Chapter 1

General introduction
and outline of this thesis
General introduction and outline of this thesis

Kidneys are complex organs that play a vital role in maintaining total body homeostasis. The unique architecture of these bean-shaped organs consists of a million microscopic filters or functional units called nephrons. These nephrons consist of glomeruli, the tubular system, interstitium, and vasculature.

One of the most critical functions of the kidneys is homeostatic regulation of water and ion content of the blood, controlled through glomerular filtration, tubular reabsorption, and tubular secretion.\(^1\,^2\) (Figure 1). The glomerular filtration rate (GFR) measures how well the kidneys filter the blood, and a healthy GFR is defined as higher than 90 mL/min.

Figure 1. Kidney reabsorption and secretion to maintain a homeostatic balance of water and ion content in the blood. Created with BioRender.com.

The natural process of aging causes loss of GFR and renal function over time. We lose about 4,500 nephrons per year per kidney, and our GFR is estimated to decrease by 10 mL/min per decade of our lives.\(^3\,^4\). However, sometimes this natural process is dysregulated, and excessive loss of kidney function occurs, also known as chronic kidney disease (CKD).
**Chronic kidney disease**

CKD is a universal name for multiple disorders marked by altered renal structure and function. CKD is characterized by a decreased renal function with a GFR of 60 mL/min or lower and/or signs of kidney injury for at least three months, such as proteinuria, albuminuria, anemia, a high blood pressure, or damage related to a history of renal transplantation\(^5,6\). There are five stages of CKD, based on GFR, as shown in Table 1. The development of CKD can occur gradually over the course of several years, and many patients are unaware of their condition as the complaints only arise when kidney function is below 30% (stage 4 CKD)\(^7\).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>GFR (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kidney damage with normal GFR</td>
<td>&gt;90</td>
</tr>
<tr>
<td>2</td>
<td>Mild reduction in GFR</td>
<td>60-89</td>
</tr>
<tr>
<td>3</td>
<td>A moderate reduction in GFR</td>
<td>30 to 59</td>
</tr>
<tr>
<td>4</td>
<td>Severe reduction in GFR</td>
<td>15 to 29</td>
</tr>
<tr>
<td>5</td>
<td>End-stage renal disease</td>
<td>&lt; 15</td>
</tr>
</tbody>
</table>

The last stage of CKD is also known as end-stage renal disease (ESRD) and is defined by a GFR below 15 mL/min. Several risk factors for ESRD include age, hypertension, diabetes mellitus, obesity, a history of renal disease, salt intake, and alcohol, tobacco, heroin, or analgesic use\(^8-10\), indicating that a person’s lifestyle significantly impacts the development of kidney failure.

During kidney failure, the filtration and reabsorption process is disturbed due to a decreased GFR, and toxic levels of metabolic waste accumulate in the blood. Regaining a balance in fluids and electrolytes requires dialysis. Unfortunately, dialysis results in a lower survival rate and a lower quality of life\(^1,2\).

**Kidney transplantation**

Therefore, renal transplantation is the best solution for patients affected by ESRD. It has proven to give long-term benefits such as better patient survival and a better quality of life\(^11,12\). In 1954, Joseph Murray and his team were the first to transplant a kidney from one identical twin to another successfully\(^13\). Today, the kidney is the most transplanted organ worldwide\(^14\).

**Different donor types**

Kidneys for transplantation can be obtained from living and deceased organ donors (Figure 2). Deceased donor organs are more commonly used\(^15\); however, living donation is also implemented to increase the donor pool. Living kidney donation generally results in better outcomes than deceased organ donation\(^16,17\).

Deceased organ donation can be divided into donation after brain death (DBD) and donation after circulatory death (DCD). Currently, DBD donation is the primary source for donor kidneys\(^15\) and can be sub-categorized into standard criteria donation (SCD) and extended criteria donation (ECD). SCD donors are preferred as they show favorable outcomes compared to ECD and DCD donors\(^18,19\). SCD donors are brain death donors below the age of 50 without comorbidities. Unfortunately, the supply of SCD kidneys is not sufficient to provide all patients on the waiting list with a kidney graft\(^11,20\). Therefore, the use of suboptimal kidneys is crucial to enlarge the donor pool.
Suboptimal kidneys, such as ECD and DCD kidneys, are more damaged and prone to post-transplant complications. ECD kidneys are obtained from a brain death donor with an age above 60 or above 50 with two of the following characteristics: history of hypertension, terminal serum creatinine levels of ≥ 1.5 mg/dL, or cerebrovascular cause of death\textsuperscript{21,22}. DCD kidneys endure more severe ischemia-reperfusion injury (IRI), which makes them more susceptible to delayed graft function (DGF), fibrosis, and chronic graft failure\textsuperscript{23,24}.

Ischemia-reperfusion injury
Ischemia is defined as the restriction of blood supply to the tissue, causing a shortage of oxygen needed to produce our primary energy source, i.e., adenosine triphosphate (ATP). As a result of oxygen deficit, the cells switch from aerobic to anaerobic respiration, leading to a decline in intracellular ATP levels, intracellular acidosis, and cell damage\textsuperscript{25–28}. After implantation of the graft, the blood supply is restored, referred to as reperfusion. However, the re-introduction of oxygen andrewarming of the kidney graft together lead to the restart of aerobic metabolism characterized by extensive ROS production, which is detrimental to the previously ischemic cells\textsuperscript{29}. This phenomenon is called IRI.

Usually, our kidneys receive 20-25% of the cardiac output for their filtration processes, although they only constitute 0.4% of total body weight. This high blood flow rate and thus high oxygen levels are also critical for renal function due to the tremendous metabolic demand to facilitate active reabsorption. Therefore, it is not strange that kidney grafts are prone to damage paired with IRI. Subsequently, a cascade of molecular processes is activated for tissue repair in order to restore kidney function.

Tissue repair, fibrosis, and chronic graft failure
Tissue repair starts with an inflammatory response, that is characterized by the secretion of inflammatory cytokines and recruitment of immune cells to the affected site to remove dead and damaged cells, as well as tissue debris\textsuperscript{30}. The cytokine transforming growth factor-beta 1 (TGF-β1) promotes the accumulation of myofibroblasts, which are key effector cells in tissue repair. Once myofibroblasts become activated, their purpose is to restore tissue integrity by producing and secreting extracellular matrix (ECM) proteins, such as collagens and fibronectins\textsuperscript{31}.

When these processes become dysregulated, ECM proteins accumulate, leading to excessive tissue scarring, fibrosis formation, and often chronic graft failure. Chapter two extensively describes the underlying pathophysiological mechanisms of ischemia-reperfusion injury and the onset of fibrosis, leading to chronic graft failure, and the importance of novel treatment strategies.

Novel treatment strategies are essential as chronic graft failure occurs in about 50% of deceased donor kidneys within ten years\textsuperscript{17}, meaning that 50% of kidney recipients need to resume dialysis or are placed back on the waiting list for a retransplant. The most promising approach to increasing the graft survival rate is limiting the damage using machine preservation.

The rise of machine perfusion
The first perfusion pump to keep an organ alive while outside of the body was designed by Alex Carrel and Charles Lindbergh in 1934\textsuperscript{32}. This sterile perfusion machine was made entirely of glass and was successfully used for over 900 normothermic machine perfusion experiments. Belzer was one of the major pioneers in the field of kidney perfusion and preservation. In 1967, he developed one of the first successful kidneys perfusion machines that could preserve canine kidneys for up to 72 hours\textsuperscript{33}.

Over the years, machine perfusion was continually studied and optimized; however, it was only scarcely implemented in the clinics. Static cold storage (SCS) with an appropriate preservation solution seemed to be sufficient for the SCD grafts that were commonly transplanted. It was not until the expansion of the donor pool that machine perfusion regained interest today.

Hypothermic machine perfusion
Currently, hypothermic machine perfusion (HMP) is the standard of clinical care for preserving and transporting deceased donor kidneys in the Netherlands, as it has shown to be superior to SCS\textsuperscript{34}. During HMP, the preservation solution is continuously circulating through the donor organ at a temperature of 0 – 4°C\textsuperscript{35}, providing the kidney with a continuous supply of nutrients and the possibility of delivering oxygen to restore ATP levels\textsuperscript{36,37}. Clinical trials have shown that HMP reduces the incidence of DGF and increases graft survival compared to SCS\textsuperscript{38–40}. Supplemenenting the perfusate with treatments that protect or repair the kidney graft could further enhance HMP techniques.

Normothermic machine perfusion
Another perfusion technique is normothermic machine perfusion (NMP). During NMP, the temperature is kept under physiological conditions (35–38°C) to support the metabolic activity of the organ\textsuperscript{41}. Studies performed by Hosgood et al. have shown that a short period of NMP is feasible as a preservation and assessment tool for suboptimal organs\textsuperscript{42,43} and lowers the incidence of DGF compared to SCS\textsuperscript{44}. Adding treatments during NMP is perhaps more efficient than during HMP as the kidney is fully metabolically active, and most pharmaceuticals have optimal kinetics at 37°C.
Although NMP is a successful clinically implemented preservation, resuscitation, and assessment technique for livers and lungs, renal NMP is only scarcely implemented in the clinics. Thus far, we have yet to decipher the exact metabolic needs of an isolated kidney, nor do we know what “good” ex vivo kidney function entails. As kidneys require a high oxygen supply to support their active processes, sufficient oxygen supply during NMP is necessary for full metabolic support. Nonetheless, research into the underlying mechanisms of renal transplant-related pathophysiology is fundamental before we can implement NMP as standard clinical care.

Unraveling the molecular footprint of each kidney graft
The pathophysiological mechanisms likely differ per donor characteristic as each donor type is exposed to damage differently. For instance, kidneys from older donors are generally more immunogenic than kidneys from younger donors. DBD kidneys undergo metabolic disturbances and alterations in the mitochondria and changes in the pulmonary, endocrine, and immunological systems. DCD kidneys are exposed to prolonged warm ischemia. If indeed the molecular footprint of each donor characteristic is unique, graft preservation and resuscitation techniques should be tailored to the needs of each donor type.

Unfortunately, many of the underlying mechanisms are still undefined. Therefore, this thesis aims to gain molecular insights into the role of protein dysregulation underlying different donor characteristics using proteomics and degradomics techniques, and to translate these insights into tailored treatment.

Proteomics as a tool for protein profiling
The term ‘proteomics’ was first defined in 1995, representing the characterization of the complete protein repertoire of a cell line, tissue, or organism. Proteomics is used to acquire a global snapshot of biology by examining as many of the cell’s proteins as possible instead of each one individually. It can be used to identify and quantify proteins in biological and clinical material, leading to multi-protein expression profiling, structures, interaction networks, and protein post-translational modifications (PTMs).

A breakthrough in the discipline of proteomics was the development of mass spectrometry (MS) technology, and antibody-based detection technologies, such as proximity extension assays (PEA) by Olink and aptamers by Somalogic. In comparison, MS remains the most comprehensive and versatile technology in large-scale proteomics today as it is not dependent on protein-specific detection tools. MS uses mass analysis to characterize peptides derived from proteolytically digested proteins, referred to as “shotgun proteomics.” It measures the mass-to-charge ratio (m/z) of gas-phase ions, and peptides detected as precursor ions are subsequently fragmented into daughter ions by tandem mass spectrometry (MS/MS), which provides the basis for protein sequencing. Proteomic profiling using mass spectrometry has been widely implemented in medical research, referred to as clinical proteomics, including in the field of transplantation.

Protein degradation
Protein degradation is a critical process in cell physiology. It is controlled by different degradation systems, of which the ubiquitin-proteasome system (UPS) and the autophagosome–lysosomal system regulate most of the cellular protein turnover. Proteases are the second-largest enzyme class in humans, counting approximately 569 members. They are described as enzymes with hydrolase activity cleaving ester, thioester, and amide (peptide & isopeptide) bonds in a process referred to as proteolysis. Proteases are one of the main drivers of post-translational protein processing and modifications, impacting protein activity, maturation, and function. It is known that precise cleavage of proteins by proteases leads to a defined regulation of multiple biological processes such as cell differentiation, cell migration, morphogenesis, wound healing, tissue remodeling, immunity, and apoptosis. Proteases and alterations in proteases also play a role in various pathological conditions such as neurodegenerative disorders, cardiovascular diseases, and cancer. In particular, organ and tissue function and integrity may be altered by accelerated proteolysis associated with tissue damage resulting from ischemia, contributing to renal diseases and transplantation-related complications.

Proteolytic activity in renal physiology
The UPS plays a vital role in renal physiology. The UPS is responsible for maintaining the glomerular cell-specific proteome in the glomerulus. Furthermore, the UPS maintains the podocyte-specific proteome, such as the actin cytoskeleton, crucial for podocyte 3D structure and function. In specialized tubulointerstitial cells, the UPS plays a role in erythropoietin (EPO) regulation and glucose transport. In the distal tubular cells, the UPS regulates salt homeostasis by controlling the levels of sodium chloride cotransporter and epithelial sodium channels, two critical tubular sodium reabsorption systems. In the cells of the collecting duct, the UPS affects water homeostasis via regulation of vasopressin-inducible water channel aquaporin 2 levels. Therefore, it is not unexpected that dysregulation of these systems leads to loss of tissue integrity and renal function. There has been some evidence that the UPS and protein degradation play a role in renal fibrosis, acute kidney injury, and chronic kidney disease. Furthermore, a recent degradomics study provides evidence that TGF-β potentially mediates cytoskeletal degradation and actin dysregulation during kidney donation after brain death. Nevertheless, research into proteolytic activity in renal pathophysiology, focused on transplantation, is quite scarce.
Degradomics to profile global proteolysis

In the past couple of years, several proteomics technologies have been developed with the aim of enriching proteolytic fragments of proteins, reflected by de novo N-termini generated after hydrolysis, for the identification of protease cleavage products in multifaceted biological samples (degradomics). More recently developed techniques, such as TAILS and HUNTER, are high throughput labeling techniques that can be used for protease substrate discovery and N-terminome analysis. These methodologies can be utilized to identify protease substrates in complex proteomes. They also allow exact mapping of the biologically generated N-termini location produced through proteolysis and identification of the responsible protease.

During TAILS and HUNTER, the α-amines of free protein N-termini and the ε-amines of lysine side chains are modified at the protein level by dimethylation or by adding isotopic mass tags. The tags distinguish the N-termini generated in the biological sample from the N-termini generated by experimental digestion with trypsin. Proteolytic digestion with trypsin only cleaves after arginine residues due to the labeled lysine residues. This results in larger peptides and so more precise MS/MS results.

Each chemically labeled N-termini is quantifiable due to the isotopic mass tag or the isotopically labeled dimethyl group. Additionally, naturally modified N-termini can be discriminated from chemically labeled N-termini. With the help of specialized software, the responsible protease can be matched to the identified cleavage site. Nonetheless, the efficient depletion of highly abundant proteins in combination with specific enrichment of N-terminal peptides increases the efficiency of comprehensive characterization of “degradomes” and their alteration in disease.

Barely of these advanced degradomics techniques have been used to explore protein degradation underlying loss of renal function and tissue integrity during kidney donation and preservation. Therefore, in chapter three, the TAILS technique was optimized and used to explore the effect of warm ischemia on protein degradation in DCD donor kidneys. In chapter four, a proteomics approach was used to try and distinguish between DBD kidneys with a good and suboptimal outcome during HMP. Chapter five compares TAILS and HUNTER, followed by an analysis of protein dysregulation and degradation in DBD and DCD kidneys during NMP. Finally, the effect of doxycycline on proteolytic degradation mechanisms and the urinary proteome of perfused kidney grafts is studied in chapter six.
kidneys during the preservation phase without systemic effects. Gene therapies, nanoparticles, and cellular therapies have been tested during NMP to attenuate IRI or instigate regeneration and repair mechanisms. However, treatments targeting fibrosis, the main burden leading to chronic graft failure, are barely explored due to the lack of proper translational models.

Models for transplant-related research
Although in vitro models are used for transplant research, multicellular and spatial interactions in cell culture are lacking. To study the effects of potential treatment, more advanced models are essential. Laboratory animals, such as rats, pigs, and dogs, are commonly used in transplant research as they provide the opportunity to (auto) transplant the kidney graft. Even though animal models have led to significant breakthroughs, they are not always necessary in the great numbers they are utilized today. Finding alternatives to animal testing is a heavily debated subject and one of the focus points of this thesis. Therefore, this thesis explores alternative models for fibrosis and transplant-related research, using tissue slices and experimental NMP for drug testing and targeted drug delivery.

Precision-cut kidney slices
An alternative to a whole animal model is precision-cut tissue slices (PCKS). These tissue slices can be obtained from any organ, including the kidney. PCKS are three-dimensional viable explants containing a large number and diversity of cells, accurately reflecting the renal architecture.

The technique of tissue slices dates back as far as the 1950s when it was used to study primary pathways of intermediary metabolism. Back then, it was challenging to create uniform slices using manual techniques, so the reproducibility of experiments was poor. It was not until the development of the Krumdieck tissue slicer in 1980 that it made it possible to rapidly obtain tissue slices with identical dimensions in a suitable dynamic organ culture system. At that time, tissue slicing was primarily used in the field of pharmacology and toxicology. Today, tissue slicing is used in all kinds of research fields. For example, to study host-pathogen interactions, respiratory disease, intestinal IRI, and renal and liver fibrosis. One huge advantage of PCKS is that the slices can stay viable for up to 72 hours.

PCKS have not been used for transplant-related research. Therefore, it was analyzed whether a porcine PCKS model provides a suitable intermediate model for IRI and transplant-related research in chapter seven. By using kidneys obtained from the local slaughterhouse, laboratory animals are limited. In chapter eight, using the porcine PCKS model, previously identified pathways were targeted with antifibrotic compounds. As the kidneys are obtained from pigs that are approximately 6 to 8 months old, aged-induced fibrosis is not yet present. Fibrosis is therefore induced experimentally by spiking the PCKS with TGF-β, a key mediator in the onset of fibrosis. In chapter three, human PCKS are used for validation studies by inhibiting protease activity with specific protease inhibitors using left-over material obtained from nephrectomy procedures.

Targeted drug delivery using machine perfusion
One of the major benefits of machine perfusion is that it provides a platform for targeted drug delivery. Experimental NMP models using porcine kidneys obtained from the slaughterhouse have proven their success as an alternative research model. Many of these studies have focused on optimizing the perfusate composition and the technique itself. However, the optimal perfusion conditions for kidney NMP are still undefined. Therefore, the metabolic needs of DCD kidneys during NMP were observed by comparing different perfusion solutions in chapter nine.

Finally, all the obtained knowledge and models from this thesis were combined in chapter ten. A new approach based on normothermic machine perfusion and precision-cut kidney slices was used. First, a fibrotic NMP kidney model using porcine slaughterhouse kidneys spiked with TGF-β was created, followed by an additional 48 hours of incubation as PCKS. Then, the antifibrotic potency of galunisertib was tested in an entire isolated organ.


