a growth factor, independent of ligands, by activating an extracellular signal regulated kinase and inducing promigratory and probranching effects (225). Hence, NGAL may protect tubular cells from ischemic injury (15, 221). However, knowledge of these non-iron related actions is still limited.

NGAL is constantly produced at low levels in neutrophils, adipocytes, and by stimulated, growing, dysplastic or involuting epithelial cells in various tissues in the skin, as well as

respiratory, gastro-intestinal and urinary tracts. That is, tissues that are constantly exposed to the external environment and thus bacteria (227–229). In healthy individuals, serum NGAL concentration in humans is approximately 20ng/mL (230). NGAL expression is induced by IL-1β, lipopolysaccharide, basic fibroblast growth factor, prostaglandin F2α, phorbol esther, dexamethasone, retinoic acid, serum and hypoxia, at the very least (15, 231). Elevated levels of NGAL have been reported in bacterial infections consistent with the bacteriostatic function of NGAL. The importance of this function has also been shown in vivo: NGAL deficient mice are more susceptible to bacterial infections and sepsis (226, 232–234). Elevated NGAL levels are also found in various systemic settings without bacterial infection, such as in the acute phase response, renal tubular injury, inflammation of the intestine, skin or airway, and cancer (14, 15, 221, 224, 229, 231, 234–239).

NGAL is freely filtered by the glomerulus due to its small size and positive charge. The kidney processes about 3.4–4mg of NGAL per day. Proximal tubule cells express a megalin receptor for NGAL uptake and NGAL is endocytosed and degraded to 14kDa fragments in lysosomes and the iron is recycled. Normal urine NGAL concentration is about 20ng/mL and it is derived from neutrophils, distal nephron cells mainly collecting ducts, and serum NGAL that has escaped the reabsorption in proximal tubulus (1/200 molecules) (230, 240).

2.8.1 NGAL in kidney injury

Increased NGAL levels have been reported in acute kidney injury caused by ischemia (surgery, sepsis, hypovolemia, heart failure), nephrotoxins (antibiotics, cisplatin, non-steroidal anti-inflammatory drugs, radiocontrast, hemoglobinuria), various chronic kidney disease (IgA nephropathy, membranous nephropathy, polycystic kidney disease), and in kidney transplantation predicting DGF and AR (11–50, 228, 237). The expression of NGAL increases rapidly–1000-fold in humans and rodents–in response to renal tubular injury (237).

Kidney injury after cardiopulmonary bypass surgery, intravenous contrast administration and neonatal sepsis are examples of clinical situations where the actual timing of the damage can be determined. Using these settings, many studies have found that NGAL can be detected in the urine and blood within three hours after the original injury (14, 241–243). The amount of NGAL synthetized in the kidney is directly proportional to the size of the injury (244). This has also been shown in clinical situations: urine NGAL (uNGAL) and serum NGAL (sNGAL) concentrations are proportional to the recovery from DGF, uNGAL concentration is twice as high in bilateral vs. unilateral ureter obstruction, and NGAL concentration correlates with the AKIN or RIFLE kidney injury grade (19, 22, 25, 245–248).

NGAL has been shown to predict AKI in several clinical settings. Firstly, serum and urine NGAL were shown to be independent predictors of AKI, morbidity and mortality in children undergoing cardiopulmonary bypass surgery (241). Serum and urine NGAL levels dramatically elevated between one to two hours after cardio-pulmonary bypass surgery in patients who later developed AKI, diagnosed by more than 50% rise in serum creatinine a few days later (14, 241, 249). This has also been shown in adults, albeit with less predictive power (11–13, 250–252). NGAL has been shown to predict contrast induced nephropathy within two to four hours after contrast (21, 242, 253). In critically ill intensive care unit (ICU) patients NGAL is associated

with AKI development (254, 255). In children, an increase in urine NGAL was associated with worsening RIFLE category (256). Makris et al. found that NGAL predicts AKI in critically ill multiple trauma patients (20) and Nickolas et al. also found that NGAL predicts AKI in patients arriving in an emergency room irrespective of diagnosis (18). In CKD, serum and urine NGAL reflect the severity and progression of kidney disease (257–259).

CKD patients have a chronic inflammatory state due to frequent infections, uremia, an increase in proinflammatory cytokines, widespread atherosclerosis, and dialysis. In these patients, the iron metabolism is off balance (260, 261). Bolignano et al. suggested that elevated NGAL in dialysis patients is due to the involvement of NGAL in iron equilibrium, and serum NGAL could be used in the assessment of iron deficiency in ESRD patients (260, 261). NGAL might contribute to the progression of CKD as a result of proinflammatory properties or iron dependent functions (262). Recently, Liu et al. found that urine NGAL in CKD patients independently associates with future ischemic events (263).

NGAL has been found to predict kidney function after heart, heart–lung and liver transplantation (39–42, 264–266). After kidney transplantation, serum/plasma NGAL has been shown to predict DGF (22–38).

NGAL is important in the diagnosis of kidney disease as it directly measures tissue damage in a dose-response fashion. NGAL expression is rapid and reversible, which means that NGAL can also be used to monitor the effect of treatment.

2.8.2 NGAL detection methods

Immunoblotting is the gold standard in NGAL analyses. However, it is time consuming, laborious and prone to human error and is not well suited for clinical work. Nowadays, various commercially available detection methods and kits exist for NGAL analyses. Enzyme-linked immunosorbent assay (ELISA) methods can be used to analyze NGAL in serum, plasma and urine. The point-of-care (POC) method can be used to analyze NGAL in whole blood and plasma, and the ARCHITECT® method to analyze NGAL in urine.

3. AIMS OF THE STUDY

The aim of the study is to prospectively analyze the role of serum and urine NGAL in kidney transplant donors and recipients and liver transplant recipients. The specific aims are to examine:

1. Whether urine and serum NGAL levels in deceased kidney donors predict DGF and prolonged DGF (I)
2. How serial urine NGAL concentrations change over time after kidney transplantation and whether recipient urine NGAL predicts DGF and prolonged DGF (II)
3. How serial serum NGAL concentrations change over time after kidney transplantation and whether recipient serum NGAL predicts DGF and prolonged DGF (III)
4. To compare the ELISA and POC methods in serum NGAL analyses (III)
5. Whether plasma NGAL concentration predicts AKI or need for renal replacement therapy after liver transplantation (IV)

4. PATIENTS AND METHODS

The study was performed at Helsinki University Hospital, which is the only transplant center in Finland providing a nationwide organ transplant service. The local ethics committee and the Department of Surgery approved the study protocol. The study was performed in accordance with the Helsinki 1975 declaration.

4.1 Study design

The study designs are summarized in Table 1.

Table 1. Summary of studies I–IV

	Patients	eGFR	Sample material Method	Sample timing
Study I	Kidney donors N=99	MDR	Urine and serum ELISA	**Urine** At the beginning of donor surgery **Serum** At donor hospital simultaneously with serum sample for HLA typing
Study II	Kidney recipients N=176	Cockroft-Gault	Urine ARCHITECT®	At arrival to the TX unit The 1st morning after TX On day 3 On day 7 On day 14
Study III	Kidney recipients N=176	Cockroft-Gault	Serum Elisa, POC	At arrival to the TX unit The 1st morning after TX On day 14
Study IV	Liver recipients N=203	MDRD	Plasma POC	On the day of TX or shortly before

eGFR= estimated glomerular filtration rate, TX = transplantation, POC = point-of-care

4.2 Patients

For the kidney studies we prospectively enrolled 100 consecutive, deceased, heart-beating donors, and their adult kidney recipients, between August 2007 and December 2008. Altogether, 196 donated kidneys were transplanted. Six kidneys were transplanted to pediatric recipients, nine kidneys were shipped to other Scandinavian transplant centers according to the Scandiatransplant exchange obligation rules, and two kidneys were transplanted for patients needing a combined kidney–liver or a kidney–lung transplantation. The remaining 179 kidney recipients were eligible for the study. Three patients refused consent. Hence, 99 donors and 176 kidney recipients were included in this study. Written informed consent was obtained from the recipients before enrolment. The primary outcome variable was the onset of graft function after kidney transplantation. The follow-up time was one year.

For the liver study, we included 211 adult liver transplant recipients between 2005 and 2010. Patients receiving combined liver–kidney transplantations (n=7) were excluded. No other exclusion criteria were used. The remaining 203 patients were included in the study, although

one did not have an adequate sample for NGAL analyses. The follow-up time was 90 days after transplantation.

After transplantation, the immediate post-operative care took place at the Helsinki University Hospital. The patients' clinical data were obtained from their hospital records, the Finnish Kidney Transplant Registry and the Finnish Liver Transplant Registry databases.

4.2.1 Kidney donors

The donors were treated in their local hospitals around Finland. This study did not affect their treatment in any way. The clinical history data of the donors were obtained from their hospital records. The following parameters were collected: age, gender, history of hypertension, need for cardiopulmonary resuscitation, need for intracranial surgery, use of vasopressor support, use of antidiuretic hormone (ADH), plasma creatinine, length of hospital stay before brain death diagnosis, cause of death and multiorgan or kidney-only donation. None of the donors had diabetes mellitus. The donors were stratified to normal criteria donors and ECDs according to Port et al. (72). The donors were regarded as ECD if they were older than 60, or older than 50 with at least two of the following: plasma creatinine more than 133μmol/L (1.5mg/dL), cerebrovascular accident as cause of death, or a history of hypertension.

According to the valid protocol, intravenous steroids were given to all donors before the organ retrieval operation. Intravenous mannitol was administrated before *in situ* perfusion. A kidney biopsy was taken before the initiation of *in situ* perfusion. The biopsy was assessed for histology according to the Banff 97 criteria (267) and the chronic allograft damage index (CADI) (268). University of Wisconsin solution was used for the *in situ* perfusion and cold storage preservation of the kidneys.

4.2.2 Kidney transplant recipients

For the kidney transplant recipients, data on the following parameters were collected: age, gender, underlying kidney disease, number of previous kidney transplants, mode of dialysis, time on dialysis before transplantation, the amount of daily urine output at arrival to the transplant unit and after transplantation, plasma creatinine level upon arrival to the transplant unit and daily after transplantation, and estimated GFR (eGFR) at three weeks, three months and one year after transplantation.

The study did not affect the recipients' treatment in any way; all the patients were treated according to the current protocol with triple immunosuppression consisting of CNI (cyclosporine A or tacrolimus), mycophenolate mofetil and steroids. CNI was started orally before transplantation, and continued after transplantation (target levels 200 to 250ng/mL for cyclosporine and 6 to 12 ng/mL for tacrolimus) for two weeks and the target levels were subsequently tapered according to treatment protocol. The target dose for mycophenolate mofetil was 1 g twice a day for patients on cyclosporine and 500 mg twice a day for patients on tacrolimus. Induction immunosuppression with IL-2R was given to 28 (basiliximab, n=19, daclizumab, n=9) highly sensitized recipients or recipients for whom steroids needed to be minimized.

4.2.3 Liver transplant recipients

For the liver transplant recipients, data on the following parameters were collected: age; gender; creatinine and eGFR at listing and transplantation day; pretransplant need for RRT; transplantation day MELD (model for end-stage liver disease) score; bilirubin, albumin and INR; amount of total bleeding; anhepatic time; day-1 lactate concentration and the highest value of bilirubin; alanine aminotransferase and INR; and the lowest eGFR value during the first week after transplantation. The three highest trough levels of cyclosporine or tacrolimus during the first week after transplantation were collected.

The study did not affect the treatment of the liver transplant recipients in any way. The patients received standard CNI based immunosuppression (cyclosporine n=140, tacrolimus n=63), with mycophenolate mofetil or azathioprine, and steroids. The target level of cyclosporine was 200 to 250ng/mL for the first month and 150 to 200ng/mL after that, and tacrolimus 5 to 15ng/mL for the first three months. The patients were considered to have excessive exposure to CNI with trough levels of >300ng/mL for cyclosporine and >20ng/mL for tacrolimus. During the first month 13 patients were switched from cyclosporine to tacrolimus due to AR, which was histologically confirmed and treated with intravenous methylprednisolone. As part of a trial, 30 patients received interleukin-2 receptor antagonist induction therapy with reduced dose of CNI or delayed initiation of CNI at day 5 after transplantation.

4.3 Definition of DGF and prolonged DGF

In studies I and II, DGF was defined according to Halloran et al. (269): urine output less than 1L/24 hours for more than two days, or plasma creatinine concentration >500μmol/L throughout the first week, or more than one dialysis session needed during the first week. As the need for dialysis during the first week after transplantation is the most commonly used definition for DGF, we used it along with the Halloran definition in Study II and as the main definition of DGF in Study III. To assess the duration of DGF we divided the transplantations in all three studies into three groups: early graft function (EGF), short DGF lasting less than 14 days, and prolonged DGF lasting 14 days or longer.

4.4 Assessment of kidney function and definition of AKI in liver transplant patients

The amount of daily urine output was measured and recorded from the donors before the organ retrieval operation and from the recipients before transplantation and daily after transplantation. Plasma creatinine was measured in the hospital laboratory using a photometric, enzymatic and accredited assay. Estimated GFR for recipients and adult donors was calculated using the MDRD equation in studies I and IV and Cockroft-Gault equation in studies II and III. For three pediatric donors, the eGFR was calculated using the Schwartz formula.

Plasma cystatin C was measured in the hospital laboratory using a photometric, immunochemical method from the liver transplant recipients and was used to calculate eGFR by the cystatin C-based chronic kidney disease epidemiology collaboration equation (CKD-EPI). Pre-transplant

cystatin C measurements were available in 174 (86%) patients and 3-month measurements in 151 (77%).

In liver transplant recipients, AKI was determined as one of the following: (1) >100% increase in transplant day creatinine, (2) >50% decrease in eGFR from transplant day to an end eGFR of <60mL/min at three months, or (3) a rise in creatinine to an end creatinine of >350μmol/L in patients with pretransplant eGFR <60mL/min. Molecular adsorbent recirculating system (MARS) therapy always included hemodialysis and in Study IV MARS treatment is considered as RRT.

4.5 Assessment of liver function

The MELD score is a chronic liver disease severity scoring system that predicts 3-month survival. It is calculated from plasma creatinine, INR, and bilirubin concentration. The highest values of INR, bilirubin and alanine aminotransferase during the first week after transplantation were used to determine liver graft dysfunction after transplantation.

4.6 NGAL sample collection

4.6.1 Donors

Serum samples were collected from the donors in their local hospitals simultaneously with the blood sample for HLA typing. Serum samples were available for NGAL analyses from 95 donors. In four cases sNGAL could not be determined due to inadequate (n=2) or incorrectly processed/stored (n=2) sampling.

Urine samples were taken at the beginning of donor surgery by the transplant team. Donor uNGAL levels could not be determined in four cases because of inadequate (n=1) or incorrectly processed/stored (n=3) samples.

4.6.2 Kidney recipients

The kidney recipient serum and urine samples were collected on arrival to the transplant unit before transplantation and serum samples in the mornings of days 1 and 14 after transplantation and urine samples in the morning of days 1, 3, 7 and 14 after transplantation. The following number of serum samples were obtained: day 0 n=141, day 1 n=170, day 14 n=166. Due to oliguria or anuria some of the recipients were not able to give a urine sample. We obtained the following number of urine samples at each time point: day 0 n=70, day 1 n= 134, day 3 n=139, day 7 n=151, day 14 n=154.

4.6.3 Liver recipients

The plasma samples from liver transplant recipients were collected on the day of transplantation (n=158), on arrival to the transplant unit, or a few days before (n=45) in cases of acute liver failure or at onset of dialysis in patients receiving pretransplant RRT.

4.7 NGAL analyses

All serum and urine samples were immediately centrifuged at 2500RPM, 4°C for 10 minutes and the plasma samples at 3000RPM. The supernatant was immediately collected, divided into tubes, frozen and stored at -70°C. No additives were used. For analysis, the urine and serum samples were thawed at room temperature. Repeated freeze–thaw cycles were avoided.

4.7.1 Serum and plasma NGAL

4.7.1.1 ELISA

In studies I and III the sNGAL assays were performed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (BioPorto Diagnostics A/S, Gentofte, Denmark) as recommended by the manufacturer. The intra- and inter-assay precision were good; the median coefficient of variation was <10% for both. The samples were diluted to 1:50 for optimal measuring feature. The screening frequency was set to 450nm and the reference frequency to 620nm. With this validated method, the measuring range was 10 to 1000ng/ml. Out of range values were re-analyzed with an individualized dilution. The measurements were performed in duplicate and blinded to clinical information.

4.7.1.2 Point-of-care

In studies III and IV the NGAL levels were analyzed using the POC fluorescence immunoassay NGAL kit and device (Triage®, Biosite, San Diego, CA, USA) as recommended by the manufacturer. The specimen can be used as such; no preliminary dilutions are needed. The specimen was added to the sample port on the test device where it reacts with fluorescent antibody conjugates. The test device was then inserted to the measuring device, which automatically measures the amount of fluorescence and calculates the NGAL concentration. The measurements were performed in duplicate and blinded to sample sources and clinical outcome.

4.7.2 Urine NGAL

In studies I and II the uNGAL assays were performed using a two-step chemiluminescent microparticle immunoassay on a standardized clinical platform (ARCHITECT® analyzer, Abbott Diagnostics, Abbott Park, IL, USA) as recommended by the manufacturer. Prior to setting up the system, we performed a 5-day precision study where three levels of controls were assayed in replicates of two on each run (total n=20 for each control). This showed good precision for the urine NGAL assay (median coefficient of variation <10%). The samples were diluted to 1:10. The sample was combined with anti-NGAL coated paramagnetic microparticles, a labeled conjugate was added, and the resulting chemiluminescent reaction was measured. The measuring range was 0-1500ng/ml and the machine further automatically diluted specimens for NGAL values exceeding 1500ng/ml. The measurements were performed in duplicate and blinded to sample sources and clinical outcome.

4.8 Statistical analyses

The SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA), was used for statistical analyses. All analyzed variables were tested for distribution. We used the T-test, ANOVA and Pearson

correlation for samples with normal distribution. Samples with skewed distribution were analyzed with the Mann-Whitney U, Kruskal-Wallis and Spearman correlation tests. Chi-square and Fisher's exact tests were employed in the analyses of contingency tables. Forward, conditional multilogistic regression analyses were used to assess DGF, prolonged DGF and AKI predictors in donors and kidney and liver transplant recipients. Factors significantly differing between the DGF and EGF groups in the univariate analyses, and also the other clinically relevant factors in this respect, were included in the multivariate analyses. These factors in the multivariate analyses consisted of categorical variables and the covariates of continuous variables. A receiver operating characteristic (ROC) analysis was performed to assess the potential of NGAL to predict DGF, prolonged DGF and AKI after liver transplantation. A p-value <0.05 was considered significant.

5. RESULTS

5.1 Kidney donors and recipients, and DGF

Studies I, II and III included 99 deceased donors and 176 adult, kidney transplant recipients. DGF was seen in 70 (39.8%) transplantations. The grafts with DGF started to function a mean 12 days after transplantation (range 3 to 38, SD 7). The EGF grafts started to function a mean 1.3 days after transplantation. The donor, recipient and transplantation characteristics are shown in tables 2, 3, and 4. The data is shown for all transplantations and stratified for EGF and DGF groups using the Halloran et al. (114) criteria.

Table 2. Clinical characteristics in all donors and differences in donor characteristics between early and delayed graft function groups. P-value indicates the difference between the EGF and DGF groups.

N	**All donors** 99	**EGF** 106	**DGF** 70	**p-value**
Mean age, years	51.8 (9–75)	49.1 (9–75)	55.8 (9–75)	0.002
Gender Male	 56 (56.6%)	 62 (58.5%)	 42 (60.0%)	 NS
Cause of death Cerebrovascular accident Traumatic brain injury	 74 (74.7%) 25 (25.3%)	 75 (70.8%) 33 (31.2%)	 57 (81.4%) 13 (18.6%)	 NS
Mean plasma creatinine, µmol/L	62 (28–143)	63 (21.0)	64 (17.4)	NS
Mean eGFR, mL/min	122 (60–263)	124 (39.2)	115 (35.0)	NS
History of hypertension	27 (27.3%)	26 (24.5%)	24 (34.3%)	NS
Expanded criteria donors	38 (38.4)	33 (31.1%)	36 (51.4%)	0.007
Need for cardiopulmonary resuscitation	21 (21.2%)	25 (23.6%)	10 (14.3%)	NS
Ante mortem intracranial surgery	30 (30.3%)	19 (17.9%)	22 (31.4%)	NS
Use of inotropes	87 (87.9%)	63 (59.4%)	62 (88.6%)	NS
Use of ADH	60 (60.6%)	50 (47.2%)	38 (54.3%)	NS
Multiorgan donor	56 (56.6%)	39 (36.8%)	38 (54.3%)	NS
Days in hospital before brain death	1.9 (1–14)	2.0 (2.5)	1.6 (1.3)	NS
Mean cold ischemia time, hours (SD)	21.8 (3.5)	21.3 (3.7)	22.9 (3.6)	0.007

eGFR=estimated glomerular filtration rate, ADH= anti diuretic hormone, EGF=early graft function, DGF=delayed graft function.

Table 3. Clinical characteristics in all kidney recipients and differences between patients with early and delayed graft function. P-value indicates the difference between the EGF and DGF groups.

	All Recipients	EGF (n=106)	DGF (n=70)	p-value
N	176	106 (60.2%)	70 (39.8%)	
Mean age, years	52.0 (19–76)	50.5 (20–70)	54.1 (19–76)	NS
Gender				
Female	66 (37.5%)	45 (42.5%)	21 (29.6%)	NS
Male	110 (62.5%)	61 (57.5%)	49 (69.4%)	
Kidney transplant number				
1st transplantation	161 (91.5%)	99 (93.4%)	62 (88.6%)	NS
2nd transplantation	13 (7.4%)	6 (5.7%)	7 (10.0%)	
3rd transplantation	2 (1.1%)	1 (0.9%)	1 (1.4%)	
Underlying kidney disease				
Polycystic disease	42 (23.8%)	26 (24.5%)	16 (22.9%)	NS
Glomerulonephritis	35 (19.9%)	21 (19.8%)	14 (20.0%)	
Diabetes mellitus	48 (27.3%)	29 (27.4%)	19 (27.1%)	
Other	51 (30.0%)	30 (28.3%)	21 (30.0%)	
Mode of dialysis				
Hemodialysis	114 (64.8%)	62 (58.5%)	52 (74.3%)	0.032
Peritoneal dialysis	62 (35.2%)	44 (41.5%)	18 (25.7%)	
Mean time on dialysis, days	850 (73–4263)	770 (73–4263)	975 (187–3361)	0.007

EGF=early graft function, DGF=delayed graft function

Table 4. Transplantation characteristics in all kidney transplantations and differences between patients with early and delayed graft function. P-value indicates the difference between the EGF and DGF groups.

	All Recipients	EGF (n=106)	DGF (n=70)	p-value
Initial calcineurin inhibitor				
Tacrolimus	41 (23.3%)	24 (22.6%)	17 (24.3%)	NS
Cyclosporine A	135 (76.7%)	82 (77.4%)	53 (75.7%)	
Mean plasma creatinine, µmol/L				
Day 1	531 (112–1383)	445 (112–1353)	664 (274–1383)	<0.001
Day 3	407 (60–1253)	250 (60–805)	644 (298–1253)	<0.001
Day 7	270 (53–925)	141 (53–333)	458 (119–925)	<0.001
3 weeks	153 (52–530)	120 (52–280)	206 (67–530)	<0.001
3 months	124 (56–525)	110 (56–200)	148 (59–515)	<0.001
1 year	116 (43–312)	109 (43–312)	128 (73–276)	0.002
Mean eGFR, mL/min				
3 weeks	57.4 (15–148)	64.2 (25–148)	46.4 (15–99)	<0.001
3 months	65.5 (17–175)	69.7 (27–175)	58.9 (17–113)	0.003
1 year	72.1 (21–153)	74.8 (21–153)	67.7 (29–132)	0.05
Mean urine output, mL				
Day 1	1756 (0–9280)	2544 (100–9280)	574 (0–2350)	<0.001
Day 3	1729 (0–5970)	2406 (150–5970)	713 (0–2880)	<0.001
Day 7	1960 (0–4630)	2412 (1500–4630)	1274 (0–3530)	<0.001
Day 14	2339 (0–4720)	2661 (1070–4720)	1888 (0–4710)	<0.001
Acute rejection	10 (5.7%)	4 (3.8%)	6 (8.6%)	NS
Mean time to rejection, days	16.8 (7–49)	8.7 (7–11)	20.8 (14–49)	NS
1-year graft survival	95.5%	99.1%	90.0%	0.005
1-year patient survival	99.4%	100%	98.6%	NS

EGF=early graft function, DGF=delayed graft function, eGFR=estimated glomerular filtration rate

In the DGF group, the donors were older and there were more ECDs compared to the EGF group. In the DGF group, the CIT was longer compared to the EGF group. Of the studied recipient characteristics there were more patients on maintenance hemodialysis (instead of peritoneal dialysis) and time spent on dialysis before transplantation was longer in the DGF group compared to the EGF group. Kidney function was better in the EGF group compared to the DGF group at all measured time points, including at one year after transplantation. Graft survival at one year was significantly better in the EGF group compared to the DGF group.

5.2 Prolonged DGF

Of the 70 DGF recipients, 26 had prolonged DGF lasting >14 days. The differences between the short and prolonged DGF groups are shown in Table 5. There were no significant differences in pretransplant or transplantation characteristics between these groups. Graft survival and function were significantly better in the short DGF group compared to the prolonged DGF group at one year after transplantation.

Table 5. Differences between the short and prolonged DGF groups

	DGF<14 days	DGF>14 days	p-value
N	44	26	
Mean recipient age, years (SD)	55.3 (11.9)	52.0 (15.4)	NS
First transplantation	38 (86.4%)	24 (92.3%)	NS
Mode of dialysis, hemodialysis	36 (81.8%)	19 (73.1%)	NS
Mean time on dialysis, days (SD)	993 (683.2)	929 (430.9)	NS
Donor age, years (SD)	55.6 (12.1)	56.0 (SD 10.7)	NS
Mean CIT, hours (SD)	22.7 (3.8)	23.3 (3.4)	NS
ECD donor status	20 (45.5%)	16 (61.5%)	NS
Day 1 creatinine, µmol/L (SD)	648 (205.4)	676 (224.6)	NS
Day 1 urine output, mL (SD)	618 (648.8)	392 (446.6)	NS
1-year creatinine, µmol/L (SD)	115 (33.4)	156 (47.3)	0.002
1-year eGFR, mL (SD)	72 (22.8)	56 (20.1)	0.009
1-year graft survival	100%	73.1%	<0.0001
1-year patient survival	100%	96.2%	NS

DGF=delayed graft function, CIT=cold ischemia time, ECD=expanded criteria donors, eGFR=estimated glomerular filtration rate

5.3 Donor kidney biopsies

All donors were biopsied before *in situ* perfusion of the kidneys. Histological evaluation was available from 97/99 biopsies. Normal histology was seen in 58/97 (59%) biopsies. The mean CADI score was 0.72 (range 0 to 5). Donor biopsies were mostly normal. Arterial changes in the form of intimal thickening, hyalinosis and global sclerosis were the most commonly observed findings in the biopsies. The histological changes found in the donor biopsies are shown in Table 6.

Table 6. Donor biopsy findings

	Number of biopsies (%)
Tubulitis	0 (0%)
Intimal arteritis	0 (0%)
Interstitial inflammation	1 (1.0%)
Glomerulitis	0 (0%)
Interstitial fibrosis	5 (5.2%)
Tubular atrophy	6 (6.2%)
Presence of sclerotic glomeruli	30 (30.9%)
Mesangial matrix increase	1 (1.0%)
Global sclerosis	21 (21.6%)
Intimal thickening	25 (25.8%)
Arterial hyalinosis	23 (23.7%)
CADI score 0–1 ≥2	 79 18

CADI=chronic allograft damage index

5.4 Donor NGAL

5.4.1 Donor uNGAL (I)

The mean donor uNGAL was 18 ng/ml (SD 26). Donor uNGAL correlated directly with plasma creatinine (r=0.37, p<0.0001) and inversely with eGFR (r=0.24, p=0.01). ADH treated donors had significantly lower mean uNGAL levels (13ng/ml, SD 14) compared to non-ADH treated donors 26ng/ml, SD 37, p=0.045). Donor uNGAL did not correlate with age (r=0.12) and was not affected by the use of vasopressors, history of hypertension, length of hospital stay, need for cardiopulmonary resuscitation or intracranial surgery, gender, ECD or standard criteria donor status, or multiorgan or kidney-only donation.

There were no significant differences in mean donor uNGAL levels between the DGF (23ng/ml, SD33) and EGF (16ng/ml, SD20, p=0.058) groups. In cases with prolonged DGF, the mean donor uNGAL was significantly higher (35ng/ml, SD49) compared to cases with short DGF (15ng/ml, SD14), or EGF (16ng/ml, SD20, p=0.002).

The donor uNGAL values were divided into High and Low according to a mean uNGAL concentration 18ng/ml. The characteristics in these groups are shown in Table 7. The High uNGAL group comprised 26 donors who donated 52 kidneys and the Low uNGAL group of 69 donors donating 116 kidneys. Graft survival was significantly better in the Low uNGAL group (97.4%) compared to the High uNGAL group (90.3%, p=0.048) at one year.

Table 7. Differences between the transplantations stratified according to low and high uNGAL concentration, using the mean concentration as a cut-off value

	Low uNGAL (<18 ng/mL) **Donors n=69** **Kidneys n=116**	**High uNGAL (≥18 ng/mL)** **Donors n=26** **Kidneys n=52**	**p-value**
Donor creatinine µmol/L (SD)	59 (17.8)	71 (21.8)	0.006
Donor eGFR ml/min (SD)	122 (35.7)	105 (31.2)	0.039
CADI score **0–1** **>2**	 61 8	 17 9	0.010
Prolonged DGF (n=25) **Short DGF (n=41)** **EGF (n=102)**	13 (11.2%) 26 (22.4%) 77 (66.4%)	12 (23.1%) 15 (28.8%) 25 (48.1%)	0.028
1-year creatinine µmol/L (SD)	117 (45.0)	114 (28.5)	NS
1-year eGFR mL/min (SD)	59 (21.4)	57 (15.9)	NS
1-year patient survival	100%	95.8%	NS
1-year graft survival	97.4%	90.3%	0.048

uNGAL=urine neutrophil gelatinase-associated lipocalin, eGFR=estimated glomerular filtration rate, CADI=chronic allograft damage index, DGF=delayed graft function, EGF=early graft function

5.4.2 Donor sNGAL (I)

The mean donor sNGAL was 212ng/ml (SD 145). Donor sNGAL directly correlated with donor uNGAL (r=0.40, p<0.0001). It also correlated directly with plasma creatinine (r=0.35, p=0.001) and inversely with eGFR (r=0.24, p=0.021). Donors treated with ADH had significantly lower mean sNGAL levels (188ng/ml, SD 125) compared to non-ADH treated donors (249ng/ml, SD 161, p=0.002). Donor sNGAL was not affected by the use of vasopressors, history of hypertension, length of hospital stay, need for cardiopulmonary resuscitation or intracranial surgery before brain death, gender, ECD or standard criteria donor status, and multiorgan or kidney-only donation. Donor sNGAL did not correlate with donor age (r=0.15).

There were no significant differences in mean donor sNGAL levels between the DGF (229ng/ml, SD 136) and EGF (206ng/ml, SD 150, p=NS) groups. Mean donor sNGAL concentration did not significantly differ between groups of prolonged DGF (220ng/ml, SD 142), short DGF (234ng/ml, SD 135), or EGF (206ng/ml, SD 150, p=NS).

We divided the donor sNGAL values into High and Low according to the mean sNGAL 214ng/ml. The characteristics of these groups are shown in Table 8. The High sNGAL group had 38 donors who donated 69 kidneys and the Low uNGAL group had 57 donors donating 99 kidneys. The 1-year graft survival rate was significantly better in the Low sNGAL group (98.0%) compared to the High sNGAL group (91.4%, p=0.050).

Table 8. Differences between the transplantations stratified according to low and high sNGAL concentration, using the mean concentration as a cut-off value

	Low sNGAL (<214 ng/mL) **Donors n=57** **Kidneys n=99**	**High sNGAL (≥214 ng/mL)** **Donors n=38** **Kidneys n=69**	**p-value**
Donor creatinine µmol/L (SD)	57 (15.1)	70 (22.8)	0.021
Donor eGFR ml/min (SD)	124 (34.5)	108 (33.9)	0.033
CADI score **0–1** **>2**	 46 11	 30 8	NS
Prolonged DGF (n=25) **Short DGF (n=41)** **EGF (n=102)**	13 (13.1%) 19 (19.1%) 67 (67.8%)	12 (17.4%) 22 (31.9%) 35 (50.7%)	NS
1-year creatinine µmol/L (SD)	115 (37.7)	117 (43.8)	NS
1-year eGFR mL/min (SD)	60 (21.1)	57 (16.9)	NS
1-year patient survival	99.0%	98.6%	NS
1-year graft survival	98.0%	91.4%	0.050

sNGAL=serum neutrophil gelatinase-associated lipocalin, eGFR=estimated glomerular filtration rate, CADI=chronic allograft damage index, DGF=delayed graft function, EGF=early graft function.

5.5 Kidney recipient NGAL

5.5.1 Kidney recipient uNGAL (II)

The mean uNGAL was high (1209ng/ml, SD 1120) in all patients before transplantation. It was not affected by recipient age, gender, underlying kidney disease, mode or length of dialysis, number of previous transplantations, pretransplant diuresis or plasma creatinine (data not shown).

Recipients with diuresis of more than 1L on day 1 had significantly lower uNGAL levels (543ng/ml, SD 642) compared to those with diuresis of less than 1L (887ng/ml, SD 613, p=0.008). The day-1 uNGAL was not affected by a decrease/increase in plasma creatinine from day 0 to day 1.

The mean pretransplant uNGAL concentration was higher in the DGF group but this difference was not statistically significant. The mean uNGAL levels decreased in both groups after transplantation, but the decrease was faster and the mean levels were significantly lower in the EGF group compared to the DGF group at all measured time points (Figure 2). In the prolonged DGF group, uNGAL levels remained high at all measured time points (Figure 2).

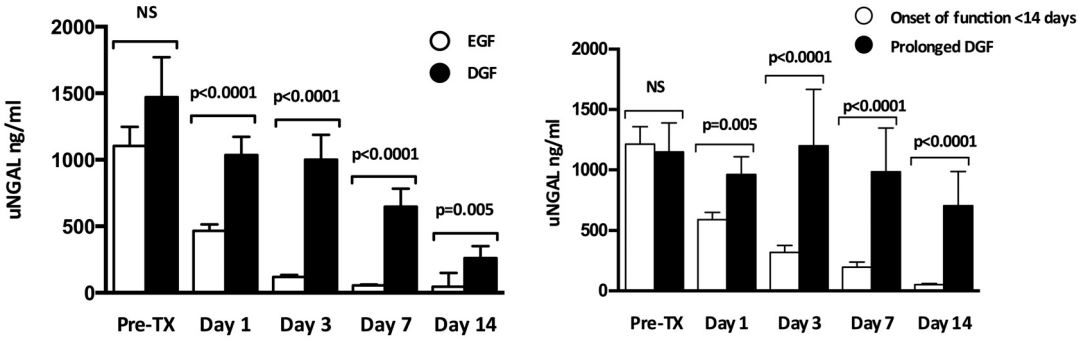

Figure 2. The mean uNGAL concentrations divided according to EGF and DGF and early vs. prolonged onset of function at all measured time points.

5.5.2 Serum NGAL analysis (II)

Both Elisa and POC methods were used to analyze pretransplant serum samples from 132 kidney recipients and day-1 samples from 128 recipients. Using the Elisa method, the mean sNGAL was 506ng/ml (SD 189). Using the POC method, the mean sNGAL concentration was 536ng/ml (SD 238). Figure 3 shows the corresponding Elisa and POC measured sNGAL values and the corresponding mean sNGAL levels with SEM. The correlation between Elisa and POC measured sNGAL levels was 0.89, p<0.0001. As the POC method is more practical and the correlation with Elisa was good, sNGAL values measured using the POC method are used for the clinical analyses in this study.

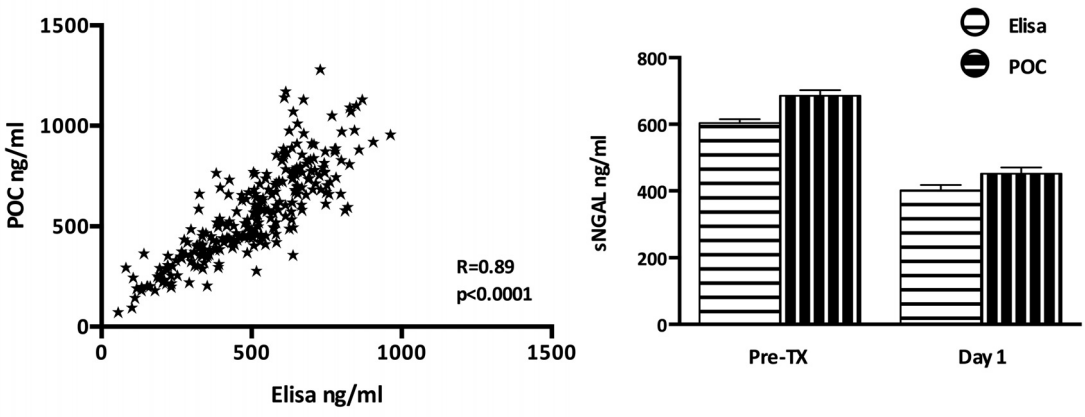

Figure 3. The correlation of sNGAL values measured using the Elisa and the POC method

5.5.3 Kidney recipient sNGAL (III)

The mean pretransplant sNGAL was 678ng/ml. It was significantly lower (356ng/ml, SD 168.1) in recipients with residual diuresis >1000ml from their native kidneys compared to those with residual diuresis <1000ml (593ng/ml, SD 186, p<0.0001). Pretransplant sNGAL was not affected by recipient age, gender, underlying kidney disease, mode or length of dialysis, number of previous transplantations, or plasma creatinine (data not shown).

The day after transplantation, recipients with decreasing plasma creatinine had significantly lower sNGAL level (382ng/ml, SD 196) compared to those with no change/increase in plasma creatinine on day 1 (547ng/ml, SD 187, p<0.0001). Recipients with diuresis of more than 1L on day 1 had significantly lower sNGAL levels (356ng/ml, SD 168) compared to those with diuresis of less than 1L (593ng/ml, SD 186, p<0.0001). There were no statistically significant differences in mean pretransplant sNGAL concentrations between the EGF and DGF groups. After transplantation, the mean sNGAL levels decreased in both groups but more rapidly in the EGF group compared to the DGF group (Figure 4). In the prolonged DGF group sNGAL levels remained high at all measured time points (Figure 4).

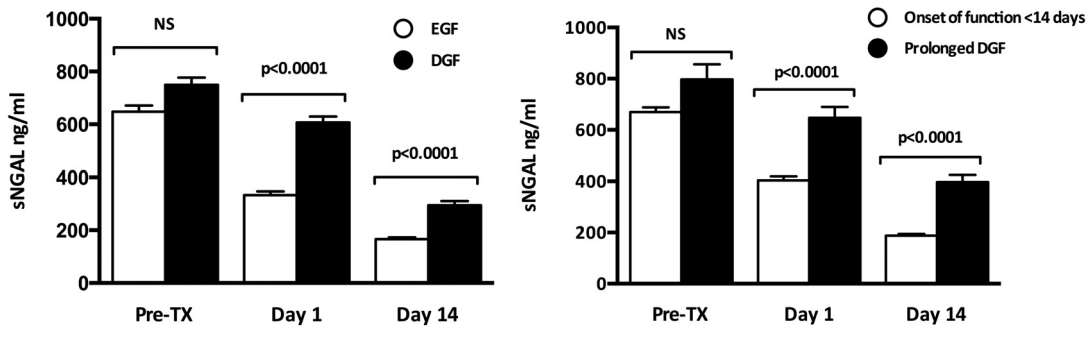

Figure 4. The mean sNGAL concentrations divided according to EGF and DGF and early vs. prolonged onset of function at all measured time points.

5.6 NGAL in the prediction of graft function onset (II, III)

5.6.1 DGF

ROC analyses were performed to assess the potential of donor and recipient uNGAL and sNGAL to predict DGF (Figure 5). Recipient uNGAL predicted DGF with an AUC of 0.750 (CI 0.663–0.837, p<0.0001). At the optimal cut off level of 560ng/ml, the sensitivity was 68% and the specificity 73%. Recipient day-1 sNGAL predicted DGF with an AUC of 0.908 (CI 0.860–0.955, p<0.0001). At the optimal cut-off level of 426ng/ml, the sensitivity was 91% and the specificity 83%. The different cut-off levels with sensitivities and specificities are shown in Table (9). Donor uNGAL and sNGAL failed to predict DGF.

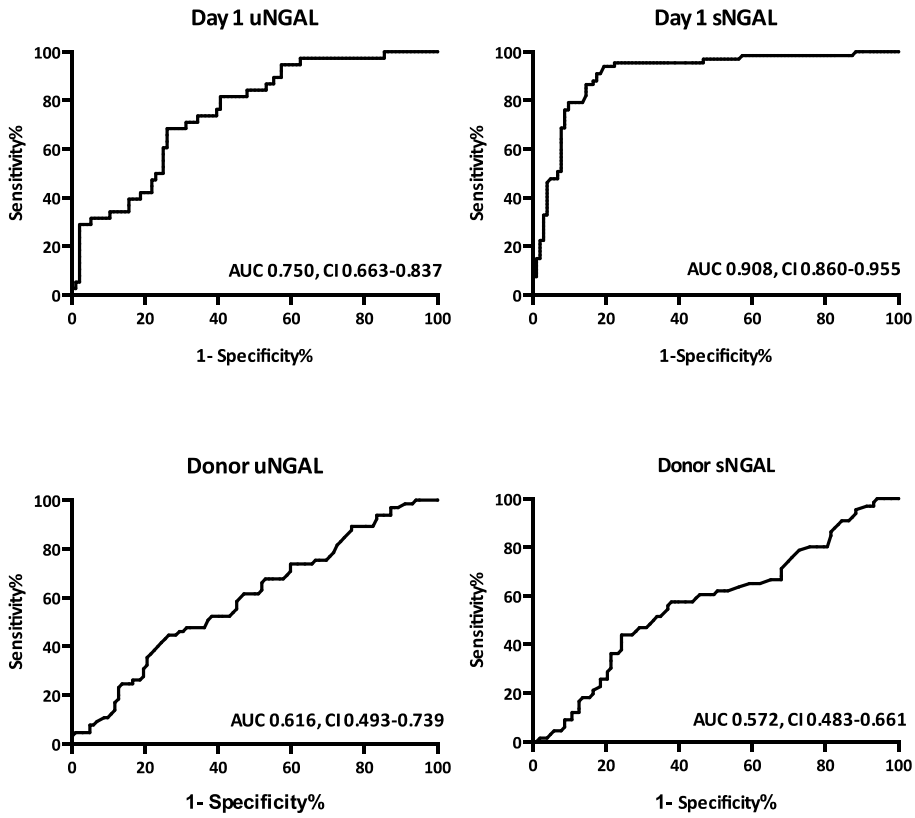

Figure 5. The day-1 uNGAL and sNGAL and donor uNGAL and sNGAL ROC curves in predicting DGF

Table 9. The sensitivities and specificities of different cut-off levels of recipient day-1 uNGAL and sNGAL in predicting DGF

uNGAL ng/mL	Sensitivity	Specificity	sNGAL ng/mL	Sensitivity	Specificity
17	1.00	0.04	71	1.00	0.00
210	0.97	0.35	201	0.99	0.14
408	0.82	0.59	323	0.97	0.51
560	0.68	0.73	426	0.91	0.83
866	0.34	0.84	568	0.51	0.92
1393	0.29	0.98	794	0.15	0.98
3185	0.06	1.00	1131	0.00	1.00

5.6.2 Prolonged DGF

ROC analyses were performed to assess the potential of donor and recipient uNGAL and sNGAL to predict prolonged DGF (Figure 6). Recipient uNGAL predicted prolonged DGF with an AUC of 0.748 (CI 0.654–0.842, p<0.0001). At the cut-off level of 560ng/ml, the sensitivity was 70% and the specificity 70%. Recipient sNGAL predicted DGF with an AUC of 0.825 (CI 0.751–0.899, p<0.0001). At the optimal cut-off level of 486ng/ml, the sensitivity was 80% and the specificity 75%. The different cut-off levels with sensitivities and specificities are shown in Table 10. Donor uNGAL and sNGAL failed to predict prolonged DGF.

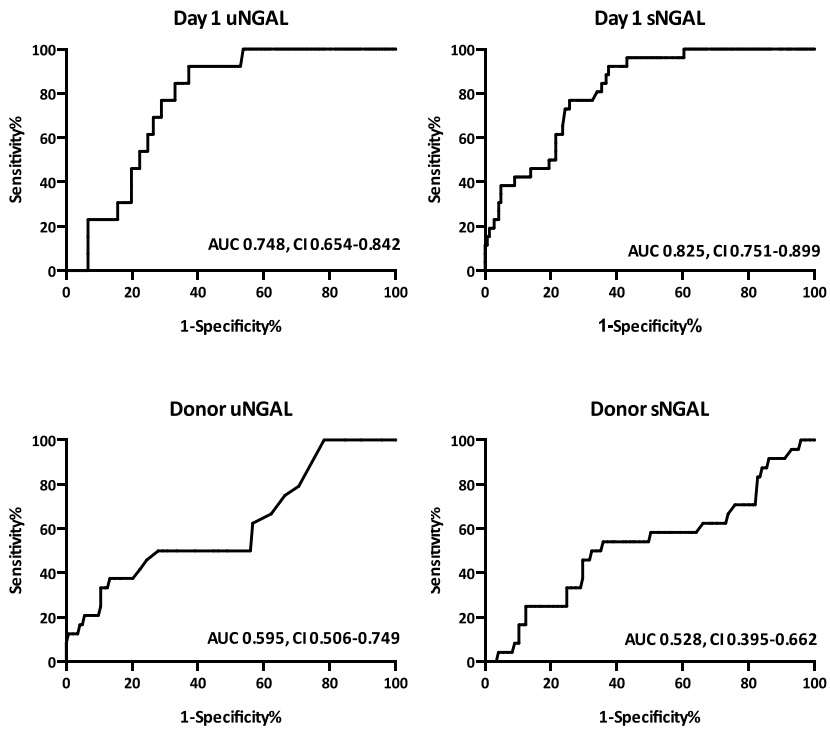

Figure 6. The day-1 uNGAL and sNGAL and donor uNGAL and sNGAL ROC curves in predicting prolonged DGF

Table 10. The sensitivities and specificities of different cut-off levels of recipient day-1 uNGAL and sNGAL in predicting prolonged DGF

uNGAL ng/mL	Sensitivity	Specificity	sNGAL ng/mL	Sensitivity	Specificity
17	1.00	0.08	83	1.00	0.01
210	1.00	0.29	201	1.00	0.13
408	0.92	0.52	323	1.00	0.37
560	0.83	0.66	486	0.77	0.74
866	0.31	0.80	568	0.50	0.80
1393	0.23	0.93	794	0.23	0.96
3185	0.06	1.00	1075	0.04	1.00

5.6.3 NGAL in cases with clinically hiding DGF

We wanted to study whether NGAL could separate DGF cases from all cases expected to have EGF on the basis of fluent urine output and decreasing creatinine. In total, 86 patients had a 50μmol/L or greater decrease in plasma creatinine from pretransplant to day 1 and 19/86 had DGF. The mean uNGAL concentration (1318ng/mL SD 1246) and sNGAL concentration (604ng/mL, SD 206) were significantly higher in the DGF group compared to the EGF group (uNGAL 398ng/mL, SD 340, p<0.0001 and sNGAL 309ng/mL, SD 125 p<0.0001). Day-1 uNGAL predicted DGF in this subgroup with an AUC of 0.744 (CI 0.570–0.918, p=0.014). Day-1 sNGAL predicted DGF in this subgroup with an AUC of 0.915 (CI 0.840–0.990, p<0.0001).

Altogether, 112 patients had a urine output (UOP) of >1L on day 1 after transplantation and 15 of these had DGF. Their mean uNGAL concentration (1217ng/mL, SD 1229) and sNGAL concentration (572ng/mL, SD 207) were significantly higher than those in the EGF group (uNGAL 460ng/mL, SD 481, p<0.0001 and sNGAL 325ng/mL, SD 136, p=0.016). Day-1 uNGAL predicted DGF in this subgroup with an AUC of 0.696 (CI 0.526–0.866, p=0.034). Day-1 sNGAL predicted DGF in this subgroup with an AUC of 0.874 (CI 0.757–0.991, p<0.0001).

5.7 DGF risk factors (I–III)

In a multivariate analysis of DGF risk factors, mode of dialysis and recipient day-1 sNGAL, uNGAL and UOP appeared as independent risk factors for DGF (Table 11).

Table 11. Multivariate analysis of different parameters in predicting DGF in studies I, II, and III

	Donor study Study I p-value	uNGAL study Study II p-value	sNGAL study Study III p-value
Donor age (years)	0.320	0.315	0.707
Donor creatinine (µmol/L)	0.807	0.891	0.622
Donor eGFR (mL/min)	0.910	0.600	0.751
Donor uNGAL (ng/mL)	0.315		
Donor sNGAL (ng/mL)	0.881		
Expanded criteria donors	0.650	0.484	0.929
Cold ischemia time (hours)	0.581	0.980	0.063
Recipient age (years)	0.261	0.958	0.994
Mode of dialysis	0.012	0.004	0.042
Time on dialysis pre-TX (days)	0.431	0.457	0.721
Δcreatinine (pre-TX to day 1)		0.891	0.586
Recipient day-1 UOP (mL)		<0.0001	<0.0001
Recipient day-1 uNGAL (ng/mL)		0.019	
Recipient day-1 sNGAL (ng/mL)			<0.0001

uNGAL=urine neutrophil gelatinase-associated lipocalin, sNGAL=serum neutrophil gelatinase-associated lipocalin, eGFR=estimated glomerular filtration rate, tx=transplantation

5.7.1 Risk factors for prolonged DGF

In a multivariate analysis of prolonged DGF risk factors, donor uNGAL and recipient day-1 sNGAL, uNGAL and UOP remained as independent risk factors for prolonged DGF in all three studies. In addition, donor eGFR was an independent risk factor in Study I and mode of dialysis in Study II (Table 12).

Table 12. Multivariate analysis of different parameters in predicting prolonged DGF in studies I, II, and III

	Donor study Study I p-value	uNGAL study Study II p-value	sNGAL study Study III p-value
Donor age (years)	0.523	0.392	0.405
Donor creatinine (µmol/L)	0.152	0.997	0.867
Donor eGFR (mL/min)	0.016	0.635	0.113
Donor uNGAL (ng/mL)	0.001		
Donor sNGAL (ng/mL)	0.096		
Expanded criteria donors	0.038	0.563	0.119
Cold ischemia time (hours)	0.066	0.754	0.416
Recipient age (years)		0.985	0.653
Mode of dialysis	0.321	<0.0001	0.861
Time on dialysis pre-TX (days)	0.460	0.457	0.867
Δcreatinine (pre-TX to day 1)		0.871	0.930
Recipient day-1 UOP (mL)		<0.0001	0.001
Recipient day-1 uNGAL (ng/mL)		0.02	
Recipient day-1 sNGAL (ng/mL)			0.042

uNGAL=urine neutrophil gelatinase-associated lipocalin, sNGAL=serum neutrophil gelatinase-associated lipocalin, eGFR=estimated glomerular filtration rate, tx=transplantation

5.8 Differences in DGF groups depending on the used DGF definition

Using the Halloran et al. (268) definition, DGF was seen in 70/176 recipients. Using the conventional definition, DGF was seen in 66/176 recipients. There were 10 recipients categorized differently depending on the used definition for DGF (Table 13). There were three patients with prompt function who required one dialysis due to high potassium levels or fluid overload (classified as EGF using the Halloran criteria and DGF using the conventional criteria). Their sNGAL levels were <400ng/ml. There were seven patients who had poorly declining plasma creatinine without a need for dialysis (classified as DGF using the Halloran criteria and EGF using the conventional criteria) who all had sNGAL levels >400ng/ml. There were no trends or significant differences between these groups in day-1 uNGAL or day-1 UOP levels or changes in uNGAL, sNGAL or creatinine levels from pretransplant to day 1.

Table 13. The 10 patients categorized differently to DGF and EGF groups depending on the used DGF definition.

Patient	DGF Halloran	Conv.	UOP 1d	Δcrea 0d–1d	uNGAL 1d	ΔuNGAL 0d–1d	sNGAL 1d	ΔsNGAL 0d–1d
1	EGF	DGF	1750	-98	455	-2904	374	-160
2	EGF	DGF	1710	-264	106	-	198	-521
3	EGF	DGF	1670	-60	96	-	221	-202
4	DGF	EGF	430	-272	2433	-	604	-266
5	DGF	EGF	2090	-74	3600	-2359	722	-75
6	DGF	EGF	2060	-319	416	-	759	-203
7	DGF	EGF	1330	-67	-	-	971	+15
8	DGF	EGF	100	-83	711	-	657	-135
9	DGF	EGF	2350	-11	217	-134	451	-
10	DGF	EGF	1030	+88	642	-	706	-

DGF=delayed graft function, UOP=urine output, uNGAL=urine NGAL, sNGAL=serum NGAL, conv=conventional

5.9 Timing of day-1 sampling

The timing of sampling was not standardized. The day-1 serum samples were taken in the transplant unit the morning following transplantation on the first laboratory round. The mean time from reperfusion to sampling was 11.8 hours (SD 5) ranging from 2 to 24 hours after reperfusion. We divided the patients into the following groups depending on the timing of sampling: <6hours (n=18), 7–12 hours (n=82), >12 hours (n=70) and analyzed whether timing had any influence on the predictive power (Table 14). Serum NGAL best predicted DGF in the group with the shortest time from reperfusion to sampling. However, the predictive power was good and roughly at the same level at all time points.

Table 14. Timing of day 1 sNGAL samples

	Mean sNGAL ng/ml EGF (SD)	Mean sNGAL ng/ml DGF (SD)	p-value DGF vs EGF	ROC-analysis AUC, p-value, CI Optimal cut-off, sensitivity, specificity
<6 hours	n=9 278 (78.8)	n=9 687 (239.4)	0.002	1.00, p=0.001, CI 1.00–1.00 436ng/ml, 100%, 100%
7 to 12 hours	n=46 365 (159.5)	n=36 594 (169.2)	<0.0001	0.864, p<0.0001, CI 0.783–0.946 420ng/ml, 94%, 73%
>12 hours	n=47 321 (125.0)	n=23 526 (187.9)	<0.0001	0.920, p<0.0001, CI 0.838–0.999 420ng/ml, 91%, 83%

The day-1 urine samples were taken by the nursing staff in the morning following transplantation. The exact time of sampling was not recorded, and hence the above analysis is not possible.

5.10 Liver transplant recipients (IV)

The characteristics of 203 liver transplant recipients are shown in Table 15. The majority of the patients received liver transplantation due to chronic liver disease: primary sclerosing cholangitis (n=38), alcoholic cirrhosis (n=32), primary biliary cirrhosis (n=16), hepatocellular carcinoma (n=18), autoimmune cirrhosis (n=10), viral hepatitis (n=7), or other (n=44). The remaining recipients had acute liver failure (n=38). Two patients needed a re-transplantation and six patients died during the follow up time.

Table 15. The characteristics of liver transplant patients

Number of patients	203
Age, years (SD)	49.3 (12)
Gender, female (n, %)	100 (49%)
Liver failure (n, %) Acute Chronic	 38 (19%) 165 (81%)
Pre-TX laboratory results Plasma creatinine at listing, µmol/L (SD) eGFR at listing, mL/min (SD)	 90 (61) 86 (34)
Pre-TX RRT (n, %)	36 (18)
TX day laboratory results Plasma creatinine, µmol/L (SD) eGFR, mL/min (SD) Cystatin C, mg/L (SD) Bilirubin, µmol/L (SD) INR (SD) Albumin, g/L (SD)	 93 (64) 83 (36) 1.4 (0.8) 113 (145) 2.0 (1.6) 32 (7)
MELD score at TX day (SD)	16 (10)
Anhepatic time, min (SD)	59 (18)
Perioperative bleeding, L (SD)	4.2 (3.6)
Lactate at day 1, mmol/L (SD)	1.5 (1.4)
IL-2R antibody induction (n, %)	30 (15)
Excessive exposure to CNI (n, %)	107 (53)
Highest value during first TX week Bilirubin, µmol/L (range) Alanine aminotransferase U/L (range) INR (SD)	 73 (13-490) 316 (51-8850) 1.7 (0.5)
Patient survival at 90 days (n, %)	197 (97)

TX=transplantation, eGFR=estimated glomerular filtration rate, RRT=renal replacement therapy, MELD= model for end-stage liver disease, IL-2R=interleukin 2 receptor, CNI=calcineurin inhibitor

5.11 Kidney function after liver transplantation (IV)

The kidney function of the liver transplant recipients is shown in Table 16. After transplantation, 17% of patients needed RRT. At three months after transplantation, 42% had eGFR<60mL/min and 8/203 (4%) eGFR <30mL/min. Two patients remained on RRT at three months after transplantation.

AKI occurred in 66/167 (40%) patients without the need for pretransplant RRT. AKI during the first week after transplantation associated with a 20% increase of eGFR <60mL/min; in other words, 57% of patients with AKI during the first week after transplantation had eGFR <60mL/min at three months compared to 37% of those without AKI (p=0.018).

Altogether, 81/203 patients had eGFR<60mL/min before liver transplantation and 36/203 (18%) patients received pretransplant RRT for a mean of four days (range 1 to 18 days). Posttransplant RRT was needed in 22/81 patients with a pretransplant eGFR of <60mL/min. At three months after transplantation, 39/81 (48%) had an eGFR <60mL/min. Of those needing pretransplant RRT, 11/36 (31%) also needed posttransplant RRT and 11/36 (31%) had eGFR of <60mL/min at three months after transplantation.

Table 16. Kidney function after liver transplantation

Highest creatinine during first week after transplantation, µmol/L	173 (106)
Lowest GFR during first week after transplantation, mL/min	45 (27)
Pre-TX NGAL, ng/mL	154 (141)
Acute kidney injury (n, %)	66 (40)
Need for renal replacement therapy within 90 days after transplantation (n, %)	35 (17)
eGFR at 90 days after transplantation >60mL/min (n, %) <60mL/min (n, %)	 113 (58) 82 (42)

5.11.1 Cystatin C

The mean cystatin C concentration at transplantation was 1.6mg/L (range 0.6 to 8.1). The mean cystatin C concentration was significantly higher (2.5mg/L, SD 1.3) in patients with eGFR <60mL/min at the time of transplantation compared to patients with eGFR >60mL/min (1.1mg/L, SD 0.4, p<0.0001). There was no significant difference in the mean cystatin C concentration in patients needing pretransplant RRT (3.0mg/L, SD 1.9) compared to those without the need for RRT (2.2mg/L, SD 0.7, p=0.06). On transplant day, in patients without the need for pretransplant RRT, cystatin C based eGFR correlated fairly well with creatinine based eGFR (R=0.71, p<0.0001). At three months after transplantation in all patients the correlation was similar (R=0.61, p<0.0001).

5.11.2 Pretransplant plasma NGAL

The mean pNGAL in all patients was 168ng/ml (range 60 to 1050). The correlation of pNGAL with creatinine (R=0.50, p<0.0001), cystatin C (0.54, p<0.0001), creatinine based eGFR (0.44, p<0.0001), and cystatin C based eGFR (R=0.38, p<0.0001) was modest.

The mean pNGAL was significantly higher (289ng/ml, SD 237) in patients with eGFR <60 mL/min at the time of transplantation compared to patients with eGFR >60mL/min (108ng/ml, SD 57, p<0.0001). In patients with pretransplant GFR <60mL/min pNGAL was significantly higher among those needing RRT (434ng/ml, SD 301), compared to those without the need for RRT (214ng/mL, SD 152, p=0.002).

There were no significant differences in the mean pNGAL concentrations between patients with (136ng/mL, SD 140) or without (112ng/mL, SD 64, p=0.26) posttransplant AKI or with (212ng/mL, SD 226) or without (159ng/mL, SD 151, p=0.33) the need for posttransplant RRT. The mean pNGAL was significantly higher among patients with eGFR <60mL/min at three months after transplantation (202ng/mL, SD 198) compared to those with eGFR >60mL/min (141ng/mL, SD 125, p=0.001).

We assessed the risk factors predicting eGFR<60mL/min at three months after transplantation in a multivariate analysis. Recipient age, IL-2R induction therapy, AKI during the first week after transplantation, highest alanine aminotransferase concentration during the first week after transplantation, and pNGAL >260ng/mL emerged as independent risk factors. The cut-off for pNGAL was set at 260ng/mL, as this is the threshold of normal and high pNGAL concentrations, as reported by the manufacturer.

6. DISCUSSION

NGAL has been found to be the earliest and highest upregulated gene in acute kidney injury caused by various insults, such as IRI or toxic nephropathy, preceding the classical markers of kidney injury such as creatinine (14, 220, 269, 270). AKI in the form of DGF is a considerable and growing problem after kidney transplantation as older recipients and donors with comorbidities are increasingly accepted for transplantation (74, 75, 271). DGF is diagnosed on a wait and see basis, and hence the diagnosis is often too late. NGAL has been proposed as a diagnostic tool for DGF. We hypothesized that measuring NGAL could help in assessing donor kidney quality and predicting DGF after kidney transplantation and kidney function after liver transplantation.

Study I is the first published study where urine and serum NGAL concentrations were systematically and prospectively measured from brain-dead donors. We found that sNGAL concentrations were high in all donors whereas uNGAL concentrations were mostly low. Brain death is a major, systemic condition affecting most vital functions. Hence, it is natural that the sNGAL levels in the donors were high and did not correlate with kidney function in the recipient. Donor sNGAL probably originates from various organs and does not describe kidney function in the donor; this is why donor sNGAL did not correlate with DGF.

Increased uNGAL can derive from increased systemic NGAL production that overrides the reabsorption capacity, impaired megalin dependent reabsorption in the proximal tubulus, or de novo synthesis of NGAL in the kidney (229). Many studies have shown that in the kidney NGAL is mainly synthetized in the loop of Henle and distal nephron in response to injury, despite the fact that the major site of acute kidney injury is the proximal tubulus (235, 236, 243, 272, 273). In CKD most of the uNGAL derives from impaired reabsorption. However, irrespective of the cause, in AKI the GFR decreases, limiting NGAL filtration and reabsorption, and most of the uNGAL originates from the distal nephron (220, 229, 243). This could explain why the donor uNGAL concentrations were mostly low: sNGAL originates from other organs than the kidneys, NGAL is filtered and reabsorbed by the donor's healthy kidneys and very little is excreted. When donor uNGAL is increased, it indicates damage in the donor kidney, and hence we can see a correlation with prolonged DGF. We found that high donor uNGAL associated with a higher CADI score, supporting the association. As IRI and recipient factors have a major impact on DGF and graft survival, a perfectly normal donor kidney can be damaged later in the transplantation process. However, high donor uNGAL concentration might reflect problems after transplantation and help with decision-making in complicated or borderline cases.

The only studied donor parameter affecting donor NGAL concentration was ADH treatment, which seemed to decrease NGAL levels. Vasopressin administration results in increased aquaporin-2 receptor concentration on distal tubule and collecting duct cells and water is progressively reabsorbed. It also increases peripheral resistance and hence increases blood pressure and improves hemodynamic stability (274), which could lead to increased renal perfusion pressure and better renal function and thus decrease uNGAL concentration. It is possible that better hemodynamic stability additionally decreases NGAL secretion in other organs, resulting in decreased sNGAL concentration. We could also speculate on the effect of vasopressin on de novo NGAL synthesis as NGAL originates from the distal tubule.

Since our study was published, there have been three other publications where the association of donor NGAL with DGF has been studied. Buemi et al. found that neither donor pNGAL nor uNGAL in 80 deceased and 17 living donor transplantations predicted kidney function after transplantation. They defined kidney function as reaching eGFR>40mL/min or serum creatinine<2mg/dL(=176μmol/L) (29). Donor sNGAL could not predict DGF, defined as the need for dialysis during the first week after transplantation, in a more recent study of 159 deceased donors (275). A panel of urinary biomarkers (Kim-1, NGAL, IFN-γ, TNF-α) was tested in another study of 182 deceased donors. The authors found that Kim-1 but not the other markers may be helpful in predicting AKI after transplantation (276). These findings are in line with our study. However, the other studies did not analyze the relationship of donor NGAL and prolonged DGF, which was the main finding in our study.

In studies II and III we investigated the role of recipient urine and serum NGAL in the prediction of DGF. The pretransplant levels were measured to get an idea of NGAL levels in ESRD patients on dialysis and to be used as a reference for posttransplant measurements. The pretransplant sNGAL concentration was significantly lower in patients with good residual diuresis. It is probable that the nonfunctioning kidneys excrete NGAL due to ongoing damage, which is then secreted into the circulation, resulting in high pretransplant serum and urine NGAL levels in all recipients. As the kidneys do not function, NGAL, originating from the kidney and other organs, circulating back to the kidney is not reabsorbed and the uNGAL concentration remains high. However, in those with fluent copious diuresis from their native kidneys, some of the systemic NGAL is filtered through the glomerulus but not reabsorbed and instead flushed away, resulting in lower sNGAL levels. This difference was not seen in uNGAL, which is consistent with the presumption that the majority of the uNGAL derives from the kidneys and is not affected by the amount of water diuresis.

After transplantation, both urine and serum NGAL levels decreased as the transplanted kidneys started to function as expected. The serum and urine NGAL concentrations were significantly higher in the DGF group as soon as the following morning after transplantation. It is most likely that the new kidney clears NGAL secreted by the native kidneys. After transplantation, the native kidneys shut down resulting in a decrease in water diuresis and probably NGAL secretion. It has previously been shown that NGAL expression is reversible and ceases when the inducing stimulus is removed (243).

In ROC analyses, sNGAL predicted DGF and prolonged DGF well, uNGAL moderately, and they emerged as independent DGF risk factors in a multivariate analysis. This finding further supports their usefulness in predicting DGF. The inferior ability of uNGAL in predicting DGF might result from the significant number of missing samples. Furthermore, the urine excreted very soon after transplantation is a mixture produced from the native and the transplanted kidneys, and it is also possible that the composition (e.g. high protein concentration) of the urine affects the uNGAL concentration or interferes with the detection method. We did not standardize uNGAL concentration to creatinine. Urine creatinine excretion is not constant and normalizing a biomarker concentration to creatinine results in under- or overestimation of the biomarker concentration, depending on the clinical situation, and complicates the determination of a threshold concentration. It has therefore been suggested that all urine biomarkers should be measured as such instead of proportioning them to creatinine (277).

In addition to our studies, so far there are nine published studies investigating the role of serum/plasma NGAL and ten to the role of uNGAL in prediction of DGF/AKI after transplantation. Serum/plasma NGAL was found useful in the prediction of DGF/AKI in eight publications (24, 26–28, 29, 34, 37, 38). The AUCs varied from 0.82 to 0.97 and the optimal cut-off value ranged from 233ng/ml to 500ng/ml, which is in line with our study. Some studies did not report AUCs. The results concerning uNGAL are not so clear. Seven publications showed that uNGAL is predictive of AKI with an AUC ranging from 0.75 to 0.9 and the optimal cut-off value from 255g/ml to 560ng/ml; in two studies uNGAL did not predict DGF (22, 25, 29–34, 36). Comparing data is impossible in some cases, as some authors did not report AUCs and in some cases uNGAL was normalized according to urine creatinine.

Additionally, sNGAL and uNGAL have been found to be of value in predicting acute rejection after transplantation (278, 279). Neither serum nor urine NGAL correlated with rejections in our studies but the number of patients with rejection was so small that no conclusions can be drawn. Additionally, there are a few other publications dealing with NGAL and kidney transplantation. Fonseca et al. and Choi et al. found that uNGAL predicts 1-year graft survival but Nauta et al. did not find any association with NGAL and graft loss after transplantation, which is in line with our results (27, 32).

Kaufeld et al. studied whether uNGAL normalized to creatinine correlated with findings in protocol biopsies at three weeks, three months, and six months after transplantation in 140 randomly chosen protocol biopsies, but could not find a link (35). In the first study ever published on NGAL and kidney transplantation, Mishra et al. stained NGAL in biopsies taken one hour after reperfusion and found that the intensity of NGAL staining correlated with peak postoperative creatinine and DGF (280). We found that donor uNGAL correlated with CADI score in donor biopsy. We did not study the correlation of NGAL and recipient biopsies.

Perioperative AKI and renal impairment at three months after transplantation are important risk factors for CKD and ESRD after liver transplantation (6, 281, 282). In our study, 40% of patients had AKI during the first week after liver transplantation, and 42% had moderate or worse kidney dysfunction at three months, thus resembling previously published rates using similar definitions (282–284). Patients with perioperative AKI have been shown to benefit from delayed CNI initiation and hence it would be very important to identify these patients early (285). Also, a combined liver–kidney transplantation could be performed for those who will remain dependent on RRT after liver transplantation (286). The problem is that it is currently impossible to identify these patients in advance.

Significant hope has been put on NGAL in predicting AKI and it has been proposed as a marker for AKI after liver transplantation. To date, there are 12 studies investigating the role of serum, plasma or urine NGAL in the prediction of AKI after liver transplantation (39–50). The number of patients in the studies varies from 19 to 107. All studies found that NGAL is useful in predicting AKI after liver transplantation. The AUCs varied from 0.68 to 0.87, but some studies did not report AUCs. In two studies NGAL was found to correlate with the severity of AKI (40, 41). However, in all of these studies the sample was taken after transplantation, which is too late for a combined kidney–liver transplantation and delayed CNI initiation would have already been done.

We were not able to predict posttransplant AKI or the need for posttransplant RRT by measuring pretransplant NGAL. Pretransplant NGAL has been studied in one other publication and, in line with our findings, it failed to predict posttransplant AKI (39). However, we found that in patients with impaired kidney function before transplantation, high pretransplant NGAL concentration was an independent risk factor for ongoing kidney dysfunction at three months after transplantation. High pretransplant NGAL concentration reflects the recipients' kidney quality and these patients are at increased risk for further kidney injury. Although no treatment as such exists, optimizing and individualizing patient care can avoid further damage.

There are several limitations in these studies that need to be addressed. First, although this is a nationwide study, it is a single center study with a limited number of patients. The timing of sampling from kidney recipients was not standardized precisely enough in any of the studies apart from donor uNGAL collection. Based on the results it would have been interesting to see whether very early sampling timing would have yielded better predictive power. In Study III it would have been better to use plasma instead of serum. In fact, it would have been best to collect plasma instead of serum as serum can be extracted from plasma but not the other way around. Moreover, we only tested one biomarker in studies I–III instead of a panel of biomarkers. Lack of urine as sample material for NGAL analyses and lack of data on UOP, proteinuria and urine sodium concentration are important limitations in Study IV. Confounding effects of, for example, dialysis, MARS treatment, surgery, anesthesia, medication, or urine composition on NGAL concentration and analysis methods are not known and could not be eliminated.

Recently, it has been shown that NGAL exists in different forms. Western blot has revealed that different forms of NGAL can be detected with different methods based on their molecular weight. Cai et al. found that monomeric NGAL is suggestive of AKI and secreted by kidney epithelial cells, whereas dimeric NGAL originates from neutrophils (287, 288). So far it is unclear which forms of NGAL the currently available tests detect, apart from western blot, so it is possible that this affects the results. Additionally, the serum and urine NGAL concentrations are not comparable as the detection method is different.

7. CONCLUSIONS

1. High donor urine NGAL is an independent risk factor for prolonged DGF (I).
2. Recipient urine NGAL on the first day after kidney transplantation predicts DGF and prolonged DGF with moderate sensitivity and specificity (II).
3. Recipient serum NGAL on the first day after kidney transplantation predicts DGF and prolonged DGF. It also predicts DGF in cases expected to have early graft function based on clinical findings (III).
4. The independent risk factors for DGF and prolonged DGF are day-1 serum NGAL, day-1 urine output and mode of dialysis before transplantation (II, III).
5. Plasma NGAL before liver transplantation associates with reduced kidney function at three months after transplantation (IV).

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