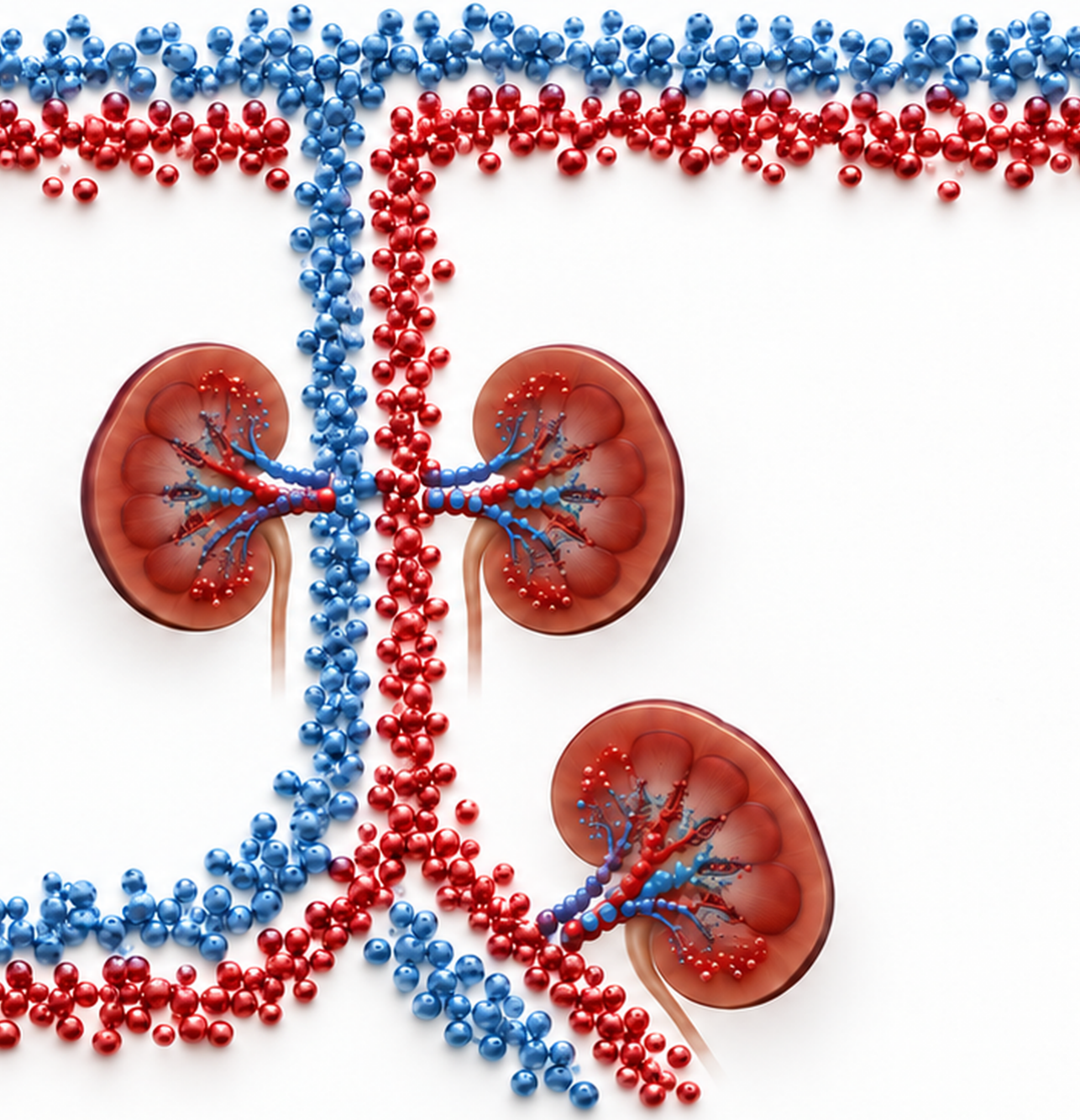
MARGINAL DONOR KIDNEYS

NOVEL STRATEGIES TO ASSESS AND IMPROVE VIABILITY



Tim C. van Smaalen

Marginal donor kidneys

Novel strategies to assess and improve viability

Tim C. van Smaalen

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Voor mijn ouders

Chapter 1

General introduction

General introduction

Functioning kidneys are vital to the humans body. They play a major regulatory role in fluid, mineral and hormone balance, contribute to acid metabolism and excretion of waste and toxins. Many diseases may lead to acute or chronic kidney failure, requiring kidney replacement therapy. For patients with end stage renal disease (ESRD) kidney transplantation is the preferred treatment. Compared to dialysis treatment, it increases both the quality of life as well as life expectancy[1,2].

For transplantation, either one kidney can be donated during life or both kidneys can be donated after death. In 1954 dr. Joseph Murray was the first to perform a successful kidney transplant from a living donor to an identical twin[3]. In the Netherlands, the first living kidney transplantation was performed in 1966. A living donor program was successfully implemented. Thereafter, the number of living donors steadily increased and numbers were further enforced by additional ways to obtain living donors. These included kidney paired donation, altruistic donation, domino-paired anonymous donation and ABO-incompatible donation[4-6]. In 2008 the number of kidney transplantations from living donors exceeded those from deceased donors for the first time. In 2018, living kidney donors still account for more than half of kidney transplantations in the Netherlands[7].

The first successful kidney transplantation from a deceased donor was reported in 1963[8]. This was a case of donation after circulatory arrest and in which the kidney still functioned 1 year after transplantation. An unusual success at the time, because in the tens of kidney transplantations thereafter the graft failed within the year. Graft failure was attributed to problems with rejection and poor preservation, in which organ quality decreases by warm ischemic injury[9]. Further attempts to improve preservation techniques and increasing use of immunosuppressive regimens led to improved results. One year graft survival improved from 12% in the mid-1960s to 46% in the beginning of the 1970s[10]. At the same time, in 1968, brain death was recognized, which lead to the introduction of brain death donors[11]. These donation after brain death (DBD) donors had an intact circulation and minimal ischemic injury, leading to improved transplant outcome. Because of the problems with poor outcome after donation after circulatory death (DCD) and the ethical questions arising regarding involuntary donation or coercion and commercialization in living donation, these types lost favour and DBD was preferred. In the following years transplant outcomes greatly improved by the introduction of new immunosuppressive regimens, i.e. ciclosporin was discovered in 1969 and clinically introduced in the 1970s[12,13]. These results were not unnoticed and a

growing group of patients with ESRD opted for transplantation[14,15]. As a result, waiting list for kidney transplantation grew and DBD could not sufficiently meet the organ demand[16]. Thus, a renewed interest in DCD and living donation arose[17,18] and ways to increase the donor pool and increase the success of kidney transplantation were explored.

Current challenges in kidney transplantation

Donor shortage and waiting lists

Given kidney transplantation is needed for patients with ESRD, ideally there would be an oversupply of kidneys for transplantation and there would be no chance of graft failure (or any complication) after transplantation. However, this is where problems arise. There is a disparity between the supply and demand of donor kidneys, with the demand being much higher[7,19]. Countries and transplant organizations try to tackle the organ shortage problem by initiating donor programs and may even change laws to increase the number of organ donors, but none have led to a final solution so far. Currently, waiting lists for kidney transplantation decrease slightly in the Eurotransplant region, while in the United States they continue to grow[19,20].

Increasing the success of kidney transplantation

The ongoing kidney shortage has forced physicians to look for alternative strategies to increase the donor pool. Not by only focussing on the actual number of people registered as donors, but also looking at ways to increase the success of kidney transplantation. These strategies focus on different aspects of kidney donation and transplantation process. Such strategies are:

- Expanding the acceptance criteria for kidney donors. Not only including all types of donors (as discussed below), but also including 'marginal kidney donors', those donors of high age and/or with comorbidities, of which kidneys otherwise would not have been accepted or discarded for transplantation[21-24].
- Improving the donor selection process. Identification of risk factors for graft dysfunction and failure may aid in the decision to either accept or decline a possible donor as suitable donor[25-29].
- Improving donor quality. In practice, this means improving donor management. After brain death donor management is initiated by means of optimising cardiocirculatory circulation and diminishing injury to the donor organs[30-37].

- Increasing the quality of procurement and allocation. By means of improved procurement protocols and different ways to allocate organs, ischemic injury (both cold and warm) is diminished and more organ recipients may be identified[4,38-42].
- Increasing organ quality. After a donors death and until actual kidney transplantation there is a window of opportunity to intervene in organ quality. Interventions during this period, such as new preservation techniques and addition of therapeutics to the kidney grafts may increase organ quality and transplant success[43-49].
- Improving the kidney graft selection process, i.e. viability testing and risk stratification. By understanding which kidneys are viable and those which are not, identifying markers for graft failure and developing means to test this, less organs may be discarded for transplantation and more donors will be accepted for donation[25-29,49,50].
- Improving post-transplant outcome. Thousands of studies have been published regarding post-transplant treatment of the recipient. All to one goal; diminishing complications after transplantation, such as rejection, to prevent graft failure and improve both short- and long-term graft and patient survival and/or improving a recipients quality of life[51,52].

Deceased donation

The introduction of deceased donation has provided a valuable expansion of the number of kidneys available to the donor pool. Alongside its growing success, waiting lists also grew. This thesis mainly focusses on strategies that increase the number of selected donors for transplantation by improving or testing graft quality. There is a particular urge for this approach in transplantation of DCD and marginal kidney donors to effectively expand the donor pool. Marginal kidney donors have often been considered as high-risk donors, due to their high age and/or having comorbidities, causing a higher chance of graft dysfunction or failure. Clinically, there is a fine and unclear line between accepting, reluctance to accept and declining donor organs for transplantation. By studying and finding objective parameters of donor and organ quality and their outcome, selection processes may be improved and less kidneys may be unnecessary discarded. To do so, a good understanding of deceased donation, its types and categories is essential.

Table 1.1 shows the types, categories and possible suitable organs which can be donated after deceased donation in the Netherlands. Figure 1.1 illustrates the differences in organ perfusion between donors. As outlined above, there are two types

of deceased donation: donation after brain death (DBD) and donation after circulatory death (DCD), the latter categorized by the Maastricht criteria (Table 1.1)[53]. These types differ in many ways and most importantly, may lead to different transplant outcomes.

Table 1.1: Organ donation from deceased donors.

Organ donation from deceased donors						
Type	Donation after brain death (DBD)	Donation after circulatory death (DCD)				
Maastricht Category		1	2	3	4	5
Remark		Uncontrolled	Uncontrolled	Controlled	Uncontrolled / Controlled	After euthanasia
Possible suitable organs	Heart Lungs Liver Kidneys Pancreas Small intestine	-	-	Lungs Liver Kidneys Pancreas Heart	Lungs Liver Kidneys Pancreas Heart	Lungs Liver Kidneys Pancreas Heart

Types and categories of deceased donors. DCD donors are categorized by the Maastricht criteria[53,54], using the following definitions: 1 = dead on arrival, 2 = unsuccessful resuscitation, 3 = awaiting cardiocirculatory arrest (including donors after euthanasia), 4 = circulatory arrest while brain dead. 5 = after euthanasia. Suitable organs are shown according to current protocols in the Netherlands[54].

Donation after brain death (DBD)

In DBD, brain death of a donor is declared by neurologic criteria according to national law[55]. Then, the donor with intact cardiovascular circulation is transported to the operating room, where procurement surgery is commenced. After careful surgical dissection without compromising organ perfusion, circulatory arrest is initiated by the surgeon and organ preservation is directly initiated. Thus, detrimental warm ischemic injury is almost nil (Figure 1.1). As result, multiple organs may be suitable for transplantation (Table 1.1). In 2018, 91% of DBD donors became multiple organ donor

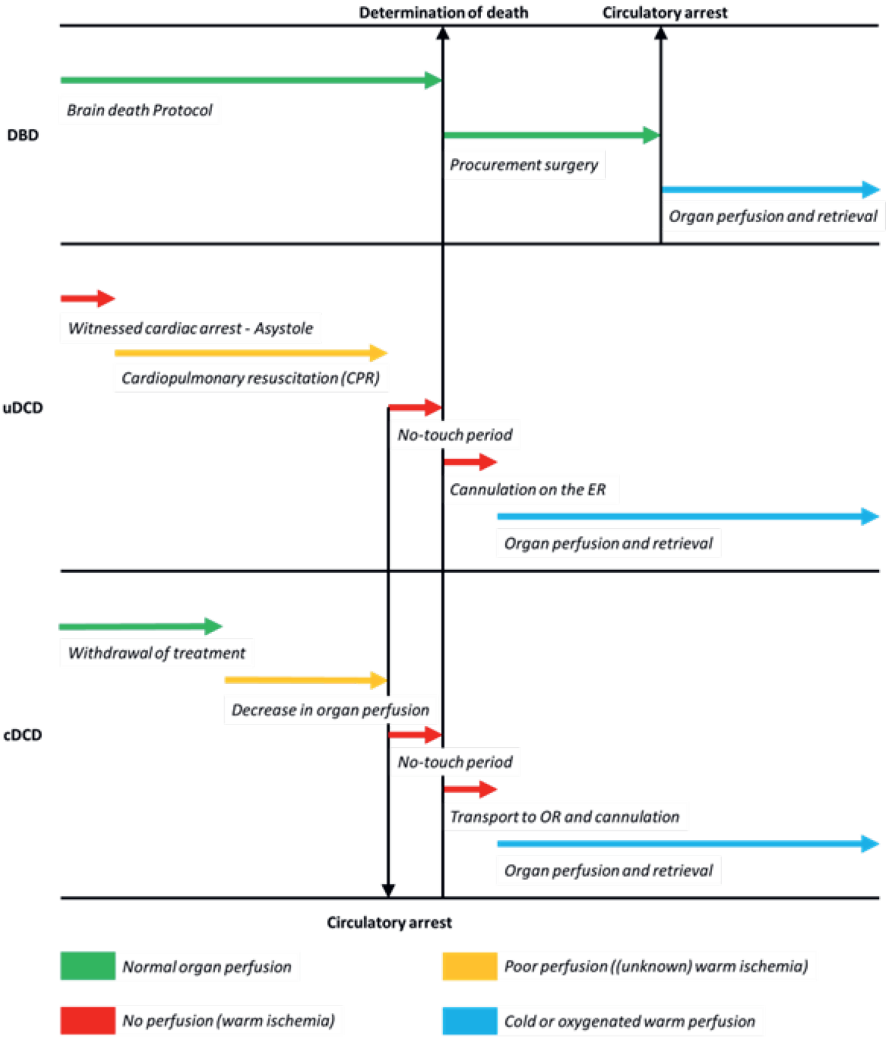
in the Netherlands[7]. In contrast, in DCD donors, this is much less. In 2018 only 50% of DCD donors became multiple organ donor, mostly they were kidney only donors[7].

In the Netherlands there is no age restriction for DBD kidney donors[56]. However, the older donors become the less likely it is that they are accepted as suitable donors. Old donor kidneys have reduced kidney function and are more susceptible to injury. Furthermore, kidneys may have suffered from diseases, especially later in life[57-59]. The growing waiting lists at the end of the last century forced physicians to explore the boundaries of acceptable donor age and comorbidity. Expanded criteria donation (ECD) was introduced and in the early 2000s part of allocation programs[60,61]. Expanded criteria donors were defined as DBD donors with a relative risk >1.7 of graft failure compared to the 'ideal' donors, which were defined as DBD donors between the ages 10 and 39 years and without a history of hypertension or CVA and a terminal serum creatinine of <133 µmol/L. Thus expanded criteria donors are donors >60 years of age, or >50 years of age with at least two of the above mentioned risk factors which ideal donors do not have[60,61]. This 'new' cohort of donors soon proved to be a significant contribution to the donor pool, but with increased risk of graft loss[62,63]. Nonetheless, for most patients acceptance of expanded criteria donors led to a survival benefit compared to remaining on dialysis treatment[64].

Donation after circulatory death (DCD)

Next to DBD, DCD donors are vital to the expansion of the donor pool. In 1997 it was estimated that DCD donors would have the potential to increase the donor pool 2.5 to 4 times[65]. Roughly twenty years later, this has not been achieved. A study of the Dutch transplant data showed that in 2014 54% of all deceased donors were DCD Maastricht category 3 donors, which are the most commonly reported DCD donors, while in the year 2000 this was 14%[66]. Thus there is an increasing role of DCD. The overall increase of deceased donor kidney transplantation from 2000 until 2018 was 27% (387 to 488 kidney transplants)[7,67].

Figure 1.1: Deceased donation process over time and quality of organ perfusion.



The 3 types of deceased donation are shown. DBD, uncontrolled DCD (uDCD) and controlled DCD (cDCD). In DBD normal organ perfusion is expected until cessation of circulation during procurement surgery. In uDCD there must be a witnessed cardiac arrest after which cardiopulmonary resuscitation must be started quickly. A period of poor and unknown circulation and therefore possible warm ischemic injury. After circulatory arrest, a no-touch time (5 min in the Netherlands) is mandatory after which death is determined. Then direct cannulation is initiated in the emergency room (ER). Together these periods count as the warm ischemic time (WIT). Then cold or oxygenated warm preservation is started. In cDCD after withdrawal of treatment donors will experience a decrease in blood pressure with poor perfusion as result; the agonal phase. Thereafter WIT consist of the no-touch period and transport to the operation room (OR) until cannulation and perfusion in started.

The vast majority of DCD donors are Maastricht category 3 donors, the so-called controlled donors (Table 1.1). In these donors, after further medical treatment has been considered futile, treatment is withdrawn on the intensive care unit and an agonal phase starts. In this period organs suffer from deteriorating perfusion and therefore (an unknown amount of) ischemic injury. After final cardiocirculatory arrest (and death is declared according to national protocols[55]), the warm ischemia time (WIT) starts, in which warm ischemic injury further harms the organs. Only then and after transport to the operating room, procurement surgery can be commenced and organs can be perfused, procured and preserved. (Figure 1.1) Prolonged WIT is associated with a higher incidence of graft failure and mortality after kidney transplantation[27,68]. For Maastricht category 1 and 2 donors, so-called uncontrolled donors, death is not declared after planned withdrawal of treatment, but after an unexpected and unsuccessful cardiovascular resuscitation. (Figure 1.1) Due to the unknown ischemic injury during resuscitation and the unplanned situation and time of death, organs may even suffer more injury compared to Maastricht category 3 donor organs. Because of the increased ischemic injury of DCD organs suffer compared to DBD organs, DCD not only leads to less multiple organ donors but also leads to poorer transplant outcomes for most organs. Kidneys suffer from increased incidence of primary non-function (PNF) and delayed graft function (DGF) compared to DBD donors[26,69-72]. Both have been associated with a higher risk of acute rejection, inferior renal function, longer hospitalisation and higher costs[73,74]. Because of this, there has been (and still is) a general reluctance to accept organs from DCD donors for transplantation, especially from uncontrolled donors. This reluctance harms patients on the waiting lists. Multiple studies have shown that DCD should be encouraged as addition to the donor pool. Snoeijs *et al.* showed that the use of DCD kidneys provides a survival benefit as compared to waiting on the waiting list for standard criteria DBD kidneys[75]. Furthermore, the long-term kidney graft and recipient survival seems to be equivalent for DCD and DBD kidneys[76].

Organ preservation

Organ preservation is a fundamental part of transplantation and key to its success. It's importance was already recognized many years before the first transplantation by dr. Murray[3,77]. Alexis Carrel and aviator Charles Lindbergh developed a pulsatile machine and experimented with hypothermic conditions in the 1930s[78]. It was acknowledged that hypothermia reduces metabolic activity and therefore leads to less ischemic injury compared to normothermia. It was only until 1960 that the first successful canine kidney transplantation using a perfusion machine and hypothermic blood was

described. At the same time, alternative solutions for cold machine perfusion were studied as well as cold preservation itself[79,80]. The development of Collins solution (1969) and University of Wisconsin (UW) solution (1987) resulted in acceptable transplant outcomes after cold storage and the possibility to transport kidneys over long distances[80,81]. Since then, cold storage has been the gold standard for kidney preservation for many years (Figure 1.2). In the last decades machine perfusion has gained a lot more interest (Figure 1.2). It not only may lead to equal or better organ preservation, but it also offers the opportunity to monitor organ quality during transport, perform viability tests, wash-out waste products or add therapeutics. A large multi-centre study by Moers *et al.* showed that hypothermic machine perfusion (HMP) was associated with a reduced risk of delayed graft function and improved graft survival in the first year after transplantation[82]. These results, along with others[83-86], led to a great increase in the use of HMP, although there is still debate on its beneficial effect on long-term graft function and survival[87].

In recent years, the development of normothermic machine perfusion (NMP) has been the focus of well-known research groups and for good reason. Preservation (cold storage or HMP) has always been used as a technique to protect kidneys from further damage during the period from organ procurement to transplantation. NMP is a technique that uses extra-corporal membrane oxygenation not only to prevent kidneys from further harm, but also to recondition and repair kidneys and to estimate organ viability. Especially in the context of marginal donor kidneys this is of interest, as it can lead to more organs being accepted for transplantation[45,48,88,89]. In 2011, the first in man renal transplantation after ex-vivo NMP was successfully performed[90]. The first results of multiple kidney transplantations after NMP show at least equal results compared to kidneys transplanted after HMP or CS preservation. Furthermore, kidneys which would otherwise have been discarded were now transplanted with good result[90,91]. This shows major clinical benefits of NMP, but large studies have not been performed yet[92,93]. Thus NMP remains the main focus of study groups developing organ perfusion techniques today.

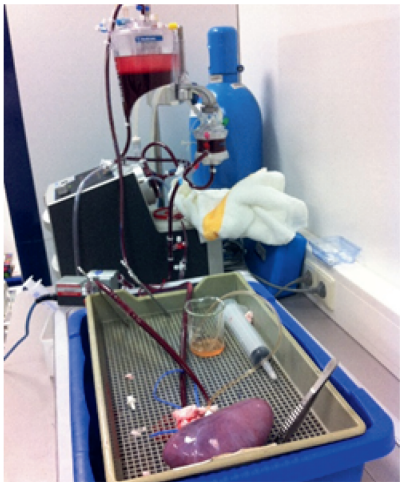
Figure 1.2: Kidney preservation modalities.



A



B



C



D

A) Top view of a Lifeport Kidney Transporter* for hypothermic machine perfusion (HMP). The pulsatile roller pump is easily visible, as well as the entire perfusion circuit. **B**) The kidney in the lifeport reservoir with perfusion solution and perfused via the arteria renalis. **C**) Our own lab setup for normothermic machine perfusion (NMP), in which the kidney is perfused with blood. The macroscopic appearance may indicate perfusion quality and urine output is monitored. **D**) Conventional cold storage styrofoam box. The kidney is preserved in 4° Celcius preservation fluid.
** LifePort Kidney Transporter, model no. LKT-100-P; Organ Recovery Systems, Des Plaines, IL*

Ischemia-reperfusion injury

Kidney function and survival after transplantation are negatively affected by the injury a kidney sustains during the period of kidney donation until and during transplantation. A major contributor is renal ischemia-reperfusion injury (IRI), which is inevitable during the donation process. IRI is associated with the increased incidence of graft rejection, PNF, DGF and graft failure. Furthermore, IRI may also affect long-term graft function

and survival by reduction of renal functional mass, graft vascular injury, chronic hypoxia and fibrosis[94-96].

IRI is the result of an inflammatory process that is triggered by the reduction or cessation of blood flow to a kidney, followed by reestablishment of kidney perfusion[96-100]. The underlying pathophysiological mechanisms of IRI are comprehensive and include a wide spectrum of processes. Understanding these pathophysiological mechanisms is important, because they may reveal options for therapeutics in order to improve kidney quality. Furthermore, the involved molecules may be used as markers for injury and therefore they may be useful in viability assessment, a focus of this thesis. Therefore, we will discuss this in more detail.

By cessation of blood circulation, there is an interruption of renal oxygen supply that leads to a reduction in oxidative metabolism, depletion of adenosine triphosphate (ATP), increase in glycolysis and inhibition of the NA/K ATP-ase pump causing impaired water and electrolyte imbalance, acidosis, with progressive injury and death of tubular epithelial cells. This is most prominently seen in proximal tubules and these may be observed after reperfusion in the urine. Furthermore, it impairs endothelial barrier function and leads to vascular permeability and leakage. The ischemia induced activation of cell death programs (e.g. apoptosis, necroptosis, ferroptosis) leads to membrane rupture, the release of cytosolic components and activation of the immune system. Clinical studies showed an increase in complement activation, with increased concentrations of complement in both DCD and DBD kidneys already in the donor, which induces tissue injury via intra-cellular pro-inflammatory signaling pathways. Dying cells may release damage associated molecular patters (DAMPs), like DNA fragments and histones. These may bind to cell-surface receptors, like Toll-like receptors 2 and 4 (TLR2/4), and trigger intracellular inflammatory and cytotoxic responses. The release of TNF-α and interleukins stimulates migration and activation of dendritic cells, which activates T-cells and macrophages. After reperfusion, formation of reactive oxygen species (ROS) and involvement of the nitric oxide synthase pathway (NOS-pathway), seem to play major roles. Also ischemia is related to transcriptional reprogramming, which can be influenced by ROS, which leads to inflammatory signaling cascades[95,97,98,101-105]. Progressive injury results in destruction of tubular endothelial and epithelial cells, widespread inflammation, loss of structure and the loss of the kidneys homeostatic control of metabolic waste products and impairment of water and electrolyte balance[96,97,99,100].

Kidney viability

It is clear that especially DCD kidneys suffer from prolonged ischemia times, due to the extended warm ischemia time in the donor, but perhaps also related to the cardiovascular cause of death. This results in an increased contribution of ischemia in IRI injury and a presumed bigger change of production of metabolic waste products compared to DBD. This makes DCD kidneys of special interest for viability testing. There are many different ways to estimate organ viability, including measurement of machine parameters, analysing machine perfusate and histopathologic examination of organ tissue.

Machine perfusion parameters

Machine perfusion offers the opportunity for dynamic evaluation of the graft. Kidneys can be monitored over time for multiple factors, such as pressure, renovascular resistance and flow. High pressure, high resistance and low flow are associated with an adverse outcome after transplantation in hypothermic machine perfused kidneys[106-110]. NMP offers, next to pump dynamics, more possibilities for viability testing. Kidney function can be assessed macroscopically by appearance of blood perfusion, renal blood flow and urine output[111-113].

Machine perfusate

In a hypothermic perfusion machine a kidney is placed in a reservoir of cold perfusate. This perfusate is pumped via a filter through the system to perfuse the kidney with cold perfusion solution (Figure 1.2a/b). Metabolic waste products and toxins are washed out and become measurable in the perfusate. Most machines have a sample port to extract perfusate for further analysis of biomarkers indicative for the extent of injury or pathophysiologic process occurring in the kidney. In this thesis there is a comprehensive review on machine perfusion viability testing using HMP. More or less the same applies to NMP, although biomarkers may not only be indicative for sustained injury. In a normothermic state, thus metabolically active state of the kidney, markers may illustrate active processes of kidney metabolism. These may be markers for injury, but also for repair and function[111-114]. Additionally, the effect of therapeutics during NMP may be evaluated.

Histology

In the beginning of the 1990s the first consensus statement for a standardized international classification of renal allograft biopsies was published by the Banff

Working Group[115]. A milestone for kidney transplantation pathology, as it lead to a homogenous approach for histologic evaluation of transplanted kidneys. These protocols were developed for post-transplant analysis, they did not focus on pre-transplant assessment. Although pre-implantation biopsies were used as a marker to accept or decline kidneys for years, it was not until recently that a consensus statement on pre-implantation biopsies was published to develop consensus to define the gold standard for interpretation of preimplantation biopsies[116]. This study describes a uniform approach on how to perform and assess kidney biopsies and compare histopathologic and clinical parameters with graft outcome. It was concluded that 'Histopathologic assessment of preimplantation biopsies is one component of donor organ assessment and is not an exclusive determinant to discard or transplant donor kidneys'[116]. Indeed, no large prospective pre-implantation biopsy studies have been performed or studies that led to a consensus for clinical practice[50]. In contrast, those studies that were performed have led to contradictory results[117-122]. Some groups even state that the use of pre-implantation biopsies may be harmful as it will lead to unnecessary discard of kidneys[117,118]. It must be concluded that the techniques used to assess pre-implantation biopsies and the role of pre-implantation biopsies in clinical practice needs further study.

Mass spectrometry imaging

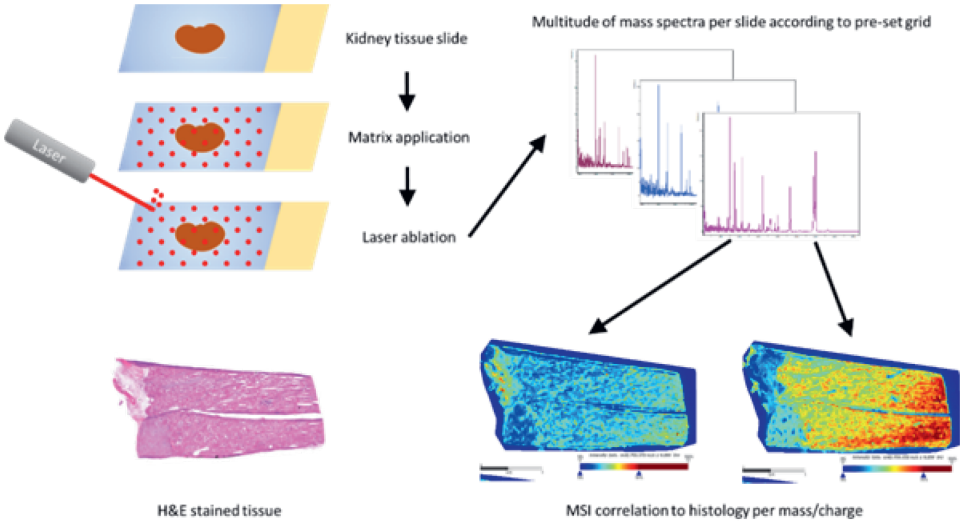
In this thesis mass spectrometry imaging is introduced as a way to rapidly identify markers of injury in renal tissue; a technique which needs extra introduction.

A mass spectrometer is an analytical instrument that uses the mass of compounds in order to identify them. After ionization of the (unknown) compound, ions are accelerated in an electromagnetic field after which they are deflected. The lighter the ions, the more they are deflected and the faster they go. A detector then detects the ions and/or time to the detector. As results the mass-to-charge ratio can be calculated and plotted. These plots are called mass-spectra. The first functional mass spectrometer was built in 1919[123].

Mass spectrometry imaging (MSI) combines the chemical specificity and parallel detection of mass spectrometry with microscopic imaging capabilities[124]. Figure 1.3 shows a schematic workflow for analysis of renal tissue using MSI. In the beginning of the 1990s, new technical developments led to a large increase of MSI as it offered the capability to analyse large intact biomolecules[125,126]. Currently, MSI is capable of recording the spatial distribution of many atoms and molecules, including lipids,

proteins, metabolites and pharmaceuticals[124]. The correlation of spatial distribution of analytes to histologic imaging opens up the path for fast and accurate molecular based histologic evaluation[127]. Moreover, recent advantages in sample preparation, spatial resolution and analysis time have made this technique accurate and fast enough for possible clinical use[128].

Figure 1.3: Mass spectrometry imaging schematic overview.



Schematic example of matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) analysis of renal tissue as used in this thesis.
After a renal biopsy, tissue is sectioned and mounted on a conductive slide. Thereafter matrix is applied. A laser ionizes the tissue in a pre-set grid and for every point where this is performed a mass-spectrum is generated. The tissue is H&E (haematoxylin and eosin) stained and prepared for conventional histologic analysis. Masses are correlated to histology slides and their spatial distribution illustrated. In this example one mass correlates to compound primarily found in the glomeruli (on the left) or to the cortex (on the right).

Aim of the thesis

The main focus of this thesis is to gain insight in the pathophysiologic aspects of ischemically injured kidneys, in order to assess or increase their viability in relation to outcome. These insights may aid in clinical decision making or provide novel methods to either increase the number or quality of organs available to the donor pool. We aim to assess kidney viability by using new techniques and look for new biomarkers of injury. Our ultimate aim is to associate clinical, histological and metabolic markers of injury to transplant outcome. For this we use a translational approach with studies in human transplantation focussing on machine preservation and on DCD donation, as well as in an ex vivo porcine model mimicking machine perfusion approaches in DCD donation.

Outline of the thesis

In chapter 2 we investigate clinical parameters of transplant outcome. We compared the outcome of uncontrolled DCD to controlled DCD kidneys and give an insight in which clinical parameters may be risk factors for graft dysfunction.

In chapter 3 we review the current scientific knowledge on viability testing used during machine perfusion. In chapter 4 we start our search for new viability test. We used a dilution technique during machine perfusion to determine if warm ischemia times are reflected by renovascular circulating volume and if this technique is reproducible. In chapter 5 and 6 we focus on the role of extracellular histones in machine perfusate and their association to transplant outcome as possible viability test.

In chapter 7 we focus on histologic assessment. We study whether histologic markers of injury, which have not been used before in clinical transplantation, are related to transplant outcome. In chapter 8 we employ mass spectrometry imaging as a new technique to assess kidney injury on a molecular basis in a pre-clinical transplant setting. To find new markers of injury and whether this test may have potential as viability test in clinical practice.

In chapter 9 we change from assessment to intervention. We study ischemically injured pig kidneys and perform a capsulotomy to see whether this may improve their perfusion.

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Chapter 2

Kidneys from uncontrolled donors after cardiac death: which kidneys do worse?

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Abstract

Background: Kidneys from uncontrolled donors after cardiac death (DCD) expand the donor pool, but are associated with more primary non-function (PNF) and delayed graft function (DGF) compared with more conventional donor kidneys. It remains unclear, which factors influence outcome of uncontrolled donation. Therefore, we studied which donor, graft, and recipient characteristics are associated with PNF in a large cohort study.

Methods: The association between different characteristics and short-term graft-function was analyzed for kidneys procured in the Maastricht region from 1 January 1981 until 1 July 2009. Patients were followed until 1 July 2010.

Results: A total of 135 uncontrolled donor kidneys were included in this study. The incidence of PNF and DGF was 22% and 61%, respectively. Increasing donor age is an independent risk factor for graft failure in a univariate analysis (OR 1.035, 95%CI 1.004–1.068, p=0.028). Donor age remains strongly associated with PNF in a multivariate analysis (OR 1.064, 95%CI 1.013–1.118, p=0.014). However, the predictive value of donor age alone is poor (AURC 0.640, 95%CI 0.553–0.721).

Conclusion*:* Increasing donor age of uncontrolled DCD donors is a major risk factor for PNF. Other clinically relevant variables were not associated with PNF. Donor age is strongly associated with PNF and remains an important parameter in donor selection.

Introduction

In the past decades, kidney transplantation using organs obtained from donors after cardiac death (DCD) has gained popularity and a wider acceptance in many countries as a valuable tool to decrease the waiting time for kidney transplantation[1-3]. Most DCD kidney donation programs only include kidneys from controlled DCD donors, donors who died after withdrawal of futile medical treatment. Uncontrolled DCD donation has not yet obtained the same wide acceptance, but its use is increasing.

In uncontrolled donors, donors who die after failed resuscitation, the period between circulatory arrest and organ preservation is often not exactly known and the efficacy of cardiopulmonary resuscitation is difficult to assess. Therefore, these kidneys are exposed to unknown ischemic injury during the inevitable period of warm ischemia, which may result in higher incidences of primary non-function (PNF) and delayed graft function (DGF). The relatively unknown ischemic injury and the unplanned situation of the procedure have led to a general reluctance to initiate uncontrolled DCD programs and to accept uncontrolled DCD kidneys for transplantation. There are a limited number of uncontrolled DCD kidney transplantation programs in Spain and France with excellent results[4,5]. Furthermore, a recent study shows that the clinical outcome of uncontrolled DCD kidneys is comparable to that of controlled DCD kidneys[6]. This justifies a more widespread use of uncontrolled DCD donors to reduce the still growing waiting list for renal transplantation and may stimulate the implementation of uncontrolled DCD kidney programs.

Most studies which assessed DCD kidney transplantation outcomes analyzed either controlled DCD kidneys or uncontrolled and controlled DCD kidneys together. Despite the different mechanism of ischemic injury, the risk factors for the outcome of uncontrolled DCD kidney transplantation have not been studied before. Because of the increased popularity, it is essential to know which uncontrolled DCD kidneys can be accepted for transplantation and which kidneys better can be discarded. Our center has a long-time experience with the use of uncontrolled donor kidneys for transplantation since 1981. To describe the opportunities and limits of uncontrolled DCD kidney transplantations, we assessed the results of our uncontrolled DCD program and determined which factors are associated with PNF or inferior graft function after transplantation of uncontrolled donor kidneys.

Materials and methods

Study design

Kidneys from uncontrolled DCD donors, procured in the Maastricht region from 1 January 1981 until 1 July 2009 and transplanted within the Eurotransplant region, were included in the current observational study. Patients were followed until the earliest of death or 1 July 2010. Kidneys were allocated according to the Eurotransplant allocation rules and were matched for Human Leukocyte Antigen (HLA) and blood group.

Donor and recipient data

Donor and perfusion characteristics of machine-preserved kidneys were routinely recorded at our institution. Recipient follow-up data were kindly provided by the recipient transplant centers, and was periodically updated in our database. The following graft characteristics were recorded: warm ischemia time (WIT), the period from circulatory arrest or stop of resuscitation until the initial cold flush of the kidneys; cold ischemia time (CIT), the period between the initial flush and the start of first anastomosis of the recipient operation; and the anastomosis period, the time to complete arterial and venous anastomoses.

Short-term graft function after transplantation was classified as 1) primary non-function (PNF): permanent inadequate renal function necessitating continuation of dialysis or retransplantation; 2) delayed graft function (DGF): renal function which was ultimately life sustaining but required temporary dialysis in the first week after transplantation; and 3) immediate function (IF): immediate renal function without the need of postoperative dialysis. Graft survival is defined as functional survival off dialysis.

Renal function after transplantation was assessed using the estimated glomerular filtration rate (eGFR), which was calculated with the abbreviated Modification of Diet in Renal Disease equation (MDRD)[7-9]. eGFR at 1 year after transplantation and the rate of decline in eGFR thereafter were studied as measures of kidney function.

Collection, storage, and use of patient data were performed in agreement with the code of conduct ‘use of data in health research’ from the Dutch Federation of Biomedical Scientific Societies (http://www.federa.org/); According to Dutch law,

Institutional Review Board (IRB) approval was not required for scientific analysis of anonymous data.

Management of uncontrolled donors after cardiac death

Kidneys were procured from uncontrolled (Maastricht category II) donors according to the Maastricht DCD categories[10,11]. Organs were preserved with the *in situ* preservation (ISP) technique, as described previously[11,12]. In short, after unsuccessful resuscitation and an obligatory no-touch period of five minutes, during which no interventions to the donor were taken, a double-balloon triple-lumen (DBTL) catheter was inserted into the aorta via one of the femoral arteries, followed by ISP of the donor kidneys with histidine-tryptophan-ketoglutarate solution (Custodiol; Dr. Franz Köhler Chemie, Alsbach, Germany)[13,14]. A large Foley catheter is placed in the femoral vein for decompression. This indispensable, minimally invasive technique may provide the opportunity to meet legal and logistical requirements for organ recovery without excessive WITs and, depending on the legal opportunities, can be initiated prior to consent for organ donation. It can be performed in the emergency department and allows fast and effective organ preservation. Insertion of the cannulas can be performed by surgeons with limited experience in donation procedures. After preservation and consent for organ donation, the organs were procured in the operating room. Following recovery of the kidneys in the operating room within two hours after the start of ISP, most of the kidneys were weighed and prepared for machine preservation and were placed in sterile organ chambers on Gambro PF-3B perfusion machines (Gambro, Lund, Sweden) using Belzer's University of Wisconsin machine perfusion solution since 1985, at a mean temperature of 4 °C. Eurocollins preservation solution was used before 1985[15]. Since 2006, the Lifeport (Organ Recovery Systems, Des Plaines, IL, USA) has been used as pulsatile perfusion machine. Added to the solution were 40 IU of insulin, 200.000 U of penicillin and 16 mg of dexamethason. During machine perfusion, pH was adjusted to values >7.10, using sodium bicarbonate.

Statistical analysis

Continuous variables are presented as mean $\pm$ standard deviation (SD) if normally distributed, and as median and interquartile range (IQR) otherwise. Categorical variables are presented as percentages. Baseline donor, preservation, and recipient characteristics are compared with the Student's *t*-tests for normally distributed continuous variables, with the Mann-Whitney *U*-test if the distribution was not normal, and with chi-square tests for categorical variables. The effects of all variables on PNF were tested with univariate logistic regression analysis. Multivariate logistic regression

analysis was performed to identify potential risk factors for PNF, with correction for those characteristics that are considered to be potential confounders, defined by the 10% change-in-estimate rule[16], or if these criteria were not met, significant variables from the univariate analyses and potential confounders based on clinical relevance were used. The same criteria were used to determine the predictive value for PNF by the area under the ROC-curve (AURC). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were analyzed using MedCalc statistical software.

Cox proportional hazards model was used to assess the effect of variables on death censored graft failure of primarily functioning kidneys, correcting for confounders as described for the multivariate analysis. Graft survival censored for recipient death with a functioning graft and patient survival was calculated with the Kaplan-Meier method.

The rate of decline in estimated glomerular filtration rate was calculated for each patient by simple linear regression from yearly glomerular filtration rate estimates, starting at 1 year after transplantation.

Machine-perfused kidneys were further an alyzed to determine machine perfusion characteristics that are associated with outcome after transplantation.

All analyses were performed using Statistical Package for the Social Sciences (SPSS) software version 16.0 for Windows, and a p-value < 0.05 was considered statistically significant.

Results

Patients

From 1 January 1981 to 1 July 2009, 280 kidneys from uncontrolled donors after cardiac death were procured in the Maastricht region, of which 133 from 85 donors were discarded based on vascular anatomy (e.g. multiple renal arteries, renal artery stenosis, N=14), macroscopic appearance (e.g. poor flush-out, cysts, renal capsula tears, N=33), long WIT (N=17), donor related problems (e.g. positive virology, diabetic nephropathy, N=14), malplacement of the cannulas (N=7), if there were no suitable recipients (N=14), if the kidneys were considered too marginal to transplant (N=24) or not reported (N=10). Ten kidneys were transplanted outside the Eurotransplant region, data were

missing of one transplanted kidney and one recipient died with an unknown graft function. Of the remaining 135 kidneys transplanted in the Eurotransplant region, 110 kidneys were machine perfused and 25 kidneys were stored on melting ice until transplantation (cold-storage).

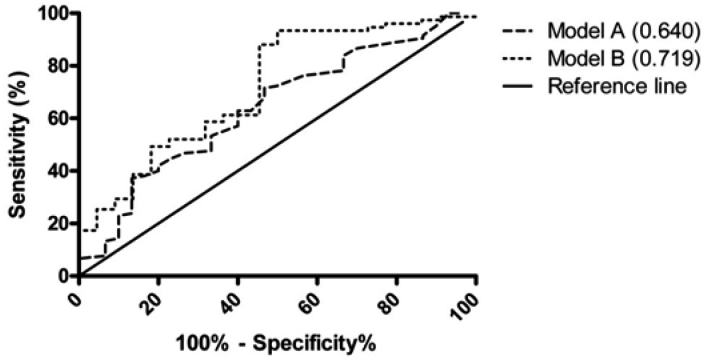
Donor, graft, and recipient characteristics are summarized in Table 2.1. Mean donor age was 45 ± 16 years. The mean WITs and CITs were 26 ± 11 minutes and 27 ± 6 hours, respectively. Donors and recipients predominantly comprised of men (73% and 68%, respectively).

Table 2.1: Baseline characteristics and their association with primary non-function

		Univariate analysis	
		OR (95% CI)	**p**
Donor related			
Age (years)	45 ± 16	1.035 (1.004 – 1.068)	0.028
Sex (male)	99 (73%)	0.658 (0.273 – 1.585)	0.351
Cause of death (cardiovascular/other)	71 / 29%	2.934 (0.843 – 6.798)	0.101
Hypertension (N = 107) (yes)	11%	1.479 (0.409 – 5.354)	0.551
Creatinine (µmol/L)	122 ± 37	1.002 (0.991 – 1.013)	0.671
Total CPR time (min)[a]	48 ± 25	1.001 (0.984 – 1.018)	0.911
Graft related			
Warm ischemia time (min)	26 ± 13	1.021 (0.988 – 1.054)	0.221
Machine perfusion / cold storage[b]	81.5 / 18.5%	1.625 (0.511 – 5.164)	0.410
Cold ischemia time (h)	27 ± 6	0.999 (0.935 – 1.067)	0.972
Of which machine perfusion time (h)	22 ± 6	1.026 (0.951 – 1.108)	0.505
Anastomosis time (min)	39 ± 14	0.998 (0.968 – 1.028)	0.878
GST T_4 (U/L/100g)	71 (52 – 111)[c]	1.005 (0.999 – 1.010)	0.089
LDH T_4 (U/L/100g)	565 (405 – 756)[c]	1.001 (1.000 – 1.001)	0.104
Renovascular resistance T_0	1.03 ± 0.63	1.542 (0.768 – 3.093)	0.223
Recipient related			
Age (years)	51 ± 13	1.012 (0.980 – 1.044)	0.472
Sex (male)	92 (68%)	2.118 (0.794 – 5.651)	0.134
Re-transplantation (yes)	15 (11%)	1.301 (0.382 – 4.424)	0.674
Total HLA mismatches	2.5 ± 1.1	1.066 (0.722 – 1.572)	0.746
Transplant center (regional / export)	64 / 36%	1.182 (0.502 – 2.782)	0.702
Kidney disease (renovascular / other) (N = 116)	18 / 82%	0.964 (0.317 – 2.931)	0.949
Dialysis time (years)	3.2 ± 2.6	1.020 (0.874 – 1.190)	0.799
Dialysis type (hemodialysis)	88 (65%)	0.773 (0.310 – 1.931)	0.582
Calcineurin inhibitor (yes)	118 (87%)	0.645 (0.208 – 2.003)	0.448
Anti-metabolite (yes)	55 (41%)	0.449 (0.183 – 1.099)	0.080
Sirolimus (yes)	29 (21%)	1.148 (0.436 – 3.022)	0.780
Induction therapy (yes)	12 (9%)	1.185 (0.300 – 4.686)	0.809

[a] Resuscitation time + CPR after declaration of death; [b] Odds ratio relates to machine perfusion; [c] Expressed as median (interquartile range).

Figure 2.1: Donor, graft, and recipient characteristics



Short-term graft function

Of the transplanted kidneys, 30 recipients developed PNF (22%), 83 recipients DGF (62%) and 22 recipients IF (16%). Donor, graft, and recipient characteristics and their association with PNF are shown in Table 2.1. Only donor age was a significant risk factor for PNF in the univariate analysis (Table 2.1) and remained an independent risk factor for PNF in the multivariate analysis (Table 2.2). Further analysis of donor age, using the ROC-curve and its accompanying table (i.e. the point closest to the [1-1]-corner), showed the strongest association with PNF from an age of 54 years and older with an odds ratio of 2.857 (95% CI 1.242 – 6.571; p=0.013). Kidneys from donors of 54 years and older have a higher percentage of PNF compared with younger donor kidneys (35% vs. 16%, p=0.012, respectively).

The predictive value of donor age on PNF was poor (AURC 0.640, 95% CI 0.553 – 0.721). Adding the variables as used in the multivariate analysis to the model, the predictive value increased to 'fair' (AURC 0.719, 95% CI 0.618 – 0.805) (Figure 2.1, Table 2.3).

Table2.2: Multivariate analysis of the risk of primary non-function and graft failure.[a]

	Multivariate logistic regression		Cox regression	
	Odds ratio (95% CI)	**p**	**Hazards ratio (95% CI)**	**p**
Donor age (years)	1.064 (1.013 – 1.118)	0.014	1.038 (1.015 – 1.060)	<0.001
Warm ischemia time (min)	1.045 (0.997 – 1.096)	0.068	1.015 (0.992 – 1.039)	0.202
Cold ischemia time (hours)	1.047 (0.946 – 1.158)	0.376	1.045 (0.996 – 1.096)	0.074
Renovascular Resistance T0 (mmHg/mL/min/100 g)	1.256 (0.602 – 2.618)	0.544	-	-

[a] A logistic regression model was used to determine the odds ratio for PNF, and a Cox proportional hazards model was used to determine the hazards ratio for death censored graft failure of primarily functioning kidneys.

Table 2.3: Predictive values of donor age for PNF.

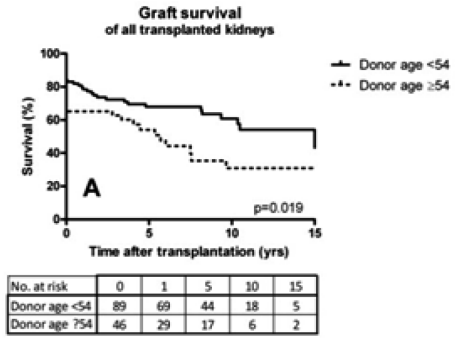
	AURC (95% CI)	Sensitivity	Specificity	PPV	NPV
Model A	0.640 (0.553 – 0.721)	0.533	0.714	0.348	0.843
Model B	0.719 (0.618 – 0.805)	0.500	0.933	0.687	0.864

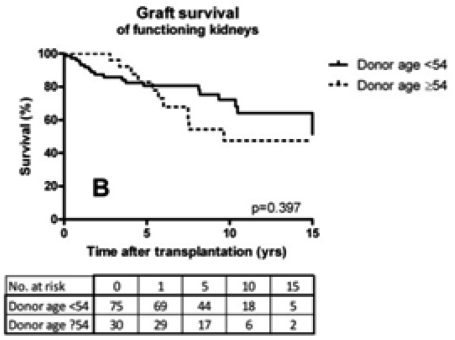
Model A: Donor age alone; Model B: Donor age, warm ischemia time, cold ischemia time, and renovascular resistance at T_0. PPV: positive predictive value, NPV: negative predictive value

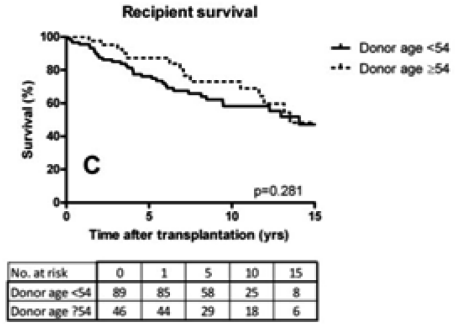
Long-term outcomes

The median period of follow-up after transplantation was 6.5 (IQR 3.5 – 12.2) years. Graft survival at 1 and 5 years after transplantation was 75% and 63%, respectively; kidneys with PNF included. Figure 2.2 shows graft survival, censored for recipient death with a functioning graft, of all transplanted kidneys (Figure 2.2A) and functioning kidneys alone (Figure 2.2B), with donor age categorized in groups. Graft survival of all transplanted kidneys was significantly lower for donor kidneys of 54 years and older, but graft survival of functioning kidneys was equivalent in both groups. Recipient survival was similar in both groups (Figure 2.2C).

Figure 2.2: Kaplan-Meier graft survival curves of A) all transplanted uncontrolled DCD kidneys, B) functioning kidneys only, all censored for recipient death with a functioning graft, and C) recipient survival.







Mean eGFR of functioning grafts was 36.2 mL/min/1.73 m^2 at 3 months, 40.7 mL/min/1.73 m^2 at 1 year, and 45.0 mL/min/1.73 m^2 at 5 years after transplantation. Donor age was significantly correlated with a decrease in eGFR (Pearson's r=-.418, p<0.001) at 1 year after transplantation and at 5 years after transplantation (Pearson's r=-.497, p<0.001).

In the Cox proportional hazards model, donor age was significantly associated with death censored graft failure of primarily functioning kidneys (HR 1.038, 95% CI 1.015 – 1.060, p<0.001) (Table 2.2).

Discussion

The use of kidneys from controlled Maastricht category III DCD donors for transplantation is generally accepted in most countries where it is legal to withdraw futile medical treatment[17,18]. The pool of controlled donors after cardiac death, however, is limited. Only a small proportion of intensive care patients, in whom further treatment is considered to be futile and who are not brain-dead, meet donation criteria. The number of patients who die after failed resuscitation is much higher[6]. Therefore, these patients can be a valuable addition to the DCD donor pool as uncontrolled Maastricht category I and II DCD donors. However, uncontrolled DCD donor kidneys are rarely used. There is a general reluctance to use uncontrolled DCD kidneys, with only a few uncontrolled DCD programs, mainly in Spain and in France[19].

In the present study, Maastricht categories I and II are analyzed together as 'uncontrolled donors'. It is difficult to distinguish category I from category II DCD donors, estimate resuscitation time, and ischemic damage. In the Netherlands, patients are not taken to the hospital after declaration of death on the street as category I donors are in Spain. It is forbidden to transport dead people to a hospital, but allowed for resuscitation only. So, potential donors are resuscitated and if the patient dies, only in the hospital death is declared. This results in longer resuscitation periods of our category II donors than the category I donors in Spain.

The use of uncontrolled kidney donors has led to an increase in the number of available donor kidneys for transplantation at our center. A recent study from our group showed no difference in transplant outcomes of uncontrolled compared with controlled donor kidneys[6].

The incidence of PNF was relatively high in this group of patients, which enabled us to adequately assess risk factors for non-function after transplantation. Reasons for the high incidence of PNF may be influenced by several factors. First, the definition of PNF used in our center is permanent inadequate renal function necessitating continuation of dialysis or retransplantation. This includes kidneys which failed because of hyperacute rejection, graft thrombosis, and surgical complications. Second, the CIT with a mean period of 27 hours is much longer than in other studies. All kidneys are allocated according to the Eurotransplant allocation rules. As many centers are reluctant to accept marginal DCD kidneys and decline an organ offer, it usually takes hours before kidneys are finally offered to our center. Third, because of the shortage of donor organs, we had a liberal acceptance strategy for DCD kidneys. Despite our finding of a higher incidence of PNF and DGF in kidneys from DCD donors, patients who receive a DCD kidney have better survival rates than patients who continue dialysis treatment while waiting for a kidney from a conventional brain-dead donor[3,20,21].

Kidneys from DCD donors that overcome the early post-transplantation period function as long as DBD (donation after brain death) kidneys. Contrary to DBD kidneys, DGF in DCD kidneys hardly affects graft survival[22,23]. As transplantation of non-viable kidneys results in unnecessary risk of surgery and immunosuppression, and sensitizes the recipient for future transplants, it is essential, particularly for DCD kidneys with a relatively high risk of PNF, to adequately assess the risk of non-function after transplantation[21].

Our results show that only donor age is independently associated with PNF. The predictive value of donor age only was poor, but adding the clinically important variables WIT, CIT and renovascular resistance during machine perfusion to the model, the predictive value increased to fair. Kidneys from donors of 54 years and older do worse than younger donor kidneys; however, like in controlled DCD kidneys, an absolute cut-off value cannot be provided. In a relatively large group of controlled and uncontrolled DCD kidneys, the results of pre-transplant biopsy increase the predictive value for graft survival in older donor kidneys[24].

Kidneys from DCD donors are inevitably subjected to a period of warm ischemia. Our data, however, shows no significant association between WIT and PNF. This may be biased as a total WIT longer than 45 minutes was a reason to discard kidneys for transplantation. Despite this potential bias, we found a trend towards significance in the multivariate analysis. In controlled DCD kidneys, a WIT longer than 45-60 minutes is also associated with an increased risk of PNF and DGF[25,26].

In our present study of uncontrolled DCD kidneys only, renovascular resistance was not associated with PNF. In a previous study from our group, we have shown that renovascular resistance of all Maastricht category II and III machine perfused DCD kidneys at the beginning of machine perfusion is independently associated with PNF, however, its predictive value is low[27].

Perfusate biomarkers of machine-perfused kidneys, including glutathione S-transferase (GST) and lactate dehydrogenase (LDH), have been used to identify risk factors for PNF and DGF in DCD kidneys[28-31]. In some studies, GST has a strong correlation with graft viability, but others have not been able to confirm this association[29-31]. For LDH concentration, no correlations were found with short-term graft function[29,31,32]. This makes it hard to discard DCD kidneys based purely on perfusate biomarker concentrations. In the present study, GST and LDH concentrations were not correlated with PNF.

Direct cannulation of the aorta is the method of choice to preserve kidneys of DCD donors[33]. In the Netherlands, minor invasive procedures are allowed to preserve the organs of potential donors before consent of the relatives and if a patient is not registered with objection in the national donor registry. Therefore, laparotomy with direct cannulation of the aorta is not feasible in uncontrolled donors. In this study, ISP with cold perfusate has been used to preserve donor kidneys. This technique is associated with longer WITs, a higher discard rate, and inferior graft survival compared to direct aortic cannulation[6]. The use of normothermic extracorporeal membrane oxygenation (ECMO) after cardiac arrest may be a valuable alternative for hypothermic ISP with the DBTL-catheter. Advantages of ECMO include recirculation of oxygenated blood until organ procurement, maintaining or restoring adenosine levels, and it may offer the opportunity of viability testing of normothermic perfused kidneys[34,35]. This technique has the potential to improve organ quality of ischemically damaged uncontrolled DCD kidneys with better graft function and graft survival[36,37]. In addition, preservation with ECMO after cardiac death may provide the opportunity to procure more organs, including the liver, so that they can be used for transplantation[34]. However, more clinical evidence of the benefits of ECMO is necessary[38].

It cannot be excluded that there are more risk factors for poor uncontrolled DCD kidney function than old age. Despite the relatively high percentage of PNF, which increases the power to identify risk factors and, compared with other studies, the large group of DCD transplantations, the number of analyzed donor kidneys remains relatively small to identify all possible risk factors for PNF.

The present study shows that donor age is associated with graft function of uncontrolled DCD kidneys, so this may influence the decision to accept or discard an uncontrolled DCD kidney.

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Chapter 3

Machine perfusion viability testing

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Abstract

Purpose of review: Pre-transplant assessment of kidney graft viability may help clinicians to decide whether to accept or discard a kidney for transplantation. With the increasing demand for donor kidneys and the increased use of marginal kidneys, the need of viability markers has increased to pursue superior transplant outcomes. Hypothermic machine perfusion (HMP) provides the theoretical opportunity to assess viability of donor kidneys. We discuss novel developments in viability testing during HMP and address future prospects.

Recent findings: HMP viability testing has focused on analysis of machine perfusion parameters and perfusate biomarkers. Renal resistance and the biomarkers LDH, AST, GST, NAG, H-FABP, LPOP, redox-active iron and IL-18 are correlated with transplant outcome in terms of development of DGF or graft survival. However, they all lack adequate predictive value for transplant outcome. New techniques including contrast-enhanced ultrasound, three-dimensional ultrasound and MR-spectrometry are promising methods to test kidney viability during HMP, but their value has to be established. The introduction of normothermic machine perfusion offers other promising opportunities for viability testing.

Summary: Machine perfusion characteristics and perfusate biomarkers have been extensively studied. They often correlate with transplant outcome, but the present viability tests are not reliable predictors of transplant outcome. New developments in kidney graft viability assessment are necessary to have a chance of being clinically useful in the future.

Introduction

Selection of suitable kidneys for transplantation is important to reduce the risk of primary non-function (PNF) and delayed graft function (DGF) after transplantation, which is associated with an increased risk of graft loss, acute rejection, longer hospital stay and high costs[1-3,4*]. The shortage of donors and the increasing use of marginal donors make donor kidney selection even more important. Although donor characteristics, including age and associated disease, and procurement methods are associated with kidney transplant function, there are no true kidney viability markers. Predicting kidney function and assessing the chance of graft survival prior to transplantation remains difficult. Due to the increasing use of marginal kidneys, there is an urgent need for better pre-transplant viability tests to avoid the risk of poor outcome after transplantation.

Hypothermic machine perfusion (HMP) is a technique to preserve donor kidneys. It has many advantages over cold storage (CS). In theory, continuous pulsatile flow through the kidney can provide nutrients and oxygen to the kidney and it can eliminate metabolic by-products and toxins. It decreases vasospasm and protects the endothelium, possibly due to flow-dependant genes. Furthermore, HMP offers the opportunity to apply therapeutic interventions, like the addition of pharmacological agents[5,6]. In a preclinical animal study, HMP was superior to CS in terms of functional recovery, oxidative stress, tissue histology and inflammatory parameters[7]. In human kidney transplantation there is high level evidence that HMP is associated with a decreased risk of DGF, improved one-year and 3-year graft survival, although the last was only proven for donation after brain death (DBD) kidneys and not for donation after cardiac death (DCD) kidneys[8-11].

HMP offers a good opportunity to test the viability of deceased donor kidneys. In the past, machine viability testing has been used to decide if kidneys should be discarded or not. Many have evaluated perfusate biomarker concentration and machine perfusion parameters as a tool to estimate graft viability. However, its use was often only justified by the argument that results of transplantation had improved after the introduction of machine perfusion viability testing. High quality evidence was usually not available[4*]. Recent literature provides more information about the association of machine perfusion viability testing and kidney function after transplantation and even more importantly, about its predictive value.

Machine perfusion parameters

During machine perfusion, pressure, flow and renal resistance (RR) are easily monitored. There is no common opinion about the best perfusion pressure of kidneys and used pressures vary between 20 mmHg and 60 mmHg[12,13]. Because pressure measurements are directly subjected to their manual setting, perfusion pressure itself has no role in outcome prediction or viability testing. Renal flow, although more often used than pressure, encounters similar problems. Flow depends on manual pressure settings, making a cut-off value for flow itself not reliable in clinical decision making[14]. Therefore, renal resistance (the ratio of perfusion pressure and renal flow) is used for viability testing instead.

It is estimated that approximately 15% of all machine perfused kidneys in the USA are discarded, partly because of high RR[15]. However, little is known about the predictive value of RR and other machine perfusion characteristics on transplant outcome or its use in selecting kidneys for transplantation[12,14,16,17]. Two studies have addressed the use of RR as viability marker without being biased by donor kidney selection prior to transplantation, i.e. discarding kidneys because of to high RR or low flow[18,19*].

The first study included 336 hypothermic machine perfused DCD and DBD kidneys of the European machine perfusion trial[8]. High RR at the end of machine perfusion was an independent risk factor of DGF (odds ratio (OR) 38.1; 95%CI 1.56 - 934); p=0.026) and for 1-year graft failure (hazard ration (HR) 12.33; 95%CI 1.11 - 136.85; p=0.004). However, the predictive value of RR on DGF was poor (area under the receiver operating curve (AURC) 0.58)[18].

The second study included 440 hypothermic machine perfused DCD kidneys. This study showed a significant and independent association of RR at the beginning of HMP with PNF (OR 2.040; 95% CI 1.362–3.056; p=0.001) and DGF (OR 2.345, 95% CI 1.110–4.955; p=0.025). However, the predictive value for PNF was poor (AURC 0.609)[19*]. This study focused on PNF instead of DGF, as DGF does not affect long-term survival in DCD kidneys[20].

Biomarkers

Machine perfusion enables viability testing by analyzing the washout of enzymes in the perfusate. These biomarkers include released contents of damaged cells leaked into the

interstitial space, free radical mediated molecules, measures of anti-oxidant status or inflammation associated proteins.

A large variety of biomarkers have been advocated as possible markers of kidney injury: lactate dehydrogenase (LDH), alanine aminopeptidase (Ala-AP), glutathione-S-transferase (GST) in different isoforms, aspartate transaminase (AST), lipid peroxidation products (LPOP), heart-type fatty acid binding protein (H-FABP), lactate, electrolytes, gas pressure (pO_2 and pCO_2), pH, osmolarity, N-acetyl-β-D-glucosaminidase (NAG), redox-active iron, malondialdehyde (MDA), total antioxidant status (TAS), glucose, acid phosphatase, analysed neutrophil gelatinase-associated lipocalin (NGAL), interleukin 18 (IL-18) and proteomics. The most important and recently studied biomarkers are discussed below. (Table 3.1[21, 22*, 26, 29, 31])

LDH was correlated with PNF in some studies and more often with DGF only, although its predictive value for DGF was poor (AURC 0.60)[4*,21]. Very recently these findings were confirmed in a unselected cohort of 335 DCD kidneys. LDH was associated with PNF as well as DGF in a multivariate analysis, corrected for confounding variables. However diagnostic accuracy for development of PNF was poor (AURC 0.699)[22*].

Ala-AP is a biomarker used in acute kidney injury and rarely described in a pre-transplant setting. Ala-AP was correlated with DGF, however with a poor predictive value (AURC 0.57)[21,23].

GST represents a family of cytosolic, microsomal and membrane-bound enzymes (total GST (tGST)), of which two of the eight cytoplasmic classes identified are of major interest. Alpha-GST, found in the proximal renal tubule, and π-GST, found in the distal renal tubule, are released during ischemia. Proximal tubules are metabolically more active, thus making α-GST in theory a more promising biomarker[23-25]. Total GST concentration was elevated in DGF and PNF kidneys, but with poor predictive values for DGF (AURC 0.67)[21] and PNF (AURC 0.58)[22*]. Analysis of GST in HMP perfusates of 111 kidneys from donors older than 55 years, showed that π-GST was not suitable to predict DGF. Alpha-GST had a better positive predictive value than tGST (71.4% and 37.5%, respectively), but is more expensive and time-consuming to measure. After multivariate analysis none did seem to be an independent predictor of DGF after transplantation[26].

LPOP, a combination of products released after oxidative stress, was also examined in the last mentioned study. LPOP concentration is proposed to be an index for the

effects of reactive oxygen species, which are thought to play a major role in ischemic injury[3,27,28]. LPOP concentration was an independent predictor of DGF after transplantation (OR 2.026; 95% CI 1.129 – 3.637; p=0.018)[26].

H-FABP was addressed in two studies. It is involved in the uptake of fatty acid from the cytosol to the mitochondria[25]. H-FABP was noted to have a poor predictive value for DGF (AURC 0.64)[21] and PNF (AURC 0.56)[22*].

AST showed a significant association with DGF after transplantation with a poor predictive value (AURC 0.61)[21].

Perfusate redox-active iron concentration was significantly higher in DCD kidneys than in DBD kidneys, explained by the increased ischemic injury of DCD kidneys. Perfusate concentration in DCD kidneys was significantly associated with DGF and PNF, with increased risk of DGF (OR 1.532; 95% CI 1.045 – 2.245; p=0.029) and PNF (OR 1.68; 95% CI 1.13 – 2.49; p=0.01). The predictive value for PNF in a univariate analysis was poor (AURC 0.625), but increased to fair (AURC 0.701) in a multivariate analysis[22*,29].

NAG was addressed in one high quality study. The perfusate sample was taken at the end of machine perfusion and showed a poor predictive value for DGF (AURC 0.64)[21].

NGAL is expressed in a wide variety of tissues, including renal tubular epithelium, and rapidly expressed after injury of tubular epithelium[24]. NGAL has been used as a biomarker for acute renal injury; the concentration in perfusate of machine perfused porcine kidneys was proportionate to the amount of prior warm ischemic injury[30]. Recently it has been studied as viability predictor in DCD kidneys. Although perfusate concentration was associated with transplant outcome, its predictive value for PNF was poor (AURC 0.503)[22*].

The pro-inflammatory cytokine IL-18 has been addressed once. In DCD kidneys it was associated with an increased risk of PNF and DGF (OR 1.001; 95% CI 1.000 – 1.002; p=0.003 and OR 1.003; 95% CI 1.001 – 1.004; p=0.002, respectively). Its predictive value for PNF in a univariate analysis was poor (AURC 0.591), but increased to fair (AURC 0.700) in a multivariate analysis[22*].

A proteomics study of machine perfusate of 18 kidneys (6 DBD, 6 controlled DCD and 6 uncontrolled DCD kidneys) identified 19 unique proteins with the use of two-

dimensional difference gel electrophoresis and mass spectrometry. In DCD kidneys, two unidentified proteins were significantly up-regulated and one protein, identified as haptoglobin, was significantly down-regulated, compared to DBD kidneys. Furthermore, two proteins were up-regulated in PNF kidneys and 1 protein, identified as α1-antitrypsin, was up-regulated in DGF kidneys. Thus, this study introduced a series of novel biomarkers of ischemic acute renal injury in human donor kidneys. However, their diagnostic value in viability testing has to be determined in future studies[31].

Table 3.1: Perfusate biomarkers.

Biomarker	Origin	Marker of	DCD/DBD kidneys[1]	Predictive value for[2] DGF	PNF	References
LDH	Cytoplasm of various body cells, including brush border of proximal renal tubule.	Non-specific cellular injury	DCD, DBD	Poor	Poor	(21, 22*, 29)
Ala-AP	Intestine and brush border of proximal renal tubule	Tubular injury	DCD, DBD	Poor	-	(21)
Total GST	Cytoplasm of various body cells, including proximal and distal renal tubule	Tubular injury	DCD, DBD	Poor	Poor	(21, 22*, 26, 29)
Alpha-GST	Cytoplasm of various body cells, including proximal renal tubule	Proximal tubular injury	DCD, DBD	-	-	(26)
Pi-GST	Cytoplasm of various body cells, including distal renal tubule	Distal tubular injury	DCD, DBD	-	-	(26)
LPOP	Various body cells	Oxidative stress	DCD, DBD	-	-	(26)
H-FABP	Heart, small intestine, skeletal muscle and distal renal tubules.	Ischemic cellular injury	DCD, DBD	Poor	Poor	(21, 22*)
AST	Several tissues, including renal parenchymal cells	Parenchymal cell injury	DCD, DBD	Poor	-	(21)
Redox-active iron	Several organ systems	Ischemia/reperfusion injury	DCD	-	Fair[3]	(22*, 29)
NAG	Lysosomes of various tissues, including proximal renal tubule	Acute oxidative stress	DCD, DBD	Poor	-	(21)
NGAL	Several tissues, Renal tubular epithelial cells	Tubular epithelial injury	DCD	-	Poor	(22*)
IL-18	Produced by macrophages and other cell types	Inflammatory reactions during ischemia/ reperfusion injury	DCD	-	Fair[3]	(22*)
Perfusate Proteomes	Unknown	Ischemic acute renal injury	DCD	-	-	(31)

[1] Kidneys on which a predictive value was based on; [2] Predictive value was derived from the area under the receiver operating curve of univariate analyses of individual biomarkers (1: perfect; 0.9-1: excellent; 0.8-0.9 good; 0.7-0.8 fair; <0.7: poor); [3] Predictive value after multivariate analysis.

Novelties in viability testing

In past decades machine perfusion viability testing mainly focused on perfusate biomarkers and machine perfusion parameters.

Contrast-enhanced ultrasound (CEUS) and three-dimensional ultrasound (3DUS) have recently been introduced as a new technique for assessing renal transplant perfusion during HMP. Five pig kidneys were procured after a period of 30 minutes of warm ischemia and placed on a hypothermic perfusion machine. Following a bolus injection of a contrast solution (tiny bubbles of inert gas, which pass through the capillary bed but not through the interstitial space or blood/brain barrier and which increase ultrasound signal return), 2D and 3D ultrasound scans were performed. These techniques were fast, safe, aseptic and non-invasive methods to assess perfusion of the kidney before transplantation[32]. In the future, this might be useful to asses organ viability, in addition to machine perfusion parameters like flow and renal resistance, albeit their value has to be assessed in large human cohorts first.

Magnetic resonance spectrometry (MRS) has been studied before to assess ischemic injury and viability of kidneys on CS[33-37]. Recently, MRS has been used to assess viability during HMP with a newly developed magnetic resonance compatible perfusion machine[38-40]. This machine has been used to oxygenate porcine kidneys during hypothermic preservation and to measure chemical shifts during HMP with the use of ^{1}H and ^{31}P-MRS. ^{31}P-MRS showed to be a valuable tool to assess ATP levels in kidneys without warm ischemia, if appropriate O_2 tension was applied to the preservation medium. MRS during HMP may also be useful to study preservation conditions and warm ischemic effects in a clinical setting[40]. Although the number of kidneys studied until now is limited, the idea of measuring ATP levels is interesting and promising as it is a measure of energy depletion after ischemic injury. Its value to predict organ viability has to be proven.

Future prospects

Kidney viability testing has benefited from the introduction of HMP, although the predictive value of machine perfusion resistance and of different perfusate biomarkers is too low to obtain a useful predictor of kidney transplant outcome. Normothermic machine perfusion (NMP) may provide new and other opportunities to assess the quality of a donor organ before transplantation. Furthermore, NMP may ameliorate ischemic damage and resuscitate the organ instead of doing further harm and preventing the graft from injury during the preservation period[41].

The Leicester group showed in a porcine kidney autotransplant model, that after a period of 20 hours of HMP, kidneys can be safely perfused with NMP for 2 hours with autologous blood[42]. Thereafter, they perfused a human kidney with a warm solution of cross-matched packed red cells with crystalloids, nutrients and some medication, shortly before transplantation, which shows that short term NMP is feasible in a clinical setting[43*]. Now NMP seems feasible and safe, it may have large potential to improve machine perfusion viability testing. The major advantage of NMP to test organ viability is the possibility of measuring functional parameters, like diuresis and creatinine clearance. In a normothermic setting with normal kidney metabolism, cellular injury may be diagnosed by cellular dysfunction instead of by markers of cell death, ischemia or inflammation. Functional measurements may provide key parameters of kidney viability and may be more accurate and useful in transplant outcome prediction than any of aforementioned viability markers[44,45].

Conclusion

We conclude that machine perfusion viability testing has not offered clinicians the expected tools to assess kidney viability before transplantation. Currently available viability tests during HMP are not reliable enough to decide to discard a donor kidney prior to transplantation. New biomarkers and novel techniques may provide tests with better predictive value than the tests used today, but their value has to be proven. Viability testing during normothermic perfusion offers a novel opportunity to assess the quality of a donor kidney, as the function of metabolically active organs is tested, instead of surrogate indicators of kidney injury.

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Novel viability assessment strategies in human renal transplantation

Chapter 4

Ischemia reperfusion injury parameters in reperfusion biopsies: association with impaired graft recovery and more IFTA at year 1 post renal transplantation

Published as

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Submitted

Abstract

Background: Ischemia reperfusion injury (IRI) causes non-immediate graft function (non-IGF = delayed graft function (DGF) or Primary non-function (PNF)) after kidney transplantation. The value of histological assessment of IRI parameters in peri-operative transplant biopsies was investigated in a retrospective single-centre observational study.

Methods: Reperfusion biopsies of 253 kidney transplantations were retrospectively scored on a semi-quantitative scale (0-3) for 'brush border loss', 'tubular cell necrosis', and 'neutrophil influx' in glomerular, and peritubular capillaries. Their relation with donor, recipient, and transplant characteristics, as well as with clinical short-term outcome and 1-year intermediate renal function (eGFR) and interstitial fibrosis and tubular atrophy (IF/TA) was analysed.

Results: Kidneys from living donors had lowest IRI scores, whereas kidneys from deceased after circulatory death (DCD) donors showed highest scores. Of the 4 IRI parameters, the presence of 'brush border loss', 'tubular cell necrosis' and 'neutrophil influx in PTCs' were related to non-IGF in univariable analysis, while 'neutrophil influx in glomerular capillaries' was not. Only 'loss of brush border' was an independent risk factor for non-IGF. 'Loss of brush border' was an independent risk factor for impaired renal function after one year, 'neutrophils in PTCs' was an independent risk factor for IF/TA.

Conclusion: 'Brush border loss' is independently related to non-immediate graft function and impaired eGFR at 1-year. 'Neutrophils in PTCs' is associated with more IF/TA at 1-year. These data suggest that assessment of IRI parameters may aid in predicting an unfavourable course posttransplant and by this guide possible interventions to prevent impaired graft recovery after transplantation.

Introduction

Ischemia reperfusion injury (IRI) is inevitable during kidney donation and transplantation. Ischemia is caused by renal hypoperfusion and hypoxia before and during donation and it induces changes in metabolism that lead to cellular injury and, when sustained, to cell death by apoptosis and necrosis[1-3]. After implantation of the kidney and restoration of the circulation, reperfusion injury is caused by increase in oxygen levels and normalising of pH in the ischemic cells which induces additional cell injury, cell death, complement activation and start of inflammation[1,2].

IRI may lead to non-immediate graft function (non-IGF, i.e. delayed graft function (DGF) or primary non-function (PNF))[1,2]. DGF is associated with longer hospitalisation, a higher risk of acute rejection, lower eGFR, and worse graft survival[4-6]. The incidence of DGF is rising as especially grafts from older postmortal donors and extended criteria donors are increasingly being accepted for transplantation[2,7]. Therefore, it is important to identify risk factors for development of DGF to take preventive measures and evaluate their effectiveness. Clinical risk factors for DGF are, amongst others, cold and warm ischemia time, donor age, and donor type, i.e. donation after circulatory death (DCD)[8,9]. Furthermore, surrogate markers of IRI are tested to predict the risk of DGF, such as serum, urine or machine perfusate biomarkers[10-12]. However, these markers do not have added value in clinical practice yet[10].

Histological assessment of chronic damage in zero-time kidney biopsies (procurement or (pre)-implantation biopsies) is used to assess graft quality which can be used as a parameter in the acceptance decisions[13]. Studies indicate that chronic histological damage parameters, including glomerulosclerosis, interstitial fibrosis and tubular atrophy, in zero-time biopsies are associated with the development of DGF[14-17] and worse graft function and graft survival (reviewed in[18]). Studies of the association of acute kidney injury parameters in zero-time biopsies with DGF, graft survival and graft function show contradictory results[18,19]. However, assessment of 'acute tubular injury' in preimplantation biopsies is at present advised by the Banff working group on preimplantation biopsy[20].

The objective of this retrospective observational single centre cohort study is to investigate the value of histological assessment of acute injury, in reperfusion biopsies, for transplant outcome the first year after transplantation. We hypothesize that the incidence and grade of the IRI parameters are associated with baseline and clinical

parameters known to be related to non-IGF and that they are related to a worse renal function and IF/TA score at 1 year.

Materials and methods

Donor, graft, recipient and biopsy selection

All single kidney transplantations of recipients older than 16 years on a tacrolimus-based immunosuppressive regimen at Maastricht University Medical Center+ (MUMC+) between April 2003 and December 2009 were retrospectively reviewed. Included were kidneys after living donation (LD), donation after brain death (DBD), and donation after circulatory death (DCD). Cases were included if there was a representative reperfusion biopsy available. Renal needle core biopsies were taken per protocol shortly after reperfusion (M0) by the transplant surgeon for all donor types. After collection, tissue is immediately placed in formalin and handed over to the pathology department for further work-up. Protocollary, at month 12 (M12) posttransplant another needle core biopsy is taken, and this was also studied. Forty cases had no (representative) M12 biopsy because of graft failure <12M (n=22), missing M12 biopsy (n=16) or non-representative M12 biopsy (n=2). Collection, storage and use of tissue and patient data were performed in agreement with the Code of conduct for 'Proper Secondary Use of Human Tissue', as described by 'The Federation of Dutch medical scientific societies' (http://www.federa.org). Permission for study of renal biopsies in relation to graft function was obtained from the Medical Ethical Committee of the MUMC+ (MEC-09-4-002).

Histological analysis of renal biopsies

Paraffin tissue sections for light microscopy were stained with haematoxylin-eosin (HE), periodic acid Schiff (PAS) and methenamine silver periodic acid-Schiff, according to standard protocols. Biopsies were considered representative if they contained at least five glomeruli and one interlobular artery. Histopathological analysis was performed retrospectively by a blinded nephropathologist (CPK). All cases with a representative reperfusion (M0) and M12 biopsy were scored by a second blinded nephropathologist (LMH) for interobserver analysis. Unless mentioned otherwise, data of CPK are given. We scored four parameters for ischemia reperfusion injury. For tubular injury, we scored two parameters, which are also components of the acute injury scoring of the Banff working group[20], i.e. 'tubular cell necrosis' and 'loss of brush border'. In addition, we scored two parameters that reflect reperfusion injury and were previously used[21,22], i.e. 'neutrophil

influx in glomerular capillaries', and 'neutrophil influx in peritubular capillaries (PTC)'. All IRI parameters were scored on a semi-quantitative 4-point scale (absent, mild, moderate, or severe). In both reperfusion and one-year biopsies, interstitial fibrosis and tubular atrophy (IF/TA) was scored according to the Banff criteria[23].

Clinical parameters

Donor, graft, and recipient characteristics were retrieved from patient files and transplant registries. The following parameters were included for analyses:

- Donor and donation characteristics: age, gender, BMI, donor type and warm ischemic time (WIT);
- Recipient characteristics: age, gender, Body surface area (BSA), baseline immunosuppression, immunisation status, and retransplantations;
- (Post)transplantation characteristics: cold ischemic time (CIT), anastomosis time, DGF, rejection within the first year and renal function after 1 year measured by the Chronic Kidney Disease Epidemiology Collaboration (CKD-epi) equation based on serum creatinine.

The immunosuppressive regimen consisted of tacrolimus (TAC) with either sirolimus (SRL) or mycophenolate mofetil (MMF) and steroid-withdrawal at day 10 in low-risk patients, as described earlier in detail[24]. No induction therapy was given. In accordance with Eurotransplant criteria, recipients were considered immunised when panel reactive antibodies (PRA) in the current serum was >5%. DGF is defined as any need for temporary dialysis treatment starting within the first week after transplantation. Within the DGF group cases that never functioned were denoted as primary non function (PNF).

Statistics

Continuous variables are presented as mean ± standard deviation (SD) and categorical parameters as frequency (percentage). The demographical, clinical, and histological data are presented for all donors as well as per donor type: LD, DBD, and DCD. Differences between donor types were tested by one-way analysis of variance (ANOVA), t-test, Chi-square test or Fisher Freeman Halton exact test, where appropriate.

Correlations were studied according to Pearson's or Spearman's rho where appropriate, and rho and p-values are given. Interobserver correlations (ICC) with 95% confidence intervals (95% CI) were analysed by using two-way random model, single measurement, absolute agreement.

Although histological IRI parameters and IF/TA are ordinal variables, they were used as continuous variables in univariable and multivariable analyses. Univariable binary logistic regression was used to test associations of IRI, histological, donor, recipient and transplant parameters with non-IGF. First, a multivariable 'clinical' model was made for non-IGF, using multivariable analysis with backward elimination. The baseline model was made based on the input parameters which had a p-value <0.2 in the univariable analysis: donor type, donor gender, anastomosis time, recipient age and gender, and retransplantation. Cold and warm ischemia times were not included in the multivariable analysis since they are strongly associated with donor type. Only donor type was significant in the 'clinical model'. As second step we tested whether IRI parameters had independent added significance by adding the IRI parameters with backward elimination to the 'clinical' model. Odds ratios (95% CI) were presented.

Relationship with renal function one year after transplantation was analysed by linear regression using univariable and multivariable analysis. First, a baseline 'clinical' model was made by backward elimination using the following parameters as input parameters: donor type, donor age, donor gender, recipient age, recipient gender, anastomosis time, retransplantation, immunisation, rejection, baseline immune-suppression and baseline IF/TA. To test whether IRI parameters are independent risk factors for renal function a multivariable linear regression with backward elimination was performed. To test the added value of IRI parameters on known risk factors for IF/TA M12, IRI parameters were added to risk factors found in Chapter 3 of this thesis and a multivariable logistic regression with backward elimination was performed.

A p-value ≤0.05 is considered statistically significant. All data analyses were performed using IBM SPSS Statistics for Windows (version 25.0, Armonk, NY: IBM Corp).

Results

Study cohort and characteristics of the study population

During the studied period, 388 kidneys were transplanted on a tacrolimus based immunosuppressive regimen, and therefore eligible for inclusion. Of these transplantations, 135 were excluded for the following reasons: in 64 transplants no reperfusion biopsy was taken, in 49 cases the T0 biopsy was not available for scoring, and in 22 cases the reperfusion biopsy was not representative. The study cohort thus consisted of 253 transplantations. Of note, donor and transplant characteristics of the 135 excluded transplants were not statistically different from the included cases. The

donor, recipient, and transplant characteristics of the study cohort are shown in Table 4.1.

Distribution of donor types in our cohort was representative of our hospital transplant program: 32% (n=80) living donors, 30% (n=75) DBD and 39% (n=98) DCD. DCD donors had a significantly longer cold ischemia time than DBD donors (p=0.014), and an elongated anastomosis time (p<0.001). Non-IGF occurred in 98 (39%) of all grafts and was mostly seen after DCD (n=73, 75%). There were 8 cases with PNF: 2 were of DBD grafts and 6 of DCD grafts. Renal function at 1 year was lowest after DCD donation (LD: eGFR 48 ml/min/1.73m^2 ± 19; DBD 50 ± 18; DCD 41 ± 17; overall ANOVA p=0.005). Acute rejection incidence in the first year was 27% (n=69) and there were no significant differences between donor types (X^2=2.832, p=0.243).

Table 4.1: Description of the cohort- clinical and demographic parameters.

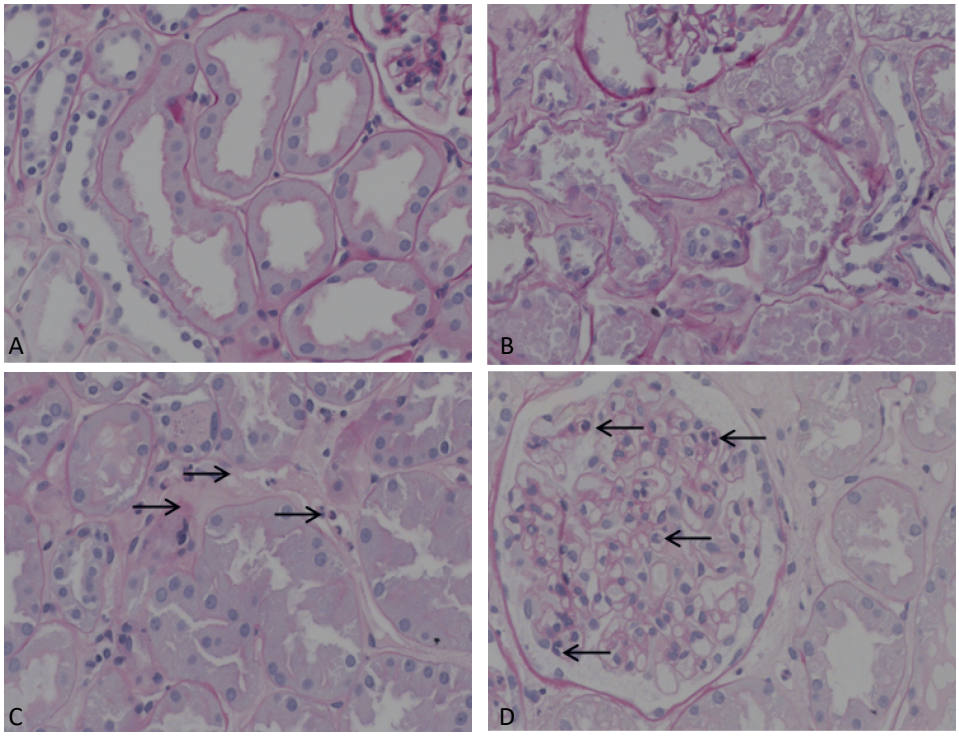
Variables	Total N=253	LD N=80	DBD N=75	DCD N=98	p-value
Donor characteristics					
Donor age (years)	50 ± 15	53 ± 12	48 ± 16	49 ± 17	p=0.110
Donor male sex (%)	139 (55%)	40 (50%)	35 (47%)	64 (65%)	p=0.029
Donor BMI (kg/m^2)	25 ± 4	26 ± 4	25 ± 4	25 ± 4	p=0.258
Recipient characteristics					
Recipient age (years)	54 ± 13	49 ± 14	55 ± 13	56 ± 13	p=0.002
Recipient male sex (%)	147 (58%)	40 (50%)	35 (47%)	72 (74%)	p<0.000
Recipient BSA (m^2)	1.86 ± 0.21	1.85 ± 0.2	1.84 ± 0.2	1.90 ± 0.22	p=0.169
Retransplantation (%)	59 (23%)	9 (11%)	30 (40%)	20 (20%)	p<0.001
Immunosuppression					p=0.711
TAC-MMF (%)	132 (52%)	44 (55%)	40 (53%)	48 (49%)	
TAC-SRL (%)	121 (48%)	36 (45%)	35 (47%)	50 (51%)	
Immunized (PRA>5) (%)	22 (9%)	2 (3%)	17 (23%)	3 (3%)	p<0.001
Transplantation characteristics					
WIT; DCD only (min) (range) (1 missing)				23 ± 10 (5-59)	
CIT; postmortal only (hours) (range) (2 missing)	20 ± 6 (0-40)		19 ± 6 (0-36)	21 ± 6 (11-40)	P=0.014
Anastomosis time (min) (range) (7 missing)	34 ± 12 (10-91)	28 ± 9 (15-71))	34 ± 12 (16-79)	38 ± 13 (10-91)	p<0.001
Post-transplant characteristics					
IGF (%)	155 (61%)	77 (96%)	53 (71%)	25 (25%)	p<0.001
Non-IGF (%)	98 (39%)	3 (4%)	22 (29%)	73 (75%)	
PNF (%)		0 (0%)	2 (3%)	6 (6%)	

Differences between the donor types were tested by ANOVA, T-test or Fisher-Freeman-Halton exact test where appropriate. Abbreviations: LD Living Donor, DBD Donation after brain death, DCD Donation after circulatory death, BMI body mass index, BSA body surface area, TAC tacrolimus, MMF mycophenolate mofetil, SRL sirolimus, WIT warm ischemia time, CIT Cold ischemia time, IGF immediate graft function, PNF primary non-function.

Frequencies of IRI parameters in M0 biopsies and their association with baseline parameters

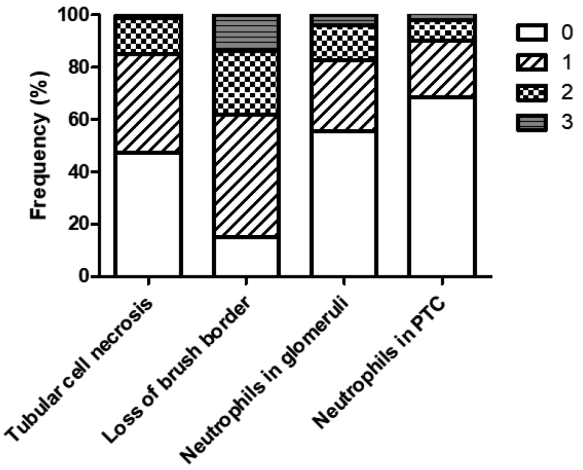
Representative examples of IRI parameters are shown in Figure 4.1. All IRI parameters were frequently detected in reperfusion biopsies (Figure 4.2). Tubular injury markers 'loss of brush border' and 'tubular cell necrosis' showed strong mutual correlation (ρ=0.649, $p<0.01$), whereas we observed a moderate correlation between both neutrophil influx markers 'neutrophil influx in glomeruli' and 'neutrophil influx in PTCs' (ρ=0.496, $p<0.01$). There were no statistically significant correlations between the tubular injury and the neutrophil influx markers. Regarding interobserver correlation: 194 biopsies were scored by two pathologists and the ICC's were poor: for 'tubular cell necrosis' ICC=0.300 (95% CI 0.169-0.421), for 'loss of brush border' ICC=0.197 (95% CI 0.029-0.348), for 'neutrophils in glomeruli' ICC=0.279 (0.147-0.402), and for 'neutrophils in PTCs' ICC=0.197 (0.061-0.327).

Figure 4.1: Histopathological examination of transplant kidney biopsy for ischemia reperfusion injury parameters.



Representative pictures of PAS stained reperfusion biopsies. Pictures were taken at objective 40x A) reperfusion biopsy without IRI parameters present. B) reperfusion biopsy with tubular cell necrosis and loss of brush border. C) biopsy with additionally neutrophils in peritubular capillaries. Arrows indicate neutrophils. D) reperfusion biopsy with also neutrophils in glomerulus. Arrows indicate neutrophils.

Figure 4.2: Presence of histological ischemia reperfusion injury parameters in reperfusion biopsies.



Distribution of ischemia reperfusion injury in reperfusion biopsies. Data is given as percentages.

The distribution of the IRI parameters over the donor types was significantly different (Table 4.2). From living to DBD to DCD grafts, there was a trend toward more 'tubular cell necrosis' (p=0.091) and 'neutrophils in glomeruli' (p=0.093). There were statistically significant more 'loss of brush border' in DBD and DCD than in LD (overall p=0.010) and statistically significant more 'neutrophils in PTC' in DCD than in LD and DBD (overall p=0.003).

Next, correlations of histological IRI parameters with baseline clinical recipient, donor and transplant factors were studied (see Table 4.3). Overall, the statistically significant correlation is at most weak, with significantly more 'tubular cell necrosis' in grafts with longer anastomosis time or from male donors, and more 'loss of brush border' in grafts with longer anastomosis time or male recipients, and more 'neutrophils in PTC' in grafts from male donor or longer cold ischemia time.

Table 4.2: Prevalence of ischemia reperfusion injury parameters in reperfusion biopsies subdivided per donor type.

	Total n=253	LD n=80	DBD n=75	DCD n=98	Fisher Freeman Halton exact p-value
TCN					
0	120 (47%)	45 (56%)	38 (51%)	37 (38%)	0.091
1	95 (38%)	29 (36%)	24 (32%)	42 (43%)	
2	35 (14%)	6 (8%)	12 (16%)	17 (17%)	
3	3 (1%)	0 (0%)	1 (1%)	2 (2%)	
LBB					
0	38 (15%)	21 (26%)	8 (11%)	9 (9%)	0.010
1	119 (47%)	39 (49%)	39 (52%)	41 (42%)	
2	61 (24%)	13 (16%)	17 (23%)	31 (32%)	
3	35 (14%)	7 (9%)	11 (14%)	17 (17%)	
PMN in glom					
0	141 (56%)	53 (66%)	43 (57%)	45 (46%)	0.093
1	68 (27%)	18 (23%)	22 (29%)	28 (29%)	
2	34 (13%)	8 (10%)	7 (9%)	19 (19%)	
3	10 (4%)	1 (1%)	3 (4%)	6 (6%)	
PMN in PTC					
0	173 (68%)	61 (76%)	58 (77%)	54 (55%)	0.003
1	55 (22%)	16 (20%)	14 (19%)	25 (26%)	
2	20 (8%)	2 (3%)	3 (4%)	15 (15%)	
3	5 (2%)	1 (1%)	0 (0%)	4 (4%)	

Differences between donor types were tested using Fisher Freeman Halton exact test. Abbreviations: LD living donor, DBD donation after brain death, DCD donation after cardiac death, TCN tubular cell necrosis, LBB loss brush border, PMN polymorphic neutrophils, glom glomeruli, PTC peritubular capillary.

Table 4.3: Correlations of ischemia reperfusion injury parameters in reperfusion biopsies with baseline clinical variables.

Variables	Tubular cell necrosis	Loss of brush border	Neutrophils in glomeruli	Neutrophils in PTC
Recipient characteristics				
Age (years)	0.105 p=0.094	0.081 p=0.197	-0.072 p=0.257	0.016 p=0.795
Gender (female reference)	0.112 p=0.075	0.123 p=0.050	0.069 p=0.272	0.092 p=0.145
Donor characteristics				
Age (years)	-0.040 p=0.527	-0.056 p=0.372	0.000 p=0.994	0.048 p=0.449
Gender (female reference)	0.024 p=0.794	-0.098 p=0.118	0.094 p=0.135	0.258 p<0.001
Transplant characteristics				
WIT; DCD only (min)	0.176 p=0.084	0.114 p=0.268	0.157 p=0.125	0.192 p=0.060
CIT; postmortal only (hour)	0.023 p=0.769	0.001 p=0.994	0.113 p=0.138	0.262 p=0.001
Anastomosis time (min)	0.173* p=0.007	0.167 p=0.008	0.028 p=0.658	0.059 p=0.355

Correlation for IRI parameters in the reperfusion biopsies with donor and transplantation characteristics were tested using Pearson's' or Spearman's rho. Abbreviations: BMI body mass index, WIT warm ischemia time, CIT cold ischemia time. * p<0.05; ** p<0.01.

Association of IRI parameters with early graft function

We studied the association of IRI parameters with early graft function (Table 4.4 and Table 4.5). There are statistically significant higher scores in the DGF and PNF group compared to the IGF group in 'tubular cell necrosis' (p=0.001), 'loss of brush border' (p=0.008) and 'neutrophils in PTC' (p=0.013), but not in 'neutrophils in glomeruli' (p=0.328). There were no significant differences in IRI scores between DGF and PNF except for 'tubular cell necrosis': the PNF group had significant higher scores than the DGF group (p=0.038).

Furthermore, we analysed univariable and multivariable associations of IRI and clinical parameters for non-IGF (Table 4.5). In line with literature, we found that donor type, cold ischemia time in postmortal donors and anastomosis time were univariable associated with non-IGF. In multivariable analysis besides donor type, 'loss of brush border' remained a significant independent predictor of non-IGF.

Table 4.4: Relationship of postoperative graft function with ischemia reperfusion parameters in reperfusion biopsies.

	IGF N=155	DGF N=90	PNF N=8	Fisher Freeman Halton exact p-value
TCN				
0	84 (54%)	35 (39%)	1 (13%)	0.001
1	55 (36%)	38 (42%)	2 (25%)	
2	16 (10%)	14 (16%)	5 (63%)	
3	0 (0%)	3 (3%)	0 (0%)	
LBB				
0	30 (19%)	8 (9%)	0 (0%)	0.008
1	79 (51%)	38 (42%)	2 (25%)	
2	31 (20%)	26 (29%)	4 (50%)	
3	15 (10%)	18 (20%)	2 (25%)	
PMNs in glomeruli				
0	94 (61%)	43 (48%)	4 (50%)	0.328
1	40 (26%)	25 (28%)	3 (38%)	
2	16 (10%)	17 (19%)	1 (13%)	
3	5 (3%)	5 (6%)	0 (0%)	
PMNs in PTC				
0	117 (76%)	53 (59%)	3 (38%)	0.013
1	29 (19%)	23 (26%)	3 (38%)	
2	7 (5%)	11 (12%)	2 (25%)	
3	2 (1%)	3 (3%)	0 (0%)	

Differences between immediate graft function, delayed graft function and primary non-function were tested using Fisher-Freeman-Halton exact test. Abbreviations: LD living donor, DBD donation after brain death, DCD donation after cardiac death, TCN tubular cell necrosis, LBB loss brush border, PMN polymorphic neutrophils, glom glomeruli, PTC peritubular capillary.

Table 4.5: Associations of histological and clinical parameters with non-immediate graft function.

Variables	Univariable analysis		Multivariable analysis	
	Unadjusted odds ratio (95% CI)	p-value	Adjusted Odds ratio (95% CI)	p-value
Histologic IRI parameters				
Tubular cell necrosis	1.793 (1.268 – 2.535)	0.001	1.235 (0.701-2.178)	0.465
Loss of brush border	1.731 (1.289 – 2.326)	<0.001	1.476 (1.016-2.145)	0.041
Neutrophils in glomeruli	1.391 (1.036 - 1.868)	0.028	1.022 (0.667-1.564)	0.922
Neutrophils in PTC	1.792 (1.250 – 2.569)	0.002	1.271 (0.801-2.015)	0.309
Chronic histologic parameters				
IF/TA at M0	0.866 (0.544 – 1.379)	0.545		
Donor characteristics				
Gender (female reference)	2.008 (1.192-3.383)	0.009		
Age (years)	0.993 (0.977-1.009)	0.401		
BMI (kg/m2)	0.998 (0.937 – 1.063)	0.950		
Donor type				
Living	0.094 (0.027 – 0.330)	<0.001	0.103 (0.029 – 0.366)	<0.001
DBD	Reference			
DCD	7.035 (3.588 – 13.793)	<0.001	6.960 (3.519 – 13.768)	<0.001
Transplant characteristics				
Warm ischemic time (min)	1.03 (0.982–1.084)	0.211		
DCD only				
Cold ischemic time (hours)	1.082 (1.021 – 1.146)	0.007		
Postmortal only				
Anastomosis time (min)	1.049 (1.024–1.074)	<0.001		
Recipient characteristics				
Gender (female reference)	2.181 (1.280–3.714)	0.004		
Age (years)	1.021(1.002–1.042)	0.034		
BSA (m2)	1.897 (0.566 – 6.362)	0.299		
Previous transplantation	1.600 (0.888 – 2.884)	0.118		
Constant			0.234	

Univariable binary logistic regression was used to test associations with non-immediate graft function. Multivariable analysis with backward elimination was used in two steps: first a clinical model was made including only clinical variables with p<0.2 in univariable analysis were included. As a second step, the IRI parameters were added with backward elimination to the 'clinical model' which includes only donor type. Abbreviations: IF/TA, interstitial fibrosis and tubular atrophy; 95% CI, 95% confidence interval; DBD donation after brain death; DCD, Donation after cardiac death; DGF, delayed graft function; PNF; primary non function.

Association of histological IRI parameters with outcome at 1 year after transplantation

To study the additional value of IRI parameters, on top of relevant clinical parameters, for the prediction of renal function at 1 year after transplantation (eGFR), we first performed a multivariable analysis of clinical parameters (Table 4.6). The final model included donor type, donor age, acute rejection in the first year, and baseline immunosuppression. The IRI parameters were added by backward elimination to this model, and only 'loss of brush border' had an independent significant added value:

eGFR decreased by nearly 4 ml/min/1.73 m^2 for every point of 'loss of brush border' (Table 4.7A).

The relation between the IRI parameters with IF/TA at 1 year was studied via a multivariable model of significant clinical donor, recipient and transplant parameters as described in Chapter 3, which contains donor type and baseline IF/TA. Adding the IRI parameters by backward elimination, only a higher score for 'neutrophils in PTCs' turned out to be an independent risk factor: Every point increase in 'neutrophils in PTCs' increased the IF/TA score at 1 year with 0.16 (p=0.039) (Table 4.7B).

Table 4.6. Associations of clinical and histological variables with eGFR at 12 months

Parameter	Univarable analysis			Multivariable analysis		
	B	95% CI	p-value	B	95% CI	p-value
Donor type						
LD	-2.086	-8.050 – 3.878	0.491	1.040	-4.228 – 6.308	0.698
DBD	Ref	Ref		Ref		
DCD	-9.155	-14.971 - -3.339	0.002	-8.564	-13.675 - -3.453	0.001
Donor age (years)	-0.533	-0.679 - -0.386	<0.001	-0.591	-0.733 - -0.449	<0.001
Donor gender (male vs female)	1.837	-3.014 – 6.688	0.456	1.623		0.454
Recipient age (years)	-0.311	-0.486 - -0.136	0.001	-0.083		0.332
Recipient gender (male vs female)	-2.821	-7.675 – 2.033	0.253	1.361		0.539
WIT1 (DCD only)	-0.263	-0.618 – 0.093	0.146			
Cold ischemia time (hours)	-0.101	-0.599 – 0.397	0.689			
Anastomosis time (min)	-0.130	-0.337 – 0.078	0.219	-0.029		0.766
Retransplantation	3.340	-2.300 – 8.979	0.244	0.947		0.733
Immunised (PRA current >5%)	7.807	-0.699 – 16.313	0.072	3.365		0.393
DGF	-8.163	-13.044 - -3.282	0.001			
Rejection	-3.711	-9.047 – 1.625	0.172	-6.987	-11.684 - -2.325	0.003
Immunosuppression at baseline (SRL vs MMF)	-3.985	-8.779 – 0.809	0.103	-4.787	-8.942 - -0.632	0.024
IF/TA t0	-7.413	-11.682 - -3.143	0.001	-1.657		0.448
Constant				82.614	74.274 – 90.955	<0.001
Tubular cell necrosis	-5.286	-8.502 – 02.070	0.001			
Loss brush border	-3.791	-6.436 - -1.146	0.005			
neutro glom	-2.218	-4.963 – 0.528	0.113			
neutro PTC	-3.191	-6.470 – 0.089	0.056			

Univarable linear regression was used to test univariable associations between clinical and histological parameters with renal function (eGFR) at one year after transplantation. Multivariable analysis with backward elimination was used to make a 'baseline clinical model'**.** Abbreviations: IF/TA interstitial fibrosis and tubular atrophy, 95% CI 95% confidence interval, DBD donation after brain death, DCD Donation after cardiac death, PRA panel reactive antibodies, DGF delayed graft function, SRL sirolimus, MMF mycophenolate mofetil, IS immunosuppression.

Table 4.7: Multivariable associations of clinical and histological factors with renal function and IF/TA 1 year after transplantation.

	Odds ratio	95% CI	p-value
A			
eGFR Year 1			
Donor type			
LD	0.076	-5.298 – 5.145	0.977
DBD	Reference		
DCD	-7.967	-13.000 - -2.934	0.002
Donor age	-0.604	-0.743- -0.464	<0.001
Rejection	-6.067	-10.682 - -1.453	0.010
Immunosuppression at baseline (SRL vs MMF)	-4.704	-8.784 - -0.624	0.024
Tubular cell necrosis	-2.736	-6.288 – 0.815	0.130
Loss of brush border	-3.645	-5.987 - -1.302	0.002
Neutrophils in glomeruli	-0.203	-2.888 – 2.483	0.882
Neutrophils in PTC	*-1.018*	*-3.933 – 1.896*	*0.492*
Constant	*87.882*		
	Odds ratio	**95% CI**	**p-value**
B			
IF/TA year 1			
Donor type			
LD	-0.021	-0.281 – 0.239	0.873
DBD	Reference		
DCD	0.453	0.194 – 0.711	0.001
IF/TA at implantation	0.292	0.106 – 0.478	0.002
Tubullar cell necrosis	-0.078	-0.224 – 0.067	0.290
Loss of brush border	0.040	-0.114 – 0.194	0.609
Neutrophils in glomeruli	0.003	-0.136 – 0.142	0.968
Neutrophils in PTC	0.160	0.008 – 0.313	0.039
Constant			

Multivariable analysis with backward elimination was perfomed**.** Table 4.7A: eGFR at year 1: covariates are significant parameters resulting of multivariable analysis of clinical and histological parameters (see Table 4.6). Table 4.7B: IFTA at year 1: covariates are significant parameters of previous multivariable analysis of clinical and demographic parameters (see Chapter 3) Abbreviations: IF/TA interstitial fibrosis and tubular atrophy, LD living donor, DBD donation after brain death, DCD donation after cardiac death, PTCs peritubular capillaries, SRL sirolimus, MMF mycophenolate mofetil, CI confidence interval.

Discussion

In this single-centre cohort study, we analysed the impact of scoring for four IRI parameters in reperfusion biopsies on early graft function and 1-year renal function and IF/TA. We show that there is a high correlation between the two tubular injury parameters ('tubular cell necrosis' and 'loss of brush border') and between the two cellular influx parameters ('neutrophils in glomeruli' and 'neutrophils in PTCs'). However, there is no correlation between the tubular injury and cellular influx markers, indicating that may be different pathophysiological mechanisms during ischemia and

reperfusion leading to the presence of these parameters in the reperfusion biopsy. Animal studies have shown that proximal tubular cells are vulnerable to hypoxic damage in the kidney[25], leading to loss of cell polarity followed by apoptosis and necrosis. 'Loss of brush border' in tubuli is an early sign of ischemic injury in animal models[25], and can be detected in human biopsies[22]. Scoring of 'loss of brush border' and 'tubular cell necrosis' is classified as mild acute tubular injury in reperfusion biopsies by the Banff working group[20]. The Banff working group on scoring reperfusion biopsies has proposed to score for vascular thrombi, coagulative type of necrosis, and infarctions as signs of more severe acute tubular injury in reperfusion biopsies[20]. However, we did not observe such lesions in our cohort.

We show that IRI parameters are associated with a higher occurrence of DGF and PNF (non-IGF) in univariable analysis. In multivariable analysis, only 'loss of brush border' is an independent risk factor for non-IGF next to donor type. Furthermore, 'loss of brush border' is an independent risk factor for worse renal function after 1 year. In contrast, only 'neutrophils in PTC' is a risk factor for more IF/TA after 1 year, which may hint towards the role of inflammation of PTCs in IRI on consecutive chronic histological damage. Results from our study give insight in potential targets related to worse outcome. Moreover, IRI parameters might serve as surrogate markers for intervention studies.

Acute tubular injury and DGF in zero-time biopsies has been shown to correlate in some studies[26-28], while others failed to detect a correlation[19,29,30]. This may be explained by differences in study design, e.g. in biopsy type (wedge or needle), fixation (frozen or paraffin-embedded), timing (procurement or reperfusion) or patient - and graft characteristics[7]. In our cohort, there is a relatively large percentage of kidneys from DCD donors, who suffer from extended warm ischemia in the donor. In line with the study of Koo *et al.*[21], we did observe more neutrophil influx in DCD than in LD grafts. Inflammation after ischemic injury occurs secondary to expression of danger signals by damaged/necrotic cells and subsequent expression of adhesion molecules and activation of complement[1-3,31-33].

Our study has several strengths and limitations. A strength of our study is the relatively large and well-defined cohort. We investigated formalin-fixed needle biopsies taken at reperfusion, in which IRI parameters are present, in contrast to procurement biopsies, where grafts have not yet experienced reperfusion injury. Two blinded pathologists scored the vast majority of biopsies. The estimated interobserver agreement was poor to moderate (ICC varies between 0.197-0.300), which is a

limitation of this study. Despite the moderate interobserver agreement, separate analysis of the data measured by the two observers provided essentially similar outcome. Up until now, there is no standardized method for scoring acute tubular injury, both in transplantation and in other settings. In addition, different approaches to classify IRI parameters are used[13,34]. We recommend that IRI scoring should be standardized before implementation in clinical practice and/or prospective trials is possible. As the number of patients with PNF in our study was very limited (n=8), detailed analysis of this group was not possible. In univariable analysis we found that 'tubular cell necrosis' was more often found in PNF cases as compared to DGF cases, therefore there may be a role for scoring for 'tubular cell necrosis'. Further research is needed to determine whether assessment of IRI injury in reperfusion biopsies aids in predicting PNF.

In conclusion, histological analysis of acute kidney injury parameters, especially 'loss of brush border' and 'neutrophils in PTC' (and possible 'tubular cell necrosis') in reperfusion biopsies is able to discriminate extent of IRI, and is related to clinical outcome after transplantation. IRI parameter assessment in reperfusion biopsies may aid in predicting kidney function after transplantation.

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Chapter 5

Presence of cytotoxic extracellular histones in machine perfusate of donation after circulatory death kidneys

Published as

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Abstract

Background: Extracellular histones are cytotoxic molecules that are related to cell stress and death. They have been shown to play a crucial role in multiple pathophysiologic processes like sepsis, inflammation, vascular dysfunction and thrombosis. Their role in organ donation and graft function and survival is still unknown. The aim of this study was to assess whether an association exists between the presence of extracellular histones in machine perfusates and deceased donor kidney viability.

Methods: Machine perfusates of 390 donation after circulatory death kidneys were analysed for histone concentration and corresponding graft function and survival were assessed.

Results: Extracellular histone concentrations were significantly higher in perfusates of kidneys with post-transplant graft dysfunction (primary non function (PNF) and delayed graft function (DGF)) and were an independent risk factor for DGF (OR=2.152 (1.199–3.863)) and one year graft failure (HR=1.386 (1.037-1.853)), but not for PNF (OR=1.342 (0.900-2.002). One year graft survival was 12% higher in the group with low histone concentrations (p=.008) as compared to the group that contained higher histone concentrations.

Conclusions: This study warrants future studies to probe for a possible role of cytotoxic extracellular histones in organ viability and suggests that quantitation of extracellular histones might contribute to assessment of post-transplant graft function and survival.

Introduction

The use of hypothermic machine perfusion (HMP) to preserve deceased donor kidneys continues to increase[1-3], likely due to the general consideration that HMP limits the effects of ischemia to the tissue[4,5]. HMP performs superior to static cold storage in terms of graft survival and post-transplant graft function[6-8]. It is assumed that especially high-risk donor kidneys, i.e. kidneys from old donors, donors with increased morbidity and donors after circulatory death (DCD), may benefit from this preservation technique[1,3,9,10]. In addition to improved transplant outcome, HMP provides an opportunity to easily perform viability tests on donor kidneys by measurement of pump parameters or analysis of machine perfusate[11-13].

Machine perfusate metabolites and other components have been studied extensively over the last decades, but controversy exists regarding the value of such analyses to assess kidney quality. Several studies have shown the increased presence of a number of different metabolites in machine perfusates and associations with higher rates of delayed graft function (DGF) or primary non-function (PNF) were made[1,11-14]. However, none of these individual molecules at present provides conclusive predictive value for short-term graft function.

Recent insights into cellular mechanisms underlying microcirculatory stress and, consequently, organ failure point towards a critical role of (extracellular) histones[15,16]. Since extracellular histones H2B, H3 and H4 are cytotoxic and cause endothelial damage[17-23], we hypothesized that extracellular histones in the machine perfusate reflect cell stress and damage (Figure 5.1) and the activation of an amplifying cascade of histone release and endothelial damage which together ultimately contribute to transplant failure. The aim of this study was to assess whether an association exists between the presence of extracellular histones in machine perfusates and kidney viability. We measured histone H3 levels in a large group (n=390) of unselected machine perfused DCD kidneys and revealed that histone H3 levels correlated positively with DGF and one year graft failure.

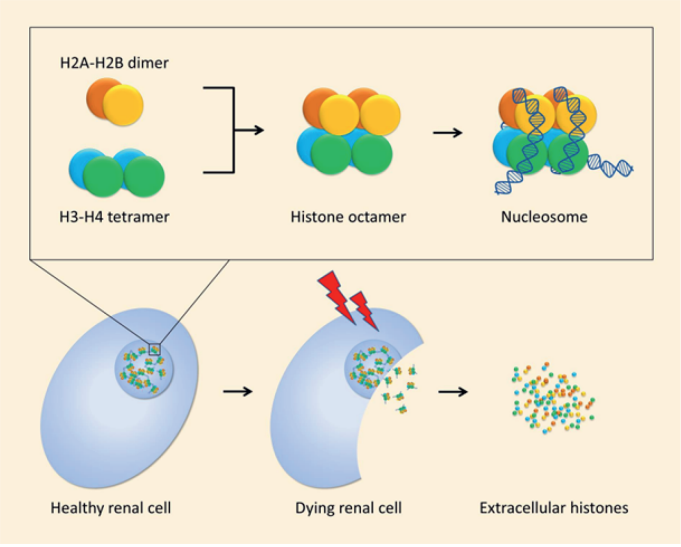
Methods

Donor, graft and recipient data

Between January 1997 and March 2013, all DCD kidneys that were preserved using hypothermic pulsatile machine perfusion in our medical centre, for at least four hours,

were used for analysis. This included kidneys procured and transplanted in our centre or elsewhere, within the Eurotransplant region.

Figure 5.1: Top half: Histones 2a (orange) and 2b (yellow) form a dimer. Histones 3 (blue) and 4 (green) also form a dimer, which associates with another H3-H4 dimer to form a H3-H4 tetramer. All combined, a histone octamer is formed, which serves as the core structure of a nucleosome. DNA is wrapped around these histone cores in order to package the genome inside the nucleus. Lower half: A healthy renal cell contains a nucleus with nucleosomes organized inside the organelle. After ischemic injury, the renal cell may die and release histones into the extracellular space.



Donor data were routinely registered and analysed for donor gender, age (years), most recent serum creatinine (µmol/L), cause of death (cerebrovascular, cardiac, respiratory, trauma, suicide, other) and DCD Maastricht category (1, 2, 3 or 4).

The following graft characteristics were recorded: left or right kidney, type of machine perfusion (Gambro (Gambro, Lund, Sweden) or Lifeport (Lifeport Kidney Transporter, model no. LKT-100-P; Organ Recovery Systems, Des Plaines, IL), duration of machine perfusion time, WIT (defined as time of death, as witnessed by an absent circulation, until time of cold perfusion), cold ischemic time (CIT, defined as the period between start of cold perfusion until start of first anastomosis) and anastomosis time.

Recipient characteristics and follow-up data were recorded and retrieved: recipient gender, age (years), primary kidney disease (renovascular or other), dialysis type (no, hemo- or peritoneal dialysis), years of dialysis, number of previous transplants, total number of HLA mismatches (including HLA-A, HLA-B and HLA-DR; scoring 0-6) and

immunosuppressive regimen. The immunosuppressive regimen changed during the study period, but mainly included use of a calcineurin inhibitor (cyclosporine or tacrolimus) and prednisolone. This was combined with sirolimus, mycophenolate mofetil or azathioprine, depending on the protocol at the time. Dacluzimab was given to patients with increased immunologic risk if needed.

The collection and storage of patient data was performed in agreement with the "Code of Conduct for Health Research" by the Dutch Federation of Biomedical Scientific Societies (http://www.federa.org/).

Kidney procurement, preservation and machine perfusion

All kidneys were procured from DCD donors who included all types of DCD donors according to the Maastricht criteria (DCD 1-4)[24]. Perfusion was either performed by using a double-balloon triple-lumen (DBTL) catheter or by direct cannulation of the aorta, as previously described[25]. After nephrectomy, kidneys were placed on pulsatile perfusion machines. Before May 2007 the Gambro PF-3B perfusion machines (Gambro, Lund, Sweden) were used with 500 mL UW-MPS (Belzer MPS; Trans-Med, Elk River, MN) as perfusion solution. This machine was set with a systolic pressure of 55 mmHg for the first hour, after which flow was kept constant. As of June 2007, kidneys were placed on the Lifeport Kidney Transporter (model no. LKT-100-P; Organ Recovery Systems, Des Plaines, IL) with 1 L of Kidney Perfusion Solution 1 (KPS-1; Organ Recovery Systems) and perfused at 30-40 mmHg pressure. All kidneys were perfused at a mean temperature of 4 degrees Celsius and pH was adjusted when lower than 7.10. Perfusion characteristics, i.e. temperature, pressure, flow, renal resistance were recorded during perfusion and any abnormalities were registered.

Sample collection and histone H3 analysis

During machine perfusion, perfusate samples were taken at 1, 2 and 4 hours after start of perfusion, centrifuged, cooled and stored at -80 °C. To ensure a balanced state of perfusion, the samples of 4 hours of perfusion were used for biochemical analysis.

Histone H3 levels were determined semi-quantitatively as previously described[18]. In brief, kidney perfusate samples were centrifuged for 4 minutes at 3000xg, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene diflouride membranes (Bio-Rad Laboratories, Hemel Hempstead, UK) by semi-dry blotting. After blocking for one hour in triethanolamine-buffered saline with Tween + 5% (w/v) nonfat dry milk (Bio-Rad Laboratories, Hemel Hempstead, UK), membranes were incubated with a rabbit polyclonal H3 antibody overnight at 4°C

(1:1000, sc-8654-R, Santa Cruz Biotechnology, Heidelberg, Germany), followed by incubation with a biotin-conjugated goat anti-rabbit IgG antibody for 30 minutes at room temperature (1:1000, Vector Laboratories Burlingame, CA, USA) and incubation with StreptABComplex/AP solution (1:3000, Dako Cytonation, Glostrup, Denmark) for 30 minutes at room temperature. Protein bands were detected by BCIP/NBT (Sigma-Aldrich, St. Louis, MO, USA) and band densities were quantified by ImageQuant TL software (GE Healtcare, Little Chalfont, UK). Purified H3 (Roche, Basel, Switzerland) served as a reference to calculate unknown H3 concentrations (µg/mL) in the perfusate by comparing the densities of all H3 specific bands in these samples with the density of known concentrations of purified H3.

Study outcome parameters

Immediate graft function (IGF), delayed graft function (DGF) and primary non-function (PNF) were scored as outcome measures for short-term graft function. DGF was scored if dialysis was needed in the first week after transplantation. PNF was scored if the graft never showed adequate function and dialysis was needed all through the follow-up period of the patient. Serum creatinine levels were analyzed for all recipients at 3 and 12 months and up to 5 years each year after transplantation if possible.

Data analysis

Numerical variables are presented as mean ± standard deviations if approximately normally distributed, and as median (interquartile range (IQR)) otherwise. Categorical variables are presented as number (percentage). Analyses were performed on all perfusates collected, irrespective of any pre-analytical variable. A p-value of 0.05 or less was considered to represent statistical significance. To allow comparison of determined concentrations between the Gambro and Lifeport perfusion systems, the relative 2-fold concentration in the former was taken into account and corrected for.

Comparison of histone H3 concentrations between groups based on graft function (PNF vs. DGF vs. IGF) or based on type of donor (uncontrolled vs. controlled) was performed using the Mann-Whitney test. Univariable and multivariable binary logistic regression analysis was used for assessment of the risk of PNF (vs. DGF or IGF) or of DGF (vs. IGF), where the effects are presented as Odds Ratio (OR) (95% confidence interval (CI)). Multivariable analysis included clinically important risk factors (i.e. WIT, CIT, anastomosis time, donor age and type of donor (uncontrolled vs. controlled), the latter was excluded in subgroup analysis for uncontrolled and controlled DCD kidneys). The discriminative capacity of histone H3 concentrations on graft function was assessed

by presenting our data as receiver operating curves (ROC) from which the area under the ROC (AURC) (95% CI) was calculated. For multivariable predictive analysis, predictive probabilities were calculated with and without Histone H3 concentrations and the added value of extracellular histones to the model was assessed. Correlations between histone H3 concentrations and WIT, donor creatinine, donor age and post-transplant serum creatinine were tested with a t-test for Spearman's Rho (r_s). Categorical comparison between groups (one and five year graft survival, histone H3 concentrations below or above the median) was performed using Pearson Chi-square test. Graft survival was censored for death, for all patients who died with a functioning graft. A Kaplan-Meier analysis was used to analyse graft survival and differences between curves were assessed using log-rank tests. A Cox proportional hazards model was applied to calculate the hazard ratio (HR) of histone H3 concentrations at one and five year graft failure. The multivariable Cox proportional hazard model included aforementioned clinical factors.

Results

Patients

From January 1997 until March 2013, 439 transplanted DCD kidneys were machine perfused by our centre. After careful assessment of machine perfusion records, 10 kidneys were excluded from analysis because of significant leakage, obstruction or documented malperfusion. Next, 10 kidneys were transplanted outside of the Eurotransplant region, and for six kidney transplantations, follow-up data could not be obtained; of another six, machine perfusion records were missing or incomplete. For 17 kidneys machine perfusate was not available, because samples were used in previous studies or sample collection was wrongly performed at the time. A remaining total of 390 perfusate samples were included.

Donor, graft and patient characteristics are shown in Table 5.1. Of the 390 kidneys entering analysis, a majority of 270 (69%) were procured from DCD-3 donors. Median donor age was 49 (IQR, 38-58) years. After death, the median WIT was 25 (IQR, 19-31) minutes, median CIT was 25 (IQR, 21-29) hours and median anastomosis time 38 (IQR, 31-47) minutes.

Table 5.1: Donor, graft and recipient characteristics.

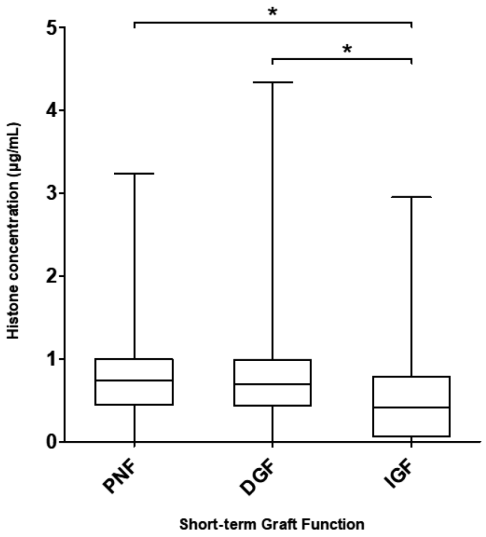
Characteristics		Univariable PNF Odds ratio (95% CI)	Univariable DGF Odds ratio (95% CI)
Donor gender			
Female/Male	143 (37%)/247 (63%)	0.756 (0.443-1.291)	0.500 (0.302-0.828) *
Donor age (years)	49 (38-58)	1.017 (0.999-1.035)	1.014 (0.998-1.031)
Donor creatinine (µmol/L)	85 (63-123)	1.004 (0.997-1.101)	1.014 (1.006-1.022) *
Donor cause of death			
Cerebrovascular	125 (32%)	1.612 (0.961-2.705)	1.586 (0.895-2.809)
Cardiac	93 (24%)	1.513 (0.867-2.640)	2.714 (1.318-5.589) *
Respiratory	6 (2%)		
Trauma	110 (28%)	0.462 (0.243-0.878) *	0.383 (0.228-0,644) *
Suicide	11 (3%)		
Other	45 (12%)		
Donor Maastricht category			
1	9 (2.3%)		
2	102 (26.2%)	1.509 (0.876-2.597)	2.459 (1.253-4.826) *
3	270 (69.2%)	0.616 (0.366-1.037)	0.431 (0.231-0.803) *
4	9 (2.3%)		
Graft			
Left/Right	199 (51%) / 191 (49%)	0.731 (0.442-1.209)	1.012 (0.616-1.663)
Perfusion machine (Gambro/Lifeport)	320 (82%) / 70 (18%)	1.075 (0.554-2.085)	2.754 (1.517-4.998) *
Machine perfusion time (hours)	19 (15-24)	1.010 (0.970-1.051)	1.044 (1.002-1.087) *
WIT (min)	25 (19-31)	1.028 (1.004-1.051) *	1.021 (0.995-1.048)
CIT (hours)	25 (21-29)	1.007 (0.969-1.046)	1.065 (1.022-1.109) *
Anastomosis time (min)	38 (31-47)	1.010 (0.995-1.025)	1.014 (0.997-1.033)
Perfusate			
Extracellular histones (µg/mL)	0.65 (0.37-0.95)	1.417 (0.974-2.063)	3.060 (1.694-5.526) *
Recipient			
Recipient gender Female/ Male	145 (37%) / 245 (63%)	1.209 (0.724-2.017)	0.675 (0.407-1.122)
Recipient Age (years)	55 (44-63)	1.013 (0.992-1.034)	1.007 (0.988-1.026)
Recipient primary disease			
Renovascular	97 (25%)	1.196 (0.679-2.104)	0.984 (0.552-1.754)
Other than renovascular	244 (63%)	0.963 (0.574-1.613)	1.142 (0.686-1.901)
Unknown primary disease	49 (13%)	0.783 (0.351-1.748)	0.786 (0.386-1.600)
Dialysis time (years)	3 (1-4)	1.133 (1.024-1.255) *	1.143 (0.999-1.309)
Dialysis type			
Hemodialysis	236 (61%)	1.072 (0.640-1,794)	1.463 (0.886-2.416)
Peritoneal dialysis	147 (38%)	0.891 (0.529-1.501)	0.810 (0.489-1.344)
No dialysis	6 (1.5%)		
Unknown	1 (0.3%)		
Retransplantation, yes/no	50 (13%) / 340 (87%)	0.762 (0.342-1.698)	1.241 (0.582-2.648)
Total HLA (A, B, DR) mismatches	3 (2-3)	0.892 (0.711-1.118)	0.878 (0.694-1.112)
Cyclosporine/Tacrolimus/none			
Cyclosporine	26 %	0.907 (0.494-1.663)	0.724 (0.401-1.308)
Tacrolimus	71 %	0.594 (0.344-1.027)	1.172 (0.648-2.119)
None	4 %		
Antimetabolite, yes/no	64 %	0.652 (0.384-1.109)	0.525 (0.285-0.966) *
Induction therapy, yes/no	11 %	1.066 (0.467-2.432)	1.143 (0.470-2.778)

Donor, graft and recipient characteristics are shown as median (interquartile range) if the variable is numerical and as number (percentage) if categorical. Univariable regression analysis of the risk of PNF and DGF is shown in the two columns on the right. For variables with multiple categories, odds ratios are calculated for single variable versus all other variables (yes/no). Odds ratios are marked with and asterisk if statistically significant (p≤.05).

Graft function

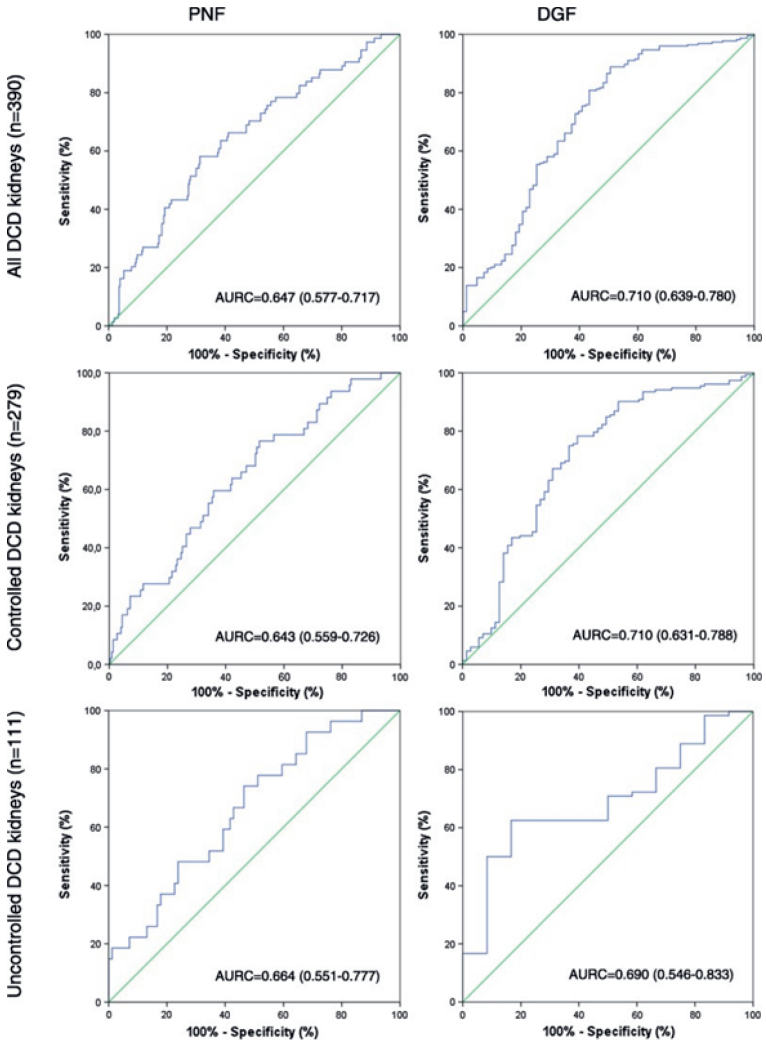
A majority of transplantations resulted in DGF: 228 of the analysed 390 (58.5%). Kidneys developed PNF in 76 (19.5%) cases and IGF in 86 (22.1%) cases after transplantation. Median overall machine perfusate extracellular histone concentration was 0.65 (IQR, 0.37-0.95) µg/mL. Extracellular histone concentration (in µg/mL) was significantly higher in kidneys which developed PNF (median 0.73 (IQR, 0.44-1.00), p<.001) and DGF (median 0.70 (IQR, 0.43–0.98), p<.001) compared to IGF (median 0.42 (IQR, 0.07–0.78)) (Figure 5.2). The difference between the histone H3 concentration in the PNF as compared to the DGF group, did not reach significance (p=.437)

Figure 5.2: Histone concentration are significantly higher in PNF (median 0.73 (IQR, 0.44-1.00) µg/mL, p<.001) and DGF (median 0.70 (IQR, 0.43–0.98) µg/mL, p<.001) groups, compared to IGF (median 0.42 (IQR, 0.07–0.78) µg/mL). Boxplots are presented as median and quartiles, and whiskers include full range of values.



The risk of PNF was not significantly increased by elevation of histone H3 levels as assessed by univariable and multivariable regression analyses (OR=1.417 (95% CI, 0.974-2.063) and 1.342 (95% CI, 0.900-2.002), respectively). Furthermore, the discriminative power of histone H3 concentrations on development of PNF was weak in univariable analysis (AURC=0.573 (95% CI, 0.502-0.645). This significance increased however after correction of the data for confounding clinical factors in the multivariable model (AURC=0.647 (95% CI, 0.577-0.717), with a minor added value of extracellular histones to the predictive model (AURC without histones = 0.640 (95% CI, 0.571-0.708)) (Figure 5.3).

Figure 5.3: The predictive value of extracellular histones for PNF and DGF, for all DCD kidneys and after subgroup analysis for controlled and uncontrolled DCD kidneys. Receiving operator curves are shown and AURC (95% CI) calculated by multivariable analysis, including clinically important risk factors.



We observed that, for development of DGF, histone H3 concentration was an independent risk factor in both univariable (OR=3.060 (95% CI, 1.694-5.526)) and multivariable analysis (OR=2.152 (95% CI, 1.199–3.863). However, the predictive value of extracellular histone concentrations in perfusates on DGF was poor (AURC=0.674 (95% CI, 0.604-0.743), but increased to fair after correction for confounding clinical factors (AURC=0.710 (95% CI, 0.639-0.780)), in which histone H3 concentration added

minor value to the predictive model (AURC without histones = 0.689 (95% CI, 0,621-0,757)) (Figure 5.3).

Extracellular histone concentrations correlated with serum creatinine at three months after kidney transplantation (r_s=0.115; p=.044). Thereafter, at one, two, three, four and five years follow-up, this correlation was no longer significant (r_s=0.111, p=.059; 0.119, p=.053; 0.110, p=.086; 0.091, p=.190; 0.103, p=.180, respectively) (Table 5.2).

Table 5.2: Correlation of histone concentration to donor, graft and outcome factors.

Factors compared to histone concentration	**Spearman correlation coefficient (r_s)**	**P-value**
Warm ischemic time	0.190	<.001
Donor serum creatinine	0.167	.001
Donor age	-0.018	.725
3 month serum creatinine	0.115	.044
One year serum creatinine	0.111	.059
Two year serum creatinine	0.119	.053
Three year serum creatinine	0.110	.086
Four year serum creatinine	0.091	.190
Five year serum creatinine	0.103	.180

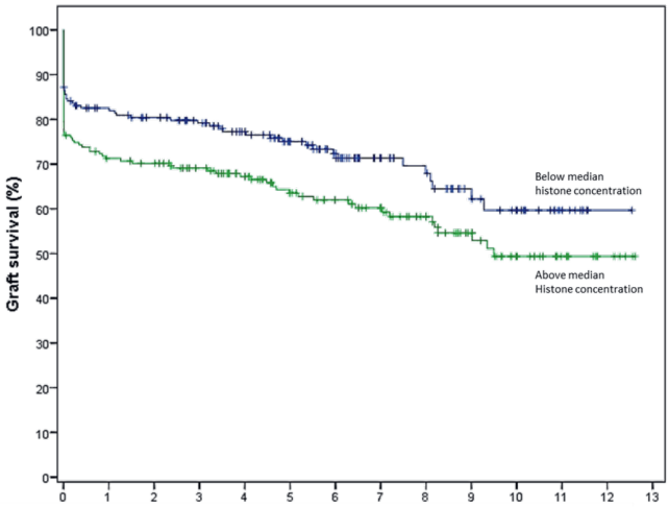
Graft survival

Median follow-up was 6.1 (IQR, 3.8-8.7) years. Overall, one year death-censored graft survival was 77% and at five years this was 71%. Figure 5.4 shows Kaplan-Meier curves with a significantly better survival of kidneys with histone H3 concentrations below the median of 0.65 µg/mL than those that have levels of extracellular histones that are above the median (log-rank, p=.026). Analysis excluding kidneys with PNF did not result in a significant difference (log-rank, p=.443). One year graft survival was significantly better for the group with a histone concentration below the median concentration than above (graft survival = 83% vs. 71%; p=.008, respectively). This difference persisted up to five years (graft survival = 76% vs. 65%; p=.014, respectively).

The risk of graft failure within one year significantly increased with increasing histone concentration (HR=1.411 (95% CI, 1.071-1.858)) in a univariable Cox regression model. Multivariable analysis also resulted in a significantly increase (HR=1.386 (95% CI, 1.037-1.853)). The risk of five year graft failure was significantly increased with increasing histone concentration in the univariable analysis (HR= 1.335 (95% CI, 1.026-1.738), but not in the multivariable analysis (HR=1.270 (95% CI, 0.960-1.680). Predictive value on graft failure within one and five years was weak after univariable analyses (AURC=0.547 (95% CI, 0.479-0.614) and AURC=0.537 (95% CI, 0.477-0.597),

respectively). Although the predictive value increased after correction for confounding clinical factors (AURC=0.637 (95% CI, 0.573-0.702) and AURC=0.648 (95% CI, 0.590-0.707), respectively), there was no added value of extracellular histones to the model for one and five year graft failure (AURC without histones = 0.641 (95% CI, 0.577-0.703) and AURC without histones=0.649 (95% CI, 0.591-0.707), respectively.)

Figure 5.4: Kaplan-Meier graph of graft survival after transplantation. After one year the group with histone H3 concentrations below the median had a 12% higher graft survival compared to group with histones H3 concentrations above the median (p=.008). Data was censored at the time of death for patients who died with a functioning graft.



Factors associated with increased extracellular histone concentrations

Extracellular histone concentrations weakly correlated to WIT (r_s=0.190, p<.001) and donor creatinine (r_s=0.167, p<.001). Histone concentrations did not correlate with donor age. The median histone H3 concentration in uncontrolled donors was significantly higher than in controlled donors (median (IQR) = 0.73 (0.52-1.1) µg/mL (n=111) vs. 0.59 (0.28-0.92) µg/mL (n=279); p<.001, respectively) (Table 5.2).

Controlled vs. uncontrolled DCD

Because there is a difference in histone concentration between uncontrolled (Maastricht category 1 and 2) and controlled (Maastricht category 3 and 4) DCD donors, we performed subgroup analyses.

Perfusates from uncontrolled DCD donor kidneys (n=111) showed no significant difference in histone H3 concentrations between the groups with PNF, DGF or IGF (PNF vs. DGF, p=.884; PNF vs IGF, p=.988; DGF vs. IGF, p=.929). Furthermore, histone H3 concentration was not associated with an increased risk of graft dysfunction (PNF or DGF). Only in multivariable predictive analyses, histone H3 concentration was predictive for PNF (AURC=0.664 (95% CI, 0.551-0.777)) and DGF (AURC = 0.690 (95% CI, 0.546-0.833)) (Figure 5.3).

Controlled DCD perfusates (n=279) contained a significantly higher median (IQR) concentration of histone H3 in kidney with PNF (0.74 (0.42-0.96) µg/mL, p<.001) and DGF (0.63 (0.39-0.97) µg/mL, p<.001) compared to IGF perfusates (0.32 (0.02-0.67) µg/mL). For controlled DCD, we found that histone H3 concentration is an independent risk factor for DGF (univariable OR=3.071 (95% CI, 1.588-5.941) and multivariable OR=2.433 (95% CI, 1.253-4.723)); for PNF however, such significance could not be shown. Furthermore, the discriminative power for development of PNF and DGF of extracellular histone levels in this subgroup is comparable to the entire group of perfusates (PNF univariable AURC=0.597 (95% CI, 0.512-0.683); PNF multivariable AURC=0.643 (95% CI, 0.559-0.726); DGF univariable AURC=0.683 (95% CI, 0.607-0.759) and DGF multivariable AURC=0.710 (95% CI, 0.631-0.788)) (Figure 5.3).

One year death-censored graft survival was 78% and at five years this was 72%. Comparing the groups with histone H3 concentrations above and below the median, Kaplan Meier analysis showed a significant (log rank, p=.012) difference in graft survival in favour of the lower concentrations. Notably, one and five year graft survival were significantly better in the group with lower concentrations (at one year 84% vs. 72% (p=.019) and at five years 79% vs. 66% (p=.015).

Discussion

Many studies have sought to identify possible relations between measurable biomolecules and organ injury, post-transplant graft function or survival. It has been shown that kidneys release a plethora of biomolecules over time during machine

perfusion. The release of these molecules appears to reflect the pathophysiologic processes that occur during organ donation.

Interest in extracellular histones has grown rapidly during the last decade. Studies have demonstrated a crucial role of extracellular histones multiple pathophysiologic processes like sepsis, inflammation, vascular dysfunction and thrombosis[15,16,18,22,26-28]. It has been shown that extracellular histones are in fact cytotoxic molecules that can kill various cell types, including endothelial cells, and contribute to organ failure [15,18,20,22,28-30]. Hence, histones once released into the circulation may initiate an amplifying cascade of cell death and histone release culminating in microcirculatory damage and consequently organ failure. Extracellular histones have not yet been studied in the pathophysiologic process of kidney injury and organ viability in a clinical setting of organ donation and transplantation. In this study we addressed the relevance of measuring extracellular histones in the machine perfusate of hypothermic machine perfused DCD kidneys before transplantation.

The incidence of PNF in this group of DCD kidneys is high. Donor kidneys in this group were procured in the period that the waitlists exceeded 5 years for kidney transplantation and we were able to extend the donor pool threefold by transplanting marginal donor kidneys. These kidneys were further characterised by a very long cold ischemia time due to allocation issues and were mainly transplanted in fragile recipients in whom a prolonged waiting time for transplantation was considered not desirable. Despite the relatively good graft survival of functioning grafts and although transplantation of marginal kidneys with a high incidence of PNF may provide a survival benefit to recipients who alternatively would wait for a standard criteria donor kidneys, we considered this percentage as too high and adapted our acceptance criteria for marginal donor kidneys[31].

Essentially, every nucleated cell contains core histones, which include the histones H2A, H2B, H3 and H4 in an equimolar ratio (Figure 5.1). Considering the equimolar presence of the cytotoxic histones H2B, H3 and H4, measurement of histone H3 is considered a proxy-measurement of H2B and H4 as well. Recently, we were able to show a correlation between disease severity and mortality in sepsis and extracellular histone H3 levels in patients in the intensive care unit[26]. Therefore we decided to measure the levels of histone H3 in our collection of machine perfusates.

To verify that differences in perfusion protocols and time of storage did not influence extracellular histone H3 measurements we compared mean histone H3

concentrations for IGF, DGF and PNF between the different perfusion protocols used here, and analysed a possible influence of storage time. We neither observed differences between perfusion protocols nor did we observe an influence of storage time. This notion is supported by several studies that show that long-term storage only results in minor decreases of nucleosome concentrations and repeated freeze-thaw cycles do not affect measured concentrations of nucleosomal DNA fragments [32-34]. We also verified the latter observation by measuring histones repeatedly in our set-up (mean average coefficient of variation = 7.24 ± 0.08 % for 3 independent experiments; *data not shown*).

The average extracellular histones concentration is strikingly higher in kidneys which showed post-transplant dysfunction. The apparent prognostic benefit that correlates with lower concentrations of extracellular histones in perfusate was especially seen in graft survival analysis, where we found a 12% higher one-year survival for the lower concentration half (Figure 5.4). The survival of functioning grafts appears to be equal. Moreover, in contrast to three recent studies[12,35,36], which did not find a significant association of any molecule measured in machine perfusate to one year graft failure, extracellular histones in this study were associated with an increased risk of one year graft failure. Also, we found that extracellular histones are an independent predictor of DGF when adjusted for relevant confounding factors. Subsequent subgroups analyses yielded similar results for controlled DCD kidney transplantation. Analyses on uncontrolled DCD only showed a significant association to graft function in the multivariable prediction model for DGF and PNF, likely due to a small group size of uncontrolled DCD and the lower amount of IGF (n=12) in this group. We have shown that extracellular histone concentration is associated with graft function and graft survival, however its value to predict the outcome after transplantation is very limited and equivalent to the predictive value of most other machine perfusate biomolecules[12-14,35,36]. Therefore the currently available data on perfusate histones concentration does not aid in the decision to either accept or refuse a kidney for transplantation.

Here, we have shown for the first time that extracellular histones are present in the perfusate of hypothermically machine perfused DCD kidneys. We observe that extracellular histones are likely to represent an independent risk factor for DGF and one year graft failure and that there is an association to PNF. DCD machine perfused kidneys with low histone concentrations have a significantly better survival after transplantation. Future studies are necessary to assess if graft survival can be improved if the extracellular histones concentration is reduced either by better organ

preservation or by therapies that may filter or neutralise these potentially cytotoxic agents.

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Novel viability assessment strategies in an ex-vivo porcine model

Chapter 6

Extracellular histone release by renal cells after warm and cold ischemic kidney injury: Studies in an ex-vivo porcine kidney perfusion model

Published as

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Abstract

Background: Extracellular histones are cytotoxic molecules involved in experimental acute kidney injury. In patients receiving a renal transplant from donors after circulatory death, who suffer from additional warm ischemia, worse graft outcome is associated with higher machine perfusate extracellular histone H3 concentrations.

Methods: We now investigated temperature-dependent extracellular histone release in an *ex vivo* porcine renal perfusion model, and subsequently studied histone release in the absence and presence of non-anticoagulant heparin. Seven pairs of ischemically damaged porcine kidneys were machine perfused at 4°C (cold ischemia) or 28°C (warm ischemia).

Results: Perfusate histone H3 concentration was higher after warm as compared to cold ischemia (median (IQR) = 0.48 (0.20-0.83) µg/mL vs. 0.02 (0.00-0.06) µg/mL; p=.045, respectively). Employing immune-electron microscopy (EM), histone containing cytoplasmic protrusions of tubular and endothelial cells were found after warm ischemic injury. Furthermore, abundant histone localization was detected in debris surrounding severely damaged glomerular cells, in a “buck shot” pattern. *In vitro*, histones were cytotoxic to endothelial and kidney epithelial cells in a temperature-dependent manner. In a separate *ex vivo* experiment, addition of heparin did not change the total histone H3 levels observed in the perfusate but revealed a continuous increase in the level of a lower molecular weight histone H3 variant.

Conclusions: Our findings show that ischemically damaged kidneys release more extracellular histones in warm ischemia, which by EM was due to histone release by renal cells. Blocking of histone-mediated damage during transplantation may be beneficial in prevention of renal injury.

Introduction

Histones are nuclear proteins, and the core histones H2A, H2B, H3 and H4 organize DNA into chromatin and regulate gene expression[1,2]. The pathologic release of extracellular histones is known to have clinical consequences as these proteins are cytotoxic towards host tissues[3–6] and serve as damage-associated molecular patterns (DAMPs)[7]. Histone release can occur through regulated or unregulated cell death[8,9] or through active immune cellmediated expulsion[10,11]. The presence of extracellular histones is linked to the initiation and detrimental progression of several clinical conditions that include organ specific events like acute kidney injury (AKI), acute lung injury (ALI) but also other conditions like sepsis, COVID-19, systemic lupus erythematosous (SLE), rheumatoid arthritis (RA), stroke and myocardial infarction[12–19]. The detrimental effects of extracellular histones have thus been studied in a variety of situations, including in renal disease. Extracellular histones have been associated with ischemia-reperfusion injury (IRI) in kidneys[5,20,21]. In IRI, an ischemic insult is followed by reperfusion with activated leukocytes, implying that circulating histones can be derived from intrarenal leucocytes and/or renal cells. Extracellular histones function as DAMPs that can activate cells through TLR2 and TLR4 receptors in post-ischemic and septic acute kidney injury and may aggravate kidney injury[20]. Direct injection of histones into murine kidneys results in increased levels of neutrophil recruitment, tubular injury and renal failure, effects that are abrogated when anti-histone antibodies are administered[20]. Moreover, histones play an important role in the adaptive immune response after kidney translation. Histone modifications contribute to the control of expression of major histocompatibility complex (MHC) I and II[22]. Serum concentrations of histone H3, as marker of circulating cell-free nucleosomes, were shown to be significantly higher in patient with acute rejection[23].

Acute kidney injury (AKI) is associated with high morbidity and mortality and is most often caused by cardiovascular or septic shock[24]. AKI also occurs after kidney transplantation, and is more severe after transplantation of kidneys from donation after circulatory death (DCD) donors, compared to kidneys from brain death (DBD) or living donors[25]. DCD kidneys suffer from additional warm ischemia in the donor, i.e. between circulatory arrest and organ preservation, leading to delayed graft function and longer hospitalisation[25]. There is a persisting donor organ shortage and a strategy to increase the donor pool may be to improve the organ quality of DCD donors[26], and thereby reduce rejection and adverse effects in the receiver. We recently found that high histone H3 concentrations in machine perfused kidneys prior to transplantation are associated with worse transplant outcome for DCD kidneys[27]. However, more

knowledge on histone release from kidneys during transplantation, and on effects of histones on kidney viability, is needed to develop strategies aimed at preventing detrimental histone effects during kidney transplantation. *In vitro* studies have shown that dying tubular renal cells can release histones[20], and we hypothesize that ischemic renal tissue injury itself plays an important role in the histone release process. We specifically aim to study differences in temperature related severity of ischemic injury.

There is a general interest in the use of glycosaminoglycans to inhibit the cytotoxicity of extracellular histones and heparin is able to neutralize histones and thereby it may prevent histone accumulation on the endothelial glycocalyx and reduce histone cytotoxicity[13,28,29]. It has recently been shown that a high molecular weight heparin reduces ischemia reperfusion injury in machine perfused porcine kidneys[30]. Our experience with an antithrombin affinity-depleted heparin, that was shown to neutralize histone-mediated cytotoxicity in vitro and in vivo, in mouse models of inflammation and sepsis[13], led to the hypothesis that we could likewise neutralize histones during machine perfusion of kidneys.

To investigate histone release from ischemically injured kidneys in a transplantation setting, we used an *ex vivo* machine perfusion model[31]. The model allows the study of the relationship of extracellular histones with severity of injury during renal ischemia, in the absence of circulating immune cells. Furthermore, we studied temperature dependent kinetics of histone release by endothelial and renal tubular epithelial cells *in vitro*.

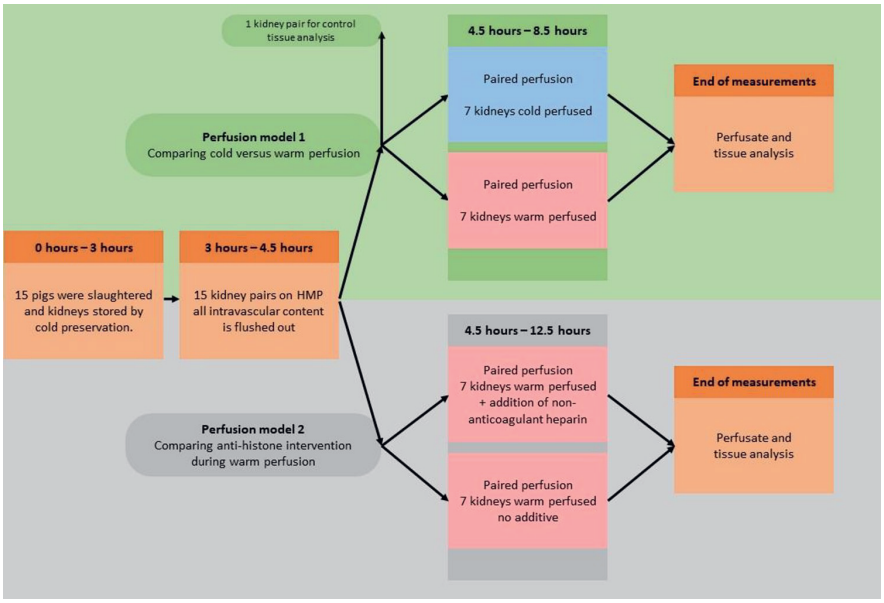
Methods

Study design

In the porcine *ex vivo* perfusion models clinically relevant differences in warm vs cold ischemia were studied as described elsewhere[31]. In brief (Figure 6.1), 7 kidney pairs underwent ischemic kidney injury, while 1 kidney pair served as control. We retrieved all kidneys from a local slaughterhouse (Slachthuis Kerkrade) from Dutch Landrace pigs between 6 and 7 months of age and with a mean weight of 105 kg. After bilateral procurement, vasculature of both kidneys was flushed simultaneously with ice cold Histidine-Tryptophane-Ketoglutarate solution (HTK, Custodiol1). After cold storage, kidneys were simultaneously hypothermically perfused at 4˚C using two Lifeport Kidney Transporters (model no. LKT-100-P; Organ Recovery Systems, Des Plaines, USA) for 1 hour with 0,5L of Kidney Perfusion Solution 1 (KPS-1; Organ Recovery Systems). After

this first period of machine perfusion with two venous flush-outs, we assumed to have a pair of identical kidneys from which all intravascular content was removed. Thereafter, each kidney was assigned to either hypothermic (HMP) or subnormothermic (SNMP) machine perfusion (cold versus warm, respectively). HMP was performed similar to the initial perfusion period, SNMP comprised of kidney perfusion for 4 hours with KPS-1 at a temperature of 28˚C. All kidneys were perfused at 40 mmHg, according to clinical standard practice. New perfusate and different perfusion packs were used for each machine perfusion period. Machine perfusate samples were taken from the Lifeport sampling port in the last 4 hour perfusion period and were immediately centrifuged at room temperature (RT) at 3000g for 3 minutes, and supernatants were aliquoted and stored at -80˚C until further analysis.

Figure 6.1: Paired ischemic porcine kidney perfusion models.



Porcine kidneys are retrieved from the slaughterhouse and flushed 25min after sacrifice (warm ischemia time). Kidneys are then put on cold storage for 3h until the first period of cold machine perfusion at 4˚C. Subsequently, kidneys are flushed one more time before being assigned to their group. **Model 1 (green)** includes 2 groups; 4h of cold perfusion vs 4h of warm perfusion. Regular perfusate sampling is done during this final perfusion period and at the end, biopsies are taken for further analysis. **Model 2 (gray)** includes 2 groups of warm perfusion; one kidney from a pair received non-anticoagulant heparin (0.31 mg/mL or 54 U/mL), whilst the other did not receive treatment. A total of 7 kidney pairs were used, from which 3 were perfused up to 4h and the other 4 pairs were perfused up to 8h. Regular perfusate sampling was done and tissue was collected at the end of perfusion.

In a second experiment, both kidneys were procured and handled in a similar manner as described above up to the final machine perfusion period (T4., Figure 6.1). Then, both kidneys from each pair were warm perfused for 4 hours (n=7 pairs). One kidney from each pair was perfused for 4 hours with perfusion liquid containing 0.31 mg/mL or 54 units/mL of nonanticoagulant heparin[13]. For 4 out of 7 kidney pairs, this was extended to 8 hours of warm perfusion time to study histone H3 levels over a longer time period.

Perfusate analysis

Differences in metabolic activity between cold and warm perfused kidneys were analysed by measuring glucose (mmol/L), lactate (mmol/L), pH, pO2 (kPa) and pCO2 (kPa) with a blood gas analyser (GEM Premier 4000, Instrumentation Laboratory, Lexington, USA) using standardized laboratory methods. Lactate dehydrogenase (LDH, U/L) and total glutathione-*S*transferase (GST, U/L) were measured using a Roche Cobas 8000 system (Roche Diagnostic International Ltd, Rotkreuz, Switzerland). Machine perfusion fluid samples taken at t=10, 60, 120, 180 and 240 minutes were analysed. Perfusate taken at different time points was analysed for the presence of histone H3 using semi-quantitative Western blotting as previously described[18,27].

Renal biopsies and electron microscopy (EM)

For all kidneys, wedge biopsies were taken of renal parenchyma at the end of machine perfusion, except for the two kidneys that were used as controls, which were biopsied immediately after transport to our lab (Figure 6.1) which thus did not undergo any preservation.

For transmission EM, sections were chemically fixed at 4˚C with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in PHEM buffer (60mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4mM MgSO4*7H2O). After washing with PHEM containing 50 mM glycine, tissues were embedded in 12% gelatine and infused in 2.3 Msucrose. Mounted gelatine blocks were frozen in liquid nitrogen after which thin sections were prepared in an ultracryomicrotome (Leica EM Ultracut UC6/FC6, Vienna, Austria).

For immuno-EM, ultrathin cryosections were collected with 2% methylcellulose in 2.3M sucrose. Then, cryosections were incubated at RT on drops of 2% gelatin/PBS for 20 minutes at 37˚C, followed by 50 mM glycine/PBS for 15 minutes, 10% BSA/PBS for 10 minutes and 5% BSA/PBS for 5 minutes. Then they were incubated with anti-histone H3 antibody (1:50 in 5% BSA/PBS for 30 minutes at RT, ab1791, Abcam). After three

washes of 10 minutes with drops of PBS, sections were incubated using protein A coupled to 10 nm diameter colloidal gold particles (1:50 in 1% BSA/PBS for 20 minutes at RT, AURION, Wageningen, the Netherlands). This was followed by another three PBS washes of 10 minutes and two washes of 10 minutes with distilled water. As a control for non-specific binding of the colloidal gold-conjugated antibody, the primary antibody was omitted. Observations were done in an Electron Microscope Tecnai G2 Spirit equipped with an Eagle 4kx4k CCD camera (FEI Company, The Netherlands).

Cell culture experiments

The human endothelial cell line EA.hy926 (CRL-2922, ATCC) and porcine kidney proximal tubular epithelial cell line PK-15 (CCL-33, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Lonza, Walkersville, USA). In addition, EA.hy926 cells were supplemented with HAT: hypoxanthine (5 mM), aminopterin (20 µM) and thymidine (0.8 mM) (Lonza).

For *in vitro* cytotoxicity assays, the EA.hy926 and PK-15 cell lines were grown to confluence. Cells were washed twice with PBS before incubation with 5–40 µg/mL purified calf histone H3, 10 µg/mL purified calf histone H1 (both Roche, Sigma-Aldrich, St. Louis, MO, USA), 10 µg/mL human recombinant histones H2A, H2B and H4 (all New England Biolabs, Ipswich, USA) or 50 µg/mL histone mix (Roche, Sigma-Aldrich, St. Louis, MO, USA) for 1 hour in serum-free DMEM (Lonza, Switzerland) at 37˚C in 5% CO2. Next, serum-free DMEM was collected in Low-Bind Eppendorf tubes and cells were washed once with PBS. Thereafter, cells were detached using 0.05% Trypsin-EDTA (0.05% Proteinase-K) for 5 min at 37˚C in 5% CO2. Next, fresh DMEM (2mM glutamine, 100 unit/mL penicillin, 100 µg/mL Streptavidin, Lonza, Switzerland) was added to the cells and cells were collected by scraping. Cells were spun down at 600 g at 4˚C and supernatant was removed. Cells were then solved in 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2 and 2.5 mM CaCl2 pH 7.4 buffer containing 0.25 µg/mL Annexin A5-FITC (PharmaTarget BV, Maastricht, The Netherlands) and 2.5 µg/mL propidium iodide (Invitrogen Life Technologies, Carlsbad, USA) and incubated for 10 minutes in the dark on ice. Cell viability was assessed by flow cytometry employing a BD Accuri C6 flow cytometer as previously described[19].

For immunofluorescence studies EA.hy926 cells were seeded and grown to confluence. Cells were equilibrated to 4˚C, 22˚C or 37˚C before incubation with 10 µg/mL histone H3 in serum-free DMEM for 1 hour. Cells were then washed, stained

with 2,5 µg/mL PI and 0,5 µg/mL Hoechst 33342 (ThermoFischer, Mannheim, Germany) for 10 minutes and imaged using a EVOS FL inverted digital microscope (ThermoFischer). Six random images were taken at 20x magnification per tested condition. The percentage of PI positive cells was quantified for each image using ImageJ software (x86, Wayne Rasband, National Institute of Health, USA) and then averaged to generate a mean PI positivity for each condition.

Data analysis

All data were analysed using IBM SPSS statistics Version 22.0 (IBM Corp., Armonk, USA) or GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA). Numerical variables are presented as mean ± standard deviation (SD) if normally distributed or else as median (interquartile range (IQR)). Categorical variables are presented as numbers (percentage). For statistical analysis of immunohistochemical, flow cytometry and immunofluorescence data, one-Way ANOVA was performed, while applying a Bonferroni correction when comparing multiple groups and a Dunnett test when comparing groups to control. For analysis of histone concentrations in the perfusate, the non-parametric Friedman test was used to test if concentration changes recorded over time for machine perfusion groups (warm and cold) were significant and presented as Chi-square and p-value per group.

To describe the difference of concentration changes over time between both groups, we used a linear mixed model. This model was applied, assuming a linear regression model, in which both types of machine perfusion (warm and cold) and machine perfusion sample time were used as fixed effect and a random intercept was included. For determination of the difference between groups, we used the interaction term of these two parameters (type of machine perfusion* machine perfusion sample time) and presented the p-value. Extracellular histonerelease over time was investigated for each type of perfusion group. To compare concentrations of GST and glucose between machine perfusion groups (warm vs. cold), the Mann-Whitney test was used per point of machine perfusion sample time (t=10, 60, 120, 180 and 240 minutes).

The Friedman test was used to test if concentrations changed over time per aforementioned marker in both machine perfusion groups. In the intervention perfusion model, histone concentrations per time point were compared by performing an independent t-test. Significant differences were indicated by a *p-value of <0.05, **p-value of <0.01 or ***p-value of <0.001.

Results

Severity of warm versus cold ischemic renal injury in the machine perfusion model

The differences in ischemic renal injury between the models are shown by quantitation of several markers in the machine perfusate. In the machine perfusate, we determined uptake of glucose, a marker of metabolic activity, by kidneys after warm and cold ischemia over time (Friedman test: SNMP Chi-square 16.000, p=.003 and HMP Chi-square 18.000; p=.001; Table 6.1). Glucose uptake was higher after warm ischemia. Furthermore, LDH, a widely used marker of tissue damage during machine perfusion[32], and lactate, as end product of renal metabolic activity, significantly increased over time, more so in warm perfusion compared to cold perfusion (Table 6.1). The markers GST, pO_2, pCO_2 did not significantly differ between groups, while pH did. However, these markers are known to be unreliable markers of viability during machine perfusion (Table 6.1).

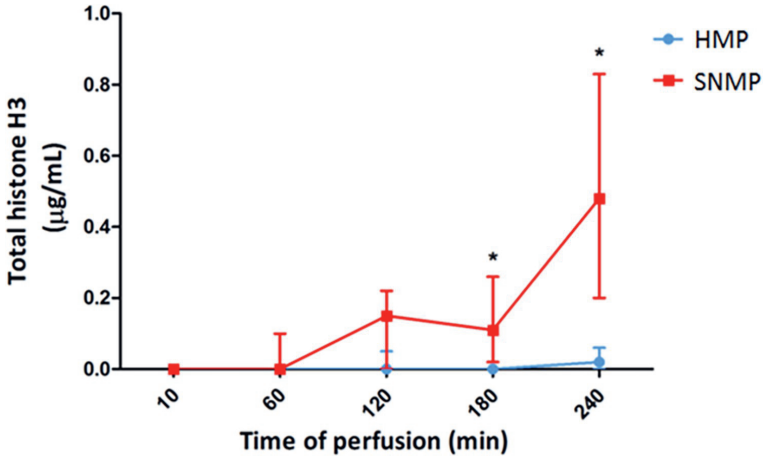
Histone detection in machine perfusate samples

Histone concentrations of the machine perfusate were measured to investigate ischemia dependent histone release into the extracellular space. During the initial stages (e.g. procurement, storage and initial flushing and perfusion stages) levels of extracellular histones in the perfusate were below the detection limit of the assay used (5 ng/ml histone H3[33]. At the final stage of the machine perfusion, extracellular histones were detected (Figure 6.1). Histone H3 concentrations were significantly higher after 4 hours of warm versus cold ischemia (median concentration = 0.48 (0.20-0.83) µg/mL) vs. 0.02 (0.00–0.06) µg/mL; p=0.045, respectively; Figure 6.2). Furthermore, there was a significant change in histone concentration over time in the warm ischemia group, while this was not the case in the cold ischemia group (Friedman test=χ^2=44.559; p<0.001 vs. χ^2=11.613; p=0.393, respectively). This was also found using a mixed linear model (p-value for the estimate of fixed effect for the interaction term of machine perfusion group * time of machine perfusion p=0.001).

Table 6.1: Friedman and Mann-Whitney tests for differences between type of perfusion group per time point.

		H3 (5 time points)	Glucose	LDH	Lactate	GST	pH	pO2	pCO2
Friedman	Chi-square	6.333	18.000	16.914	24.361	1.172	11.075	9.257	7.387
HMP	p-value	0.176	.001*	.002*	<.001*	.883	.026*	.055	.117
Friedman	Chi-square	18.719	16.000	23.886	28.000	5.600	14.071	8.547	1.640
SNMP	p-value	.001*	.003*	<.001*	<.001*	.231	.007*	.073	.802
10 min	HMP median (IQR)	0.00 (0.00-0.00)	9.9 (9.8-10.3)	5 (4-11)	0.6 (0.3-0.7)	7.9 (3.5-9.9)	6.99 (6.96-7.08)	26.0 (24.6-27.6)	2.9 (2.3-3.8)
	SNMP median (IQR)	0.00 (0.00-0.00)	9.3 (9.1-9.9)	11 (8-23)	1.0 (0.4-1.0)	7.9 (6.4-10.2)	6.84 (6.82-6.87)	25.6 (24.4-26.1)	5.6 (4.9-5.6)
	Mann-Whitney p-value	1.000	.035*	.165	0.259	.818	.001*	.383	.017*
60 min	HMP median (IQR)	0.00 (0.00-0.00)	8.7 (8.4-9.2)	21 (17-25)	0.6 (0.6-0.8)	6.5 (4.1-11.7)	6.89 (6.81-6.97)	23.5 (21.7-24.9)	4.0 (2.8-6.9)
	SNMP median (IQR)	0.00 (0.00-0.10)	8.1 (7.7-8.3)	40 (32-47)	2.2 (1.5-2.7)	10.3 (7.3-12.2)	6.80 (6.80-6.84)	23.5 (20.9-25.3)	5.4 (4.6-9.9)
	Mann-Whitney p-value	.805	.015*	.001*	.001*	.366	.038*	.902	.128
120 min	HMP median (IQR)	0.00 (0.00-0.05)	8.7 (8.1-8.8)	36 (34-48)	0.8 (0.7-1.1)	10.0 (5.0-10.9)	6.89 (6.80-6.90)	24.8 (24.2-25.7)	4.3 (3.5-6.4)
	SNMP median (IQR)	0.15 (0.00-0.22)	7.5 (7.0-7.7)	60 (58-85)	4.0 (2.6-4.4)	11.4 (6.3-13.7)	6.80 (6.80-6.80)	23.7 (22.8-24.6)	7.9 (4.5-9.3)
	Mann-Whitney p-value	.097	.001*	.038*	.001*	.318	.038*	.128	.073
180 min	HMP median (IQR)	0.00 (0.00-0.00)	8.2 (7.6-8.6)	48 (35-51)	1.0 (0.7-1.0)	8.5 (8.4-10.2)	6.86 (6.80-7.00)	24.0 (22.6-26.0)	4.9 (2.9-7.0)
	SNMP median (IQR)	0.11 (0.02-0.26)	6.9 (6.2-7.2)	95 (76-117)	5.7 (3.6-6.4)	10.9 (6.6-12.8)	6.80 (6.80-6.80)	22.9 (21.1-24.9)	7.1 (4.8-7.7)
	Mann-Whitney p-value	.011*	.008*	.004*	.001*	.383	.026*	.456	.209
240 min	HMP median (IQR)	0.02 (0.00-0.06)	8.2 (8.1-8.5)	56 (41-62)	1.0 (0.9-1.1)	11.3 (5.5-12.6)	6.90 (6.80-6.94)	22.8 (22.0-24.7)	4.3 (2.8-8.6)
	SNMP median (IQR)	0.48 (0.20-0.83)	6.0 (5.7-7.1)	118 (61-130)	7.4 (5.2-9.0)	10.4 (7.5-13.2)	6.80 (6.80-6.81)	24.1 (23.1-25.8)	7.9 (5.6-8.7)
	Mann-Whitney p-value	.004*	.001*	.038*	.001*	.836	.165	.456	.165

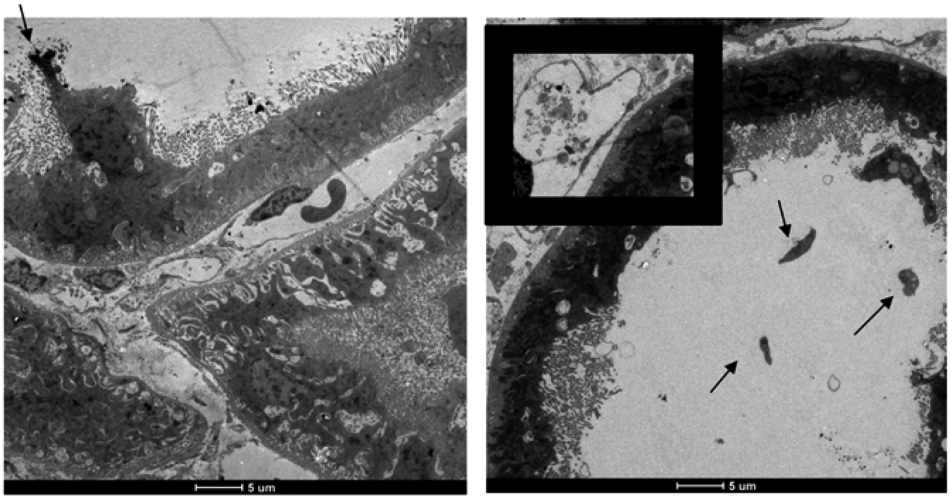
Figure 6.2: Release of extracellular histone H3 is dependent on the amount of ischemic injury. Extracellular histone H3 was determined semi-quantitatively using Western blotting. Perfusate levels of total histone H3 increased over time in the warm ischemic injury group, whilst remaining constant in the cold ischemia group. Data are presented as median and whiskers include interquartile range.*Indicates significant differences between groups (p<0.05).



Electron microscopy (EM) studies of histone expression in ischemic renal injury

Transmission EM of kidney tissue after warm ischemia showed intraluminal spheroid or oval blebs protruding from tubular and endothelial cells in tubuli and peritubular and glomerular capillaries (Figure 6.3), as described previously in ischemic renal injury[34,35]. With immunogold electron microscopy, histone H3 was detected in nuclear chromatin of all cells, which thus served as internal positive control. After warm ischemia, histones H3 were additionally found in the cytoplasmic blebs protruding in lumina of glomerular and peritubular capillaries as well as in tubular lumina (Figure 6.4). Moreover, abundant localization of histones was detected in extracellular debris of severely damaged glomerular cells. Figure 6.5 shows a podocyte with extensive foot process effacement after warm ischemic injury with localization of histones by immuno-gold labelling in extracellular debris in a “buck shot pattern”. This pattern was also seen surrounding a mesangial cell. A similar pattern of histone expulsion was found surrounding endothelial cells with loss of fenestrations and subendothelial accumulation of electronlucent material, while the adjacent podocyte still has intact foot processes.

Figure 6.3: Transmission EM images after 4 hours of cold (left) or warm ischemia (right). In cases with warm ischemia, cytoplasmic blebs (arrows) are seen in lumina of proximal tubuli with cytoplasmic blebbing and swelling in peritubular capillaries (surrounded by square). Scale bar represents 5 μm.



Cytotoxic effects of extracellular histones *in vitro*

Endothelial and renal epithelial cell viability decreases upon histone exposure in a concentration-dependent manner (Figure 6.6A). Endothelial cells incubated with different types of histones revealed a variable effect on cell viability for the different histone isotypes (Figure 6.6B). For these cells, histone-mediated cytotoxicity appears to be isotype specific, with the linker histone H1 being the least cytotoxic, followed by histone H2A, H2B, H3 and H4. The mixture of histones (consisting of all histone subtypes at equal molar ratio) induces the most cell death.

To investigate whether the cytotoxicity of extracellular histones is dependent on temperature, EA.hy926 cells were incubated with a fixed concentration of histone H3 for 1h at 4˚C, 22˚C or 37˚C. Immunofluorescence analysis show that the number of PI positive nuclei, as a measure of histone-induced cell death, increases with increasing temperature (Figure 6.7), with incubation at 37˚C resulting in significantly more cell death than incubation at 4˚C (27.6%, $p<0.01$). Incubation at an intermediate temperature of 22˚C induced a moderate level of cell death (16.6%) even though this was not significantly different from the levels of induced cell death for the other temperatures tested.

Figure 6.4: EM images of anti-histone H3 immunolabelling after 4 hours of warm ischemia in moderately damaged renal cells. Histone H3 (few gold particles indicated with black arrows) was scarcely detected by immuno-EM using antibody labelling with 10 nm gold particles. Top row: representative images are shown for histone H3 localization in a proximal tubule with histone localization in an ovoid cytoplasmic structure (left low magnification, right high magnification). Bottom row: extracellular histone H3 is present in ovoid and spheroid in glomerular endothelial cytoplasmic structures (left low magnification, right high magnification). Scale bar represents 2 µm or 500 nm as indicated.

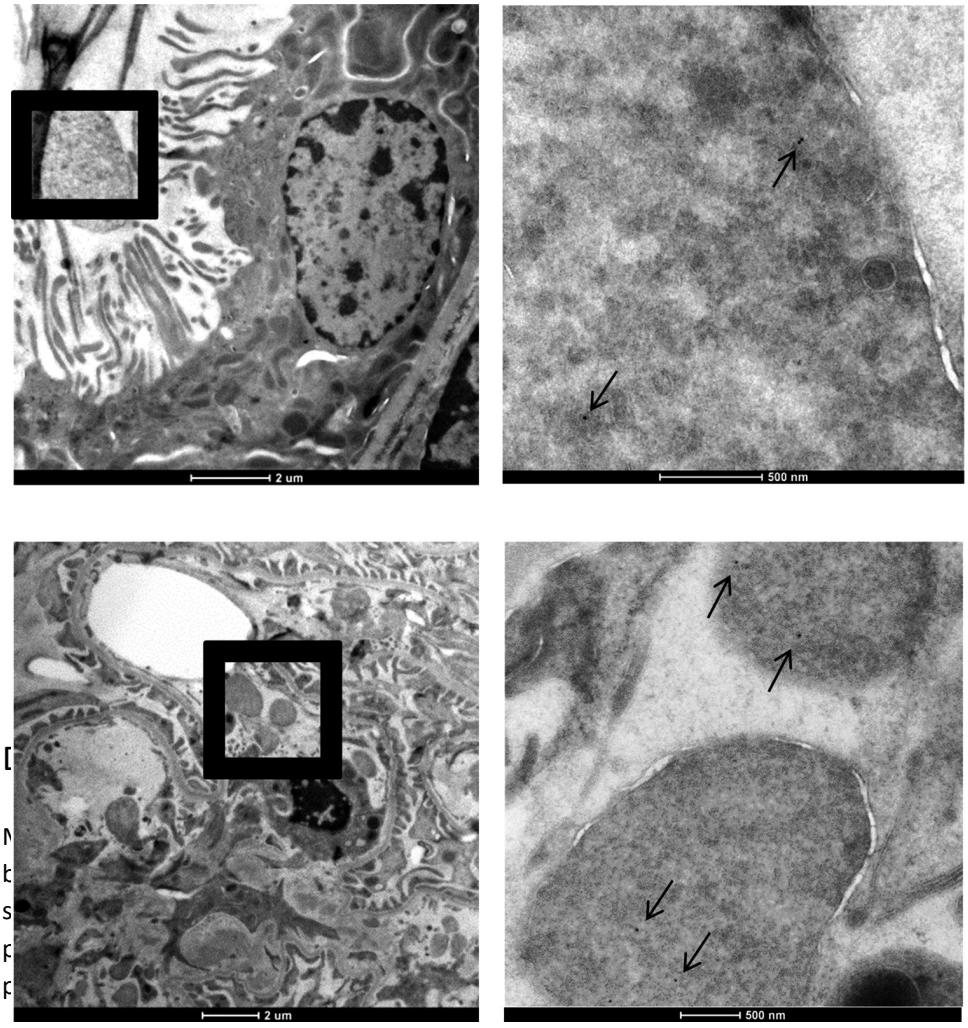


Figure 6.5: EM images of anti-histone H3 immunolabelling after 4 hours of warm ischemia in severely damaged glomerular cells. Histone H3 was detected by immuno-EM using antibody labelling with 10 nm gold particles (visible as small black dots). Top row: representative images are shown for histone H3 localization surrounding a podocyte with foot process effacement (asterisk, left image), with histones in the cytoplasm (thick arrow and in extracellular debris (thin arrows) in a "bug shot pattern", right image). Bottom row: extracellular histone H3 is released from an endothelial cell with loss of endothelial fenestrations and subendothelial accumulation of electronlucent material (left image enlarged area in right image) with histone localisation in extracellular debris (thin arrows, right image). Scale bar represents 1 µm or 200 nm or 500 nm as indicated.

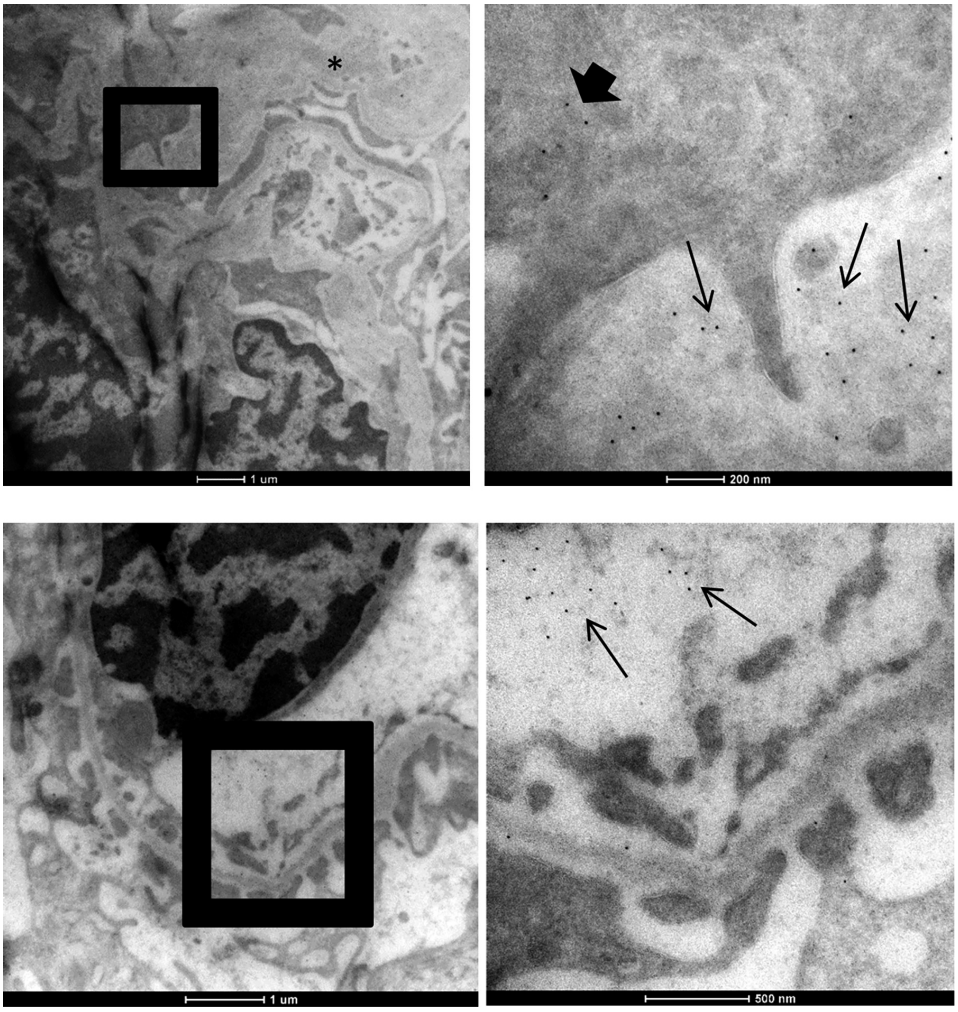


Figure 6.6: Cytotoxicity of extracellular histones to endothelial and kidney epithelial cells. (A) Endothelial cells (EA.hy926) and porcine kidney epithelial cells (PK-15) were incubated with 5-40 µg/mL histone H3 in serum-free DMEM for 1 hour at 37 °C. (B) PK-15 cells were incubated with 10 µg/mL of individual histones H1, H2A, H2B, H3 and H4, or a mixture of these histones (Hmix) under the same conditions as in (A). Cell viability was assessed using flow cytometry with PI and annexin-A5 FITC. Data is shown as the mean + SD. *p<0.05 and ***p<0.001 as compared to control.

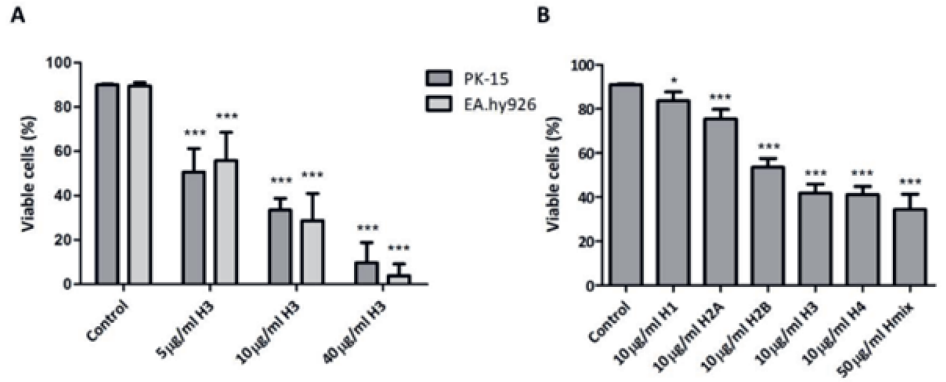
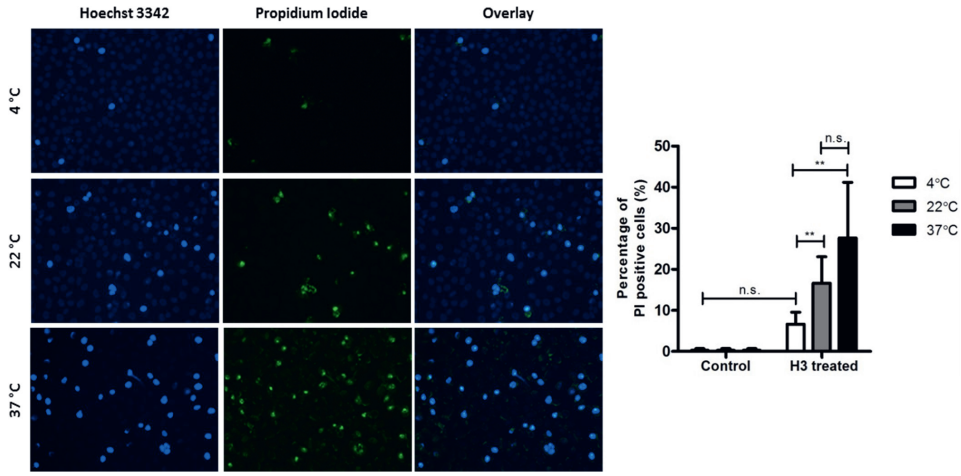


Figure 6.7: Temperature-dependent cytotoxicity of extracellular histone H3. Endothelial cells (EA.hy926) were incubated with 10 µg/mL histone H3 in serum-free DMEM for 1 hour at 4 °C, 22 °C or 37 °C. Left panel: representative images from histone treated cells. Nuclei were stained using Hoechst 33342 (in blue) and necrotic cells were stained using PI (in green). Right panel: The number of PI positive cells were quantified in 6 random image fields using ImageJ software. Data is expressed as the mean ± SD of the average percentage of PI positive cells per condition (n=5).



Nonanticoagulant heparin and histone presence in kidney perfusates

Addition of heparin at the start of warm perfusion did not result in changes in perfusion parameters and glucose perfusate levels as compared to kidneys that did not receive heparin. Western blot analysis of machine perfusate showed a continuous increase in the total amount of extracellular histone H3 that was detected in the perfusate up to the last measurement at 8 hours. However, no significant differences in the total amount of extracellular histone H3 between heparin treated or untreated kidneys at any time point up to 8 hours of perfusion were observed (Figure 6.8A). Interestingly, as was found by Western blotting, in both groups a histone H3 band of an apparent lower molecular weight of about ±14–15 kDa was found which gradually appeared over the course of perfusion time, clearly discernible from the full-length protein band with an apparentMWof ±17 kDa (UniProt F1RVA0 determinedMWof 15.4 kDa) (Figure 6.8C).

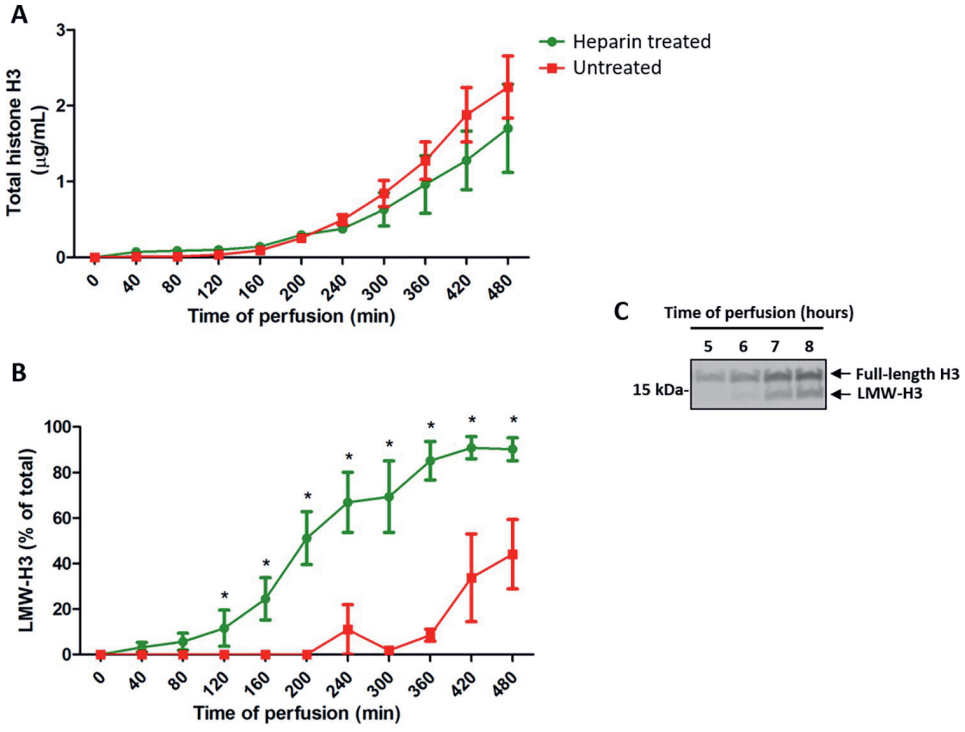
While the total amount of histones that were measured in the perfusate was equivalent for both groups, the LMW-H3 was released significantly faster and more pronounced in kidneys with circulating heparin compared to their counter parts (Figure 6.8B). The increase in LMW-H3 became apparent for the heparin kidneys after 2 hours of perfusion whilst this only started to occur after 6 hours of perfusion for the untreated counterparts. For both groups, this increase was exponential up to at least 8h of perfusion. Significant differences in LMW-H3 levels between both groups were observed for all time points between 2 and 8 hours of perfusion.

Discussion

Acute kidney injury can lead to delayed graft function and primary non function after kidney transplantation, especially in the context of DCD donors that suffer from extended warm ischemia in the donor[36,37]. As high histone H3 concentrations in machine perfused kidneys prior to transplantation are associated with worse transplant outcome for DCD kidneys[27], we now investigated the pathophysiological role of histone release in ischemic kidney injury in an *ex vivo* porcine model with perfusion at 4˚C (cold ischemia) and at 28˚C (warm ischemia). We find that extracellular histones are released from ischemically damaged kidneys during machine perfusion into the perfusate in a time- and temperature-dependent manner. With EM immunolabelling, histones were located in cytoplasmic protrusions of tubular and endothelial cells of moderately damaged cells after warm ischemia. Furthermore, abundant histone localisation was found in extracellular debris surrounding severely damaged glomerular cells. In *in vitro* experiments we found that the cytotoxic effect of histones on renal cells depends on

both temperature, histone concentration and histone isotype. While we have shown earlier[13] that nonanticoagulant heparin is able to neutralize histone mediated cytotoxicity towards endothelial cells *in vitro* and in an *in vivo* sepsis model, *ex vivo*, addition of nonanticoagulant heparin did not result in different overall histone H3 levels, but resulted in an earlier shift from a full length histone H3 release towards the appearance of a lower molecular weight histone H3 variant in the circulating perfusate. Our results show that histones can be released from ischemically injured renal cells, and that histones induce direct damage to renal cells in a temperature dependent manner. Thus, histone release can be an indicator of ongoing tissue damage, and actively contribute to warm ischemic kidney injury.

Figure 6.8: Time-dependent release of histone H3 in heparin treated and untreated kidneys with warm ischemic injury. Extracellular histone H3 was determined in kidneys with or without circulating nonanticoagulant heparin. Kidneys treated with heparin showed a similar release in total histone H3 (A) but a more rapid and pronounced increase in levels of a lower molecular weight histone H3 (LMW-H3) variant (B) compared to untreated counter kidneys. (C) A representative Western blot image of full-length histone H3 (17 kDa apparent MW) and LMW-H3 (15 kDa apparent MW) in perfusate of untreated SNMP kidney at t=5, 6, 7 and 8 hours of perfusion. *Indicates significant differences between groups (p<0.05).



Extracellular histones were found in the machine preservation fluid of kidneys with substantial warm ischemic damage, whilst this release was not seen after cold ischemic injury. Previously, we found expression of lipid-degradation products in kidney tissue after warm but not cold ischemia with mass-spectrometry imaging in this model[31]. In the various experiments performed with the present in vivo model, we found higher levels of lactate and LDH and lower glucose levels in the preservation fluid as sign of increased renal injury. As we verified that the kidneys in our model were depleted of neutrophilic granulocytes due to the perfusion prior to ischemia, our findings indicate that the ischemically damaged renal cells themselves release histones. This in line with *in vitro* studies where dying tubular cells were capable of releasing histones[20]. While in our study we specifically looked at histone H3, we regard this isotype as a proxy for other isotypes as all core histone isotypes are complexed in nucleosomes and are expected to be released as such.

Histones have been detected by immunofluorescence in human biopsies of patients with post-transplant acute tubular necrosis and kidneys of mice with ischemia/reperfusion injury[5,38]. In the human biopsies neutrophilic granulocytes were found in close proximity to the histones, indicative that the detected histones likely originate from neutrophil extracellular traps (NETs). Also in biopsies of patients with ANCA vasculitis, NETs have been detected adjacent to infiltrating neutrophils[39]. With EM immunolabelling we now find intracellular and extracellular histone H3 localization in glomerular and tubular cells of kidneys with warm ischemia, in a setting that is neutrophil-independent. In moderate warm ischemic injury, histones are localized in cytoplasmic blebs/protrusions of tubular cells[40] and endothelial cells in peritubular as well glomerular capillaries. Abundant extracellular localization of histones was found in severely damaged glomerular cells in a “buck shot” pattern within cellular debris. This pattern of histone expulsion resembles that of ETosis, as has been visualized in neutrophilic granulocytes (NETosis)[10] and macrophages (METosis)[40] with immuno EM. Given the absence of blood flow, and verified absence of neutrophils, our findings suggest that the histones are derived from renal cells and may form renal cell derived extracellular traps (RETs), parallel to NETosis or METosis pathways. These histones, may be equally harmful and can further aggravate renal injury by damaging adjacent cells in an autoamplification loop.

Our *in vitro* experiments confirm that extracellular histones exert cytotoxic effects on endothelial and tubular epithelial cells[20], which has also been found for glomerular endothelial cells and podocytes[41]. Cytotoxicity was observed most at 37˚C and only minimal cell death at 4˚C. Histone-mediated toxicity is known to progress through

several pathways, including histone-mediated membrane disruption[17,42], histone-mediated NOD2/NLRP3 activation[43,44] and histone-induced TLR-activation[20,45,46]. Of relevance, it has been described that TLR receptors are upregulated in DCD kidneys[47]. Machine perfusion, one of the first preservation methods[48], is widely used as preservation method for donor organs[49,50]. Hypothermic machine perfusion (HMP) shows superior transplant outcome compared to cold storage[51–53] and has the advantage that it reduces renal resistance during perfusion and flushes out vasoactive and inflammatory mediators, while oxygen and nutrients may be delivered[54,55]. In contrast to HMP, normothermic machine perfusion (NMP) is recently gaining popularity, because it adds the advantage of perfusing organs while they are metabolically active and therefore injury may be prevented[56–58]. The clinical introduction of NMP[59,60] makes our findings relevant for transplant immunology, because we show that histones are not only more cytotoxic at higher temperatures, but their presence is more pronounced after prolonged warm perfusion. Recent preclinical and clinical studies show the feasibility and safe use of normothermic perfusion up to 48 hours[61], which emphasizes the relevance of studying histone release during prolonged warm perfusion. Synchronously to the increase of normothermic perfusion, there is an increase of oxygenation during machine perfusion. In normothermic perfusion, oxygenation is necessary for graft viability. However, oxygenation during hypothermic machine perfusion is arbitrary. Many preclinical and clinical studies have been conducted, questioning the use of oxygen in a hypothermic environment[62]. While it should provide a benefit in theory, its clinical benefit is less clear. The only clinical publication using oxygenation in DCD kidneys was recently published in the Lancet[63]. The primary outcome measure, eGFR at one 1 year, did not differ significantly between oxygenated and non-oxygenated groups. Oxygenation was not an option in our perfusion model, using kidneys which were deprived of all intravascular content, because the use of the oxygen carriers may interfere with histone measurements and lead to additional bias. Further studies should clarify the role of histone release in oxygenated machine perfused kidneys.

The increasing appearance of cytotoxic proteins in the preservation fluid led us to test charge-mediated neutralization of these toxic molecules with nonanticoagulant heparin, which has reported histone-neutralizing properties *in vitro* and has previously been shown to reduce histone-induced cell death in inflammation *in vivo*[13]. Unexpectedly, within the timeframe of this experiment, the total amount of detected extracellular histone H3 did not differ between both groups, nor did markers of cellular damage in the preservation fluid. Histones are known to bind cell surface proteoglycans[64], but also to plastic tubing. We cannot exclude that heparin could

possibly lead to an apparent increase in the circulating histone pool as it can serve as a carrier molecule able to liberate cell-bound histones or stabilize histones that were released and retain them in the circulating perfusate. Interestingly, we observed a progressive appearance of a lower molecular weight histone H3 variant (LMW-H3) in warm perfused kidneys, with heparin accelerating this phenomenon. The presence of this variant is hypothesized to be the result of histone proteolysis and/or modification. Binding of histones to heparin could lead to an increased affinity of (circulating) protease(s) for histone substrate and initiate proteolysis faster than in untreated kidneys. In the literature, several examples of histone-cleaving proteases have been described: trypsin(-like) enzymes[65], granzyme A[66], cathepsins[67], and activated protein C (APC) (own observations and[68], plasma hyaluronan-binding protein[69], neutrophil elastase (NE)[19,70], mast cell tryptase[71], plasmin[72]. In addition, many other proteins can react with histones in plasma, including proteinase inhibitors and MMP-2 which might be blocked by heparin binding[73]. Interestingly, MMP-2 expression is linked to delayed graft function after kidney transplantation[74] while cleavage of histones has been associated to reduced development of acute kidney injury in severe COVID-19 ICU patients[19]. Future studies are needed to clarify the exact origin and role of histone variants after addition of heparin, and cytotoxicity of the smaller histone variant, also in the context of ischemic kidney injury.

A limitation of this study is that our model did not allow the accurate measurement of functional markers such as creatinine, blood urea nitrogen, urinary markers and urine output[75,76], as kidneys are deprived from blood and do not function as *in vivo*. Novel preservation strategies such as oxygenated NMP with autologous blood should be considered in order to better assess graft function[77]. A more direct translational approach would be the use of a porcine transplantation model. Finally, as we did not label our heparin, we could not investigate in more detail the localization of this polysaccharide in the machine perfused kidney. Sedigh et al. have shown that a biotinylated heparin conjugate bound to the inner surface of the vessel wall during machine perfusion of porcine kidneys and could restore the endothelial glycocalyx[78]. Although they did not observe this for unfractionated heparin, it remains unclear whether the unfractionated heparin could be visualized with fluorescently labelled streptavidin.

In conclusion, our findings substantiate the role of histones during warm ischemic kidney injury, and show that damaged renal cells themselves can release extracellular histones. This histone release can further aggravate ischemic renal injury. Translational studies should now be encouraged to investigate the clinical effect of histone release

and histone neutralization, because of the increasing use of donor kidneys with increased warm ischemic injury and the exponential use of normothermic perfusion in the field of kidney transplantation.

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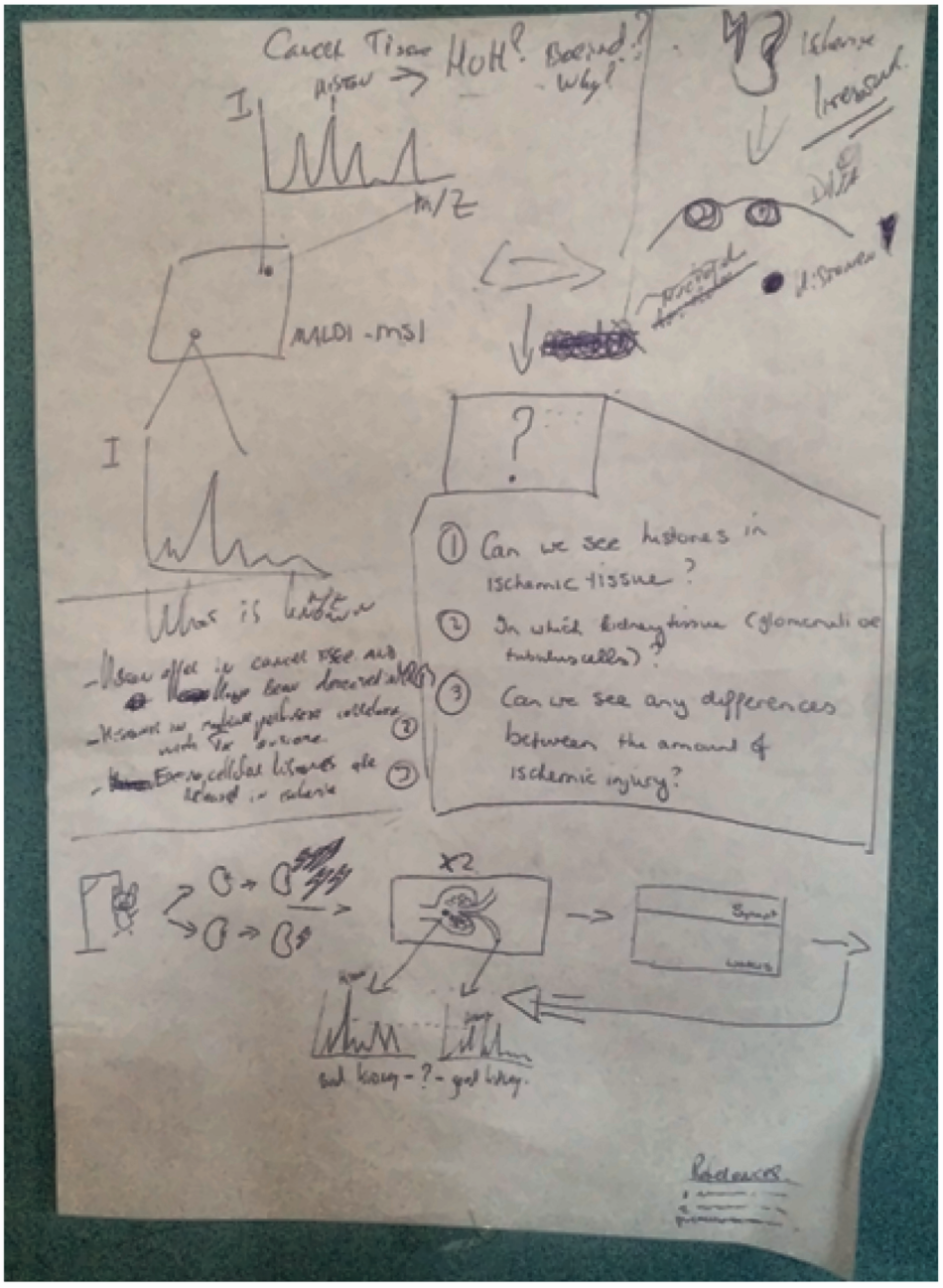
Chapter 7

Rapid identification of ischemic injury in renal tissue by mass-spectrometry imaging

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Cancer Tissue
HOH?
Ischemic
Increased
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?
① Can we see histones in ischemic tissue?
② In which kidney tissue (glomeruli or tubulus cells)?
③ Can we see any differences between the amount of ischemic injury?
What is histone
X2
Sham
Ischemia
Results

Poster

A poster presentation during the combined Maastricht M4I and general surgery grouptrip to Fiesch in Austria 2015. This brainstorm was the kick-off for the project leading to chapter 7.

Abstract

The increasing analytical speed of mass spectrometry imaging (MSI), leads to growing interest in the medical field. Acute kidney injury is a severe disease with high morbidity and mortality. No reliable cut-offs are known to estimate the severity of acute kidney injury. Thus, there is a need for new tools to rapidly and accurately assess acute ischemic, which is of clinical importance in the intensive care and in kidney transplantation. We investigated the value of MSI to assess acute ischemic kidney tissue in a porcine model. A perfusion model was developed where paired kidneys received warm (severe) or cold (minor) ischemia (n=8 per group). First, ischemic tissue damage was systematically assessed by two blinded pathologists. Secondly, MALDI-MSI of kidney tissues was performed to study spatial distributions and compositions of lipids in the tissues. Histopathological examination revealed no significant difference between kidneys, while MALDI-MSI was capable of a detailed discrimination of severe and mild ischemia by differential expression of characteristic lipid degradation products throughout the tissue within 2 hours. In particular lysolipids, including lysocardiolipins, lysophosphatidylcholines and lysophosphatidylinositol, were dramatically elevated after severe ischemia. This study demonstrates the significant potential of MSI to differentiate and identify molecular patterns of early ischemic injury in a clinically acceptable time frame. The observed changes highlight the underlying biochemical processes of acute ischemic kidney injury and provide a molecular classification tool that can be deployed in assessment of acute ischemic kidney injury.

Introduction

Acute kidney injury (AKI) is associated with high morbidity and mortality, and is most often caused by hypotension or hypoxia in cardiovascular or septic shock[1]. AKI also occurs after kidney transplantation and its extent is associated with later graft function[2]. After successful recovery of AKI there is an increasing incidence of CKD, with increased risk for cardiovascular disease[3]. At present, the pathophysiology of AKI is not fully understood, and good biomarkers to predict severity of AKI are lacking[1].

The aim of this study was to investigate the value of mass spectrometry imaging (MSI) in assessment of extent of acute ischemic kidney damage. MSI is an innovative approach enabling the unlabelled molecular imaging of hundreds of molecules in a single experiment, and can allow correlation of localized molecular profiles to tissue state and morphology[4-7]. MSI is now finding its way into clinical analyses due to its ability to reveal localised chemical profiles throughout tissue sections and discriminate between diseased and healthy tissue by virtue of these molecular patterns that are characteristic of cell and tissue types[4,8,9]. Recent advances in instrumentation enable high-throughput tissue imaging and analysis times on a scale compatible with conventional histological analysis[10]. This now opens up the path of using localised molecular information to aid in tissue diagnosis. Lipids present an exciting molecular class to target with MSI due to the relative ease of detection of many abundant lipid classes and simple and rapid sample preparation[11]. In renal research MSI has been applied to study localised sphingolipid changes in sphingomyelin synthase 2-deficient mice and lipidomic changes after cisplatin chemotherapy. A recent study has also highlighted lipidomic changes associated with acute kidney injury[12-14].

In this study we confirm earlier findings that conventional histopathologic examination is not sufficient to accurately discriminate between extent of acute ischemic renal injury[15,16]. In contrast, studying localised lipid profiles with MALDI-MSI provided accurate discrimination of cold and warm ischemia via observation of dramatically elevated levels of lysophospholipids in the case of severe ischemia, with total analysis time of one hour per tissue, including sample preparation. We propose this discriminative capacity opens up the door for molecular-guided quality assessment and can provide new insight into the pathophysiologic processes of ischemic kidney injury.

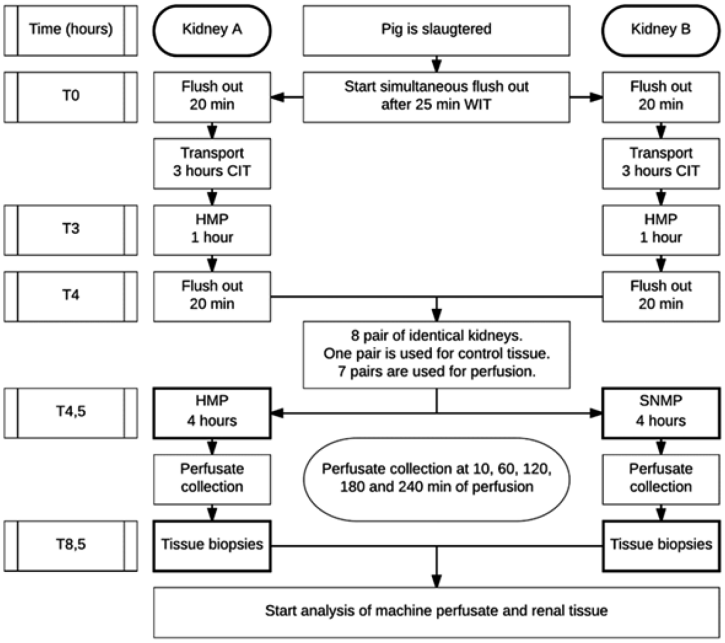
Experimental section

Kidney procurement and ischemia model

We used a paired porcine kidney model to distinguish between ischemic injury in kidneys (Figure 7.1). We aimed to develop a system which can be extrapolated to clinical transplantation, where, in light of the increasing need of donor organs, kidneys of deceased donors with warm (i.e. in the donor) and cold ischemia (i.e. during transportation) are increasingly used. Kidneys were procured from a local slaughterhouse from 8 pigs. Both kidneys of one pig were simultaneously procured 15 minutes after death by electroshock and exsanguination. Perirenal fat was removed and renal arteries were dissected, cannulated and attached to an infusion bag. Kidneys were flushed 25 minutes after death, with cold histidine-tryptophan-ketoglutarate preservation solution of 0-4°C for 20 minutes. The start of perfusion was documented as the end of the warm ischemia time (WIT) and start of cold ischemia time (CIT). Simultaneously, topical cooling was performed using cold sodium chloride solution. Thereafter, both kidneys were stored in bags with sodium chloride solution and put in a Styrofoam box with ice for transport to the laboratory (according to regular organ transport protocols).

In our laboratory, kidneys were removed from cold storage and exactly after 3 hours of CIT simultaneous hypothermic machine perfusion (HMP) was started, using two Lifeport Kidney Transporters (model no. LKT-100-P; Organ Recovery Systems, Des Plaines, IL) and 0.5L of Kidney Perfusion Solution 1 (KPS-1; Organ Recovery Systems, Des Plaines, IL) per kidney at a perfusion pressure of 40 mmHg, as currently used in clinical transplantation practice. Kidneys were hypothermically perfused for one hour at 4°C. Thereafter, they were flushed for 15 minutes to remove all intravascular content and Lifeport perfusion cassettes and KPS-1 was discarded. Kidneys of seven of eight pairs were then randomly assigned to either of two perfusion groups: HMP for 4 hours at 4°C (cold or mild ischemia) or subnormothermic machine perfusion (SNMP) (warm or severe ischemia) for 4 hours at 28°C. One pair was not perfused thereafter and directly biopsied (as described below) to serve as control tissue.

Figure 7.1: Vertical time-line in hours of the study set-up using 8 pigs. A pig is slaughter and its kidneys randomly assigned in either of two groups (kidney A or B). Twenty five minutes after death kidneys are simultaneously flushed using cold solutions and put in cold storage. After 3 hours of cold ischemia time, simultaneous HMP is started for 1 hour after which another flush out follows. One pair of kidneys is directly biopsied, serving as control tissue. The remaining 7 pair of kidneys are assigned in a HMP (cold ischemia) and SNMP (warm ischemia) group for a subsequent 4 hours of perfusion, in which perfusate is sampled. After perfusion, biopsies are taken and analysis of machine perfusate (lactate and LDH) and renal tissue (histopathologic examination and MALDI-MSI) is performed.



HMP was performed as described above but with the use of 1L of KPS-1, with a mean temperature of 4°C. For SNMP a Lifeport Kidney Transporter was used. The reservoir, normally filled with icewater for HMP, was filled with water and continuously heated with a heat exchanger at a constant temperature of 35°C. This lead to a constant temperature of KPS-1 of 28°C, in which the kidney was placed and perfused. Higher (normothermic) temperatures were not possible, as it would stop the perfusion machine from pumping. After 4 hours of simultaneous machine perfusion (HMP or SNMP) wedge biopsies were taken. For the two kidneys which were not subject to the two groups, this was done directly after their first hour of machine perfusion (Figure 7.1). Two large (>1 cm^2) wedges were cut in a manner that both medullar and cortical renal tissue was included. One wedge biopsy was formalin fixed and paraffin embedded (FFPE) and stored for histopathologic examination. The other wedge biopsy was snap

frozen using a 2-methylbutane solution and stored at -80 °C for MSI analysis (without the use of OCT compound).

To test our presumption of different metabolic activity between groups and subsequent ischemic injury, machine perfusate was gathered during machine perfusion and tested for lactate and lactate dehydrogenase (LDH) as markers of metabolic activity and cellular injury. During the 4 hours of HMP and SNMP machine perfusate samples were taken at 10, 60, 120, 180 and 240 minutes. After withdrawal from the Lifeport sampling port, samples were centrifuged at 3000g for 3 minutes, aliquoted, and stored at -80°C. After the samples were thawed, lactate was measured (in mmol/L) using a blood gas analyser (GEM Premier 4000, Instrumentation Laboratory, Lexington, MA, USA). Lactate dehydrogenase was measured (in U/L), using a Roche Cobas 8000 system (Roche Diagnostic International Ltd, Rotkreuz, Switzerland).

Histopathologic scoring of tissue biopsies

FFPE tissues were sectioned at 4 µm, twice for every kidney. Subsequently they were stained with Hemotoxyllin and eosin (H&E) or Periodic Acid Schiff Diastase (PAS-D) according to standard protocols[17]. Two nephropathologists (CJPK and LMH), blinded for ischemia groups, performed histopathological examination of all biopsies using light microscopy. Of each tissue section 10 random fields of renal cortex containing at least 1 glomerulus were assessed at 200x enlargement and scored (0-3 point scale: none, mild (0-30%; moderate (30-60%) and severe (>60%)) for five parameters, associated with ischemic injury: tubular cell necrosis, loss of brush border, oedema, tubular casts, blebs in tubular epithelial cells. A total injury score was calculated by adding the scores of all parameters.

Mass spectrometry imaging (MSI)

Tissue preparation

Wedge biopsies were collected from -80 °C storage, mounted on the specimen stage with H_2O and sectioned at 12 µm at -21 °C using a Cryostat HM525 (Microm, Walldorf, Germany), thaw mounted onto conductive indium-tin-oxide (ITO) coated glass slides which were purchased from Delta Electronics (Loveland, CO, USA, 4–8 Ω resistance) and dried under vacuum. On each slide both kidneys of the same pig were mounted to ensure a similar and comparable analysis during MSI. Norharmane matrix (Sigma Aldrich, Zwijndrecht, The Netherlands) at 7 mg/mL in chloroform:methanol (2:1, v:v) was applied to sections using a SunCollect automatic pneumatic sprayer (Sunchrom

GmbH, Friedrichsdorf, Germany). 15 layers were applied at following speeds: first layer: 10 µL/min; second layer: 20 µL/min; third layer: 30 µL/min and fourth layer and additional: 40 µL/min.

Mass spectrometry analysis

MALDI-MSI data were acquired on a Bruker rapifleX MALDI Tissuetyper™ (Bruker Daltonik GmbH, Bremen, Germany) in reflectron mode using a nominal acceleration potential of ± 20kV[10]. Data was acquired in the *m/z* range of 400-1000 and *m/z* 600-1600 for positive and negative ion mode, respectively. Mass calibration was performed before each analysis using red phosphorous clusters in positive and negative ion mode. Each tissue was imaged sequentially in positive and negative ion mode as recently described[18]. Briefly positive ion data was first acquired using a 20×20 µm^2 laser scan area and a 50×50 µm^2 step size of the stage. After which negative ion data was acquired with identical parameters but with a 25×25 µm^2 offset in stage position applied to produce an interlaced acquisition pattern. For each slide the laser focus position was optimized and 200 laser shots were acquired at each position. Total analysis time was approximately 1 hour per tissue in both polarities including sample preparation time. The identification of selected lipid species was based on both accurate mass measurements and collection of tandem mass spectrometry data using collision-induced dissociation acquired from adjacent tissues using an Orbitrap Elite mass spectrometer (Thermo Fisher, Bremen, Germany) coupled to a reduced pressure MALDI-MSI source as recently described[19]. Data visualisation and analysis was performed using FlexImaging 5.0 (Bruker Daltonik GmbH) and SCiLS lab 2016a (SCiLS GmbH, Bremen, Germany).

Histological imaging of tissue sections slides after MSI analysis

After MSI, matrix was washed off using 2 x 30 second submersions in 100% EtOH. Thereafter, H&E staining was performed using a rapid staining protocol with the use of a Leica linear stainer (Leica ST4020) including 10 second submersions in solutions in the following order: 2 x formaldehyde, 1 x H2O, 3 x haematoxylin, 1 x H2O, 1 x ammoniac water, 1 x tap water, 1 x eosine, 1 x tap water, 2 x 100% EtOH, 2 x Xylene.

Statistical analysis

Numeric variables are presented as mean ± standard deviation (SD), if approximately normally distributed and as median (IQR) otherwise. To measure the difference between perfusate concentrations at the measured timepoints (10, 60, 120, 180 and 240 min) we used the Wilcoxon signed rank test. To test for differences in cold and

warm ischemic injury by histological parameters, we used a Mann-Whitney-U test for all parameters scored, including the total score. All statistical analysis of MSI data was performed using SCiLS lab 2016a software (SCiLS GmbH, Bremen, Germany). For ROC analysis the mean spectrum from all 16 tissues was exported to mMass software[20]. Peak picking was performed using a signal-to-noise threshold of 5 with no baseline corrections or smoothing applied. The generated peaklist was then imported into SCiLS lab 2016a for all subsequent analysis using an interval width of ± 0.25 Da. This was repeated for both positive and negatives ion mode. ROC analysis was performed using the warm and cold ischemia tissues (7 tissues for each per polarity) as two groups and peaks assigned to be discriminatory with an AUC >0.8 or <0.2.

Results and discussion

Severity of ischemia is associated with machine perfusate markers, but not with histology

Ten minutes after the start of perfusion lactate levels (mmol/L) in the perfusate were not different between HMP and SNMP (i.e. cold versus warm ischemia) kidneys (median= 0.6 (interquartile range (IQR), 0.3-0.7) vs. 1.0 (IQR, 0.4-1.0), respectively, p=.293). As sign of a higher metabolic activity, the flush out of lactate was significantly higher in SNMP kidneys compared to HMP kidneys over time (median lactate at 240 min HMP vs. SNMP=1.0 (IQR, 0.9-1.1) vs. 7.4 (IQR, 5.2-9.0), p=.018). As sign of increased ischemic injury in SNMP kidneys, LDH concentrations (U/L) at baseline were not significantly different (median LDH at 10 min HMP vs. SNMP=5 (IQR, 4-11) vs. 11 (IQR, 8-23), p=.236) but after 4 hours of perfusion they were (median LDH at 240 min HMP vs. SNMP=56 (IQR, 41-62) vs. 118 (IQR, 61-130), p=.028). These results are also depicted in Figure 7.2.

A systematic histopathologic examination of the renal tissue sections by two blinded renal pathologists did not discriminate between the different types of ischemia. There were no significant differences found for scoring of tubular cell necrosis, oedema, tubular casts, blebs in tubular epithelial cells, nor for combining these scores. One pathologist was able to find a significant difference between ischemic groups by scoring the loss of brush border (scores of HMP vs SNMP=18 (IQR, 16-25) vs. 25 (IQR, 23-29), p=.024). The results of this pathologist are depicted in Figure 7.3.

Figure 7.2: Renal flush out of lactate and LDH during machine perfusion. Red lines resemble flush out of SNMP kidneys with warm ischemia, the blue line flush out in HMP kidneys with cold ischemia. 1a: After four hours there is a significant difference in median lactate concentration between groups (median lactate: HMP vs. SNMP=1.0 (IQR, 0.9-1.1) vs. 7.4 (IQR, 5.2-9.0), p=.018). 1b: After four hours there is a significance difference in LDH concentration between groups (median LDH: HMP vs. SNMP=56 (IQR, 41-62) vs. 118 (IQR, 61-130), p=.028).

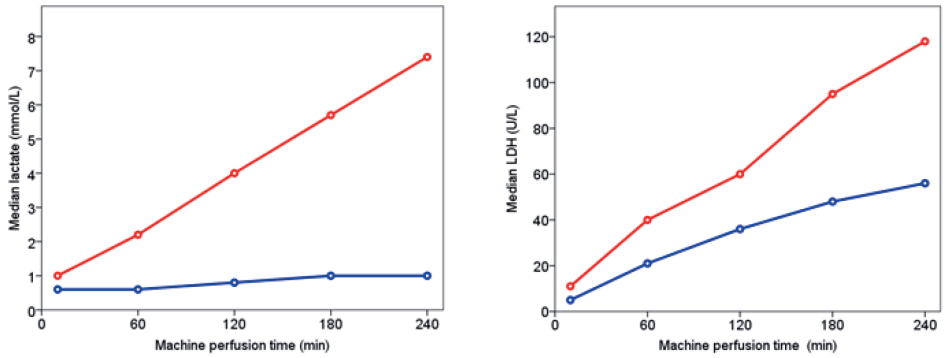
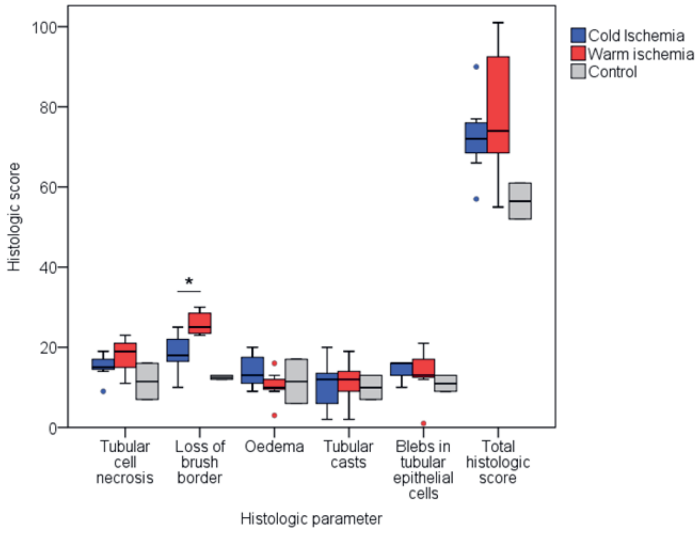


Figure 7.3: Comparison of histologic scores of cold and warm ischemia by a blinded pathologist. Five parameters were scored and a total histologic score parameter was calculated by the sum of all five scored parameters. The Mann-Whitney-U test was applied to test for differences between groups. A p-value ≤.05 was found significant and annotated (*) if so. * Median score for HMP=18 (IQR, 16-25) vs SNMP 25 (IQR, 23-29); p=.024.



We have tested the extent of ischemic kidney injury especially in a pre-clinical porcine model mimicking transplantation. At present, it is not possible to determine the exact viability of an ischemically damaged kidney and to estimate whether

transplantation will lead to a successful outcome. There is no consensus on how to select kidneys suitable for transplantation, because many of the used selection criteria are only poor or moderate predictors of graft outcome[15,16,21-23]. Normothermic machine perfusion, a promising organ preservation strategy, is also used to assess kidneys before transplantation and is increasingly performed to assess AKI[24-26]. In normothermic machine perfusion, extracorporeal membrane oxygenation is utilized to avoid warm ischemia. Preliminary results show that NMP may even recondition and repair ischemically damaged tissue[27]. We used machine perfusate markers to validate the presumed ischemic injury in our perfusion model. Lactate and LDH confirm ischemic injury and may therefore also have potential as viability marker. However, these two markers have often been studied for their potential role as viability marker and are indeed associated with ischemic injury and functional outcome, but they do not have predictive value to be used as a clinical marker, as with most perfusate biomarkers thus far[28]. At present, renal biopsies are increasingly used to assess the quality of the potential donor kidney, in which the extent of chronic histological damage is associated with long term graft function[15,29]. Assessment of acute ischemic damage in pre-transplant procurement biopsies does not have clear additional value[16]. This emphasizes the need for additional viability tests for acute kidney injury, which can be used in transplantation. A possible test may be MSI.

MSI can discriminate between severe and mild ischemic injury via lipid degradation products

In positive ion mode analysis of porcine tissues, phosphatidylcholines (PC) and sphingomyelins are mostly observed as a mixture of protonated, sodiated and potassiated forms, a common observation in MSI. Negative ion mode allowed detection of a wide variety of acidic lipids, including phosphatidylserines (PS), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), phosphatidylinositols (PI), sulfatides (ST) and cardiolipins as predominantly deprotonated ($[M-H]^-$) ions. The average positive and negative ion spectra acquired from the warm ischemia, cold ischemia and control kidneys are showed in Figure 7.4 and 7.5. We performed a receiver-operator characteristic (ROC) analysis to elucidate the discriminatory lipid MSI signals for ischemic injury in negative ion mode between *m/z* 600 *and* 1600. In total three monoisotopic *m/z* signals were found with an area under the curve (AUC) value greater than 0.8 (elevated in warm ischemia) which were attributed to the three monoisotopic lipids described above and listed in Table 7.1.

Figure 7.4: **Average positive ion spectra from warm ischemia (a), cold ischemia (b) and control (c) tissues.**

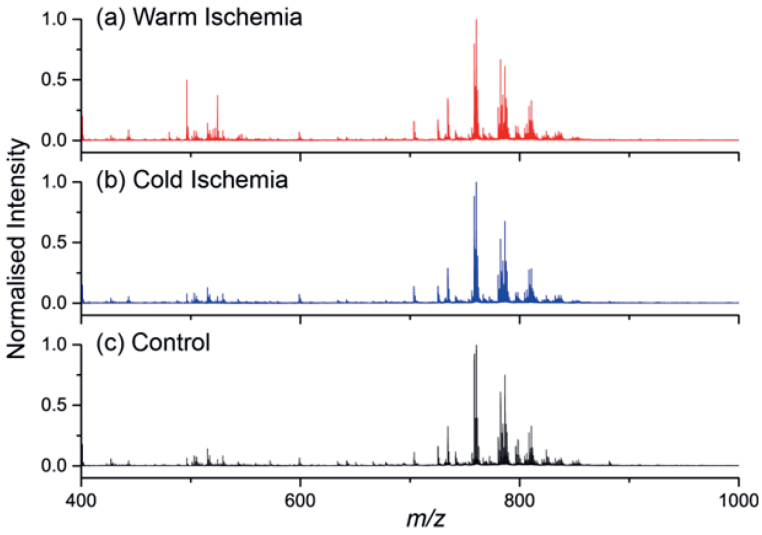


Figure 7.5: Average negative ion spectra from warm ischemia (a), cold ischemia (b) and control (c) tissues.

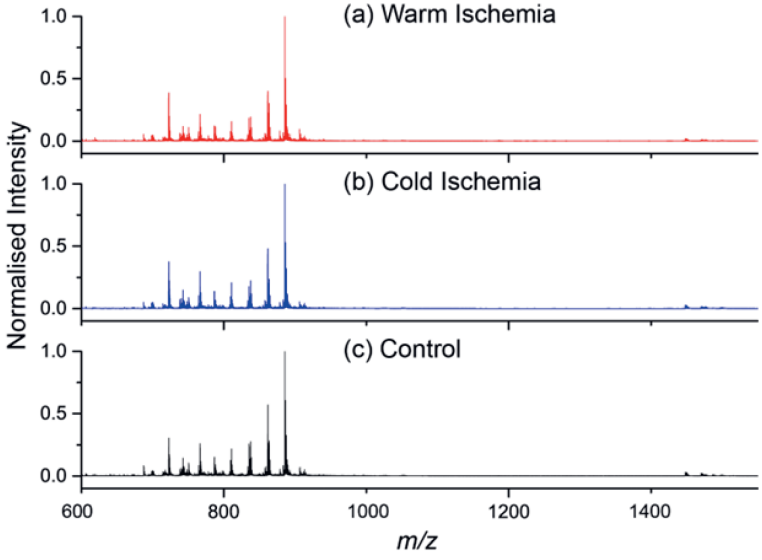


Table 7.1: Discriminatory *m/z* values that are significantly elevated (AUC>0.8) in ischemically damaged renal tissue. Only monoisotopic lipids are shown. Lipids are identified following ROC analysis of tissues exposed to cold and warm ischemia. No discriminative ions elevated in the cold ischemic tissue were observed.

MALDI-ToF *m/z*	AUC	Orbitrap *m/z*	Lipid Sum-Composition	PPM *m/z* error
Negative Ion Mode				
619.3	0.85	619.28815	$[LPI(20:4)-H]^-$	-1.2
1185.8	0.89	1185.73349	$[MLCL(54:6)-H]^-$	-1.5
1207.8	0.85	1207.71537	$[MLCL(54:6)-2H+Na]^-$	-1.5
Positive Ion Mode				
480.8	0.87	480.34436	$[LPC(O-16:1)+H]^+$	-1.0
496.4	0.81	496.33935	$[LPC(16:0)+H]^+$	-0.8
520.4	0.84	520.33924	$[LPC(18:2)+H]^+$	-1.0
522.4	0.82	522.35495	$[LPC(18:1)+H]^+$	-0.9
524.4	0.82	524.37059	$[LPC(18:0)+H]^+$	-0.9
544.4	0.80	544.33805	$[LPC(18:1)+Na]^+$	1.3
546.4	0.82	546.35264	$[LPC(18:0)+H]^+$	-0.7

In the negative ion mode an increase in ion signal between *m/z* 1180-1240 was observed for all tissues with induced warm ischemic damage (Figure 7.6). In particular ions observed at *m/z* 1185.8 and 1207.8 were elevated in the warm ischemia tissue (Figure 7.6a and 7.10a). The discriminatory *m/z* 1185.8 ion is assigned by accurate mass to monolyosocardiolipin $[MLCL(54:6)-H]^-$ with the primary molecular species assigned to $[MLCL\ (18:2\_18:2\_18:2)-H]^-$ using tandem mass spectrometry. The *m/z* 1207.8 is assigned to the analogous sodium adduct $[MLCL(18:2\_18:2\_18:2)-2H+Na]^-$. Interestingly, no corresponding decrease in the precursors to these MLCL signals, namely CL(72:8) as $[CL(72:8)-H]^-$ and $CL(72:8)-2H+Na]^-$ at *m/z* 1447.9 and 1669.9, respectively, was observed (Figure 7.7). Furthermore, in the negative mode lysophosphatidylinositol (LPI), namely $[LPI\ (20:4)-H]^-$ at *m/z* 619.3 was also elevated in tissues exposed to warm ischemia (Figure 7.8). No ions elevated in cold ischemic or control tissues relative to warm ischemic tissues were observed. Ischemia-related elevation of lysophosphatidylcholine (LPC) lipids was also observed in the positive ion mode (Table 7.1 and Figure7.9). The distribution of the most abundant LPC, observed at *m/z* 496.3 ($[LPC(16:0+H]^+$) throughout all 16 tissues is provided in Figure 7.10b.

Figure 7.6: Average negative ion MALDI mass spectra acquired from each individual warm ischemia (a), cold ischemia (b) and control (c) tissue in the *m/z* range 1180-1300. Data show the elevation of MLCL(54:6)-related ion signal in the case of warm ischemia.

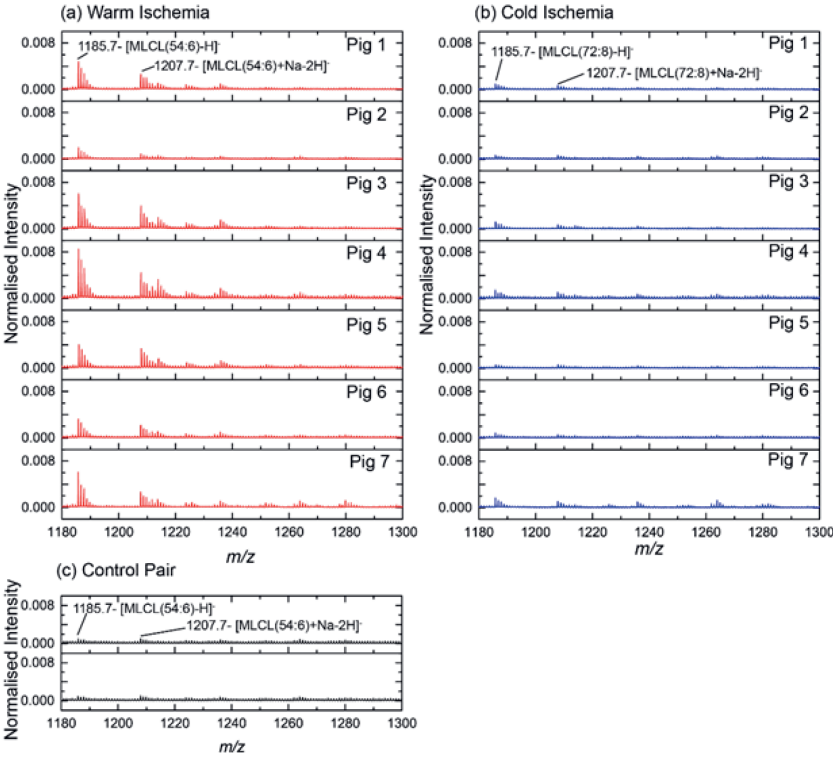


Figure 7.7: **Average negative ion MALDI mass spectra acquired from each individual warm ischemia (a), cold ischemia (b) and control (c) tissue in the *m/z* range 1420-1540. Data show the signal intensity of cardiolipin signal (mostly originating from CL(72:8) across each tissue.**

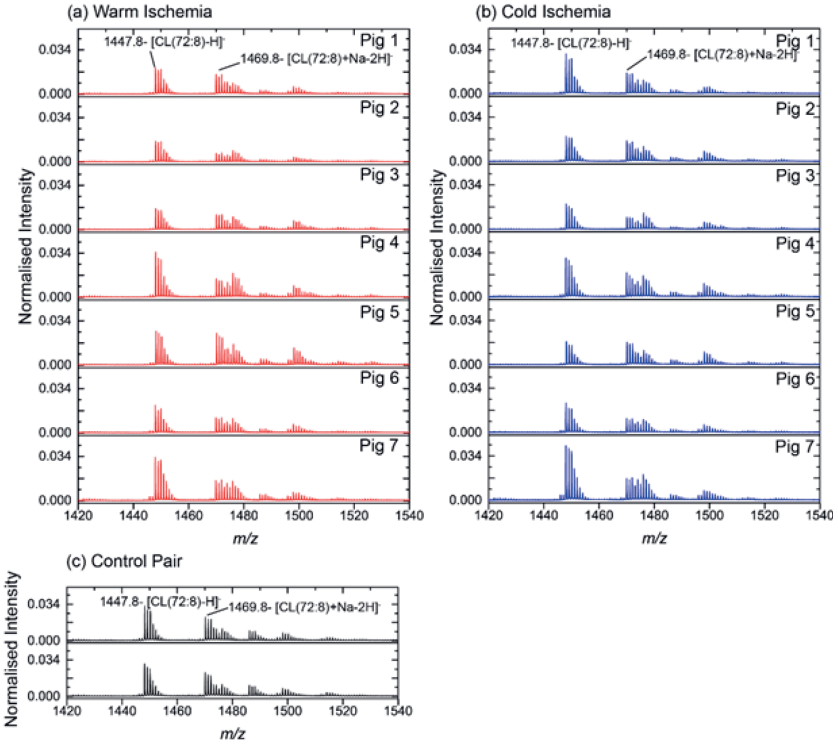


Figure 7.8: Average negative ion MALDI mass spectra acquired from each individual warm ischemia (a), cold ischemia (b) and control (c) tissue in the *m/z* range 610-630. Data show the elevation of $[LPI(20:4)-H]^-$ signal in the case of warm ischemia).

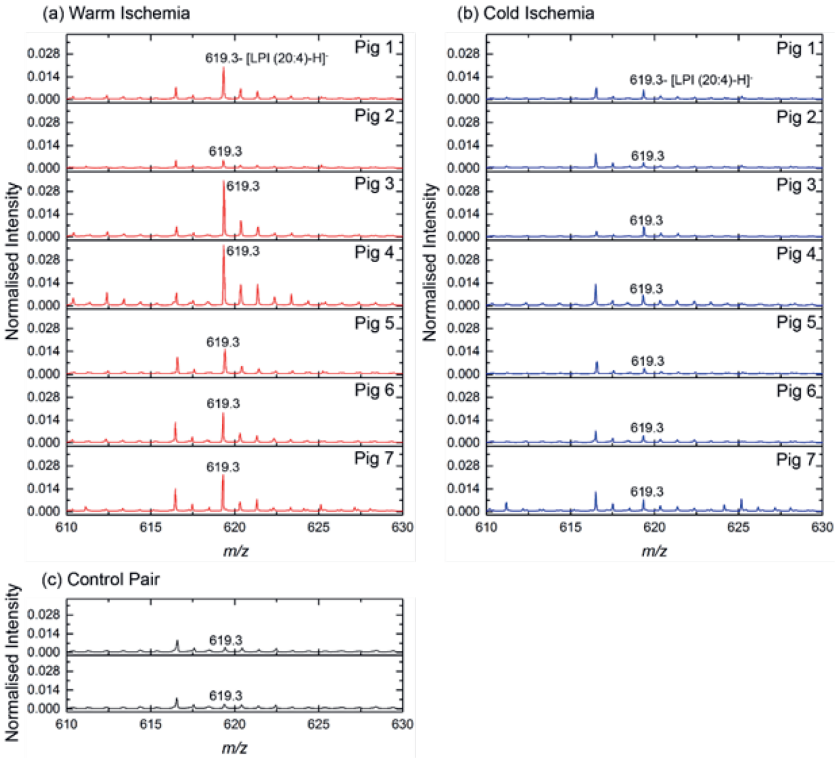


Figure 7.9: Average positive ion MALDI mass spectra acquired from each individual warm ischemia (a), cold ischemia (b) and control (c) tissue in the *m/z* range 490-530. Data show the elevation of lysophosphatidylcholine signals in the case of warm ischemia.

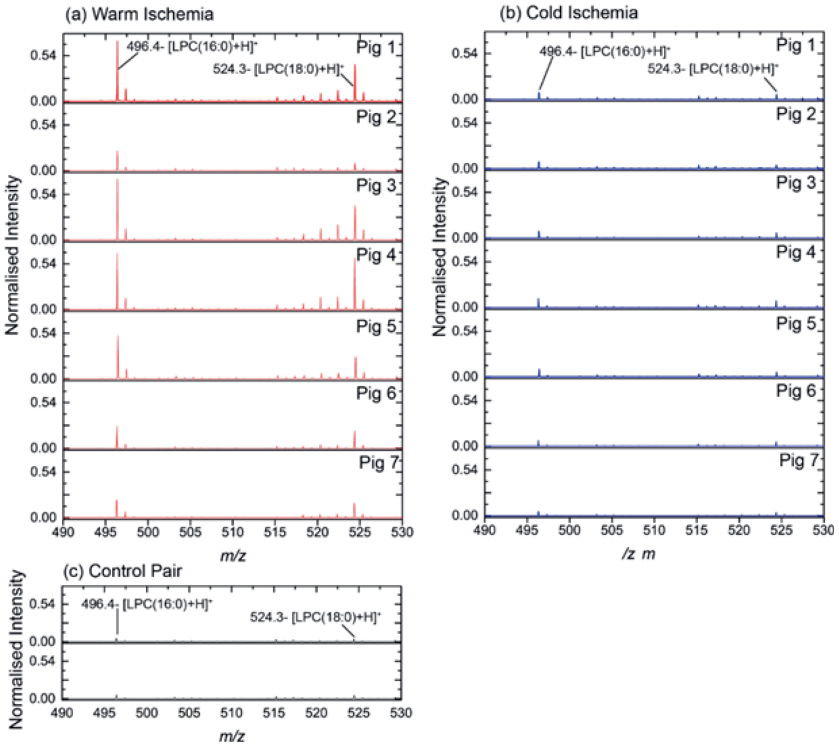
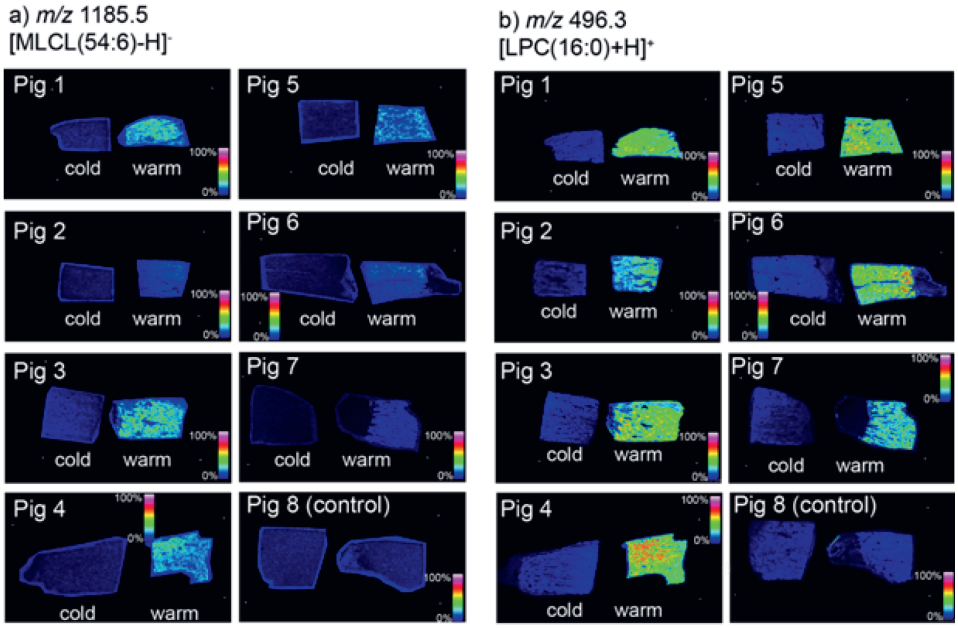
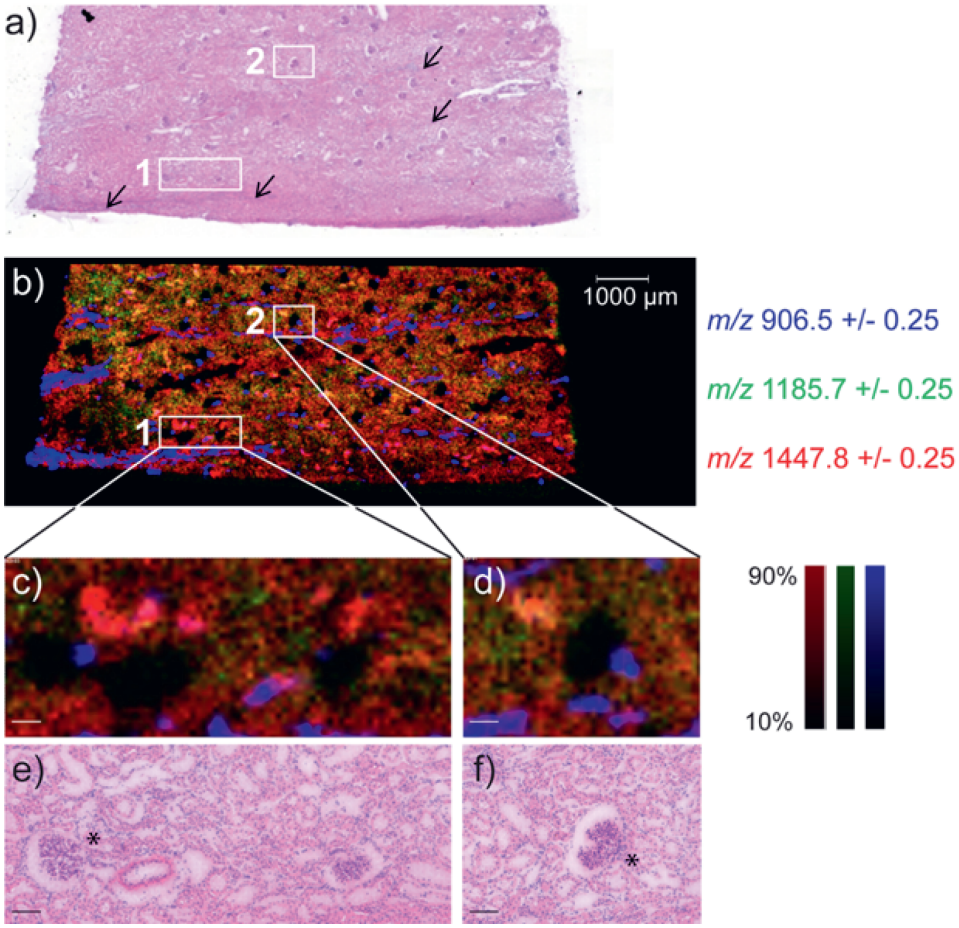


Figure 7.10: MS images of discriminatory structures. Mass spectrometry imaging data acquired at 50 µm resolution revealing spatial distributions of the lysolipids, $[MLCL(54:6\text{-}H]^-$ (a) and $[LPC(16:0+H]^+$ (b) through all 16 tissues (7x warm ischemia, 7x cold ischemia and 2x control). All images are normalised to the total ion count.



MSI results also suggested a spatial heterogeneity in the degradation of cardiolipins in ischemic tissue. To further investigate this we imaged a warm ischemic tissue at 20 µm spatial resolution (Figure 7.11). The distribution of the $[M\text{-}H]^-$ ions of CL(72:8), MLCL(54:6) and the sulfatide ST(d42:1(2OH)) are shown in red, green and blue, respectively. Co-registration of the post-MSI H&E stained tissue (Figure 7.11b, c and d) revealed the sulfatide signal at *m/z* 906.5 to be localised in the area of the medullary rays (arrows Figure 7.11a) and in the juxtaglomerular areas (astericces Figures 7.11e and f). Interestingly the ischemia-related MLCL(54:6) degradation product is not exclusively co-localised with its precursor Cl(72:8), suggesting that ischemia-related breakdown of mitochondrial-specific cardiolipin occurs to greater extent in distinct tubular areas of the renal cortex (Figure 7.11b, c and d).

Figure 7.11: Lipid degradation and distribution in renal tissue after warm ischemic injury. Post-MSI H&E stained tissue (a, e, f) used to generate data in (b, c, d). (b) Mass spectrometry imaging data acquired at 20 µm resolution revealing spatial distributions of [ST(d42:1(2OH)-H]$^-$ (*m/z* 906.5, blue), [MLCL(54:6-H]$^-$ (*m/z* 1185.7, green) and [CL(72:8-H]$^-$ (*m/z* 1447.8, red). Two regions of interest and marked as 1 and 2 are enlarged (in (c) and (d), respectively) to show the heterogeneous MLCL(54:6) formation relative to its main precursor CL(72:8) in distinct tubular cortical areas. All images are normalised to the total ion count. Medullary rays are indicated with black arrows in (a). Asterix (*) in (e) and (f) indicate juxta glomerular areas.



MSI shows potential as viability test and offers new insights in pathophysiology of ischemic injury

In a porcine donor kidney model, we show that it is feasible to rapidly visualize ischemic damage in renal tissue on the basis of local lipid profiles. We are able to visualize and

identify alterations in the lipidome between kidneys with mild or severe ischemic injury using MSI. The advantage of such molecular analysis likely arises by detection of the rapid biochemical alterations occurring during acute tissue injury. This short time frame may not allow significantly histological changes to be induced, leading to the poor accuracy of histological identification of acute ischemia (e.g. Figure 7.3). Using MALDI-MSI in this context has two distinct advantages. First, by rapid assessment of ischemically damaged kidneys using MSI, it may provide a practical tool to assist in viability assessment and thereby it holds the potential to improve donor kidney selection. With the development of rapid MALDI-MSI instrumentation and automated matrix application, such as those employed in this study, a single tissue could be analysed with 50 µm spatial resolution in 30 minutes in both positive and negative ion modes, which is within the critical time frame needed for e.g. graft viability assessment in clinical transplantation. Total analysis time, including matrix application, is approximately 1 hour. Second, in this study we have identified multiple discriminative molecules of ischemic injury, not yet described in AKI. In particular, a suite of lysophospholipids have been identified as significantly elevated in severe ischemia, compared to mild ischemia. The source of these lysolipids is likely lipid degradation by increased activation of phospholipase A2 (PLA2) enzymes which have been previously been shown to be exhibit elevated activation in during ischemia[30-33]. The observation of both degradation of mitochondrial lipids (cardiolipin) and membrane lipids (PC, PI and PE; also observed in the high resolution Orbitrap analysis) may be reflective of different forms and activations of PLA2 in renal ischemia that lead to the disruption of cell membranes[34-36]. Thereby, MSI shows potential to identify new molecules which may play an important role in AKI assessment. Furthermore, it offers new insights in molecular pathways of ischemic injury and may even reveal novel targets for diagnostics and therapeutics that reduce ischemic injury.

MSI identifies lipid degradation products as ischemic marker

Using MSI we were able to identify surrogate markers of ischemic processes such as MLCL, LPC and LPI in kidney tissue with severe ischemia. Interference with cardiolipin activity has already shown to be beneficial in chronic renal damage in tubular and endothelial cells in the context of ischemia reperfusion as well as in the context of revascularisation in renal artery stenosis[37,38]. Cardiolipins are located in the inner membrane of mitochondria and are needed for protection of mitochondrial cristae and ATP generation. Ischemia reperfusion induced mitochondrial injury plays a pivotal role in organ viability in transplantation[39] and is associated with kidney injury[40,41]. Next to the kidney, cardiolipins are also involved in ischemia reperfusion injury in other organs

like the brain[32], heart[42] and liver[43]. Moreover, action of mitochondrial-specific PLA2 forms has been shown to result in further lipid mediators in ischemia which have further detrimental effects on tissue quality[44]. We now are the first to rapidly measure and visualize elevated levels of MLCL in ischemic renal tissue in a clinically acceptable timeframe, which may pave to the way to cardiolipin measurements in for instance assessment of graft viability.

Other important lysolipids that we identified in association with severe renal ischemia are LPCs and LPIs. Increased expression of LPCs has been described in ischemic tissue, like liver tissue (including graft biopsies of DCD donors)[45] and heart tissue[46]. In renal ischemia, proximal tubules show increased expression of PI on their apical membrane[47,48]. Elevated LPC levels were also found in renal ischemia[49,50]. The function of LPCs is not fully understood, but in ischemic conditions they can e.g. increase tubular secretion of the pro-fibrotic factors PDGF-B and CTGF[51]. LPCs are hydrolysed PCs following removal of *sn*-2 fatty acyl chains by PLA2. PLA2 activity has been described to be altered in renal ischemia reperfusion injury, which can lead to differential cytotoxic or cytoprotective effects on renal tubular cells[30,34-36]. Thus, we have visualised expression of compounds in ischemic processes, which are confirmed by literature[37,38,47-50], in an early stage of loss of organ viability within a very short time frame. A time frame in which morphological changes are difficult to observe. This has not been done before and holds the potential to aid in assessment of AKI, risk prediction for CKD and ultimately improved outcome.

Limitations of the study model

Within the current porcine model, we compared two degrees of ischemic injury which were based on clinical transplantation: four hours of cold perfusion (mild ischemia) versus four hours of subnormothermic (warm) perfusion (severe ischemia). The difference in metabolic activity and cell injury is shown by our perfusate analyses. The clinical value of the current study needs further investigation, in animal models of AKI in relation to outcome, and in human kidney biopsies. MSI has great potential in its application on human tissue biopsies. Thus, future studies are needed to study alterations in human biopsies, in recipients of whom transplant outcome is known. Furthermore, in human biopsies also chronic histological damage, in relation to inflammation and fibrosis, may be present (in contrast to the young pigs we studied), and thus lipid patterns may differ. Also studies with MSI in relation to the pathophysiology of AKI to CKD transition will increase our pathophysiological insights herein, and provide novel diagnostic strategies to decrease kidney failure.

Conclusions

In conclusion, we have shown that MSI shows great potential as new tool to assess ischemic injury. In this study we were able to discriminate moderately and severely ischemically injured kidneys for every pair analysed. MSI has the capacity of identifying discriminatory molecular patterns of ischemic kidney injury in a clinical acceptable time frame. In addition to histopathological examination, it provides useful information on the biochemical changes during ischemia relative to tissue morphology. Furthermore, we have identified lipid molecules using MSI, which may serve as biomarker for acute kidney injury as future outcome prediction.

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Chapter 8

Measurement of renovascular circulating volume during hypothermic organ perfusion

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Abstract

Background. Kidney donation after cardiac death leads to vascular damage as a result of warm ischemia, affecting renovascular circulating volume. Novel ultrasound dilution techniques may be used to measure renovascular circulating volumes during hypothermic machine perfusion of donor kidneys.

Methods. Renovascular circulating volumes of machine perfused porcine kidneys were repeatedly measured by ultrasound dilution at different perfusion pressures (30, 40, 50, and 60 mmHg), durations of perfusion (1 and 24 h) and warm ischemia times (15 and 45 min). Validity of ultrasound dilution was assessed by comparing volume changes after clamping of renal artery branches.

Results. Repeatability of ultrasound dilution measurements of renovascular circulating volumes was good (mean coefficient of variation: 7.6%). Renovascular circulating volumes significantly increased with higher perfusion pressures, remained constant over time, and significantly decreased with longer warm ischemia times. Changes in ultrasound dilution measurements after renal artery branch clamping did not correlate with changes in actual perfused volumes.

Conclusions. Ultrasound dilution is a reproducible method to assess renovascular circulating volumes in machine perfused kidneys that is susceptible to changes in warm ischemia times. Future studies should evaluate the value of renovascular volume in pre-transplant kidney viability testing.

Introduction

Kidney transplantation in patients with end-stage renal disease leads to superior life expectancy and better quality of life compared to dialysis[1]. In the past decades there is an increasing shortage of donor kidneys. Organs from donors after cardiac death (DCD) have become generally accepted to expand the donor pool and meet the demand for additional donor kidneys. However, transplantation of DCD kidneys is associated with an increased incidence of initial graft dysfunction[2,3].

The higher incidence of graft dysfunction in DCD kidneys, compared to kidneys from donation after brain death (DBD) donors, can be attributed to the prolonged period of warm ischemia before organ procurement. This period of warm ischemia leads to more capillary damage due to thrombosis, vasoconstriction and oedema, and may consequently result in a decreased renovascular circulating volume. Therefore, pre-implantation renovascular circulating volume of the kidney may be associated with the severity of warm ischemic injury and could be a valuable predictor of transplant outcome.

The dilution technique is used to measure circulating volume in hemodialyzer fibre bundles. It determines the dilution of an injected saline bolus with ultrasound flow probes before and after transit of the dialyzer. In hemodialyzer volume assessment this technique is accurate and its results reproducible[4-6]. The same technique can be used to measure renovascular circulating volume in donor kidneys. Renovascular circulating volume may be a valuable viability marker in ischemically damaged donor kidneys if measured before transplantation. Therefore, we studied the dilution technique to measure renovascular circulating volume in machine perfused porcine kidneys and determined if renovascular circulating volume is associated with warm ischemia time.

Methods

The experiments were carried out with porcine kidneys, which were recovered from a regional slaughterhouse. Pigs were between 6 and 7 months of age and had a mean weight of 105 kg. In the highly standardized slaughtering process, the time from death until recovery of the kidneys took exactly 15 minutes. After receiving an ‘en bloc’ pair of kidneys, kidneys were separated and then flushed with 0.5 L of Histidine-Tryptophan-Ketoglutarate (HTK) preservation solution for 20 minutes at the site. For transport to the preservation laboratory kidneys were temporarily stored on ice. Two hours after

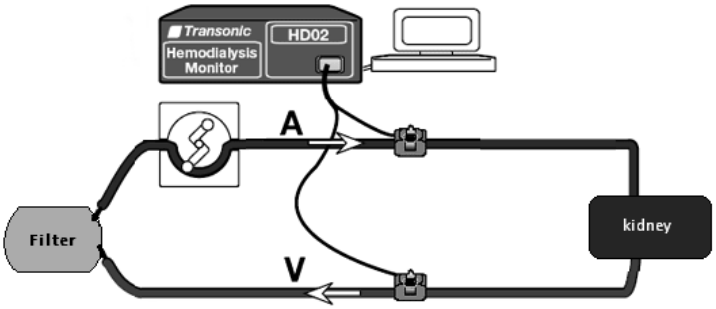
recovery, kidneys were connected to a pulsatile perfusion machine (LifePort Kidney Transporter (model # LKT-100-P), Organ Recovery Systems, Des Plaines, USA) and perfused with 500 mL University of Wisconsin (UW) solution. During machine perfusion, pressure (mmHg), flow (mL/min), and resistance (mmHg/mL/min) were recorded using the Lifeport Kidney Transporter.

According to national legislation, study approval by the local animal experiments committee (DEC) was not required. Our study did not interfere with the standardized slaughtering process; kidneys were obtained after death.

Dilution technique

Renovascular circulating volume was assessed with the dilution technique. This technique has been described in detail before by others[6]. Briefly, the renovascular circulating volume was measured with a dilution monitor (HD02 haemodialysis Flow-QC monitor, Transonic Systems Inc, Ithaca, NY) via two reusable ultrasound flow probes (Transonic H4E Flow Probes) installed at 2 cm distance from the kidney on the arterial tube and on a tube connected to the renal vein. The dilution monitor was connected to a computer installed with HD02 Dialysis Monitoring Software (Figure 8.1). The system was calibrated for UW solution. To obtain measurements, we injected a saline bolus of 2 mL into the arterial injection port. The indicator is first sensed by the arterial sensor that records the time and volume of injection. The indicator then travels through the renovascular system and is recorded by the venous sensor on the renal vein. The venous sensor measures the change in concentration of saline in the preservation fluid and the transit time through the renovascular system. The renovascular circulating volume is calculated by the equation $V = Q \times MTT$. In this equation Q is defined as the flow in the system and MTT (mean transit time) as the average transit time of the indicator. The first bolus was injected one hour after the start of perfusion (T1), to ensure a balanced state of perfusion. The additional volume within the tubing between vein or artery and sensors was kept constant by placing the sensors at a fixed distance to the kidney.

Figure 8.1: Schematic research setting. Sensors were placed in the perfusion machine on the arterial (A) tube, and on the venous tube (V), connected to the renal vein. The dilution monitor was connected to a computer installed with HD02 Dialysis Monitoring Software.



Flow measurements

To assess the correlation between measured flow by ultrasound flow probes and derived from roller pumpspeed, we performed simultaneous measurements of flow in six kidneys using at pressures of 40, 50 and 60 mmHg. In total 87 paired measurements were done. Given the very strong correlation between flow measured by ultrasound flow probes and derived from roller pump speed, only flow data derived from roller pump speed was used in the calculations.

Repeatability and validation

To verify repeatability in measuring renovascular circulating volume, we performed sets of 6 measurements at different perfusion pressures (30, 40, 50 and 60 mmHg) in 9 kidneys. Per kidney and per pressure, 6 boluses of saline were injected at one hour after the start of perfusion (T_1) and after 24 hours of machine perfusion (T_{24}). A total of 426 measurements (71 data sets) were done. In addition, of all measurements in this dataset we plotted the correlation of measured volume against flow as well as volume against resistance.

Although the dilution technique has shown to be accurate in estimating perfused volume in dialyzer circuits, there is no golden standard of measuring renovascular circulating volume. Therefore we verified validity of the dilution technique by comparing volume ratio and weight ratio in 6 partially perfused kidneys after the repeatability measurements were done, at a pressure of 60 mmHg. One of the renal arterial branches was clamped and the measurements repeated. Next, a bolus of methylene blue was injected into the system, colouring the perfused part of the

vascular system. The coloured tissue was macroscopically divided from the non-coloured tissue and the percentage of the weight of each part was assessed. Validity was determined comparing the renovascular circulating volume ratio and weight ratio of perfused and unperfused parts of the kidney.

Effect of warm ischemia time on renovascular circulating volume

To assess the effect of warm ischemia on renovascular circulating volume, we performed measurements in 5 kidney pairs. Each pair contained a kidney exposed to a mean of 15 minutes and a kidney exposed to a mean of 45 minutes of warm ischemia. After retrieval, this was realized by immediate cannulation of one of the renal arteries of the 'en-bloc' kidneys and connecting only this kidney to the perfusion fluid for initial flush. After the initial flush (20 min), kidneys were separated; the first kidney was stored on melting ice, and the contralateral kidney was stored at room temperature until 45 minutes of warm ischemia and then flushed. Measurements were performed after one hour of machine perfusion and after 24 hours (T_1 and T_{24}) with a perfusion pressure of 40 mmHg.

Data analysis

Continuous variables are presented as means $\pm$ standard deviations (SD) if normally distributed (histogram). Correlation coefficients were calculated with the Pearson test for normally distributed variables, and with the Spearman test otherwise. To determine repeatability, the coefficient of variation was calculated as the ratio of mean volume and standard deviation per dataset. Paired sample t-test was used to calculate the significance of differences between time points. Renovascular volume differences after clamping were calculated using the Wilcoxon Signed Rank test. We used a paired-samples t-test to analyse differences between the 15 and 45 minutes WIT groups.

Data analysis was done using SPSS 18.0 and Microsoft Excel 2007 for Windows. A p-value below 0.05 was considered evidence of a significant difference.

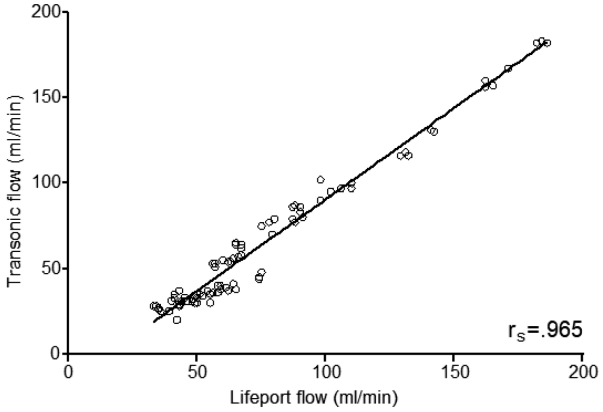
Results

Flow measurements

The correlation between perfusate flow measured by ultrasound flow probes and derived from roller pump speed was very strong (R=0.97, 95% CI: 0.95-0.98, p<0.001;

Figure 8.2). Perfusate flow derived from roller pump speed was on average 11.0±8.0 mL higher than perfusate flow measured by ultrasound flow probes.

Figure 8.2: Correlation of flow between the machine perfusion system and ultrasound flow probes using spearman correlation coefficient.



Repeatability and validation

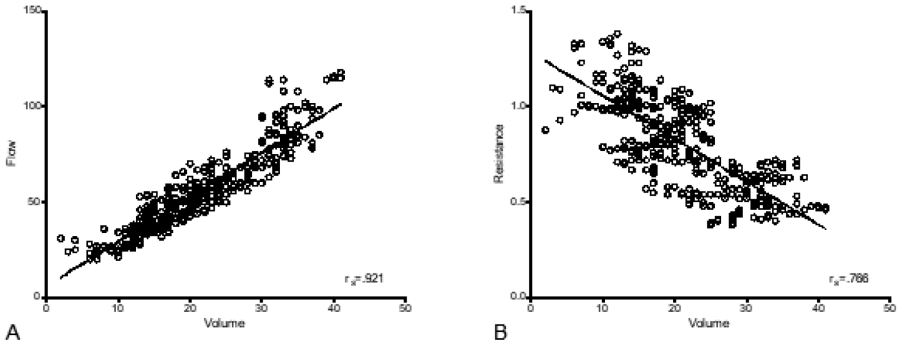
In one kidney, the series of measurements at a pressure of 30 mmHg at T_1 failed because flow was too low. The mean coefficient of variation (CV) of renovascular circulating volume measured by ultrasound dilution was 7.6 ± 6.2 % (Table 8.1). The CV did not significantly change with different durations of perfusion (1 and 24 hour; mean CV 6.8 ± 4.5% and 8.4 ± 7.5%, respectively; p=0.166). The mean CV at a perfusion pressure of 30 mmHg was higher than at 40 mmHg (p=0.007), but did not significantly change comparing other pressures (40 vs 50 mmHg; p=0.229; 50 vs 60 mmHg; p=0.978). Excluding the 30 mmHg measurements from the analyses showed a decrease of mean CV and SD from 7.6 ± 6.2% to 6.2 ± 3.6%. Correlation of all volume measurements with flow and with resistance was good (r_s=.921 and r_s=.766, respectively) (Figure 8.3).

Renovascular circulating volumes measured by ultrasound dilution decreased after clamping of one of the renal arterial branches (from 25.6 ± 8.9 mL to 16.3 ± 7.6 mL; p=0.028). However, these changes in renovascular circulating volume were not correlated with changes in actual perfused volume as measured by methylene blue staining (Figure 8.4).

Table 8.1. Characteristics of measurements at different pressures. Variables as mean ± standard deviation. N=number of measurements. V=volume (mL). CV=coëfficient of variation (%). Q=flow (mL/min). R=resistance (mmHg/mL/min).

P (mmHg)	Time	N	V (mL)	CV (%)	Q (mL/min)	R (mmHg/mL/min)
30	T_1	8*6=48	15.3 ± 4.8	11.5 ± 6.7	37.5 ± 9.6	0.76 ± 0.20
	T_{24}	9*6=54	15.9 ± 8.2	12.8 ± 12.3	39.6 ± 15.3	0.78 ± 0.31
40	T_1	9*6=54	18.9 ± 6.2	6.8 ± 2.1	46.4 ± 11.4	0.82 ± 0.19
	T_{24}	9*6=54	21.0 ± 8.1	7.1 ± 6.8	51.7 ± 18.9	0.79 ± 0.30
50	T_1	9*6=54	21.8 ± 6.6	4.9 ± 2.3	55.9 ± 11.2	0.85 ± 0.18
	T_{24}	9*6=54	23.4 ± 7.9	6.6 ± 2.5	62.9 ± 22.4	0.81 ± 0.28
60	T_1	9*6=54	25.2 ± 7.7	4.7 ± 2.1	64.9 ± 13.3	0.88 ± 0.18
	T_{24}	9*6=54	24.9 ± 8.1	6.9 ± 3.8	75.1 ± 24.3	0.79 ± 0.24

Figure 8.3: Correlation of flow (mL/min) (A) and resistance (mmHg/mL/min) (B) measurements with renovascular circulating volume (mL).



Effect of perfusion pressure, duration of perfusion and warm ischemia time on renovascular circulation volume

Per pressure (30, 40, 50 and 60 mmHg), renovascular circulating volume measured by ultrasound dilution did not differ between T_1 and T_{24} (p=0.557; p=0.084;p=0.126; p=0.833, respectively; Table 8.1). Renovascular circulating volume increased significantly with every 10 mmHg increase of pressure at T_1 and for the increase from 30 mmHg to 40 mmHg at T_{24}. Increasing the duration of hypothermic perfusion from 1 hour to 24 hours did not change renovascular circulating volumes (mean renovascular circulating volume 20.4 ± 7.3 mL and 21.5 ± 8.7 mL, respectively; p=0.078).

The mean renovascular circulating volumes of kidneys with 15 minutes of warm ischemia were higher than of kidneys with 45 minutes of warm ischemia (34.1 ± 6.0 mL

vs. 26.8 ± 5.3 mL, respectively; p= 0.005) at T_1, but did not significantly differ at T_{24} (36.7 ± 11.4 mL vs. 27.3 ± 8.0 mL, respectively; p=0.059).

Discussion

Organs from DCD donors have rapidly increased the deceased donor pool, but suffer from warm ischemic injury resulting in a higher incidence of postoperative graft dysfunction. Pre-transplant assessment of DCD kidneys in order to select viable grafts for transplantation is crucial to obtain the best results after transplantation. Warm ischemia can lead to microvascular damage, affecting renovascular circulating volume. The dilution technique is a relatively simple method to measure intravascular volume and has not been used before in transplant surgery. As intravascular volume may be associated with organ viability, the dilution technique is a potentially valuable method to use in organ viability assessment. Therefore we determined the repeatability and the validity of this technique and we assessed the effect of warm ischemia on renovascular circulating volume in machine perfused porcine kidneys.

In 1897 the dilution technique was introduced for cardiac output measurement[7]. More recently this method has been applied to measure regional blood flow in heart and lungs and this application has gained wide acceptance[8]. Since 1995 a modification of the dilution technique has been used to measure shunt flow, recirculation and cardiac output during hemodialysis[4,9]. The current study is the first that applies the dilution technique to measure renovascular volume of machine perfused kidneys. Given the importance of pre-transplant viability testing in DCD kidneys, we have studied thefeasibility of the dilution technique to measure renovascular circulating volume as a new potential viability marker.

We have shown that the dilution technique is a reproducible technique to measure renal blood flow in machine perfused porcine kidneys. The repeatability of the dilution method was good, with an acceptable CV of 7.6%. Repeatability did not change with duration of perfusion, but did show changes at different perfusion pressures. CV at a pressure of 30 mmHg showed a significant higher value than at 40 mmHg, indicating that measurements at low pressure and flow are less reliable. This is probably due to equipment adjustments and should be optimized.

If one of the renal arterial branches of the kidney was clamped, measured renovascular volume decreased for all kidneys. Nonetheless, a correlation between

volume and weight ratio could not be demonstrated. In four of six kidneys, volume and weight ratio measurements after clamping one arterial branch were almost equivalent, but ratio's in two other kidneys were slightly off the line of equality of ratio's. One of the limitations is that, in all six kidneys, between 30 to 50% of the kidney was excluded from perfusion. Moreover, it is not possible to assess if differences between volume and weight ratio measurements are caused by differences in the initial perfusion of one kidney. One part of a kidney may be better perfused with a higher intravascular volume than another.

We think that pre-transplant viability assessment by the dilution technique could have advantages as compared to viability assessment by machine perfusion. Machine perfusion has two potential advantages over cold storage. Firstly, machine perfusion improves early graft function compared to cold storage by preventing interstitial oedema, decreasing oxidative stress, improving adenosine triphosphate (ATP) availability and by washing out thrombi and metabolic products[10-16]. Secondly, machine perfusion allows early viability assessment by measuring perfusion characteristics, including flow, pressure and renovascular resistance, or biomarkers[17-22]. However, recent studies show that the value of these parameters to predict kidney transplant outcome is limited[21,23,24]. Machine perfusion characteristics could be influenced by leakage from the arterial connection due to atherosclerosis of the patch or by leakage from small arterial branches that have been damaged during procurement. In the dilution technique however, leakage cannot influence the measurements as an exact bolus of saline is assessed. However, the theoretical advantage of the dilution technique in viability testing has to be proven in clinical practice.

Renovascular volume decreased after a prolonged period of warm ischemia. This may be explained by the manifestation of oedema. Cellular volume regulation depends on mechanisms that require sodium-potassium ATP-ase activity, which is limited by decreased ATP during ischemia[25,26]. The significant differences between renovascular circulating volumes between short and long ischemia did not sustain until 24 hours of machine perfusion. This is in line with previous studies, which demonstrate that perfusion differences decrease over time[23,27]. In the study we only compared different warm ischemia times at a machine perfusion pressure of 40 mmHg. This is the maximum perfusion pressure used at our and many other transplant centres to avoid microvascular injury. Higher perfusion pressures may open capillaries, with better perfusion but are also associated with vascular injury due to shear stress[28-30]. The finding that renovascular circulating volume is associated with warm ischemia time confirms our hypothesis on the relevance of renovascular circulating volume.

Further studies are necessary to assess the value of the dilution technique and renovascular volume measurements in human transplantation. Sterile flow probes for the renal artery and vein are available which makes testing in human kidneys possible. The assessment of the effect of different pharmacological interventions during machine perfusion including vasodilating and antithrombolytic drugs on circulating volume could be a future application of the dilution technique.

We conclude that the dilution technique is a reproducible way to assess renovascular circulating volume in machine perfused porcine kidneys, that the technique is suitable to detect differences in warm ischemia time.

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Chapter 9

Capsulotomy of ischemically damaged donor kidneys: a pig study

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Abstract

Background: Ischemia reperfusion injury of donor kidneys may worsen transplant outcome. Kidneys with severe injury, like kidneys of donation after circulatory death donors, develop edema, which may lead to renal compartment syndrome with reduced tissue perfusion. We studied the effect of capsulotomy during hypothermic machine perfusion of ischemically damaged porcine kidneys.

Methods: Eight pairs of kidneys from slaughterhouse pigs were assigned into two groups: 20 min and 45 min of warm ischemia. After 21 hours of hypothermic machine perfusion, capsulotomy was performed, and perfusion was continued for two hours. During perfusion, flow (Q), renal resistance (RR), renovascular circulating volume (RCV), intraparenchymal pressure (IPP) and weight were recorded. Parenchymal injury was examined with methylene blue infusion.

Results: Mean flow and RCV increased directly after capsulotomy (percentage increase (95% confidence interval), ΔQ = 32% (17, 47), p=.001 and ΔRCV = 19% (3, 35), p=.023). Mean RR decreased (ΔRR = -23% (-31, -15), p<.001). Subanalysis, comparing both warm ischemia groups showed no significant different effect of capsulotomy between groups. There was no methylene blue leakage after capsulotomy in any kidney.

Conclusions: Renovascular perfusion can be improved with capsulotomy during hypothermic machine perfusion, without damaging the renal parenchyma. Follow-up studies need to determine which donor kidneys may benefit from capsulotomy.

Introduction

Kidney transplantation provides a survival benefit for patients with end stage renal disease compared to dialysis treatment. However, not all candidates for transplantation can be transplanted, because of the persistent shortage of donor kidneys[1,2]. The relative shortage has led to the exploration of alternative sources of donors, including donors after circulatory death (DCD)[3]. These provide a valuable extension of the donor pool, but suffer from an increased risk of graft dysfunction because of the inevitable period of warm ischemia[4,5].

Ischemia reperfusion injury (IRI) causes endothelial cell dysfunction and capillary damage, which result in increased permeability of the renal vascular bed and edema of the graft[6]. Tissue swelling causes vascular compression with reduced tissue perfusion and additional ischemic damage to the organ[6]. Swelling of the parenchyma with an unyielding capsule has been described as renal compartment syndrome[7]. It may lead to reduced or absent (re)perfusion of the kidney, impaired tubular function after transplantation and structural damage[8]. For the three most well-known compartment syndromes, i.e. abdominal compartment syndrome, limb compartment syndrome and intracranial compartment syndrome, there is consensus that tissue should be decompressed to regain adequate tissue perfusion and avoid detrimental effects[9-12]. In analogy with this concept, capsular incision of ischemically damaged and edematous kidneys is a logical technique to improve renal perfusion. However, it has hardly been studied and outcomes are contradictory[7,8,13-15]. It has not or only hardly been used in clinical practice. Thus, we hypothesized that capsulotomy may improve renovascular perfusion. Therefore, we studied the effect of capsulotomy on renal flow and intravascular volume in machine perfused ischemically damaged porcine kidneys.

Materials and methods

Kidney selection and machine perfusion

All experiments were carried out with porcine kidneys. These were retrieved from a local slaughterhouse after a highly standardized slaughtering process. In a paired kidneys design, both kidneys of one pig were flushed with histidine-tryptophan-ketoglutarate preservation solution after 20 and 45 minutes of warm ischemia, respectively. A 20-minute initial flush per kidney resulted in a colorless venous output. Thereafter, kidneys were packed, put on cold storage and transported to our research center. After 3 hours of cold ischemia, both kidneys were put on a hypothermic

perfusion machine (LifePort Kidney Transporter, model no. LKT-100-P; Organ Recovery Systems, Des Plaines, IL) and perfused with 1 L of University of Wisconsin solution at a pressure of 50 mmHg.

Capsulotomy

Twenty-four hours after the initial flush, kidneys were briefly taken off of the perfusion machine. With a surgical blade the capsule was carefully punctured so that a pair of Metzenbaum dissecting scissors would fit under the capsule. With scissors, the capsule was easily opened along the entire great curvature of the kidney (Figure 9.1).

Figure 9.1: Renal capsulotomy.



Measurements

Measurements were performed during hypothermic machine perfusion (HMP) at five time points (Table 9.1). The first and second measurements were performed 1 hour (T_4) and 2 hours (T_5) after the start of HMP, respectively. The third measurement was performed after 21 hours HMP, shortly before capsulotomy (T_{24}). The fourth and fifth measurements were performed directly (T_{25}) and 2 hours (T_{27}) after capsulotomy. Machine flow (Q), renal resistance (RR), renovascular circulating volume (RCV) and intraparenchymal pressure (IPP) measurements were performed four times at each time point and recorded, after which the mean of the four measurements was used for analysis.

Table 9.1: Measurement time points.

Time points	Explanation
T_3	Start of HMP. Three hours of cold ischemia.
T_4	One hour after start of HMP. Four hours of cold ischemia.
T_5	Two hours after start of HMP. Five hours of cold ischemia.
T_{24}	Twenty-one hours after start of HMP. 24 hours of cold ischemia.
T_{25}	Directly after capsulotomy. 22 hours of HMP. 25 hours of cold ischemia.
T_{27}	Two hours after capsulotomy. 24 hours of HMP. 27 hours of cold ischemia.

Time points of flow, RR, RCV, IPP and weight measurements.

Kidneys were weighed and measurements of Q (mL/min), RR (mmHg/mL/min) and RCV (mL) were performed in analogy with our previously described protocol [1]. Briefly, flow and RR are read from the display of the perfusion machine. RCV is measured using a dilution technique. A dilution monitor (HD02 Flow-QC Hemodialysis Monitor; Transonic Systems Inc., Ithaca, NY) is connected to machine tubing by two reusable ultrasound flow probes (Transonic H4E Flow Probes), installed close to the renal artery and renal vein, respectively. The monitor was connected to a computer with HD02 Dialysis Monitoring Software (Transonic). After infusion of an indicator (2 ml of normal saline), the computer automatically calculated RCV by multiplying the flow in the system with the mean transit time of the indicator through the renovascular system, as measured by the ultrasound flow probes.

IPP was measured with a Codman ICP microsensor (Johnson & Johnson Medical Limited, Berkshire, UK). The sensor was introduced after a mini-incision of the capsule and advanced just below the surface.

To assess the integrity of the renal parenchyma after capsulotomy, 50 cc of methylene blue was injected into the renal artery after all measurements. Leakage from the parenchyma was visually assessed by swiping a white tissue over the renal parenchyma. Change in the color of the tissue was noted.

Data analysis

To measure the effect of capsulotomy, the in- or decrease over time periods was expressed in absolute numbers (e.g. T_{24}-T_4) as well as percentage (= 100%*[T_{24}-T_4]/T_4). To check whether there was a prolonged HMP effect (T_5 versus T_{24}), a capsulotomy effect (T_{24} versus T_{25}), and whether the effect persisted for at least two hours after capsulotomy (T_{25} versus T_{27}), linear mixed model analysis was applied to these absolute and percentage changes (Δ) in Q, RR, RCV and IPP (Table 9.2). Linear mixed models were used to account for the correlation between the kidneys from the same pig by

including a random pig effect. To compare these effects between the two groups (20 versus 45 min warm ischemia), group was included as fixed factor (Table 9.3). The same model was applied to the outcomes at each specific time point (T_4, T_5, T_{24}, T_{25}, T_{27}) separately to assess the group differences at these time points (Table 9.4). To analyze the change in weight over time (ΔW), i.e. before HMP (T_3), after start of HMP (T_4), after 21 hours of HMP just before capsulotomy (T_{24}), two hours after capsulotomy (T_{27}), the same linear mixed model as for overall change in other outcomes was used. For each linear mixed model analysis, the estimated mean (change), 95% confidence interval and p-value were reported. Data analysis was performed using IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. P≤0.05 was considered as statistically significant.

Table 9.2: The effect of capsulotomy.

	Time periods	Absolute values		Percentage	
		In- or decrease (95% CI) (N=16)	p-value	In- or decrease (95% CI) (N=16)	p-value
ΔQ (mL/min)	$T_5 – T_{24}$	6 (1, 10)	.021	8 (2, 13)	.012
	$T_{24} – T_{25}$	25 (12, 37)	.002	32 (17, 47)	.001
	$T_{25} – T_{27}$	-1 (-5, 3)	.624	0 (-4, 3)	.795
ΔRR (mmHg/mL/min)	$T_5 – T_{24}$	-0.05 (-0.09, -0.01)	.013	-8 (-14, -2)	.013
	$T_{24} – T_{25}$	-0.14 (-0.19, -0.09)	<.001	-23 (-31, -15)	<.001
	$T_{25} – T_{27}$	0.00 (-0.02, 0.02)	.832	1 (-3, 4)	.643
ΔRCV (mL)	$T_5 – T_{24}$	5 (3, 6)	<.001	23 (18, 29)	<.001
	$T_{24} – T_{25}$	5 (1, 8)	.019	19 (3, 35)	.023
	$T_{25} – T_{27}$	1 (-1, 2)	.234	3 (-3, 10)	.253
ΔIPP (mmHg)	$T_5 – T_{24}$	2 (-2, 7)	.269	25 (-14, 65)	.172
	$T_{24} – T_{25}$	-3 (-5, -1)	.015	-7 (-21, 7)	.259
	$T_{25} – T_{27}$	0 (-2, 2)	.861	1 (-12, 15)	.803

In- or decrease (Δ) of Q, RR, RCV and IPP are presented for all kidneys (N=16) for different time periods. Mean in- decrease was analysed with linear mixed model analysis and presented as mean (95% confidence interval) and p-value. Capsulotomy was performed just before T_{25}. Both differences in absolute change and change in percentage are presented.

Ethical approval

According to national legislation, study approval by the local animal experiments committee (DEC) was not required. Our experiments did not interfere with the standardized slaughtering process and kidneys were obtained after death.

Table 9.3: Differences between warm ischemia groups.

	CIT	Absolute values			
		20 min WIT (N=8)	45 min WIT (N=8)	Difference (95% CI)	p-value
Q (mL/min)	4	72	61	-11 (-19, -4)	.009
	5	74	67	-8 (-15, 0)	.056
	24	80	73	-7 (-20, 6)	.230
	25	101	101	0 (-14, 15)	.969
	27	102	98	-4 (-14, 7)	.436
RR (mmHg/mL/min)	4	0.62	0.77	0.15 (0.06, 0.23)	.004
	5	0.61	0.71	0.10 (0, 0.20)	.045
	24	0.56	0.66	0.10 (-0.01, 0.21)	.063
	25	0.45	0.49	0.04 (-0.3, 0.10)	.193
	27	0.44	0.49	0.05 (-0.01, 0.10)	.078
RCV (mL)	4	24	20	-5 (-10, 1)	.080
	5	22	20	-2 (-6, 2)	.247
	24	27	25	-2 (-5, 1)	.131
	25	31	30	-1 (-6, 4)	.692
	27	32	32	0 (-5, 5)	.890
IPP (mmHg)	4	20	23	3 (-1, 7)	.161
	5	19	23	3 (-1, 8)	.091
	24	24	23	-1 (-8, 7)	.834
	25	21	21	0 (-5, 5)	.964
	27	18	23	5 (0, 10)	.058

Mean Q, RR, RCV and IPP of the two groups (20 and 45 minutes of warm ischemia time (WIT)) after different lengths of cold ischemia time (CIT) are presented as mean. Differences were calculated using linear mixed models and presented as mean (95% confidence interval) and p-value).

Table 9.4: Difference of the effect of capsulotomy between warm ischemia groups.

	Time periods	Absolute values				Percentage			
		20 min WIT (N=8)	40 min WIT (N=8)	Difference (95% CI)	p-value	20 min WIT (N=8)	40 min WIT (N=8)	Difference (95% CI)	p-value
ΔQ (mL/min)	$T_5 - T_{24}$	5	5	0 (-11, 12)	.929	8	8	0 (-15, 15)	.994
	$T_{24} - T_{25}$	21	28	7 (-3, 18)	.138	26	38	12 (-3, 28)	.107
	$T_{25} - T_{27}$	1	-3	-4 (-10, -3)	.228	1	-1	-2 (-8, 3)	.360
ΔRR (mmHg/mL/min)	$T_5 - T_{24}$	-0.05	-0.05	0 (-0.08, 0.08)	.993	-8	-8	0 (-12, 13)	.968
	$T_{24} - T_{25}$	-0.11	-0.17	-0.06 (-0.14, 0.02)	.108	-20	-27	-7 (-17, 3)	.140
	$T_{25} - T_{27}$	0	0.01	0.01 (-0.02, 0.04)	.464	-1	2	3 (-2, 8)	.197
ΔRCV (mL)	$T_5 - T_{24}$	5	5	0 (-4, 3)	.895	22	25	3 (-16, 22)	.724
	$T_{24} - T_{25}$	4	5	1 (-4, 6)	.565	15	23	8 (-11, 27)	.367
	$T_{25} - T_{27}$	0	1	1 (-4, 7)	.611	1	6	5 (-13, 23)	.515
ΔIPP (mmHg)	$T_5 - T_{24}$	4	0	-4 (-13, 5)	.304	42	8	-34 (-79, 11)	.118
	$T_{24} - T_{25}$	-3	-2	1 (-7, 8)	.813	-11	-4	7 (-36, 50)	.702
	$T_{25} - T_{27}$	-2	3	5 (0, 9)	.054	-12	14	26 (-2, 54)	.061

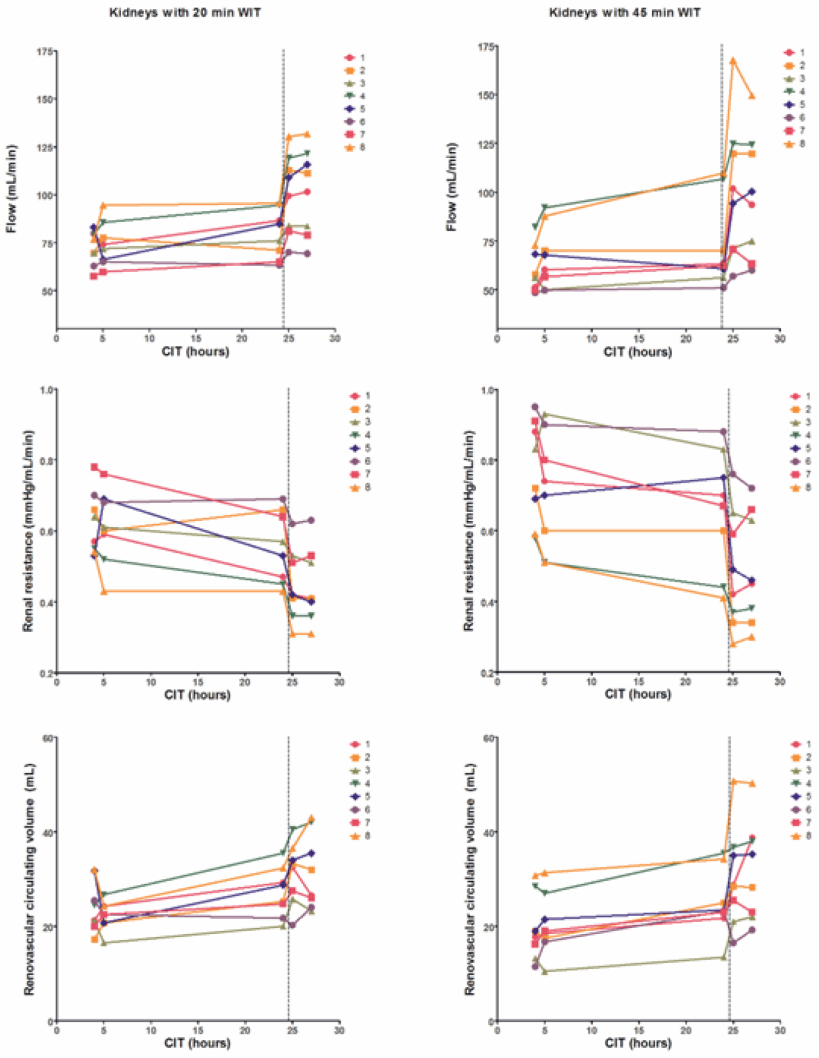
Mean in- or decrease (Δ) of Q, RR, RCV and IPP are presented per warm ischemia group (20 min WIT [N=8] and 40 min WIT [N=8], respectively) for different time periods. Difference between these two groups was analysed with linear mixed model analysis and presented as mean (95% confidence interval) and p-value. Capsulotomy was performed just before T_{25}. Both differences in absolute change and change in percentage are presented.

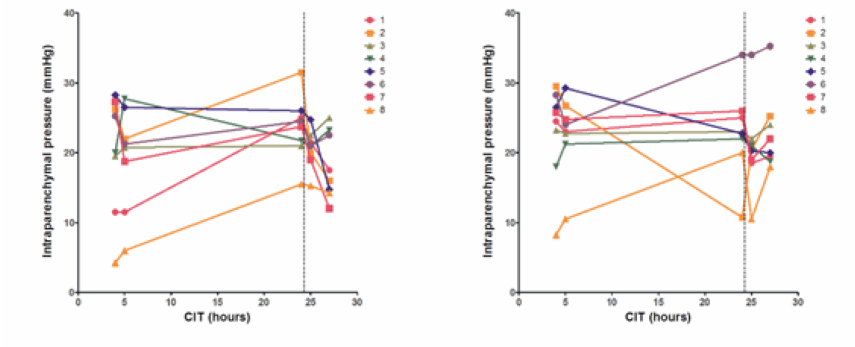
Results

Effect of capsulotomy

The effect of capsulotomy on flow, RR, RCV and IPP is graphically shown in Figure 9.2.

Figure 9.2: Course of flow, RR, RCV and IPP ovr time. Plotted courses of flow, RR, RCV and IPP over time and during HMP, shown in two groups; 20 min WIT on the left (N=8) and 45 min WIT on the right (N=8). Color and number of the lines represent kidneys from the same pig. Kidneys were put on HMP after 3 hours of CIT, and first measurements started one hour afterwards at T_4. Capsulotomy was performed just before T_{25}, as indicated by the dotted line.





The mean renal flow of all kidneys (n=16) increased with prolonged HMP (ΔQ from T_5 to T_{24}=6 (1, 10) mL/min; p=.021). Directly after capsulotomy, there was a large additional increase in renal flow (ΔQ from T_{24} to T_{25}=25 (12, 37) mL/min; p=.002), which persisted for at least two hours (ΔQ from T_{25} to T_{27}=-1 (-5, 3) mL/min: p=.624) (Table 9.2).

The RR decreased during HMP (ΔRR from T_5 to T_{24}=-0.05 (-0.09, -0.01) mmHg/mL/min, p=.013). Directly after capsulotomy, RR decreased even further (ΔRR from T_{24} to T_{25}=-0.14 (-0.19, -0.09) mmHg/mL/min; p<.001) and did not change significantly thereafter (ΔRR from T_{25} to T_{27}=0 (-0.02, 0.02) mmHg/mL/min; p<.832) (Table 9.2).

As evidence of improved intravascular volume after capsulotomy, the increased flow and decreased RR were accompanied by an increased RCV (ΔRCV from T_{24} to T_{25}=5 (1, 8), p=.019). Again, the effect of the capsulotomy did not change significantly thereafter (ΔRCV from T_{25} to T_{27}=1 (-1, 2); p=.234) (Table 9.2).

IPP did not significantly change during prolonged HMP (ΔIPP from T_5 to T_{24}=2 (-2, 7) mmHg, p=.269). Directly after capsulotomy, IPP decreased significantly (ΔIPP from T_{24} to T_{25}=-3 (-5, -1) mmHg, p=0.015). IPP did not change significantly thereafter (ΔIPP from T_{25} to T_{27}=0 (-2, 2) mmHg; p=.861) (Table 9.2).

Mean weight (W) before HMP was 235 (222, 249) grams. Weight increased directly after the start of HMP (ΔW from just before T_3 to T_4=56 (42, 70) grams; p<.001). The weight increased further during the following 21 hours HMP (ΔW from T_4 to just before T_{24}=34 (19, 49) grams; p=.001). There was no significant effect after capsulotomy (ΔW from just before T_{24} to T_{27}=8 (-4, 20) grams; p=0.172).

20 min vs. 45 min of warm ischemia

Figure 9.2 lists flow, RR, RCV and IPP at different time points per warm ischemia group. Renal flow was higher and the RR lower after 20 minutes warm ischemia compared to 45 minutes of warm ischemia, which was significant at T_4 (Q=72 ± 9 vs. 61 ± 12 mL/min; p=.009 and RR=0.62 ± 0.09 vs. 0.77 ± 0.14 mmHg/mL/min; p=.004, respectively) and at T_5 (RR=0.61 ± 0.10 vs. 0.71 ± 0.16 mmHg/mL/min; p=.045, respectively). After prolonged HMP, measurements for both groups were similar (Table 9.3). The effect of capsulotomy on flow, RR, RCV and IPP was not significantly different between groups (Table 9.4).

Methylene blue infusion

Capsulotomy did not injure the underlying renal parenchyma, as shown with infusion of methylene blue. All paper tissues remained white.

Discussion

Renal compartment syndrome

The etiology of compartment syndrome is variable and includes trauma, infection and ischemia-perfusion injury[2,3]. Increased pressure within a fixed anatomic space leads to a vicious circle of tissue compression and blood flow obstruction with ischemia, dysfunction of the tissue and eventually necrosis [2]. The etiology of renal compartment syndrome is obvious: particularly in DCD kidneys, IRI causes edema with compression of renal tissue within its capsule. Because IRI cannot be entirely avoided, we focused on methods to decrease detrimental effects of compartment syndrome and studied if capsulotomy might be beneficial to enhance renovascular flow in ischemically damaged donor kidneys.

Measurements

At given time points, repeated measurement of flow, RR, RCV and IPP were performed to minimize the influence of measurement errors. Flow and RR have frequently been studied and recording these data after two hours of HMP ensures reliable data from a balanced state of perfusion. RCV is a new viability test which we have used before with good reproducibility[1]. Pressure measurements have not been described in a porcine kidney model yet. Our IPP measurements showed large dispersion, making them unreliable. This could have been the result of multiple influencing factors. Firstly, it is

unclear whether dynamic perfusion characteristics are the same in the entire kidney, because they can easily be affected by thrombosis or vasoconstriction. Secondly, without imaging, it is impossible to know where exactly the pressure probe is positioned.

Capsulotomy during machine perfusion

We chose to do a capsulotomy after prolonged cold ischemia, because this resembles current practice. It has been shown that the weight of DCD kidneys increases during cold ischemia due to edema[4]. HMP offers the opportunity to measure renal flow and intravascular volume during the cold ischemic period. It is feasible to do a capsulotomy before or during HMP and the effect of capsulotomy can be measured accurately. As result, the decision to transplant or discard a kidney before transplantation may be made easier for the transplant clinician. Furthermore, HMP is used for viability testing of donor kidneys, although most tests have not shown to be very useful so far[5]. The advantages of HMP, like the wash-out of metabolic waste products and the possibility to provide nutrients or oxygen to the renal tissue, may be increased by capsulotomy, because of the improved renovascular flow[6].

The no-reflow phenomenon can occur after reperfusion of a transplanted kidney, because of IRI induced endothelial damage and vascular obstruction[7-9]. In this situation capsulotomy may be beneficial to the graft. By improving renovascular perfusion before reperfusion, reperfusion itself may be improved, although this has to be shown in further studies including reperfusion of the graft.

Selection of suitable kidneys

We have shown that capsulotomy during HMP improves renal perfusion. Ischemic injury is associated with the duration of ischemic time and severity of edema. Therefore, we hypothesized that kidneys with prolonged warm ischemia times would benefit more from capsulotomy than kidneys with shorter warm ischemia times. We did not find any differences between both warm ischemia groups after capsulotomy (Table 9.3) and the effect of capsulotomy was equivalent (Table 9.4). The question which kidneys benefit most from capsulotomy remains.

Capsulotomy should only be performed if the benefit of the procedure is larger than its risk. Therefore, kidneys with little IRI and edema, like live donor kidneys, should not be subjected to this intervention. Kidneys which are susceptible to IRI and possible

renal compartment syndrome, such as kidneys from old DCD donors, may benefit from capsulotomy.

Risks

Capsular tears after transplantation of donor kidneys are not uncommon. In kidneys with high intracapsular pressures the capsule may tear and cause a blow-out with a poor prognosis for eventual kidney function. Graft rejection, renal vein thrombosis and acute tubular necrosis have been reported as causes for capsular tears and renal allograft rupture[10-13]. The capsule may also be damaged during organ procurement, often with associated injury to the underlying renal parenchyma. Because of the risk of parenchymal damage and possible bleeding, it is not surprising that surgeons are reluctant towards kidneys with capsular damage. In our porcine model, we performed a controlled capsulotomy. It was possible to incise the complete capsule without damage to the underlying tissue. Methylene blue infusion confirmed that there was no leakage from the renal parenchyma, so that the observed increase of arterial flow cannot be explained by parenchymal leakage. We observed an increase in RCV which indicates that capsulotomy opens capillaries or other blood vessels which were collapsed before.

The renal capsule consists of strong fibrous tissue with arterial, venous and lymphatic plexuses and supports the integrity of the renal tissue[14,15]. A large capsulotomy attenuates the support of the renal tissue itself and jeopardizes the integrity of the renal parenchyma. As reported for other compartment syndromes, the decompressive effect largely depends on the size of the opening and should incorporate all sites of potential compression[16,17]. In contrast, Herrler and colleagues recently showed, in a murine model, that a much smaller capsulotomy may already increase renal function and perfusion, with a decrease of injury markers. Furthermore, they showed that a capsulotomy in combination with supportive cell therapy works synergistically to improve aforementioned outcome parameters[18].

Despite the safety of capsulotomy in the present study, capsulotomy is a potentially dangerous procedure which has to be performed with caution. Despite our positive experiences with our capsulotomy model for renal compartment syndrome, further studies may be required before the intervention is routinely used in clinical situations. These may include animal studies, comparing capsulotomy to no intervention in ischemically damaged kidneys, safety studies or interventional studies.

Conclusions

Capsulotomy improves renal flow, renal resistance and renovascular circulating volume in ischemically damaged machine perfused porcine kidneys. Capsulotomy may benefit grafts with extensive IRI, edema and high intrarenal pressures. It has to be determined at which renal flow capsulotomy improves renal perfusion and outweighs the potential risk of parenchymal damage.

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Chapter 10

General discussion and summary

General discussion and summary

Kidney transplantation remains the treatment of choice for patients with end stage renal disease. While patients die and suffer on the waiting list, transplantation provides the best chance of restoring a normal lifestyle. In 65 years of kidney transplantation there have been many medical, technical and logistic improvements which led to a tremendous increase in patient and graft survival[1]. However, since the first human renal transplant, the same barriers still exist; organ shortage and graft failure[2]. The ongoing shortage of kidneys and the limitations to perfect functional outcome warrant studies which may aid in overcoming these problems.

In this thesis "Marginal donor kidneys; novel strategies to assess and improve viability" we have employed novel strategies to assess and improve viability. Good renal allograft viability is a prerequisite to perform transplantation of a kidney graft. However, the definition of renal viability is subjective, criteria unclear, and different per country, transplant centre or doctor. It is still impossible to exactly determine a kidney's viability and thus, transplant outcome. Evidently, kidneys with minor injury and of ideal donors (young healthy living donors) will likely function after transplantation. Therefore, we have focussed in this thesis on a group of kidneys of which it is hard to estimate viability and transplant outcome: marginal donor kidneys. These are kidneys from patients of old age and/or with increased morbidity or kidneys which have sustained substantial (ischemic) injury.

It is currently almost impossible to predict transplant outcome for marginal donor kidneys. Thus, choosing which marginal kidney can be safely transplanted remains difficult and a struggle for transplant clinicians. Although more patients may receive a transplant kidney when a liberal acceptance strategy for marginal kidneys is used, it may also lead to poor transplant outcomes including post-transplant graft failure. Patients are unnecessarily exposed to risks of surgery, immunosuppression, sensitization of donor antigens or even death. On the other hand, a conservative strategy may lead to unnecessary discard of possibly viable kidneys and a smaller number patients will be transplanted. This results in an increase in waiting time and an increase of patients suffering and dying on the waiting list. '*Primum non nocere – First, to do no harm (Hippocrates)'* But how do we do no further harm, when we do not know what to choose? In this thesis we aim to get insight in the pathophysiological processes of kidney transplantation and which factors influence viability and transplant outcome, so that transplant clinicians may have tools to make a well informed decision. To do so we have performed translational studies, using both human and animal models.

Furthermore, we tried to translate our results from the laboratory to the clinic and the other way around, to explain wat we have observed in a clinical situation.

The marginal kidney donor

To expand the donor pool, kidneys of marginal quality have been increasingly used[3]. The introduction of donation after brain death (DBD) donors, the use of expanded criteria donors (ECD) and the increasing use of donation after circulatory death (DCD) donors have shown to be successful strategies with improvement of graft and patient survival[4-11]. There is a general consensus that, because of the survival benefit and positive effect on the waiting list, kidneys from these donors should be used for transplantation[12]. Unfortunately, there is no general consensus on the use of marginal donor kidneys, but there are studies, which define those kidneys which are acceptable for transplantation and those that are not[13-18]. Marginal donor kidneys includes a very large and heterogenous group of kidneys. These include kidneys from old living donors or those with comorbidities, such as obesity, hypertension, microalbuminuria or borderline-normal glomerular filtration rates[19]. Furthermore, they include DBD and DCD donors with comorbidity and of old age or DCD donors themselves may be seen as marginal quality donors, especially uncontrolled donors, because of the large amount of warm ischemic injury[3,20].

Uncontrolled donation after circulatory death

Maastricht category 1 and 2 DCD donors, uncontrolled DCD donors (uDCD), have been part of ongoing discussion whether they should be part of transplant programs throughout the world. In Europe, there is a variation in DCD protocols and not all countries have a uDCD program[21]. In uDCD, after unsuccessful resuscitation, organ perfusion is promptly initiated by either cold or normothermic perfusion[22]. The process of unsuccessful resuscitation and prompt recirculation for organ preservation purpose has been subject of ethical discussion and an argument for procurement organizations not to implement uDCD programs[23]. Furthermore, logistic difficulties make it hard to implement uDCD programs. It is advised that, when implementing a uDCD program, strict national and regional protocols are needed[24].

Although there may be (some) reluctance towards uDCD, outcome has proven to be acceptable. A study of our own cohort in 2011 showed similar results comparing uDCD and DCD in terms of primary non-function (PNF): 22% vs. 21%, respectively) and delayed graft function (DGF, 61% vs. 56%, respectively). Ten-year graft and recipient survival were also similar in uDCD and DCD groups (50% vs 46% and 61% vs 60%,

respectively)[25]. The Madrid group reported similar results in the largest uDCD group ever reported (N=774). Ten-year survival rates for uDCD were better than those of ECD DBD donors (72% vs 61%, p=0.02, respectively). The main cause of graft loss was PNF of which donor death by pulmonary embolism, extra-hospital cardiopulmonary resuscitation (CPR) ≥75 min and in-hospital CPR >50 min were predictive factors[26]. The results of multiple studies were recently reviewed in a systematic review and meta-analysis and showed that uDCD is inferior in the short-term, but outcomes are comparable to controlled DCD in the long-term[27]. In this thesis, in Chapter 2, we show that for uncontrolled DCD, age was the only risk factor for PNF and strongly associated with graft failure, while resuscitation times were not recorded. The differences between our results and those in Spain are a good example of the heterogenicity of protocols and laws regarding uDCD, determination of death and transport of deceased patients. Furthermore, the mean donor age in Madrid was only 40 years, while this is a risk factor in our own cohort, especially for the group older than 54 years. Unfortunately, while general uDCD results are good, the Maastricht group had to abandon their uDCD protocol, as it is costly, time consuming, requires a lot of expertise and effort and uDCD donors rates were continuously low.

A promising intervention that may increase the use of uDCD donors is the introduction of a new *in situ* preservation technique, normothermic regional perfusion (NRP). This is in line with the increasing use of normothermic perfusion in organ preservation[28]. NRP uses extra corporal membrane oxygenation techniques to perfuse and resuscitate organs *in situ* after circulatory death while at the same time the brain is excluded from perfusion. The number of clinical studies is still low , but is increasing. A study 2019 reported a cohort of 499 kidneys transplantations of which 50% were preserved with NRP. *In situ* preservation with a double-balloon triple lumen catheter (DBTL[29]) resulted in a higher risk of PNF (OR 4.5, p=0.015) and worse renal function after one year compared to NRP[30]. The Spanish also showed that NRP led to superior results compared to in-situ preservation, with an increased risk of graft loss (OR 5.6; 95% CI 2.7-11.5) after in-situ preservation during the first year after transplantation[31]. Because of the promising results, NRP has now also been used in controlled DCD donors with success[32]. Next to impact on graft quality, NRP has the advantage of quality assessment during perfusion, already in the donor. Hemodynamic parameters and markers of injury, inflammation, haemostasis, coagulation perturbation may be measured to estimate graft viability[33].

When Maastricht decided to stop their uDCD program, the use of uDCD in the Netherlands came to a halt. This is unfortunate, because transplant waiting lists tend to

grow and studies show favourable outcome[31,34-36]. The reluctance towards uDCD is attributable to the uncertainty of its cost-effectives as well as the clinical doubt in a good outcome, which can be very subjective and differs per team or doctor. However, the technical and clinical developments in extra-corporal membrane oxygenation (ECMO) in general, may lead to new insights regarding the use of uDCD in the Netherlands. First, the clinical use of ECMO (or similar techniques) is increasing and the array of indications to use ECMO has broadened[37-40]. Thus, there is an increasing number of medical personnel with expertise and training programs are successfully developed to facilitate efficient use of oxygenation machines and proper management of organ or donor viability[41]. Because uDCD programs require a specialist team to be available 24/7, the increase of trained staff may lower the burden for centres who are willing to (re)start a uDCD program. Second, the increasing use of ECMO and ex-vivo perfusion of all transplantable organs, leads to a growing pool of evidence in favour of its clinical use. Furthermore, the use of these techniques in multiple settings and for multiple organs, leads to cross-pollination of knowledge and confidence therein. Hopefully, this may lead to the renewed and successful use of uDCD in the Netherlands in the future. In addition, viability assessment may contribute too, as it may be used as final tool to decide whether or not to transplant an organ. Therefore, studies on viability assessment are warranted.

Viability assessment

Viability assessment of donor kidneys has been performed for many years, with the aim to predict post-transplant graft function and survival and thereby simplifying the decision to accept or decline a donor kidney. Furthermore and theoretically, it may provide valuable knowledge on a graft quality, so that to recipient management can be customized per case. Also, the obtained knowledge regarding graft quality and especially graft injury may be used to develop new interventions to prevent further injury or even develop targeted resuscitation strategies. Unfortunately, while viability assessment theoretically has great potential and seems harmless (most test are non-invasive), its clinical implementation has been difficult. Not only did studies show that many viability tests have a poor predictive value on transplant outcome, it was also argued that assessment may have negative affect on the waiting lists. Improper use of viability assessment may lead to unnecessary discard of organs which may aggravate the organ shortage. Furthermore, viability test are time consuming and may lead to delay and increased detrimental ischemia times[42-44].

Viability assessment has most often been performed by means of machine perfusion viability testing, histologic procurement biopsy assessment and donor risk scores[45-48]. In chapter 3 we have reviewed the current methods of viability assessment during machine perfusion and come to a similar conclusion as many. There were (and still are) no singular viability tests which have a desired predictive value to be of clinical use. This repetitive negative conclusion has always been accompanied by another statement, which is the need for further studies towards the understanding of the mechanisms involved in graft failure and the need for new techniques to improve prediction accuracy[48-51]. We share the same conclusions and have therefore performed multiple studies addressing mechanisms of kidney injury during the transplant process and viability assessment.

Molecular mechanisms of graft injury differ in DCD and DBD

In chapter 1 the differences between DCD and DBD were explained in terms of types, categories and their impact on donor logistics and organ perfusion. To get a better understanding of kidney viability, it is of importance to understand the underlying mechanisms that are associated to the development of graft failure. They are complex, because both donor and recipient factors contribute to the decay in graft viability. These mechanisms include immunological and non-immunological processes and differ per type of deceased donation, i.e. DCD vs. DBD[52-54].

Several clinical studies have shown that the pathophysiological processes of organ injury and repair differ for DCD and DBD. This was foremost shown by the finding that graft survival was not affected by DGF in patients who received a kidney from DCD donors, while it was affected by those who received kidneys from DBD donors[5,55-59]. Furthermore, although DCD kidneys have twice the risk on DGF, long-term graft survival is equivalent to DBD kidneys[5,57,58]. These clinical differences reflect a variation of pathologic processes during the process of kidney transplantation and thereafter.

Although DBD is preferred over DCD due to presumed better organ quality, several specific detrimental processes have been attributed to DBD. Brain death is induced by increased intracranial pressure and herniation of the brain stem, which triggers the release of catecholamines and increases parasympathetic activity. The negative impact on hemodynamic donor stability will result in decreased organ perfusion and the need for fluid resuscitation and inotropic support in the donor, with negative consequences for the grafts. Furthermore, the impaired function of the hypothalamus and pituitary gland causes hormone imbalance in the donor, such as decreased cortisol, ADH, T3 and

insulin release[60-64]. Brain death may induce an inflammatory response through several mechanisms. The ischemic brain releases inflammatory mediators and catecholamine release induces aerobic metabolism or causes shear stress of the endothelium and cytokine release. Changes in hormones, like insulin, are directly related to the inflammatory response[62]. The pathophysiologic changes after brain death have detrimental effect on kidneys. Circulation of cytokines (TNF-α and interleukins), chemokines and adhesion molecules (VCAM-1, ICAM-1, E-selectin, P-selectin) promote leukocyte influx in kidney grafts, which is aggravated after reperfusion[61,62,65]. Histological examination of kidneys after brain death showed arterial intimal proliferation and degenerative changes, such as vacuolar degeneration, atrophy and necrosis, especially in distal tubuli[66]. A study on the effect of brain death duration on kidney graft function surprisingly showed that prolonged brain death duration may be somewhat protective. This may indicate that after the direct changes of brain death a resuscitative period may arise in the donor[65]. Indeed, in prolonged brain death cytoprotective genes are upregulated[67,68]. These findings instigate the implementation of specialized donor management to adequately preserve organs in the donor[60,69].

For DCD kidneys, the cessation of blood after circulatory arrest has both advantages and disadvantages. As compared to DBD, the absence of circulation prevents toxins and immunoactive compounds to circulate through the body. However, traumatic injury, cardiovascular resuscitation and brain injury may also lead to detrimental effects on kidneys in DCD donors, similar to DBD, such as an inflammatory response. Furthermore, an agonal phase in controlled donors may lead to irreversible inflammatory and ischemic injury. For this reasons there are strict protocols as to under which conditions an agonal phase may exist[70]. The high risk of DGF and worse short-term graft survival in DCD kidney transplantation has mostly been attributed to warm ischemic injury (chapter 1, Figure 1.1). Ischemia-reperfusion injury (IRI) was discussed in depth in chapter 1 and further explains the biological difference between DCD to DBD kidneys.

Thus, the biological differences between DCD and DBD kidneys are significant, the understanding and awareness of these principles of importance and thus they should be further studied. Not only do these differences lead to a different transplant outcome, they change the way on how we should treat donors, employ preservation strategies and treat kidney recipients. As consequence, we may need to revise our thoughts regarding viability assessment of deceased donor kidneys and perhaps employ different strategies for different kidney types in the future. Moreover, with the development of molecular based viability assessment, as we will discuss later, this may be of special interest.

Novel viability assessment strategies in human renal transplantation

The difficulties with the current viability assessment strategies is highlighted in Chapter 4, as we discuss the controversies of the use of procurement and implantation biopsies. In Maastricht, colleague Snoeijs studied the role of histologic assessment of pre-implantation biopsies with the use of the Remuzzi score[71] and Banff classification[72]. He concluded that there is an association with kidney function and graft survival in DCD donors older than 60 years, although the predictive value on graft loss was poor. For this older group it was suggested that careful histologic assessment may improve donor selection[73]. Thereafter, the use of pre-implantation biopsies decreased, due to the risk of discarding kidneys which would otherwise have functioned. Therefore, biopsies are currently only taken on indication. One of the difficulties of histologic assessment is the interobserver variability, which can be lowered when there is a special team trained for histopathologic analysis[74,75]. Also, there is discussion as to which technique (needle vs wedge biopsy) is best[76,77]. However, optimizing quality by implementation of a special 'donor kidney analysis team' may present clinical difficulties due to the acute setting of transplantation and the need for on-call pathologists 24/7. The use of a first digital histopathology service in the United Kingdom is currently studied and may overcome such problems[78]. In addition and to anticipate on future developments, the first papers have been published to address the role of artificial intelligence in renopathology and transplantation. They state that artificial intelligence has the potential to significantly improve transplant outcome[79,80]. In chapter 7 we have used mass-spectrometry imaging (MSI) assisted molecular assessment in combination with digital histopathologic examination. The additional value of these techniques should be further studied in a clinical setting. Furthermore, studies on pre-transplant donor kidney biopsies are limited and include retrospective studies with a high risk of selection bias and prospective clinical studies are lacking. Thus, there is debate on the quality of evidence based selection and decision making and the use of biopsies in clinical situations[42,43,81,82]. Studies on implantation biopsies (at the time of reperfusion) are even more limited and of questionable quality. Post-reperfusion biopsies have a different meaning, as they are not used for the decision to accept or decline a kidney for transplantation. However, they may show changes after reperfusion, like neutrophil influx and alloimmune processes, as compared to biopsies before reperfusion[77]. These insights may aid in the development of targeted therapy of a donor organ or recipient or lead to identification of viability markers. One implantation biopsy study on short-term function showed that arteriolar hyaline thickening and interstitial fibrosis were the only factors associated with outcome in multivariate analyses[83]. In addition, the combination of a histologic score and donor risk score may have the best predictive

value on graft loss so far[84,85]. Although sceptics on procurement and implantation biopsies argue their uselessness and the recent published British guidelines state that "None of the kidney transplant biopsy scoring systems - alone or in combination - have sufficient predictive value to mandate organ discard"[86], Angeletti et al. stated that the lack of good quality studies should prompt more studies aimed at optimizing the strategies to score renal samples[87]. In chapter 4 we have aimed to broaden the tools for histopathologic examination of kidney biopsies as regularly performed. We introduced a set of histologic markers, related to IRI, in contrast to the regularly performed analysis according to the Banff criteria[72] and show that these are associated to DGF. As such, they may be potential predictive markers, but prospective randomized studies need to be performed. The increased presence of histologic IRI parameters in chronically injured donor kidneys also indicates a higher susceptibility to acute injury. These findings are in line with clinical studies, which show that ECD kidney transplantation has worse outcome than those of 'regular' DCD or DBD kidneys[26]. Furthermore, this emphasizes that the increased susceptibly of marginal donor kidneys to IRI warrants the need for resuscitative strategies to prevent them from further injury, like NRP or normothermic machine perfusion (NMP)[30,88,89].

Next we focussed on viability assessment by the use of biomarkers during machine perfusion. As reviewed in chapter 3, current biomarkers are associated with transplant outcome but the predictive power is unsatisfactory. In collaboration with the department of biochemistry we studied the association of extracellular histones and transplant outcome in an unselected cohort of 390 DCD kidneys in Chapter 5. We showed that extracellular histones were independent risk factors for DGF and 1 year graft failure. The predictive value on DGF was equivalent to the predictive value of most other machine perfusate biomolecules, thus we had to conclude that this marker (also) cannot be used as stand-alone clinical predictive marker of outcome. Extracellular histones had not yet been studied in the pathophysiologic process of kidney injury and organ viability in a clinical setting of organ donation and transplantation before. Therefore, these findings show that histones reflect organ injury and further studies may test whether extracellular histones can be of value in viability assessment. Furthermore, because our study was performed with DCD kidneys, further studies should also focus on DBD kidneys. Extracellular histones are known to be cytotoxic and could have a different effect on DBD donors and kidneys, while cardiovascular perfusion is intact and renal injury ongoing. Also, because histones are associated with cell death and plasma membrane rupture[90], they may be of value for monitoring the quality of organ resuscitation after procurement. This prompted us to go back to the

laboratory and perform additional tests in an animal model to get insight in the kinetics of extracellular histone release after renal injury.

Novel viability assessment strategies in an ex-vivo porcine model

In chapter 6 and 7 we developed a new perfusion model, using porcine kidneys from the slaughterhouse which, after death, were flushed several times so that all intravascular content (proinflammatory molecules and toxins) was removed. Thereafter, we were able to perform paired kidneys analysis comparing kidneys with severe and mild ischemic injury.

The kinetics of extracellular histones release were studied in chapter 6. We showed that histones are exponentially released over time and depend on the amount of ischemic injury. The used techniques were able to measure histones 1 hour after perfusion. Additionally, *in vitro* tests showed that histones are more cytotoxic to renal cells in increased temperature and concentration. Thus, ischemia alone may lead to cytotoxic histone release, which, especially in a warm environment, may trigger a vicious circle of cell death, increased release of cytotoxic molecules and loss of graft quality. These findings confirm that warm ischemia should be minimized. Furthermore, during intact circulation in the donor or during NMP, extracellular histones can cause renal injury, therefore it may be of interest to either minimize the cytotoxic effects or decrease their release. The Maastricht biochemistry department earlier showed that heparin can neutralize cytotoxic histones both *in vitro* an *in vivo*[91] and Sedigh *et al.* showed beneficial effects of a heparin conjugate in ischemically injured kidneys[92]. Therefore, we tested whether the addition of non-anticoagulant heparin would decrease histone release. Addition of heparin did not change the total histone H3 levels. However, we did observe a continuous increase in the level of a lower molecular weight histone H3 variant, which we presume is an effect of increased protease activity by histone-heparin binding. All in all, histone release is directly related to ischemic kidney injury and further studies should be performed to explain their contribution to renal injury and ways to neutralize their adverse effects in order to optimally preserve transplant kidneys.

In collaboration with The Maastricht Multimodal Molecular Imaging Institute (M4I), Division of Imaging Mass Spectrometry, we attempted to use mass spectrometry imaging (MSI) for the purpose of organ quality assessment, which had never been done before (Chapter 7). This was made possible by the introduction of a new type of imaging mass spectrometer, which allows to assess spatial distribution of biomolecules

20 to 50 times faster than other commercial instrumentation available at the time. Therefore, high-throughput analysis of samples was possible and moreover, because of the speed, it becomes possible to perform tissue analysis in a clinical acceptable time frame for viability assessment[93]. Thus, we used MSI to identify ischemic injury between mild and severely injured pig kidneys. While our first tests, focussing on proteins were not successful due to the difficulty to assess and identify molecules with high molecular mass and their diversity. The subsequent analysis of lipids (having a much smaller molecular mass) was successful. First, a blinded MSI analysist could rapidly and easily differentiate between severe and mild ischemic injury. Interestingly, histopathologic examination, being the gold standard, could not differentiate between amount of ischemic injury, which may be explained by the following factors. First, it has repetitively been shown that microscopic assessment is not accurate for assessment of ischemic injury after kidney donation (as described above). Furthermore, MSI allows molecular based identification of injury during donation, in a time frame in which microscopic and morphologic changes may not be detectable yet. Of clinical interest is that the speed of MSI may be faster than histopathologic examination and that, being a digital technique, interpretation of MSI output may be performed on a topographic different location. These promising results should be interpreted with caution and translation to the clinical setting is preliminary, because the analysis on human tissue is expected to be more difficult. This is because of the heterogeneity of human renal tissue. Human kidneys have sustained injury over years (senescence) and suffer from injury different per type of donor (i.e. DCD and DBD), compared to a relatively homogenous group of young pig kidneys. Next to the capacity of MSI to differentiate between mild and severely injured kidneys, this study also led to insights in the underlying biochemical processes of acute ischemic kidney injury. We found that a suite of lipid degradation products is elevated after severe ischemia, such as lysocardiolipins, lysophosphatidylcholines and lysophosphatidylinositol. These lipids are associated to both mitochondrial and cell membrane integrity. The increased concentration of these lipids confirms that they have a role in renal injury. Furthermore, using MSI, we were able to appoint these injury markers to distinct tubular areas of the renal cortex, which may make future analysis of renal injury distribution after ischemia possible. In conclusion, MSI may be a new promising technique in the field of viability testing. It may provide information in an early stage of injury on a molecular level. While new preservation techniques, such as NMP emerge, the use of high-throughput MSI for viability testing is of even more interest, because it may test the effect of interventions on tissue in a pre-clinical setting and reveal pathophysiologic processes which are altered. This needs to be further investigated.

Furthermore, future clinical studies may provide insight in the role of MSI in human tissue analysis and its possible clinical value[94].

The next novel method, with a potential to test viability in a porcine perfusion model, is the use of renovascular circulating volume (chapter 8). We used this dilution technique, used in circulating volume assessment of hemodialyzer fibres, to test renovascular circulating volume during machine perfusion with different perfusion time, pressure and different ischemia times. Because this test was not performed before, we focussed on its repeatability and validation. The dilution technique was reproducible. Furthermore, renovascular circulating volumes differ per group of ischemia (15 vs 45 min), showing that circulating volume decreases when the ischemic injury is more severe. Although the results of this study show that the technique is reproducible, it did not result in a follow-up study. This is mostly due to our focus on different techniques, which we felt are more promising, such as MSI or pathologic examination. However, the dilution technique remains non-invasive and easily to perform. As such, it may be of interest to perform further studies with an improved technical setup; even better it would be when the technique is incorporated into perfusion machines. Furthermore, with the introduction of NMP (with higher flows, temperature and circulating of blood), the sensitivity and specificity of the dilution technique may be improved. It may also be an addition to monitor organ quality during these resuscitative preservation techniques.

In chapter 9 we performed capsulotomy of ischemically injured porcine kidneys. The idea arose from the observation that severe ischemically injured kidneys were often swollen and renovascular circulating volume and perfusion reduced. We showed, using 16 kidneys, that capsulotomy along the greater curvature of the kidney immediately alleviates the compromised kidney and parameters, such as flow, resistance, renovascular circulating volume and intraparenchymal pressure, changed in all kidneys for the better. Surprisingly, we did not find a significant difference between kidneys with minor (20 min) or severe (45 min) ischemia. The positive effect of this intervention on renal perfusion seems evident, but its clinical use is however debatable and needs further study on its safety. Although we did not observe any parenchymal injury of the intervention in our model, transplant surgeons feel reluctant to perform this manoeuvre. Especially, because there are no reliable viability tests to select those kidneys which are viable and thus need no intervention with possible complications. Therefore, capsulotomy should only be performed in kidneys susceptible to renal compartment syndrome and the risk of further decline in viability. Irrespective of the risk, capsulotomy may improve renal perfusion and thereby preservation quality by

means of faster cooling, improved perfusion of therapeutics and better wash-out of waste products. As result of the latter, viability assessment may be improved when injury markers in the perfusate are measured. Furthermore, the study was performed when NMP was not performed yet. It would be of interest to repeat the study using NMP, which offers a better setting to observe relevant changes in a metabolic active organ as well as the possible risk of parenchymal injury and haemorrhage.

Future prospects

Since January 2016 hypothermic machine perfusion (HMP) has been implemented as standard preservation technique for all kidneys donated after death in the Netherlands[95]. From a clinical point of view this was necessary because it was concluded that HMP is superior to cold storage. However, the use of viability assessment during the perfusion period is not implemented in protocols, because, as we have reviewed, there is no scientific basis to do so. In this thesis we have used multiple novel strategies to improve viability testing, but were not able to find a single test with clinical useful predictive power. In addition, we have used novel strategies, like the use of renovascular circulating volume or the use of MSI, in animal studies. Especially MSI seems promising, but results are preliminary and the technique should be further developed and studied to estimate its potential. We conclude that the future of viability testing is unclear and no one has shown otherwise so far. After more than 20 years of use and research of HMP, we may argue that HMP is not suitable for viability assessment of donor kidneys. Donor kidneys in a hypothermic hypometabolic state may not properly reflect their viability and chance of post-transplant function well enough. Also, the aetiology of graft failure is mostly multifactorial and prediction of which kidneys will function or fail, especially with regards to marginal donor kidneys, is difficult when kidneys are preserved at 4° C.

The use of NMP has theoretically many advantages of HMP with regard to viability assessment[96]. Compared to HMP, kidneys are perfused with blood-based perfusates and with higher flows and they are metabolically fully active. Of all viability assessments strategies during HMP we discussed, i.e. measurement of perfusion parameters, perfusate biomarkers and histologic and molecular tissue analysis, all are theoretically more promising when performed in a normothermic state[97]. The improved perfusion mimics in-vivo perfusion and parameters like renal resistance and renovascular circulating volume may be much more reliable[98]. If perfusion is compromised it will be easier to pin-point the problem, maybe even by macroscopic inspection. Furthermore,

increased perfusion leads to a better inflow of therapeutics and/or oxygen and better outflow of waste-products[99]. As results, biomarkers may give a better reflection of kidney viability. Using blood-based perfusate, more biomarkers may be measured Additionally, the kidney will produce urine if properly perfused, and production of and biomarkers therein can be measured to estimate viability[100-102]. As result, normothermic machine perfusion, as a new platform to perform pre-transplant viability assessment, has led to a rapid increase in studies using interesting novel strategies to determine renal viability. Using a porcine perfusion model, it was shown that laser speckle contrast imaging linearly correlates to renal blood flow and microcirculation of absent perfusion can be visualized and semiquantified[103]. The group of Thomas Minor used the metabolic turnover of 13C-acetate as marker during pre-transplant isolated perfusion and could discriminate between "good", "acceptable" and "critical" quality grafts (AURC area under the ROC curve 0.91; $p<0.001$)[104]. The group from Rotterdam also used photoacoustic imaging in a porcine model, which is easily performed using an modified ultrasound devices, to assess cortical oxygenation. They showed that it effectively distinguishes between functional and non-functional kidneys and conclude that this is a promising tool that has a high potential for supporting organ selection for kidney transplantation[105]. In line with non-invasive assessment during NMP, Hamerlink et al. used magnetic resonance imaging to assess renal function. They found different blood flow distribution, comparing in-vivo and ex-vivo with minimal or severe warm ischemic injury[106]. Analysis of tissue after NMP has not been performed much[107]. However, in theory, tissue analysis after a normothermic resuscitative period may give a better reflection of organ viability than after a hypothermic period.

After the first kidney transplantation after NMP in 2011 in Leicester[108], only a few groups have been able to achieve the same results . Clinical data is scarce, almost solely coming from the Nicholson group[109,110]. They recently performed a high-quality randomized controlled trial, comparing static cold storage (SCS) to SCS plus one hour NMP in a total of 338 DCD kidneys. The found no beneficial nor detrimental effect of NMP, with a DGF rate of 60.7% for NMP and 58.5% in SCS (adjusted odds ratio (95% confidence interval) 1.13 (0.69–1.84); p=0.624). Interestingly, for viability assessment, they only used a NMP binary assessment score, including macroscopic appearance, renal blood flow and urine output, and found no difference in observed DGF for high or low scores[110]. In the near future, more clinical studies are expected, comparing NMP to HMP (ClinicalTrials.gov identifier: NCT04882254)[111] or NMP tot SCS (ClinicalTrials.gov identifier: NCT05031052)[89]. In contrast to clinical studies, there are multiple study groups in a pre-clinical phase. They focus on improving NMP technical set-up, optimising perfusion solution and devising new renoprotective strategies and

interventions for NMP, using animal or discarded human kidneys and comparing them to HMP or SCS[112-121]. For viability assessment, the widespread implementation of NMP in renal transplantation has just begun. Therefore, clinical data on viability assessment is still hardly available[89,122]. In conclusion, NMP is a new technique which needs further development and study to clarify its additional clinical value. Viability assessment slowly follows and enters a new 'warm era', of which the theoretical advantages seem obvious, but the data to support this are still lacking.

Irrespective of the technical developments in donor kidney viability improvement and testing, their use heavily depends on the developments in clinical kidney transplantation. A recent paper describes the successful developments in pre-clinical xenotransplantation[123]. This led to the first pig-to-human genetically modified kidney transplant on the 16th of March 2024 and it demonstrates that, at least in the short term, the kidney is safe and functions[124,125]. Regretfully, the recipient died after 52 days from sudden cardiac arrest, but with a kidney without serologic of histologic evidence of antibody-mediated rejection[125,126]. If xenotransplantation proves to yield clinical acceptable long-term results, it may greatly change the availability of transplantable organs. However, these follow-up results are not available, thus this remains unclear. Another interesting future prospect was recently discusses by Hosgood et al.[127]. With the use of vitrification (the rapid cooling of organs to a stable, ice-free, glass-like state) and nanowarming (the heating of nanoparticles within organ vasculature to achieve a rapid and uniform warming) it was shown that rat kidneys sustained function up to 30 days after transplantation after they had been cryogenically stored for 100 days[128]. The concept of cryopreservation and organ banking would allow a more elective approach to kidney transplantation[127,129]. The concepts of successful vitrification, nanowarming, cryopreservation, organ banking and xenotransplantation will inevitably impact the field of organ viability improvement and testing, a field which will have to adept and advance to these possible clinical changes. These upcoming developments are exiting and hopeful for future patients with end-stage renal disease.

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Chapter 11

Nederlandse discussie en samenvatting

Algemene bespreking en samenvatting

Niertransplantatie blijft de voorkeursbehandeling voor patiënten met eindstadium nierziekte. Terwijl patiënten overlijden en lijden op de wachtlijst, biedt transplantatie de beste kans op herstel van een normaal leven. In 65 jaar niertransplantatie hebben talrijke medische, technische en logistieke verbeteringen plaatsgevonden die hebben geleid tot een enorme toename in zowel patiënt- als transplantaatoverleving[1]. Echter, sinds de eerste menselijke niertransplantatie bestaan dezelfde barrières nog steeds: orgaantekort en transplantaatfalen[2]. Het aanhoudende tekort aan nieren en de beperkingen om een perfect functioneel resultaat te bereiken, rechtvaardigen studies die kunnen bijdragen aan de oplossing van deze problemen.

In deze thesis, getiteld “Marginale donornieren; nieuwe strategieën om levensvatbaarheid te beoordelen en te verbeteren”, hebben we nieuwe strategieën toegepast om de levensvatbaarheid te beoordelen en te verbeteren. Een goede levensvatbaarheid van een nier is een vereiste om een niertransplantatie uit te kunnen voeren. Echter, de definitie van levensvatbaarheid is subjectief, de criteria zijn onduidelijk en verschillen per land, transplantatiecentrum of arts. Het is nog steeds onmogelijk om de levensvatbaarheid van een nier exact te bepalen en daarmee ook het transplantatie succes. Het is evident dat nieren met minimale schade en afkomstig van ideale donoren (jonge, gezonde levende donoren) hoogstwaarschijnlijk na transplantatie zullen functioneren. Daarom ligt de focus van deze thesis op een groep nieren waarvan het moeilijk is de levensvatbaarheid en het transplantatie succes te voorspellen: marginale donornieren. Dit betreft nieren afkomstig van oudere patiënten en/of met een verhoogde morbiditeit, of nieren die aanzienlijke (ischemische) schade hebben opgelopen.

Het is momenteel bijna onmogelijk om de uitkomst van transplantatie voor marginale donornieren te voorspellen. Het kiezen van welke marginale nier veilig getransplanteerd kan worden, blijft daarmee een uitdaging en een worsteling voor transplantatieartsen. Hoewel meer patiënten een niertransplantatie kunnen ontvangen wanneer een liberale acceptatiestrategie voor marginale nieren wordt gehanteerd, kan dit ook leiden tot slechte transplantatie uitkomsten, waaronder transplantaatfalen. Patiënten worden onnodig blootgesteld aan de risico’s van de operatie, immunosuppressie, sensibilisatie tegen donorantigenen of zelfs overlijden. Aan de andere kant kan een conservatieve strategie leiden tot onnodig weggooien van mogelijk levensvatbare nieren, waardoor minder patiënten getransplanteerd worden. Dit resulteert in een langere wachttijd en een toename van het aantal patiënten dat lijdt en overlijdt op de wachtlijst. ‘Primum non nocere – Ten eerste geen kwaad doen

(Hippocrates)'. Maar hoe kunnen we geen verder kwaad doen, als we niet weten wat te kiezen? In deze thesis streven we ernaar inzicht te krijgen in de pathofysiologische processen van niertransplantatie en welke factoren de levensvatbaarheid en het transplantatie succes beïnvloeden, zodat transplantatieartsen over de benodigde instrumenten beschikken om een weloverwogen beslissing te nemen. Hiertoe hebben we translationele studies uitgevoerd, waarbij zowel menselijke als diermodellen werden gebruikt. Daarnaast hebben we geprobeerd onze resultaten vanuit het laboratorium naar de kliniek te vertalen en vice versa, om te verklaren wat we in een klinische situatie hebben waargenomen.

De marginale nierdonor

Om het donorpotentieel te vergroten, worden nieren van marginale kwaliteit steeds vaker gebruikt[3]. De introductie van donoren na hersendood (DBD), het gebruik van donoren met uitgebreidere criteria (ECD) en het toenemend gebruik van donoren na circulatoire dood (DCD) hebben aangetoond succesvolle strategieën te zijn met een verbetering van zowel transplantaat- als patiëntoverleving[4-11]. Er is algemene consensus dat, vanwege het overlevingsvoordeel en het positieve effect op de wachtlijst, nieren van deze donoren voor transplantatie zouden moeten worden gebruikt[12]. Helaas is er geen consensus over het gebruik van marginale donornieren, maar er zijn wel studies die aangeven welke nieren acceptabel zijn voor transplantatie en welke niet[13-18]. Marginale donornieren vormen een zeer grote en heterogene groep nieren. Hierbij gaat het om nieren van oudere donoren bij leven of van donoren met comorbiditeiten, zoals obesitas, hypertensie, microalbuminurie of matige nierfunctie[19]. Daarnaast omvat het zowel DBD- als DCD-donoren met comorbiditeit en van oudere leeftijd, of worden de DCD-donoren zelf als van marginale kwaliteit gezien, met name bij "ongecontroleerde" donoren na reanimatie, vanwege de grote hoeveelheid aan warme ischemische schade[3,20].

Ongecontroleerde donatie na circulatoire dood

Maastricht categorie 1 en 2 DCD-donoren, de ongecontroleerde DCD-donoren (uDCD), zijn voortdurend onderwerp van discussie over of zij deel zouden moeten uitmaken van transplantatieprogramma's wereldwijd. In Europa bestaat er variatie in DCD-protocollen en niet alle landen beschikken over een uDCD-programma[21]. Bij uDCD wordt na een mislukte reanimatie de orgaanperfusie direct gestart door middel van koude of normotherme perfusie[22]. Het proces van een mislukte reanimatie en de daaropvolgende snelle recirculatie met als doel orgaanbehoud is onderwerp van ethische discussie en wordt door transplantatie stichtingen aangehaald als reden om

geen uDCD-programma's te implementeren[23]. Bovendien maken logistieke moeilijkheden de implementatie van uDCD-programma's lastig. Er wordt geadviseerd dat bij de implementatie van een uDCD-programma strikte nationale en regionale protocollen noodzakelijk zijn[24].

Hoewel er (enige) terughoudendheid kan bestaan over uDCD, hebben uitkomsten aangetoond dat succespercentages acceptabel zijn. Een studie in ons eigen cohort in 2011 liet vergelijkbare resultaten zien tussen uDCD en DCD wat betreft primaire non-functie (PNF: 22% versus 21%, respectievelijk) en vertraagde transplantaatfunctie (DGF: 61% versus 56%, respectievelijk). De tienjaarsoverleving van zowel het niertransplantaat als de ontvanger was eveneens vergelijkbaar in de uDCD- en DCD-groepen (50% versus 46% en 61% versus 60%, respectievelijk)[25]. De Madrid-groep rapporteerde soortgelijke resultaten in de grootste gerapporteerde uDCD-groep (N=774). De tienjaarsoverlevingspercentages voor uDCD waren beter dan die van ECD DBD-donoren (72% versus 61%, p=0,02). De belangrijkste oorzaak van graftverlies was PNF, waarbij donorsterfte door longembolie, extramurale cardiopulmonaire reanimatie (CPR) ≥75 minuten en intramurale CPR >50 minuten voorspellende factoren waren[26]. Meerdere studies zijn recent samengevat in een systematische review en meta-analyse, waaruit bleek dat uDCD op de korte termijn inferieur is, maar dat de uitkomsten op de lange termijn vergelijkbaar zijn met gecontroleerde DCD[27]. In deze thesis, in hoofdstuk 2, laten we zien dat bij ongecontroleerde DCD de leeftijd de enige risicofactor voor PNF was en sterk geassocieerd werd met transplantaatfalen, terwijl reanimatietijden niet geregistreerd werden. De verschillen tussen onze resultaten en die in Spanje illustreren de heterogeniteit van protocollen en wetgeving omtrent uDCD, de vaststelling van de dood en het transport van overledenen. In Madrid was de gemiddelde donorleeftijd slechts 40 jaar, terwijl leeftijd in ons cohort een risicofactor bleek te zijn, vooral voor de groep ouder dan 54 jaar. Helaas, ondanks de goede algemene resultaten van uDCD, moest de Maastricht-groep hun uDCD programma staken, omdat het te kostbaar en tijdrovend was, en veel expertise en inzet vereiste waarbij de aantallen uDCD donoren continu laag waren.

Een veelbelovende interventie die het gebruik van uDCD donoren zou kunnen vergroten, is de introductie van een nieuwe in-situ preservatietechniek, normotherme regionale perfusie (NRP). Dit sluit aan bij het toenemende gebruik van normotherme perfusie bij orgaanpreservatie[28]. NRP maakt gebruik van extracorporele membraanoxygenatie technieken om organen in-situ te perfunderen en te reanimeren na circulatoir overlijden, terwijl het brein tegelijkertijd wordt uitgesloten van perfusie. Het aantal klinische studies is nog beperkt, maar groeit gestaag. Een studie uit 2019

rapporteerde een cohort van 499 niertransplantaties, waarvan 50% gepreserveerd werd met NRP. In-situ preservatie met een dubbelballon drievoudig lumen katheter (DBTL[29]) resulteerde in een hoger risico op PNF (OR 4,5, p=0,015) en slechtere nierfunctie na één jaar vergeleken met NRP[30]. Ook de Spaanse studie toonde aan dat NRP leidde tot superieure resultaten vergeleken met in-situ preservatie, met een verhoogd risico op transplantaatverlies (OR 5,6; 95% CI 2,7-11,5) tijdens het eerste jaar na transplantatie[31]. Vanwege de veelbelovende resultaten is NRP inmiddels ook met succes toegepast bij gecontroleerde DCD-donoren[32]. Naast de impact op de kwaliteit van het transplantaat heeft NRP het voordeel dat er tijdens de perfusie al een kwaliteitsbeoordeling kan plaatsvinden, al in de donor. Hemodynamische parameters en markers van schade, inflammatie, hemostase en coagulatiestoornissen kunnen worden gemeten om de levensvatbaarheid van het transplantaat in te schatten[33].

Toen Maastricht besloot hun uDCD programma te staken, kwam het gebruik van uDCD in Nederland tot stilstand. Dit is jammer, omdat de wachtlijsten voor transplantatie groeien en studies juist gunstige uitkomsten laten zien[31,34-36]. De terughoudendheid ten aanzien van uDCD is toe te schrijven aan de onzekerheid over de kosteneffectiviteit en de klinische twijfel over een goede uitkomst, wat zeer subjectief kan zijn en per team of arts verschilt. De technische en klinische ontwikkelingen op het gebied van extracorporele membraanoxygenatie (ECMO) kunnen echter nieuwe inzichten bieden over het gebruik van uDCD in Nederland. Ten eerste neemt het klinische gebruik van ECMO (of vergelijkbare technieken) toe en is het scala aan indicaties voor ECMO toegenomen[37-40]. Hierdoor is er een groeiend aantal medisch personeel met expertise en worden trainingsprogramma's succesvol ontwikkeld voor een efficiënt gebruik van oxygenatiemechanismen om orgaan- of donorlevens-vatbaarheid te verbeteren[41]. Aangezien uDCD-programma's een specialistisch team vereisen dat 24/7 beschikbaar is, kan de toename van getraind personeel de drempel verlagen voor centra die een uDCD-programma willen (her)starten. Ten tweede leidt het toenemende gebruik van ECMO en ex-vivo perfusie van alle transplantabele organen tot meer bewijs voor het klinisch gebruik ervan in de volle breedte. Bovendien zorgt het gebruik van deze technieken in meerdere setting en voor meerdere soorten organen voor een kruisbestuiving van kennis en vertrouwen daarin. Hopelijk leidt dit in de toekomst tot het hernieuwde en succesvolle gebruik van uDCD in Nederland. Daarnaast kan levensvatbaarheidsbeoordeling ook bijdragen, omdat het als extra instrument gebruikt kan worden om te beslissen of een orgaan wel of niet getransplanteerd kan en moet worden. Daarom zijn studies naar levensvatbaarheids-beoordeling gerechtvaardigd.

Levensvatbaarheidsbeoordeling

De beoordeling van de levensvatbaarheid van donornieren wordt al jarenlang uitgevoerd, met als doel de post-transplantatie transplantaatfunctie en overleving te voorspellen en daarmee de beslissing om een donor nier te accepteren of af te wijzen te vereenvoudigen. In theorie kan deze beoordeling waardevolle kennis opleveren over de kwaliteit van de nier, zodat de behandeling van de ontvangend patiënt per casus kan worden afgestemd. Ook kan de verkregen kennis over transplantaatkwaliteit en transplantaatschade worden gebruikt om nieuwe interventies te ontwikkelen die verdere schade voorkomen of gerichte resuscitatiestrategieën mogelijk maken. Helaas blijkt uit studies dat veel levensvatbaarheidstesten een slechte voorspellende waarde hebben voor transplantatie uitkomsten en werd betoogd dat deze beoordeling een negatief effect kan hebben op de wachtlijsten. Onjuist gebruik van levensvatbaarheidsbeoordeling kan namelijk leiden tot onnodig afwijzen van organen, wat de orgaantekorten verder verergert. Bovendien zijn levensvatbaarheidstesten tijdrovend en kunnen ze vertraging en nadelige ischemische tijden veroorzaken[42-44].

De levensvatbaarheidsbeoordeling is meestal uitgevoerd door middel van machineperfusie testen, histologische beoordeling van transplantaatbiopten en donorscore's[45-48]. In hoofdstuk 3 hebben we de huidige methoden voor levensvatbaarheidsbeoordeling tijdens machineperfusie onderzocht en kwamen we tot een conclusie die overeenkomt met die van anderen. Er waren (en zijn nog steeds) geen enkele levensvatbaarheidstesten beschikbaar met de gewenste voorspellende waarde voor klinisch gebruik. Deze herhaalde negatieve conclusie ging altijd gepaard met de constatering dat er meer studies nodig zijn om de mechanismen achter transplantaatfalen te begrijpen en om nieuwe technieken te ontwikkelen die de voorspellingsnauwkeurigheid verbeteren[48-51]. Wij delen deze conclusies en hebben daarom meerdere studies uitgevoerd die gericht zijn op het begrijpen van de mechanismen van nierschade tijdens het transplantatieproces en op levens-vatbaarheidsbeoordeling.

Moleculaire mechanismen van transplantaatschade verschillen tussen DCD en DBD

In hoofdstuk 1 werden de verschillen tussen DCD en DBD toegelicht in termen van types, categorieën en hun impact op donorlogistiek en orgaanperfusie. Voor een beter begrip van de levensvatbaarheid van de nier is het van belang de onderliggende mechanismen te begrijpen die samenhangen met de ontwikkeling van transplantaatfalen. Deze mechanismen zijn complex, omdat zowel donor- als

ontvangerfactoren bijdragen aan de afname van de transplantaatlevensvatbaarheid. Ze omvatten immunologische en niet-immunologische processen en verschillen per type overleden donor, oftewel DCD versus DBD[52-54].

Verschillende klinische studies hebben aangetoond dat de pathofysiologische processen van orgaanschade en -herstel verschillen voor DCD en DBD donoren. Dit werd met name geïllustreerd door de bevinding dat de transplantaatoverleving niet beïnvloed werd door DGF bij patiënten die een nier van een DCD donor ontvingen, terwijl dat wel het geval was bij ontvangers van DBD nieren[5,55-59]. Daarnaast hebben DCD nieren, hoewel ze een tweemaal hoger risico op DGF hebben, op de lange termijn een equivalente transplantaatoverleving als DBD nieren[5,57,58]. Deze klinische verschillen weerspiegelen de variatie in de pathologische processen tijdens en na niertransplantatie.

Hoewel DBD de voorkeur geniet boven DCD vanwege de veronderstelde betere orgaankwaliteit, worden verschillende specifieke nadelige processen toegeschreven aan DBD. Hersendood wordt ingeleid door een verhoogde intracraniële druk en inklemming van de hersenstam, wat de afgifte van catecholamines triggert en de parasympathische activiteit verhoogt. De negatieve impact op de hemodynamische stabiliteit van de donor resulteert in een verminderde orgaanperfusie en de noodzaak tot vochttoediening en inotrope ondersteuning, met nadelige gevolgen voor de organen. Bovendien zorgt de verminderde functie van de hypothalamus en hypofyse voor hormonale disbalans bij de donor, zoals een verminderde afgifte van cortisol, ADH, T3 en insuline[60-64]. Hersendood kan een inflammatoire reactie veroorzaken via meerdere mechanismen. Het ischemische brein geeft inflammatie mediatoren af en de catecholamineafgifte veroorzaakt ofwel een verhoogde aerobe stofwisseling ofwel shear stress van het endotheel en de afgifte van cytokines. Hormonale veranderingen, zoals verandering in insuline afgifte, houden direct verband met de inflammatoire reactie[62]. De pathofysiologische veranderingen na hersendood hebben een nadelig effect op nieren. De circulatie van cytokines (TNF-α en interleukines), chemokines en adhesiemoleculen (VCAM-1, ICAM-1, E-selectine, P-selectine) bevordert de instroom van leukocyten in niertransplantaten, wat na reperfusie nog verergerd wordt[61,62,65]. Histologisch onderzoek van nieren na hersendood toonde arteriële intimale proliferatie en degeneratieve veranderingen, zoals vacuolaire degeneratie, atrofie en necrose, vooral in de distale tubuli[66]. Een studie naar de invloed van de duur van hersendood op de nierfunctie liet verrassend genoeg zien dat een verlengde hersendoodperiode enigszins beschermend kan werken, wat erop kan wijzen dat na de directe veranderingen van hersendood een resuscitatie periode in de donor kan ontstaan[65].

Het blijkt dat bij langdurige hersendood cytoprotectieve genen worden opgewekt[67,68]. Deze bevindingen laten zien dat behandeling van een donor complex is en verricht zou moeten worden door gespecialiseerde artsen, zodat de organen optimaal gepreserveerd kunnen worden[60,69].

Bij DCD nieren heeft het stoppen van de bloedsomloop na circulatiestilstand zowel voordelen als nadelen. In vergelijking met DBD voorkomt de afwezigheid van circulatie dat toxines en immunoactieve stoffen door het lichaam verspreid worden. Echter, traumatisch letsel, cardiovasculaire reanimatie en hersenletsel kunnen ook nadelige effecten op DCD nieren hebben, vergelijkbaar met DBD, zoals een inflammatoire reactie. Bovendien kan een agonale fase (de fase voor de circulatiestilstand maar na het staken van de actieve behandeling) bij gecontroleerde donoren leiden tot onomkeerbare inflammatoire en ischemische schade. Om deze reden bestaan er strikte protocollen omtrent de voorwaarden waaronder een agonale fase mag bestaan[70]. Het hoge risico op DGF en slechtere korte termijn transplantaatoverleving bij DCD-niertransplantatie wordt voornamelijk toegeschreven aan warme ischemische schade (hoofdstuk 1, Figuur 1.1). Ischemie-reperfusieschade (IRI) werd uitvoerig besproken in hoofdstuk 1 en verklaart verder de biologische verschillen tussen DCD en DBD nieren.

De biologische verschillen tussen DCD en DBD nieren zijn evident en significant. Het begrijpen en bewust zijn van het bestaan van deze principes is van groot belang en moet verder bestudeerd worden. Niet alleen leiden deze verschillen tot een verschillend transplantatie uitkomst, maar ze veranderen ook de wijze waarop we orgaandonoren en hun ontvangers moeten behandelen en hoe we preservatie-strategieën moeten inzetten. Hierdoor moeten we wellicht onze gedachten over de levensvatbaarheidsbeoordeling van overleden donornieren herzien en in de toekomst mogelijk verschillende strategieën hanteren voor verschillende type donornieren. Bovendien kan, met de ontwikkeling van levensvatbaarheidsbeoordeling op moleculair niveau, dit van bijzonder belang zijn.

Nieuwe strategieën voor levensvatbaarheidsbeoordeling in niertransplantatie

De beperkingen van de huidige strategieën voor levensvatbaarheidsbeoordeling worden belicht in hoofdstuk 4, waarin de controverses rond het gebruik van preservatie- en implantatiebiopten worden besproken. In Maastricht bestudeerde collega Snoeijs de rol van histologische beoordeling van pre-implantatiebiopten met behulp van de Remuzzi-score [71] en de Banff-classificatie[72]. Hij concludeerde dat er een

associatie bestaat tussen nierfunctie en transplantaatoverleving bij DCD donoren ouder dan 60 jaar. Al was de voorspellende waarde voor transplantaatverlies gering. Voor deze oudere groep werd gesuggereerd dat een zorgvuldige histologische beoordeling de donorselectie kan verbeteren[73]. Vervolgens nam het gebruik van pre-implantatiebiopten af, vanwege het risico dat nieren die anders functioneel zouden zijn, onterecht afgekeurd zouden worden. Biopten worden derhalve momenteel uitsluitend op indicatie afgenomen. Eén van de moeilijkheden van histologische beoordeling is de interobserver-variabiliteit, wat verminderd kan worden door een gespecialiseerd team in histopathologische analyse in te schakelen[74,75]. Daarnaast is er discussie over welke techniek (naald- versus wigbiopsie) de voorkeur heeft[76,77]. Het optimaliseren van de kwaliteit door implementatie van een gespecialiseerd 'donornier-analyseteam' kan echter klinische uitdagingen opleveren vanwege de acute setting van transplantatie en de noodzaak van 24/7 beschikbare pathologen. Momenteel wordt de eerste digitale histopathologieservice in het Verenigd Koninkrijk bestudeerd, wat dergelijke problemen mogelijk kan oplossen[78]. Tevens zijn de eerste publicaties verschenen over de rol van kunstmatige intelligentie in de nierpathologie en transplantatie. Zij stellen dat kunstmatige intelligentie transplantatie-uitkomst aanzienlijk zou kunnen verbeteren[79,80]. In hoofdstuk 7 hebben we met behulp van beeldvormende massa spectrometrie (MSI) een moleculaire beoordeling gemaakt in combinatie met digitaal histopathologisch onderzoek. De toegevoegde waarde van deze technieken dient in een klinische setting verder onderzocht te worden. Verder zijn studies met pre-transplantie donornierbiopten beperkt en omvatten zij retrospectieve studies met een hoog risico op selectiebias; prospectieve klinische studies ontbreken. Er is dus discussie over de wetenschappelijke kwaliteit van de selectie criteria en besluitvorming en het gebruik van biopten in klinische situaties[42,43,81,82]. Studies met implantatiebiopten (op het moment van reperfusie) zijn nog beperkter en van twijfelachtige kwaliteit. Post-reperfusiebiopten hebben een andere betekenis, aangezien zij niet gebruikt worden voor de beslissing om een nier voor transplantatie te accepteren of af te wijzen. Wel kunnen zij veranderingen na reperfusie aantonen, zoals de instroom van neutrofielen en allo-immuunprocessen, vergeleken met biopten vóór reperfusie[77]. Deze inzichten kunnen helpen bij het ontwikkelen van gerichte therapieën voor een donororgaan of ontvanger, of leiden tot de identificatie van levensvatbaarheidsmarkers. Eén implantatiebioptstudie naar korte termijn functie toonde aan dat arteriolaire hyaline verdikking en interstitiële fibrose de enige factoren waren die in multivariate analyses geassocieerd waren met het resultaat[83]. Bovendien zou de combinatie van een histologische score en een donorscore tot dusver de beste voorspellende waarde hebben voor transplantaatverlies[84,85]. Hoewel critici van preservatie- en implantatiebiopten hun gebruik betwisten en de relatief recent

gepubliceerde Britse richtlijnen stellen dat “geen van de niertransplantatiebiopt-scoringssystemen, alleen of in combinatie, voldoende voorspellende waarde heeft om het afwijzen van organen te rechtvaardigen”[86], stelden Angeletti et al. dat het gebrek aan kwalitatief goede studies aanleiding zou moeten geven tot meer onderzoek om de strategieën voor het scoren van nierweefsel te optimaliseren[87]. In hoofdstuk 4 hebben we getracht de mogelijkheden voor histopathologisch onderzoek van nierbiopten, zoals die routinematig worden uitgevoerd, te vergroten. We introduceerden een reeks histologische markers, gerelateerd aan IRI, in tegenstelling tot de reguliere analyse volgens de Banff-criteria[72] en lieten zien dat deze geassocieerd zijn met DGF. Daarmee zouden zij potentiële voorspellende markers kunnen zijn, maar hier moeten prospectieve gerandomiseerde studies voor worden uitgevoerd. De toegenomen aanwezigheid van histologische IRI-parameters in chronisch beschadigde donornieren duidt tevens op een verhoogde vatbaarheid voor acute schade. Deze bevindingen sluiten aan bij klinische studies, waaruit blijkt dat ECD-niertransplantatie slechtere uitkomsten heeft dan ‘normale’ DCD of DBD nieren[26]. Tevens benadrukt dit dat de verhoogde gevoeligheid van marginale donornieren voor IRI noodzaak geeft tot resuscitatiestrategieën om verdere schade te voorkomen, zoals NRP of normotherme machineperfusie (NMP)[30,88,89].

Vervolgens richtten we ons op levensvatbaarheidsbeoordeling door het gebruik van biomarkers tijdens machineperfusie. Zoals onderzocht en uiteengezet in hoofdstuk 3, zijn de huidige biomarkers weliswaar geassocieerd met het transplantatie uitkomst, maar de voorspellende kracht is onvoldoende voor betrouwbaar gebruik. In samenwerking met de afdeling biochemie bestudeerden we de associatie van extracellulaire histonen en transplantatie uitkomst in een niet-geselecteerde cohort van 390 DCD-nieren in hoofdstuk 5. We toonden aan dat extracellulaire histonen onafhankelijke risicofactoren waren voor DGF en transplantaatfalen na 1 jaar. De voorspellende waarde voor DGF was vergelijkbaar met die van de meeste andere biomoleculen in het perfusaat, waardoor we moesten concluderen dat (ook) deze marker niet als op zichzelf staande klinische voorspeller van uitkomst kan worden gebruikt. Extracellulaire histonen waren in de context van nierschade en orgaanlevensvatbaarheid bij orgaandonatie en -transplantatie in een klinische setting tot dusver nog niet bestudeerd. Deze bevindingen tonen aan dat histonen orgaanschade weerspiegelen en dat verder onderzoek moet worden verricht naar de bijdrage van extracellulaire histonen aan levensvatbaarheidsbeoordeling. Bovendien, omdat onze studie werd uitgevoerd met DCD-nieren, zouden verdere studies zich ook moeten richten op DBD-nieren. Het is bekend dat extracellulaire histonen cytotoxisch zijn en een ander effect kunnen hebben op DBD-donoren en nieren, terwijl

cardiovasculaire perfusie intact is en nierbeschadiging voortduurt. Omdat histonen geassocieerd zijn met celdood en plasmamembraanruptuur[90], kunnen ze van waarde zijn voor het monitoren van de kwaliteit van orgaan resuscitatie na uitname. Dit zette ons aan om terug te gaan naar het laboratorium en aanvullende tests uit te voeren in een diermodel om inzicht te krijgen in de kinetiek van extracellulaire histonafgifte na nierschade.

Nieuwe strategieën voor levensvatbaarheidsbeoordeling in een ex-vivo varkensmodel

In hoofdstukken 6 en 7 ontwikkelden we een nieuw perfusiemodel, waarbij varkensnieren uit het slachthuis werden gebruikt. Na overlijden werden deze nieren meerdere keren doorgespoeld om alle intravasculaire inhoud (pro-inflammatoire moleculen en toxines) te verwijderen. Vervolgens konden we een gepaarde nieranalyse uitvoeren waarbij nieren met ernstige en milde ischemieschade met elkaar werden vergeleken.

De kinetiek van de afgifte van extracellulaire histonen werd bestudeerd in hoofdstuk 6. We toonden aan dat histonen exponentieel vrijkomen over de tijd en afhankelijk zijn van de mate van ischemieschade. De gebruikte technieken waren in staat om histonen al 1 uur na perfusie te meten. Daarnaast toonden in-vitro tests aan dat histonen cytotoxischer zijn voor niercellen bij hogere temperaturen en concentraties. Dit duidt erop dat ischemie op zichzelf kan leiden tot de afgifte van cytotoxische histonen, die, vooral in een warme omgeving, een vicieuze cirkel van celdood, toegenomen afgifte van schadelijke moleculen en verlies van transplantaatkwaliteit kan veroorzaken. Deze bevindingen bevestigen dat warme ischemie geminimaliseerd dient te worden. Bovendien kunnen extracellulaire histonen tijdens een intacte circulatie in de donor of tijdens NMP nierschade veroorzaken. Daarom is het interessant om ofwel de cytotoxische effecten te minimaliseren of hun afgifte te verminderen. De biochemische afdeling van Maastricht had eerder aangetoond dat heparine cytotoxische histonen zowel in vitro als in vivo[91] kan neutraliseren en Sedigh et al. toonden gunstige effecten van een heparineconjugaat in ischemisch beschadigde nieren aan[92]. Daarom testten wij of de toevoeging van niet-anticoagulerende heparine de histonafgifte zou verminderen. De toevoeging van heparine veranderde de totale histon H3-concentraties niet, maar we observeerden wel een continue stijging in de concentratie van een histon H3-variant met een lager moleculair gewicht, wat we toeschrijven aan een toegenomen protease-activiteit door de interactie tussen histon en heparine. Alles bij elkaar genomen is de afgifte van

histonen direct gerelateerd aan ischemische nierschade en dienen verdere studies de bijdrage van histonen aan nierschade en methoden om hun nadelige effecten te neutraliseren te onderzoeken, teneinde transplantatienieren optimaal te preserveren.

In samenwerking met het Maastricht Multimodal Molecular Imaging Institute (M4I), Division of Imaging Mass Spectrometry, hebben we geprobeerd massa-spectrometrie imaging (MSI) toe te passen voor de beoordeling van orgaankwaliteit, wat nog niet eerder is gedaan (hoofdstuk 7). Dit werd mogelijk gemaakt door de introductie van een nieuw type imaging massa spectrometer, die de ruimtelijke distributie van biomoleculen 20 tot 50 keer sneller kan beoordelen dan commerciële instrumenten die op dat moment beschikbaar waren. Hierdoor was analyse van monsters met een hoge doorvoer mogelijk en kan bovendien door de hoge snelheid weefselanalyse binnen een klinisch acceptabele tijdsduur voor levensvatbaarheidsbeoordeling worden uitgevoerd[93]. We gebruikten MSI om ischemische schade te identificeren tussen milde en ernstig beschadigde varkensnieren. Terwijl onze eerste tests, gericht op eiwitten, niet succesvol waren vanwege de moeilijkheid om moleculen met een hoge moleculaire massa en hun diversiteit te beoordelen en identificeren, was de daaropvolgende analyse van lipiden – met een veel lagere moleculaire massa – succesvol. Allereerst kon een geblindeerde MSI-analist snel en gemakkelijk onderscheid maken tussen ernstige en milde ischemische schade. Interessant genoeg kon de histopathologische beoordeling, die als de gouden standaard wordt gezien, geen onderscheid maken in de mate van ischemische schade. Dat kan verklaard worden door een aantal factoren. Ten eerste is herhaaldelijk aangetoond dat microscopische beoordeling niet accuraat is voor het beoordelen van ischemische schade na orgaandonatie. Daarnaast maakt MSI het mogelijk om op moleculair niveau ischemische schade te identificeren in een tijdsbestek waarin microscopische en morfologische veranderingen nog niet detecteerbaar zijn. Van klinisch belang is dat de snelheid van MSI mogelijk hoger is dan die van de histopathologische beoordeling en dat, als digitale techniek, de interpretatie van MSI-resultaten op een andere locatie topografisch kan plaatsvinden. Deze veelbelovende resultaten dienen met voorzichtigheid te worden geïnterpreteerd en de vertaling naar de klinische setting is voorlopig, omdat de analyse op menselijk weefsel naar verwachting moeilijker zal zijn. Dit komt doordat humaan nierweefsel jarenlang schade heeft opgelopen (senescentie) en lijdt aan schade die per donor (DCD versus DBD) verschilt, vergeleken met een relatief homogene groep jonge varkensnieren. Naast het vermogen van MSI om onderscheid te maken tussen milde en ernstig beschadigde nieren, leidde deze studie ook tot inzichten in de onderliggende biochemische processen van acute ischemische nierschade. We vonden dat een reeks afbraakproducten van lipiden verhoogd is na ernstige ischemie, zoals lysocardiolipines,

lysophosphatidylcholines en lysophosphatidylinositol. Deze lipiden zijn geassocieerd met zowel mitochondriale als celmembraanintegriteit. De toegenomen concentratie van deze lipiden bevestigt dat zij een rol spelen bij nierschade. Bovendien konden we met MSI deze schade markers toewijzen aan specifieke tubulaire gebieden in de renale cortex, wat toekomstige analyses van de distributie van nierschade na ischemie mogelijk maakt. Concluderend lijkt MSI een nieuwe, veelbelovende techniek op het gebied van levensvatbaarheidstesten. Het kan informatie verschaffen in een vroeg stadium van schade op moleculair niveau. Nu nieuwe preservatietechnieken op komst zijn, zoals NMP, is het gebruik van snelle en geavanceerde MSI voor levensvatbaarheidsbeoordeling des te interessanter, omdat het de uitkomsten van interventies op weefsel in een preklinische setting kan testen en verandering in pathofysiologische processen kan onthullen. Dit moet verder onderzocht worden. Bovendien kunnen toekomstige klinische studies inzicht bieden in de rol van MSI bij de analyse van humaan weefsel en de mogelijke klinische waarde ervan[94].

De volgende nieuwe methode, met het potentieel om levensvatbaarheid te testen in een varkensperfusiemodel, is het gebruik van het renovasculair circulerend volume (hoofdstuk 8). We gebruikten deze verdunningstechniek, zoals die wordt toegepast bij de bepaling van circulerende volume van dialysatorvezels, om het renovasculair circulerend volume te testen tijdens machineperfusie met verschillende perfusietijden, -drukken en verschillende ischemietijden. Omdat deze test nog niet eerder is uitgevoerd, lag de focus op de reproduceerbaarheid en validatie ervan. De verdunningstechniek bleek reproduceerbaar te zijn. Bovendien verschillen de renovasculaire circulerende volumes per ischemiegroep (15 versus 45 minuten), wat aantoont dat het circulerend volume afneemt naarmate de ischemieschade ernstiger is. Hoewel de resultaten van deze studie aantonen dat de techniek reproduceerbaar is, heeft dit niet geleid tot een vervolgstudie. Dit is grotendeels te wijten aan onze focus op andere technieken, die wij meer veelbelovend achten, zoals MSI of pathologische beoordeling. Desalniettemin blijft de verdunningstechniek niet-invasief en eenvoudig uit te voeren. Het kan daarom interessant zijn om verdere studies uit te voeren met een verbeterde technische opzet; nog beter zou het zijn als de techniek in perfusiemachines wordt geïntegreerd. Bovendien kan met de introductie van NMP (met hogere stroomsnelheid (flow), temperaturen en circulatie van bloed) de sensitiviteit en specificiteit van de verdunningstechniek worden verbeterd. Het kan tevens een aanvullende methode zijn om de orgaankwaliteit tijdens deze preservatietechnieken te monitoren.

In hoofdstuk 9 voerden we een kapsulotomie uit bij ischemisch beschadigde varkensnieren. Het idee ontstond uit de observatie dat ernstig ischemisch beschadigde nieren vaak gezwollen waren en het renovasculaire circulerend volume en de perfusie verminderd waren. Met 16 nieren toonden we aan dat een kapsulotomie langs de grotere kromming van de nier de gecompromitteerde nier direct verlichtte, waarbij parameters zoals flow, weerstand, renovasculair circulerend volume en intraparenchymateuze druk in alle nieren verbeterden. Verrassend genoeg vonden we geen significant verschil tussen nieren met milde (20 minuten) of ernstige (45 minuten) ischemie. Het positieve effect van deze interventie op de renale perfusie lijkt evident, maar het klinische gebruik ervan is ter discussie en dient verder onderzocht te worden wat betreft de veiligheid. Hoewel we in ons model geen parenchymateuze schade observeerden door de interventie, zijn transplantatiechirurgen terughoudend bij het uitvoeren van deze manoeuvre. Vooral omdat er geen betrouwbare levensvatbaarheidstesten zijn om te selecteren welke nieren levensvatbaar zijn en dus geen interventie met mogelijke complicaties behoeven. Daarom zou een kapsulotomie alleen moeten worden uitgevoerd bij nieren die vatbaar zijn voor een renaal compartimentsyndroom en het risico op verdere daling in levensvatbaarheid lopen. Ongeacht het risico kan een kapsulotomie de renale perfusie verbeteren en daarmee de preservatie kwaliteit verbeteren door middel van snellere afkoeling, betere perfusie van therapeutica en efficiëntere afvoer van afvalproducten. Hierdoor kan de levensvatbaarheidsbeoordeling eveneens verbeterd worden wanneer schade markers in het perfusaat worden gemeten. Bovendien werd deze studie uitgevoerd in een tijdperk waarin NMP nog niet gangbaar was. Het zou interessant zijn deze studie te herhalen met NMP, wat een betere setting biedt om relevante veranderingen in een metabolisch actief orgaan te observeren en om het mogelijke risico op parenchymateuze schade en bloedingen te onderzoeken.

Toekomstperspectieven

Sinds januari 2016 is hypotherme machineperfusie (HMP) in Nederland als standaard preservatietechniek geïmplementeerd voor alle post-mortale donornieren[95]. Klinisch gezien was dit noodzakelijk omdat werd geconcludeerd dat HMP superieur is aan koude statische preservatie. Echter, het gebruik van levensvatbaarheidsbeoordelingen tijdens de perfusieperiode is niet in de protocollen opgenomen, omdat er geen wetenschappelijke basis voor bestaat. In deze thesis hebben we meerdere nieuwe strategieën toegepast om de levensvatbaarheid te testen, maar we konden geen enkele test vinden met klinisch bruikbare voorspellende kracht. Daarnaast hebben we nieuwe strategieën ingezet, zoals het gebruik van renovasculair circulerend volume of MSI, in

dierstudies. Vooral MSI lijkt veelbelovend, maar de resultaten zijn nog niet klinisch getest en de techniek dient verder ontwikkeld en onderzocht te worden om het potentieel ervan in te schatten. We concluderen dat de toekomst van levensvatbaarheidsbeoordeling onduidelijk is en tot dusver niemand het tegendeel heeft aangetoond. Na meer dan 20 jaar gebruik en onderzoek naar HMP kan men stellen dat HMP niet geschikt is voor de levensvatbaarheidsbeoordeling van donornieren. Donornieren in een hypotherme, hypometabole toestand weerspiegelen mogelijk niet voldoende hun levensvatbaarheid en kans op post-transplantatiefunctie. Ook is de etiologie van transplantaatfalen grotendeels multifactorieel en is de voorspelling welke nieren zullen functioneren of falen, met name met betrekking tot marginale donornieren, moeilijk wanneer nieren bij 4 °C worden bewaard.

Het gebruik van NMP heeft theoretisch gezien veel voordelen ten opzichte van HMP wat betreft levensvatbaarheidsbeoordeling[96]. Vergeleken met HMP worden nieren bij NMP geperfundeerd met een op bloed gebaseerd perfusaat en bij hogere stroomsnelheden, waardoor zij metabool volledig actief zijn. Van alle levensvatbaarheidsbeoordelingsstrategieën tijdens HMP, oftewel de meting van perfusieparameters, perfusaatbiomarkers en histologische en moleculaire weefselanalyses, zijn al deze methoden theoretisch gezien veelbelovender wanneer ze in een normotherme toestand worden uitgevoerd[97]. De verbeterde perfusie bootst de in-vivo situatie beter na, waardoor parameters zoals renale weerstand en het renovasculair circulerend volume waarschijnlijk betrouwbaarder zijn[98]. Als de perfusie verstoord is, is het eenvoudiger om het probleem te lokaliseren, wellicht zelfs door macroscopische inspectie. Bovendien zorgt een verbeterde perfusie voor een betere instroom van therapeutica en/of zuurstof en een efficiëntere afvoer van afvalstoffen[99]. Hierdoor kunnen biomarkers een betere weergave geven van de renale levensvatbaarheid. Met een op bloed gebaseerd perfusaat kunnen bovendien meer biomarkers gemeten worden. Daarnaast zal de nier urine produceren, indien deze goed wordt geperfundeerd, en kan de urineproductie en de daarin aanwezige biomarkers gemeten worden om de levensvatbaarheid in te schatten[100-102]. Hierdoor is normotherme machineperfusie, als nieuw platform voor pre-transplantatie levensvatbaarheidsbeoordeling, aanleiding geweest voor een snelle toename van studies die nieuwe strategieën onderzoeken om de renale levensvatbaarheid te bepalen. In een varkensperfusiestudie werd aangetoond dat “laser speckle contrast imaging” lineair correleert met de renale bloedstroom en dat microcirculatie van afwezige perfusie kan worden gevisualiseerd en semi kwantitatief beoordeeld[103]. De groep van Thomas Minor gebruikte e metabolische omzetting van 13C-acetaat als marker tijdens pre-transplant perfusie en kon daarmee onderscheid maken tussen

"goede", "aanvaardbare" en "kritieke" kwaliteit van nieren (AURC: area under the ROC-curve 0,91; p<0,001)[104]. Ook de groep uit Rotterdam gebruikte photoacoustische beeldvorming in een varkensmodel, een techniek die eenvoudig uit te voeren is met aangepaste echografietoestellen, om de corticale oxygenatie te beoordelen. Zij toonden aan dat deze methode effectief onderscheid maakt tussen functionele en niet-functionele nieren en concludeerden dat dit een veelbelovend hulpmiddel is met een goed potentieel voor ondersteuning bij de orgaanselectie voor niertransplantatie[105]. In lijn met niet-invasieve beoordeling tijdens NMP heeft Hamerlink et al. magnetische resonantiebeeldvorming gebruikt om de nierfunctie te beoordelen. Zij vonden een verschillend bloedstroomdistributie in-vivo versus ex-vivo, met minimale of ernstige warme ischemieschade[106]. Tot nu toe is weefselanalyse na NMP nauwelijks uitgevoerd [107], maar in theorie kan een weefselanalyse na een normotherme resuscitatie periode een betere weergave geven van de orgaanlevensvatbaarheid dan na een hypotherme periode.

Na de eerste niertransplantatie na NMP in 2011 in Leicester[108], is dit resultaat slechts door enkele groepen gerepliceerd. Klinische data is schaars en komt vrijwel uitsluitend van de Nicholson-groep[109,110], die recentelijk een hoogwaardig gerandomiseerde gecontroleerde studie publiceerde waarin statische koude opslag (SCS) werd vergeleken met SCS plus één uur NMP in een totaal van 338 DCD-nieren. Zij vonden geen significant voordeel of nadeel van NMP, met een DGF-percentage van respectievelijk 60,7% voor NMP en 58,5% voor SCS (aangepaste odds ratio (95% betrouwbaarheidsinterval) 1,13 (0,69–1,84); p=0,624). Opvallend is dat zij voor de levensvatbaarheidsbeoordeling uitsluitend een binaire NMP-beoordelingsscore gebruikten, die bestond uit macroscopie, renale bloedstroom en urineproductie, en zij vonden geen verschil in de geobserveerde DGF voor hoge of lage scores[110]. In de nabije toekomst worden meer klinische studies verwacht waarin NMP wordt vergeleken met HMP (ClinicalTrials.gov identifier: NCT04882254)[111] of NMP met SCS (ClinicalTrials.gov identifier: NCT05031052)[89]. In tegenstelling tot klinische studies bevinden zich meerdere onderzoeksgroepen in een preklinische fase. Zij richten zich op het verbeteren van de technische opzet van NMP, het optimaliseren van de perfusieoplossing en het ontwikkelen van nieuwe renoprotectieve strategieën en interventies voor NMP, door diermodellen of afgekeurde menselijke transplantatie nieren te vergelijken met HMP of SCS[112-121]. Wat betreft levensvatbaarheidsbeoordeling is de wijdverspreide implementatie van NMP in de niertransplantatie nog maar net begonnen. Klinische data over levensvatbaarheidsbeoordeling is derhalve nog nauwelijks beschikbaar[89,122]. Concluderend is NMP een nieuwe techniek die verdere ontwikkeling en onderzoek behoeft om de aanvullende klinische waarde vast te stellen.

De levensvatbaarheidsbeoordeling komt langzaam op gang en gaat een 'warm tijdperk' in, waarvan de theoretische voordelen evident lijken, maar waar nog onvoldoende data voor is.

Ongeacht de technische ontwikkelingen in het verbeteren en testen van de levensvatbaarheid van donornieren, hangt het gebruik hiervan sterk af van de ontwikkelingen in de klinische niertransplantatie. Een recent artikel beschrijft de succesvolle ontwikkelingen in preklinische xenotransplantatie[123]. Dit leidde tot de eerste varken-naar-mens genetisch gemodificeerde niertransplantatie op 16 maart 2024 en toont aan dat, ten minste op korte termijn, de nier veilig is en functioneert[124,125]. Helaas overleed de ontvanger na 52 dagen vanwege een hartstilstand, maar met een nier zonder serologische of histologische tekenen van afstoting[125,126]. Als xenotransplantatie op de lange termijn klinisch aanvaardbare resultaten oplevert, kan dit de beschikbaarheid van transplantabele organen aanzienlijk veranderen. Die langetermijnresultaten zijn echter nog niet beschikbaar, waardoor dit voorlopig onzeker blijft. Een ander interessant toekomstperspectief werd recentelijk besproken door Hosgood et al.[127]. Met behulp van vitrificatie (de snelle afkoeling van organen naar een stabiele, ijsvrije, glasachtige toestand) en nanowarming (het verwarmen van nanodeeltjes binnen de vasculatuur van organen om een snelle en uniforme opwarming te realiseren) werd aangetoond dat rattennieren tot 30 dagen functioneerden na transplantatie, nadat zij 100 dagen cryogeen waren opgeslagen[128]. Het concept van cryopreservatie en orgaanbankieren zou een meer electieve benadering van niertransplantatie mogelijk maken[127,129]. De concepten van succesvolle vitrificatie, nanowarming, cryopreservatie, orgaanbankieren en xenotransplantatie zullen onvermijdelijk invloed hebben op het veld van orgaanlevensvatbaarheid en -behoud, een veld dat zich zal moeten aanpassen en verder ontwikkelen om met deze mogelijke klinische veranderingen om te gaan. Deze opkomende ontwikkelingen zijn spannend en bieden hoop voor toekomstige patiënten met een eindstadium nierziekte.

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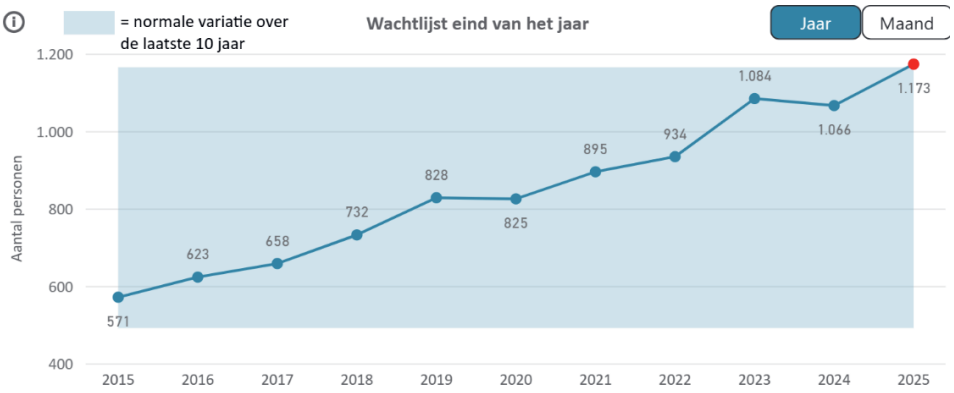
Chapter 12

Impact paragraph

The transplant waiting list and organ shortage

At the end of 2024, there were an estimated 1066 patients on the active waiting list for kidney transplantation in the Netherlands. There is a steady increase over the years, almost doubling in ten year time (Figure 12.1)[1]. Of these patients 488 (46%) were already on the list for more than two years, 84 patients died while on the waiting list and 103 were removed from the list, because there medical condition deteriorated and could not be transplanted anymore[1]. The inflow of patients on the waiting list in 2024 was 1659 in total and there was an outflow 1576[1].

Figure 12.1: Number of patients on the active waiting list for kidney transplantation from 2014 to 2025.



Kidney transplantation remains the treatment of choice for patients with end stage renal disease. While patients are dying on the growing waiting list, we can only conclude that novel strategies to increase the amount of successful kidney transplantations are crucial. These strategies are outlined in Chapter 1 of this thesis. There are two main responsible entities to implement these strategies. The Dutch government and society in general are responsible for increasing the number of organ donors and facilitating the medical field financially to strive for progression. Secondly, the medical field itself is responsible for improving transplant success and quality, to gain more donor organs.

Due to the organ shortage the Dutch government has explored many ways to increase the organ shortage. This first giant step was the implementation of the Dutch Organ Donation Act (Wet op Orgaandonatie) in 1998[2], including a national Donor Register, based on an opting in consent system. However, this did not lead to a structural increase of donors. A further attempt, a Masterplan Organ donation did not

result in the minimal desired 25% increase in donors[3]. On the 13th of February 2018 a proposition for a new law, describing an opt-out consent system, was approved by the Dutch Senate (Eerste Kamer) and was implemented in July 2020. The effect on the donor pool has to be evaluated thereafter. These changes took 8 years from first proposition to implementation, due to the major societal impact.

Kidney transplantation not only leads to an increase of quality and expectancy of life, it also leads to an economic benefit compared to dialysis[4,5]. This benefit accounts for both living and deceased donation[6]. A cost-benefit analysis in 2014 showed that dialysis treatment costs on average €80.000 per year. Deceased kidney donation cost the same in the first year (€80.000), but thereafter €8.000 per year of nephrological care. A €72.000 yearly save from the second year of transplantation, with an average graft function of deceased donors of 10-15 years. Thus, a deceased donor kidney transplant may lead to a €1.000.000 cost reduction as compared to ongoing dialysis. Living kidney donation saves even more, as recipients are mostly transplanted before dialysis, while recipients otherwise wait 3 years while having dialysis[4]. Additional cost-benefit studies on hypothermic machine preservation showed a beneficial effect on costs compared to static cold storage[7,8].

Relevance of this thesis

This thesis “Marginal donor kidneys; novel strategies to assess and improve viability” focusses on the medical aspect of improving the success of kidney transplantation. We have performed clinical and experimental studies in both human and animal setting to gain insight in factors that relate renal injury or transplant outcome. As outlined in Chapter 1 and above, the socioeconomic impact of kidney donation and transplantation is substantial. The organ shortage has to be addresses and the search for new techniques to either assess or improve viability are vital to the growing success of kidney transplantation. In part I of this thesis we studied which factors are attributed to graft failure and whether viability assessment can be useful. In part II and III we have used novel techniques, of which most have never been used in the setting of kidney donation and transplantation, to test if they have potential as viability assessment tool or may improve organ quality. The outcomes are promising and may lead to improvement of organ viability assessment and thus a better understanding of which kidneys will function or fail. This again may aid clinicians in the organ selection process, increase the number of organs selected for transplantation which has a beneficial effect

on the organ shortage. Subsequently, this may lead to shorter waiting list and the additional financial benefits as outlined above.

It is worrisome that viability assessment is used on a bread scale throughout the world, while the scientific evidence is poor. Researchers in the US studied a cohort of 212305 deceased donor kidneys of which 36700 were discarded. More than 14000 kidneys (38.2%) were discarded after histopathologic viability assessment, which was the most common reason for discard[9]. Others concluded that procurement biopsies may therefore lead to unnecessary discard[10-12]. Currently, the use of routine histologic examination is abandoned in the Netherlands. However, Dutch nephrologists may still use histologic assessment as viability tool and base their decision to discard or transplant on this outcome. In Maastricht hypothermic machine perfusion parameters in combination with machine perfusate biomarkers have been used to test kidney viability, especially of marginal donor kidneys and transplant decisions were (in part) based on these parameters. After concluding that possible transplantable kidneys were unnecessarily discarded, this strategy was discarded. Although the idea of viability assessment seems promising, the scientific evidence to use predictive tools in a clinical situation is poor. We therefore conclude that further studies are warranted.

The possible hazard of unnecessary discard and (in contrast) the opportunity to select those kidneys which would normally have been discarded but may seem viable, highlight the clinical potential and possible impact of viability assessment. Both the development of novel strategies for viability assessment as well as subsequent prospective randomized trials aid in better decision making and a possible increase of suitable kidneys for transplantation. Chapters 3, 4, 5, 7, 8 and 9 are additions to viability assessment evidence. Moreover, we have not only confirmed findings by others, but we included new techniques for viability assessment. Renovascular volume measurement during machine perfusion, introduction of mass spectrometry imaging (MSI) for histologic analysis of donor tissue and the association of histones with transplant outcome are good examples.

This thesis shows multiple innovative approaches to test viability of donor kidneys in clinical and preclinical experimental settings. In part I we shown that clinical parameters are of interest when selecting uncontrolled donor kidneys. In part II we have used novel assessment strategies, i.e. a new set of histologic markers and their association to delayed graft function and a new marker ‘extracellular histones’, in a human cohort. These markers show that there is indeed an association to clinical outcome and may add to the growing literature on viability test in order to make this

entity an evidence based clinical tool. Furthermore, our pre-clinical studies with pig tissue, i.e. studies on the release of extracellular histones, the use of MSI, renovascular volume measurement during machine perfusion and the use of a capsulotomy in severely ischemic injured kidneys, show that these novel strategies may aid in better quality assessment or improvement of renal flow. However, these should be studied further and validated in a clinical setting.

In conclusion, efforts to alleviate the donor kidney shortage are warranted and positive results will have socioeconomic impact. This thesis adds to the existing literature, as it implements new strategies to assess and improve renal quality before transplantation, which may lead to improved selection strategies and more kidneys suitable for transplantation. Our findings may be of interest to researchers as it offers insight into pathophysiologic processes during ischemia-reperfusion injury. It may also be of interest to physicians, who are in need of clinical tools to select suitable kidneys and may wish to perform additional studies.

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Curriculum vitae

Curriculum vitae



Tim van Smaalen werd samen met zijn tweelingzus Annelieke geboren op 19 maart 1985 te Rotterdam, als jongere broer van Mart en Ruben en als zoon van Henk en Monique. Hij groeide op in Bergschenhoek en ging na het behalen van het Atheneum aan de Montessori School te Rotterdam (2004) geneeskunde studeren in Maastricht. De voorliefde voor de heelkunde groeide tijdens een tussenjaar in een regionaal ziekenhuis in Tamale in Ghana (Tamale Teaching Hospital). Hij vervolgde deze weg door een klinisch en wetenschappelijk oudste co-schap transplantatie chirurgie te volgen onder begeleiding van prof.dr. Ernst van Heurn. Daarmee was de interesse voor de chirurgie definitief. In september 2011 behaalde hij zijn Master Diploma Geneeskunde aan de Universiteit Maastricht en begon hij als arts-onderzoeker nierdonatie en -transplantatie onder begeleiding van prof.dr. Ernst van Heurn en dr. Carine Peutz-Kootstra. Gedurende deze periode combineerde hij dit met een functie als transplantatiecoördinator in het MUMC+. Begin 2016 verhuisde hij naar Rotterdam om als ANIOS chirurgie in het Franciscus Gasthuis & Vlietland te werken. In juli 2017 startte hij met de opleiding tot chirurg in het Erasmus MC te Rotterdam. Tijdens de opleiding werkte hij in het Erasmus MC (2017-2018 o.b.v. prof.dr. Bas Wijnhoven en 2022 o.b.v. dr. Sjoerd Lagarde), het Franciscus Gasthuis en Vlietland (2018-2020 o.b.v. dr. Taco Klem en 2022-2023 o.b.v. dr. Marijn Poelman) en het Catharina Ziekenhuis Eindhoven (2020-2022 o.b.v. dr. Simon Nienhuijs). Hij rondde de opleiding tot algemeen en gastro-intestinaal chirurg af in juni 2024 in het Amphia ziekenhuis te Breda onder begeleiding van prof.dr. Lyckle van der Laan en George van der Schelling (2023-2024). Met grote interesse in robot geassisteerde chirurgie werkte hij nog ruim een jaar als chirurg in het Amphia ziekenhuis, een robot expertise centrum, om zich verder te bekwamen in met name de colorectale robot geassisteerde chirurgie. In september 2025 kon hij zijn passie voor de buikwandhernia chirurgie nastreven en begon hij als Fellow complexe buikwandhernia chirurgie met sterke nadruk op robot geassisteerde behandelingen en innovatieve technieken in het Reinier de Graaf Gasthuis in Delft o.b.v. dr. Bob Bloemendaal. Tevens specialiseert hij zich verder in de chirurgische behandeling van diepe endometriose met darmbetrokkenheid.

In 2015 ontmoette hij zijn vrouw Judith, tevens GE-chirurg, met wie hij tijdens hun gezamenlijke opleiding tot chirurg in 2018 een tweeling kreeg (Noraly en Julia) en in 2021 werd een derde dochter geboren (Yara). Na verhuizingen van Rotterdam naar Breda, naar Eindhoven en weer terug naar Rotterdam is het gezin in augustus 2026 (hopelijk voor de laatste keer) verhuisd naar Leiden.

Dankwoord

Dankwoord

Het proefschrift is gedrukt. Een hele reis, die langer heeft geduurd dan vooraf gepland. Desalniettemin is het zover en ben ik blij dat het zo een mooi boekje is geworden. Vooral ben ik trots op het multidisciplinaire aspect en dat ik met zoveel vakspecialisten heb mogen werken. Chirurgen, nefrologen, pathologen, intensivisten, biochemici, analisten, massa-spectrometristen, statistici, technici, slachters, verpleegkundigen, transplantatiecoördinatoren, onderzoekers, studenten, kidneyracers, allen moet ik daarvoor danken en dan vergeet ik vast nog iemand. Maar niet voordat ik hén dank die alles mogelijk maken zonder daar iets voor terug te krijgen: de donoren en hun naasten. Verder gaat mijn dank uit naar:

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Pieter en Jasper, wat een uren hebben we kunnen maken zonder resultaat! BB3, de -80 vriezer, slachthuis ritjes, een perfusielab bouwen en pompen, pompen, pompen én we claimen nog altijd officieus de eerste warm gepreserveerde nier in Nederland. Dat

wat niet op papier kwam is minstens zo belangrijk als dat wat er wel kwam, dankzij al die ontdekkingen.

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