Molecular signatures underlying kidney transplantation
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DOI:
10.33612/diss.248377050

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2022

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 11

Summary
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Summary

By performing the studies described in this thesis, the principal goal was to improve our understanding of the unique pathophysiology underlying different donor types in renal transplantation and attempt to translate these results into tailored treatment.

Chapter two focused on the molecular processes from organ procurement until graft failure in donation after circulatory death (DCD) kidneys. One key aspect that emerged was that the damage already starts upon the withdrawal of life support and slowly progresses over the course of many years. For this reason, tissue damage, leading to the onset of fibrosis, could already be treated while the organ is outside of the body. This has the advantage that more potent drugs, for example targeting the transforming growth factor-beta 1 (TGF-β1) signaling pathway, can be used as systemic effects are limited. Ultimately, the combination of hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP) with added pharmacologically-active substances could lead to better long-term outcomes for renal transplant patients and is therefore extensively studied in this thesis.

But before treating the kidney grafts, there was more to learn about the underlying mechanisms of ischemia using advanced mass spectrometry technology. Chapter three aimed to map out the degradation mechanisms underlying ischemia. First, the N-Terminal Amine Isotopic Labelling of Substrates (TAILS) degradomics enrichment technique was established. TAILS allowed mapping of the exact cleavage sides for the first time in degradomics workflows, providing more detailed insights into underlying degradation mechanisms. The preliminary experiments revealed that degradation occurred mainly during warm ischemia compared to cold ischemia. This was expected as the metabolism at 4°C is significantly lower. Interestingly, protease activity was observed under hypothermic conditions.

As warm ischemia revealed more protein degradation, a short (10 min), medium (20 min), and a long (≥ 30 min) period of warm ischemia time (WIT) in DCD kidneys were then compared. Medium and long WIT resulted in more protein degradation as compared to short WIT. Medium and long WIT exhibited degradation of metabolic proteins, cytoskeletal proteins, and extracellular matrix proteins. Protease analysis revealed collagen cleavage by matrix metalloprotease (MMP) 2/9, calpain activity and the degradation of its activator calpastatin, and extensive cathepsin D/L activity. Inhibiting MMP2/9 in human tissue slices led to trends of higher collagen levels, lower MMP activity, and better preserved tissue integrity.
In **chapter four**, a different proteomic approach was applied to focus on molecular alterations underlying donation after brain death (DBD) kidney grafts. HMP secretome profiles of kidneys with a good and suboptimal transplant outcome one-year post-transplantation were compared. The rationale of this study was to see if biomarkers that predict transplant outcomes in a non-invasive manner could be identified. A clear distinction was observed between kidneys with a good versus a suboptimal transplant outcome. Interestingly, elevated levels of proteins involved in classical complement activation in the secretome of kidneys with a good outcome and proteins involved in lipid metabolism and cytoskeletal proteins in the secretome of kidneys with a suboptimal outcome were identified. Furthermore, ATP-citrate synthase and fatty acid binding protein 5 and immunoglobulin heavy variable 2-26 and desmoplakin presented predictive values for kidney transplant outcome.

**Chapter five** aimed to reveal the unique proteome and degradome underlying different donor and perfusion characteristics. First, a novel degradomics enrichment technique with an improved experimental protocol compared to TAILS was explored. High-efficiency Undecanal-based N Termini EnRichment (HUNTER) is a robust technique that allows N-terminal enrichment in microscale samples. Indeed, a higher enrichment efficiency and more identified degradation products were observed when comparing HUNTER to TAILS. For this reason, HUNTER was implemented for all consecutive degradomics experiments.

Next, the different donor characteristics were analyzed. Metabolic process enrichment and degradation of structural proteins were associated with “more optimal” donor characteristics, such as a younger donor age (<60), DBD donor, and donors with a healthy BMI (<25). Proteomic enrichment of immune and complement-related pathways was associated with older (>60) and male donors. Older donors also displayed degradation of apoptosis-related proteins. Kidneys obtained from donors with a high BMI (>25) showed degradation of proteins involved in lipid homeostasis. When looking at the temporal changes during NMP, we observed an association between enrichment of immune-related proteins in DBD and kidneys deemed transplantable. An association between enrichment of ECM proteins and DCD kidneys, kidneys considered not transplantable, and kidneys obtained from older donors was observed. When looking at perfusion factors over time, Kidneys that had no urine production during NMP showed enrichment of proteins located in Henle’s loop. After 6h of NMP, kidneys with a lower flow (<80 mL/min/100g.) showed gluconeogenesis and glycolysis-related protein degradation.

As ischemia resulted in increased collagen cleavage by MMP 2 and 9 shown in **chapter three**, inhibition of these proteases was attempted using doxycycline in **chapter six**. Additionally, the impact of doxycycline on proteolytic degradation mechanisms and the urinary proteome of perfused porcine kidney grafts was investigated. The administration of doxycycline during HMP was found not to inhibit MMP2/9 activity. This could be due to the dose-response, the thermodynamics, or the bioavailability of doxycycline under hypothermic conditions. However, significantly lower urinary NGAL levels were observed, indicating that doxycycline did have somewhat protective effects. Proteomic analysis revealed distinctive clustering profiles between urine samples collected at the beginning of NMP and towards the end of NMP. Furthermore, degradomics analysis indicated that metabolic, complement, and coagulation pathways were altered during machine perfusion.

After the studies focused on molecular profiling of the proteome and degradome of ex vivo renal transplants, the next step was to explore potential tailored treatment strategies. For this purpose, in **chapter seven**, we examined the utility of porcine precision-cut kidney slices (PCKS) as an ex vivo ischemia-reperfusion injury model. Porcine kidneys were obtained from the abattoir and preserved using oxygenated hypothermic machine perfusion. The results show that slices can be cultured for up to 72 hours while remaining viable. William’s medium E supplemented with ciprofloxacin provided the most optimal incubation condition to preserve tissue viability.

The PCKS model was then used to test different anti-fibrotic drugs in **chapter eight**. Previous studies have shown that galunisertib, doxycycline, taurine, and febuxostat inhibit fibrosis via different pathways. Our results showed that only galunisertib exhibited significant anti-fibrotic effects, and doxycycline only trended towards lower levels of anti-fibrotic markers. Therefore, the effects of galunisertib in fibrotic PCKS cultured with TGF-β1 were analyzed. TGF-β1 significantly promoted fibrosis in the slices and galunisertib clearly attenuated the expression of all fibrosis-related genes.

Chapters three, five, and six revealed an altered metabolism due to ischemic injury, emphasizing the importance of an adequately supported metabolism during NMP. For this reason, a blood-based solution was compared to a serum-like preservation solution (AQIX® RS-I) enriched with colloids and red blood cells to see if alternatives to blood provide enough support for the renal metabolism in **chapter nine** during NMP of porcine kidneys. The results revealed that a blood-based perfusion solution remained superior when looking at renal function and metabolism. The addition of red blood cells during NMP reduced renal injury, improved function, and was associated with increased renal metabolism.
Finally, in chapter ten, all obtained knowledge and optimized techniques were combined to create a novel drug testing and delivery platform: Machine perfusion and Organ slices as a Platform for Ex vivo Drug delivery (MOPED). MOPED is a robust technique to explore fibrogenesis suppression strategies as pretreatment with TGF-β is an effective method to quickly induce the onset of fibrosis.

Galunisertib was the most promising drug tested in chapter eight and was therefore tested in whole isolated porcine kidneys using MOPED. This study revealed that galunisertib suppresses the onset of fibrosis in kidney allografts without compromising renal viability, functionality, and injury as assessed by oxygen consumption, tissue ATP levels, histological structure, lipid peroxidation, urine production, proteinuria, creatinine clearance, fractional sodium excretion, metabolic coupling, urinary NAG, LDH, and ASAT levels. These results illustrate the value of targeted drug delivery, using isolated organ perfusion for reducing post-transplant complications.

General discussion

In the United States alone, approximately one patient dies every hour because he/she could not receive a donor organ in time. And although each year over one hundred thousand kidneys are transplanted worldwide, this is only 20-30% of the patients waiting on a donor kidney. Living donors and standard criteria donors are considered optimal donors. However, the gap between demand and supply is still rapidly increasing, urging the need to increase the donor pool.

Suboptimal donor organs, such as extended criteria donors (ECD) and DCD donors, are already used to increase the donor pool. But as mentioned many times before in this thesis, these donor organs are more prone to damage, resulting in more post-transplant complications and a lower graft survival rate. One thing we know for sure, these suboptimal donor kidneys require better preservation than provided by static cold storage (SCS) if we want to reduce complications and improve survival. Thus it is not strange that, after almost 100 years of research, machine perfusion has been reinvented and is moving its way into the clinics.

Hypothermic machine perfusion (HMP) has been extensively studied. It shows benefits such as a continuous supply of nutrients and oxygen, restoration of ATP levels, protection of endothelial cells, and a reduced inflammatory response, compared to SCS. Over a dozen clinical trials have been performed using SCD, ECD, and DCD donors, and all point towards superior preservation with HMP when looking at delayed graft function (DGF) and graft survival. For this reason, it is advocated that HMP should be used to preserve all deceased donor kidneys. Whether all donor kidneys benefit from oxygenated HMP is yet to be determined.

Besides better preservation, HMP also provides the opportunity to assess injury by measuring biomarkers in the HMP perfusate, as shown in chapter four and previous studies. Using HMP as a treatment platform in chapter six proved to be less successful. This could be explained by pharmaceuticals’ altered dose-response and thermodynamics under hypothermic conditions. Therefore, HMP may not be suitable for pharmaceutical interventions to repair suboptimal donor organs.

Normothermic machine perfusion (NMP) is a more advanced technique designed to support renal metabolism and restore cellular function fully instead of suppressing the metabolism. This could be considered as an advantage as functionality assessment can be performed. However, the disadvantage is that restoration of function may also induce harmful inflammatory responses and that it can be challenging to fully support the renal metabolism, as shown in chapter nine. Not to forget the relatively
high costs of NMP and the risk of additional warm ischemia time in case of a technicality malfunction.

Although NMP is widely studied in an experimental setting, the evidence proving superior preservation is scarce. Studies have shown that NMP is superior to SCS, but evidence that NMP or HMP and NMP combined is superior to HMP alone is scarce. So, the question of whether NMP on itself provides better preservation than HMP is yet to be answered. Perhaps the strength of NMP is the unique opportunity to treat an isolated organ.

The main aim of this thesis was to gain more knowledge about underlying molecular mechanisms of the different donor types, with the idea of translating these results into tailored treatment during machine perfusion. Combining all the results of this thesis, several key pathways proved to be dysregulated. Pathways related to metabolism, complement and coagulation cascades, TGF-β signaling, and intracellular and extracellular structure were identified. Therefore, these pathways or protein classes should be carefully considered when developing a suitable perfusion solution and when considering treatment.

As renal cells need high energy levels due to their ATP-dependent functions such as reabsorption, secretion, and filtration, it is not strange that metabolic processes are disturbed by ischemia and reperfusion injury. The results of chapters three, five, and six displayed disruption and degradation of proteins related to metabolic processes. Chapter three revealed extensive degradation of proteins related to ATP synthesis, oxidative phosphorylation, and oxidoreductase activity after a more extended period of warm ischemia in DCD kidneys. Interestingly, more metabolic protein enrichment in DBD kidneys than DCD kidneys was observed in chapter five. Moreover, younger donors aged 60 or under showed more metabolic protein enrichment than older donors. Furthermore, urinary secretion of metabolic proteins was observed at the beginning of NMP in chapter six.

These results show the essence of fully supporting renal metabolism during NMP as the underlying metabolic processes are already altered. In chapter nine, different compositions of perfusion solutions were compared. The results showed that only the red blood cell-containing groups displayed sufficient oxygen consumption, indicating the importance of an oxygen carrier during NMP. Although AQIX® RS-I appears to have essential components to fuel oxidative phosphorylation, the solution did not support ATP production and subsequent sodium reabsorption at the same level as plasma did. These results express that multiple plasma components are essential for oxidative phosphorylation, and all kidney grafts require a perfusate composition that fully supports the renal metabolism during NMP.

Another pathway that appeared to be dysregulated was the complement system. Previous research has shown that brain death is associated with complement activation, and chapter four revealed secretion of complement factors C1r, C1s, C1q, and C4BPA in perfusate samples of DBD kidneys was associated with a good outcome post-transplantation. Similar results were obtained in chapter six, where doxycycline treated kidneys showed urinary excretion of complement protein C1s. Together, these results suggest that a washout of tissue-resident complement proteins, perhaps due to detachment of remnant unproductive complement attack complexes, could have protective effects on kidney function.

Degradation of complement C3 was observed after 4 hours of NMP in chapter six, and degradation of C3 is associated with podocyte damage. Additionally, enrichment of processes related to immune response and complement activation in kidney tissue obtained from both older and male donors was observed in chapter five. Unfortunately, we could not link these findings to transplant-outcome as the kidneys were not transplanted. However, previous research has shown that complement activation is associated with the inflammatory response to ischemia-reperfusion injury and transplant-related pathologies.

Furthermore, it has been shown that male donor kidneys exhibit more graft rejection and have a higher chance of returning to dialysis after renal transplantation. Complement inhibitors could therefore be promising drugs to administrate during NMP. Studies have shown that treatment with a C1 inhibitor has potential protective effects during renal transplantation. Perhaps DBD kidneys with a suboptimal outcome, older donors, and male donors would benefit most from treatments targeting the complement system.

TGF-β is one of the most important cytokines involved in developing renal allograft fibrosis—one of the most significant burdens of chronic graft failure, as we extensively described in chapter two. The TGF-β pathway drives the differentiation of fibroblasts into myofibroblasts—key effector cells that produce large quantities of matrix proteins, especially collagens and fibronectins, eventually leading to tissue scarring and fibrosis. For this reason, TGF-β could be a potential drug target for attenuating fibrosis during NMP.

Significant dysregulation of TGF-β itself was only observed in chapter six. The doxycycline-treated group revealed more excretion of TGF-β as compared to the control. In line with the excretion of C1s, this washout could have protective effects or represent an onset of tissue repair & healing.
Chapter two discusses potential TGF-β inhibitors that could be promising in a renal transplant setting. Chapters eight and ten extensively focus on inhibiting the TGF-β signaling pathway. Of all the drugs tested in chapter eight, only galunisertib significantly attenuated fibrosis and TGF-β gene expression. It should be mentioned that galunisertib was the only specific TGF-β inhibitor tested in these experiments. As galunisertib showed such promising results, the anti-fibrotic potency of galunisertib was also assessed in an entire isolated porcine kidney. Again, galunisertib exhibited considerable anti-fibrotic effects, emphasizing its potential application to attenuate fibrosis in renal allografts.

The structural components of the kidney, both intracellular and extracellular, are essential for tissue integrity and normal renal function. Chapter three revealed extensive degradation of extracellular matrix (ECM) proteins during extended warm ischemia. Numerous collagen, laminin, and fibronectin substrates were identified. These collagen substrates were probably cleaved by MMPs. MMPs are responsible for the degradation of collagen and other components of the ECM. They also play a role in the onset of fibrosis and could serve as a potential drug target for attenuating fibrosis. In chapters three, six, and eight, MMP2 and 9 were targeted with MMP2/9 Inhibitor I and doxycycline. MMP2/9 Inhibitor I resulted in higher collagen levels, lower MMP activity, and better-preserved tissue integrity. Doxycycline yielded in a lower expression of fibrosis markers when administrated under normothermic conditions and resulted in significantly lower urinary NGAL when administrated under hypothermic conditions. Taken together, kidneys exposed to extended warm ischemia may benefit most from MMP2/9 protease inhibition. However, the delicate balance between ECM degradation and accumulation should be closely monitored, as both phenomena contribute to fibrosis formation.

The podocyte cytoskeleton is a regulated target of proteolytic modification, and proteolysis is altered upon podocyte damage. Chapter three revealed extensive degradation of cytoskeletal proteins in DCD kidney grafts. Cathepsin L1 was one of the proteases responsible for actin degradation. For this reason, we tried inhibiting cathepsin L1. Unfortunately, this did not result in better tissue integrity. Perhaps a long-term outcome study is needed to reveal potential benefits. Chapter five revealed degradation of vimentin and plectin, two cytoskeletal proteins that are associated with apoptosis, in grafts obtained from an older donor (>60). In chapter four, secreted actinin-1 and talin-1 in the perfusate of DBD kidneys with a suboptimal outcome one-year post transplantation was observed. Enhanced excretion of cytoskeletal proteins could cause lower eGFR rates in this suboptimal group due to loss of tissue integrity and glomerular filtration barrier disruption. Degradation of actin and talin-1 was also observed in DBD donors with a suboptimal function in a study performed by Vaughan et al.

They suggest that TGF-β induces calpain-1 protease activation leading to dysregulation of the actinin cytoskeleton. Targeting calpain-1 in vitro seemed to have protective effects. However, in chapter three, inhibition of calpain-1 in ex vivo tissue slices did not result in better-preserved tissue integrity. Degradation of actin was also observed after puromycin adenonucleoside induced renal damage in a degradomics study performed by Demir et al. Taken together, preserving the podocyte cytoskeleton would potentially benefit DCD and suboptimal DBD kidneys.
Future perspectives

Normothermic machine perfusion

Over the past ten years, many research teams across the globe have performed numerous experiments to optimize and implement normothermic machine perfusion for kidney transplantation. But even after all these years, we can conclude that renal NMP is still in its infancy. Several important questions remain unanswered and should be further explored before implementing NMP as standard clinical care.

One of the main unresolved questions is to what extent does ex vivo renal physiology resembles in vivo renal physiology? What are the exact metabolic needs of an isolated perfused kidney, and do all kidney grafts have the same needs? In this thesis, we performed extensive proteomics and degradomics analyses to unravel the underlying molecular mechanisms of perfused kidney grafts. Although the proteome reveals important mechanistic pathways, it is not the only level of -omics that provides information about renal function and tissue integrity. Post-translational modifications (PTMs) could be additionally explored, and genomics, epigenomics, transcriptomics, and metabolomics techniques could be combined into a multi-omics approach to gain an even deeper understanding of ex vivo renal physiology and the underlying mechanisms of each donor type. Ultimately, all of these layers could be combined into an integrative tool that recommends the appropriate treatment for each individual kidney graft.

Another question that remains is, what are optimal conditions during NMP? There are many aspects of NMP that cause the significant heterogeneity of NMP protocols. For example, the type of machine, arterial pressure, oxygenation, temperature, gradual rewarming, the duration of NMP, and perfusate composition can be altered. Currently, each research group is using its own designed protocols based on their expertise and personal preference. Perhaps not all kidney grafts may benefit from the same NMP conditions and require tailored perfusate compositions based on graft quality. However, a universal protocol is greatly desired with regards to mapping out general renal function and viability criteria during NMP.

Which brings me to my next question: what does “good” ex vivo renal function entail? We are currently assessing renal function during NMP by looking at the arterial flow, urine production, oxygen consumption, and creatinine clearance. However, renal function can significantly differ when altering the perfusate composition. The results of chapter 9 indicate that the addition of red blood cells clearly influences oxygen consumption, and the addition of different colloids affects tissue swelling and urine production. To address these two questions, our group is performing numerous
experiments, testing different pressures, rewarming techniques, oxygen concentrations, and perfusate compositions. Furthermore, we are working on a database combining all our performed porcine NMP experiments, including over 300 kidneys. A universal database with NMP experiments can compare all the different conditions that have already been tested and shed some light on ex vivo renal function and the absolute critical components of a perfusion solution.

Another important question to ask is whether NMP itself is beneficial for the kidney graft. Countless studies have focused on optimizing the NMP platform, but only a few have shown the benefits in a (pre)clinical setting. Additional animal studies and randomized controlled trials will have to demonstrate whether NMP or HMP combined with NMP is more beneficial to SCS or HMP alone. A large randomized controlled trial (ISRCTN15821205) including 400 patients comparing SCS versus NMP has recently been completed in Cambridge, and the results of this trial will be available soon. The Prolonged ex-vivo normothermic machine perfusion for kidney regeneration (PROPER) trial (NCT04693325) is currently performed in Leiden and Groningen to test the feasibility and effect of normothermic machine perfusion. Additionally, the upcoming trial Renal Ex vivo Warm Advanced Resuscitation through Machine perfusion (REWARM) (https://pre-image.eu/) will compare HMP versus HMP and NMP combined in a multicenter study, and further unravel the underlying molecular mechanisms in a clinical setting.

Whether or not NMP itself is beneficial for improving clinical transplant outcomes is something we should further explore. However, the experiments in this thesis have shown the potential of NMP as a drug delivery platform. Several groups have tested NMP as a delivery platform for pharmaceuticals, stem cells, antibodies, and silencing RNAs. This thesis reveals the anti-fibrotic potency of galunisertib administration during NMP, and these results should be further validated using donated human kidneys unsuitable for transplantation for more translatable results. Additionally, pharmaceuticals targeting the complement system, preserving the extracellular matrix and podocyte skeleton, and inhibiting the TGF-β signaling pathway could be tested using our MOPED platform. These pharmaceuticals (combined) can then be implemented during NMP to attenuate the damage induced by brain death and ischemia and prevent the onset of fibrosis. Ultimately, tailored treatment could be implemented for each individual kidney graft.

**Replacing lab pigs with pig waste**

The intention of this thesis was to perform all experiments without the use of laboratory animals. All the experiments in this thesis were performed using porcine waste material obtained from the abattoir, human waste material obtained after nephrectomy procedures or from unsuitable donor kidneys, and human tissue/perfusate samples obtained from a biobank.

The use of abattoir kidneys is becoming increasingly popular, especially as the ethical and social concerns regarding the wellbeing and need for laboratory animals are rising. Advantages of using abattoir material are an almost infinite supply of kidneys at relatively low costs and that it is timesaving as ethical approval is not needed. But an abattoir model also has its disadvantages. As the pigs are bred for food consumption, there is no option to assess injury or treat kidneys before retrieval. The pigs are bred in a less controlled setting compared to laboratory animals, leading to more heterogeneity, although this could also be seen as an advantage. The kidneys cannot be obtained in a sterile manner, increasing the chances of infections, especially when preserving the organs for a longer time. Lastly, the organs cannot be (auto)transplanted, so the renal function can only be monitored for a limited period.

The latter is one of the reasons why we developed Machine perfusion and Organ slices as a Platform for Ex vivo Drug delivery (MOPED). MOPED combines machine perfusion techniques with precision-cut tissue slices and provides the opportunity to incubate renal tissue for an additional 24 to 72 hours. This allows for more extended observation of, for example, fibrosis development.

The MOPED platform can be used to answer all sorts of transplant and non-transplant-related questions. It can be used to optimize NMP conditions with a more extended read-out period. It can be used to study the complexities of brain death, ischemia, reperfusion, fibrogenesis and the onset of fibrosis. Furthermore, it can be used to test other anti-fibrotic therapies or to target the previously described potential drug targets. MOPED could also be implemented using human tissue for more translational results, and other organs, such as lungs and livers. Finally, MOPED could be utilized for drug development, toxicity testing, drug kinetics, studying organ physiology and pathophysiology, studying tumor growth, and the efficiency of novel chemotherapies.

**Concluding remarks**

This thesis aimed to unravel molecular mechanisms that can be targeted to increase kidney graft quality. We unraveled key pathways that are dysregulated during organ donation and machine perfusion and exhibited that NMP can be used as a drug targeting platform to attenuate fibrosis. However, additional research is needed to translate these results into a clinical setting. In an ideal future, hypothermic and normothermic machine perfusion is tailored to each unique donor graft, and where there was once a huge gap in the donor pool, now only a tiny hole remains.
References


