Use of Mesenchymal Stem Cells for Preconditioning of Kidney Grafts in an Ex-vivo Kidney Perfusion Model

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Declaration of Originality

I, Natalie Vallant, hereby declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Information derived from the published and unpublished work of others has been acknowledged in the text and a list of references is given in the bibliography.

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Abstract

Introduction: One of the biggest issues in transplantation today is the lack of suitable transplantable organs for an increasing number of patients on the transplant waiting lists. Efforts to address this problem are made by the inclusion of organs from extended-criteria and donation-after-cardiac death donors, however these organs show inferior outcomes. Advances in organ preservation are needed to focus on techniques to assess and optimise organ viability prior to transplantation.

Methods: This research focuses on organ preconditioning using hypothermic- and normothermic ex-vivo machine perfusion technology in combination with stem cell treatment. A translational approach ranging from small animal models via porcine to human models was used with intent to facilitate translation of findings into clinical practice. A portable rapid sampling micro-dialysis (rsMD) device was tested which might enable live monitoring of kidney grafts during preconditioning periods and help estimate the condition of organs.

Results: In the first head to head comparison between hypothermic and normothermic machine perfusion for organ preconditioning ex-vivo, an advantage for hypothermic perfusion was detected in the porcine model. Contrary to this, in the translational model using human organs, the outcome was dependent on demographic backgrounds of the organs; especially the cold ischemia time organs were exposed to, had an impact on which perfusion modality was better. RsMD can successfully provide detailed real-time information on tissue and organ viability at all time points of organ preservation. Delivery of Mesenchymal Stem Cells (MSC) into grafts ex-vivo by machine perfusion was possible for the hypothermic- as well as the normothermic setting. MSCs could be traced within the grafts, but the cells did not immediately influence their function. Conditioned Mesenchymal Stem Cell media can influence the phenotype of immune cells, e.g. macrophages. Dependent on the genetic background of MSCs, the obtained conditioned media showed to influence macrophages in different ways.

Conclusion: This research has been successful in its overall objective to introduce Mesenchymal Stem Cells into ex-vivo machine perfusion settings. By doing so the goal would be to alleviate ischemia reperfusion injury of organs for transplantation and to thereby expand the pool of acceptable donor organs.

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Table of abbreviations

AKI	Acute kidney injury
AMP	Adenosine monophosphate
ANPEP	Alanyl amino peptidase
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
CD	Cluster of differentiation
CIT	Cold ischemia time
CKD	Chronic kidney disease
CM	Conditioned media
CSS	Cold Static Storage
CVA	Cerebrovascular accident
CXCR	Chemokine receptor
CyA	Cyclosporine A
DAMP	Damage associated molecular pattern
DBD	Donation after brain death
DCD	Donation after cardiac death
DGF	Delayed graft function
DLC	Dynamic lung compliance
ECD	Extended criteria donor
ECMO	Extracorporeal membrane oxygenation
EDN-1	Endothelin-1
END	Human endoglin gene
ESC	Embryonic Stem Cells
ESRF	End stage renal failure
EVHMP	Ex-vivo hypothermic machine perfusion
EVMP	Ex vivo machine perfusion
EVNMP	Ex-vivo normothermic machine perfusion
GGT	Gamma GT
GPI	Glycophosphatidylinositol
GSH	Glutathione
GVHD	Graft versus host disease
HD	Haemodialysis
HES	Hydroxyethyl starch
HGF	Hepatocyte growth factor
HIF	Heat inducible factor
HMGB1	High-motility group box 1
HMP	Hypothermic machine perfusion
HO-1	Haem oxygenase 1
HSP	Heat shock protein
HTK	Histidine-tryptophane-ketoglutarate
HTN	Hypertension
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon γ

IGL	Institute George Lopez
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent Stem Cells
IRI	Ischemia-reperfusion injury
KHB	Krebs-Henselleit buffer
LDH	Lactate dehydrogenase
MAPK	Mitogen activated protein kinase
MP	Machine perfusion
Mrc-1	Mannose receptor-1
MSC	Mesenchymal Stem (-Stromal) cells
NEVKP	Normothermic ex-vivo kidney perfusion
NGAL	Neutrophil gelatinase-associated lipocalin
NHBT	Non heart beating donor
NHS	National Health System
NHSBT	NHS blood and transplantation
NMP	Normothermic machine perfusion
NO	Nitric oxide
NOD	Nucleotide-binding ologomerization domain
PCR	Polymerase chain reaction
PD	Peritoneal dialysis
PEG	Polyethylene glycol
PGE2	Prostaglandin E2
POPS	Pulsatile organ perfusion system
PRR	Pathogen recognition receptor
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
RRT	Renal replacement therapy
RT-PCR	Real time polymerase chain reaction
SCD	Standard criteria donors
SCS	Static cold storage
SDF1	Stromal derived factor 1
SOT	Solid organ transplantation
TGF-β	Transforming growth factor β
Thy-1	Thymocyte differentiation antigen
TLR	Toll-like receptors
TNFα	Tumour necrosis factor α
uDCD	uncontrolled donation after cardiac death
UW	University of Wisconsin solution
UW-SCS	University of Wisconsin solution for static cold storage
VCA	Vascularized composite allografts
VCAM-1	Vascular cell adhesion molecule
WIT	Warm ischemia time
5’NT	5’ nucleotidase

Chapter 1: Introduction

1.1. The History of Renal Transplantation [1–3]

1.1.1. Early attempts of transplantation

Solid organ transplantation is a procedure in which an organ is removed from one body and placed into the body of a recipient in order to replace a damaged or missing organ. Organs or tissues, which are transplanted within the same individual's body, are called autografts, organs/tissues coming from a different donor of the same species are called allografts and organs/tissues coming from a different species are called xenografts. Allografts can either come from a living donor or from a cadaveric donor. So far, organs that have been successfully transplanted in humans are: heart, liver, kidneys, lungs, pancreas, intestine and thymus. Furthermore, also vascularized composite allografts (VCAs) have been successfully transplanted, including forearms, faces and the uterus. Worldwide, kidneys are the most commonly transplanted organs, followed by the liver and the heart.

The first case of kidney transplantation was reported at the Vienna Medical Society meeting in January 1902 by the Hungarian surgeon Emerich Ullmann. A dog's kidney had been transplanted into another dog's neck. In the same year, the French surgeon, Alexis Carrell (1873-1944) published a paper with the title ‚The Operating Technique for Vascular Anastomosis and Organ Transplantation' in the Journal *Lyon Médicine.* 2 years later, Carrell left France and went to live in Chicago, where he worked in close collaboration with Charles Guthrie (1880-1963). The two investigators contributed significantly in the field of vascular grafts and organ transplantation.

In 1906, the first documented xenotransplantation, a transplantation of a porcine kidney into the elbow of a woman suffering from nephrotic syndrome, was performed by the French surgeon Mathieu Jaboulay (1860-1913). Of course, the graft failed due to early vascular thrombosis, most likely caused by hyper acute immunological rejection.

Following these first unsuccessful attempts of transplantation, developments in the field paused until 1936, which is when the first unsuccessful kidney transplant between two humans was reported by Yurii Voronoy (1895-1961) in the Soviet

Ukraine. Voronoy transplanted a kidney from a 60-year-old man who had died from a fracture of the base his skull into a 23-year-old woman, who was in uremic coma after swallowing mercury in suicidal intention. Unfortunately, also in this case, acute rejection led to the patient dying within 48 hours. In the years around 1950, the American surgeon David Hume (1917-1973) had performed several human-to-human kidney transplants with different levels of success. The recipient who survived the longest was a patient who died 5 months after his transplant with accelerated hypertension and heart failure being the cause of death. Around the same time, Alexis Carrell described a theory of, biological incompatibility' for the first time and the Australian virologist Sir Frank Macfarlane Burnet (1899-1985) and the British biologist, Sir Peter Medawar (1915-1987) confirmed this theory by describing an ability of the human's immune system to distinguish between self vs. non self. This, together with the unsuccessful cases of transplantation led to the conclusion that transplantations between unrelated or unmatched individuals were doomed to fail. Only 2 years after these failures, in 1954, the first truly successful human-to-human kidney transplantation was performed by Dr. Joseph Murray (1919-2012). The transplant was performed at the same hospital in Boston as where David Hume had not been successful before, with the difference of that transplant being performed between two 23-year old identical twins. Obviously, due to this perfect matching, both patients survived, with the recipient later returning to work and raising a family. This case led to the desire to apply the findings of Medawar and Burnet and to find a way how to tolerize potential recipients to kidneys from unrelated donors.

Murray continued to perform kidney transplants on identical twins, and in 1959 recorded the first successful transplant to a non-identical recipient: a patient who received a kidney transplant from a fraternal twin. He had received radiation and a bone marrow transplant to suppress his immune response and lived for 29 more years. Finally, with the arrival of drugs to suppress the immune response, Murray successfully transplanted a cadaver kidney in 1962. This was the start into the modern age of kidney transplantation.

1.1.2. First Developments in the Field of Transplant Immunology

As mentioned above, it was Alexis Carrel, who first recognised and described a theory of ‚biological incompatibility' after the first unsuccessful attempts of kidney

transplantations. He also contributed to the ancient developments in ex-vivo machine perfusion of organs, which will be described in Chapter 1.2. He won the Nobel Prize in Medicine in 1912 for his contributions.

The breakthrough to describing the reasons behind Carrel's theory of ‚biological incompatibility' were made by Sir Frank Macfarlane Burnet and Sir Peter Medawar in the years around 1950. Burnet first introduced the concept of ‚self vs. non-self' and Medawar and colleagues described a ‚second set response' after performing skin graft experiments in mice. They subsequently learned that the skin grafts were rejected from incompatible recipients due to a cell mediated rejection process. In 1953, they could finally show that the immune system of one mouse could be induced to ‚tolerate' the skin of another mouse by exposing it to certain cells from that donor mouse around the time of birth. Medawar and Burnet were awarded the Nobel Prize in 1960 for their pioneering work in the field of immunology.

1.1.3. Important Developments underlying modern Immunosuppression

After Murray's first successful kidney transplant between identical twins and above mentioned discoveries in the field of Immunology had been made, the next aim was to find a way how to tolerize recipients to be able to perform transplantations between unrelated individuals. Joseph Murray, who transplanted non identical twins and used pre-operative radiation to cause immunosuppression in the recipients, undertook one step towards that goal, again. This led to two of his patients surviving long term. By the year 1961 Murray and his colleagues were finally able to expand their successes to non-twin siblings as well as to live unrelated donors by applying the previously described technique. In 1959, the ground stones for the modern immunosuppression were laid with the work of the British surgeon Sir Roy Calne (1930-). He recognised that a rapid proliferation of T- and B-lymphocytes was required for an immune response against ‚foreign' tissue. Therefore, his work began with the use of ‚anti-metabolites'. These are drugs that inhibit DNA synthesis, and as a consequence, reduce immune cell proliferation around the time of transplantation. Using the anti-metabolite 6-mercaptopurine at a time when this was an experimental drug, Calne was able to demonstrate prolonged kidney transplant survival in dogs. The drug was then used to complement total body irradiation but this was lethal in too many cases, so alternative treatment regimens had to be found.

Calne visited Boston, where he continued his work together with Joseph Murray. There, they experimented with a less toxic anti-metabolite and a pro-drug form of 6-mercaptopurine, Azathioprine. After some successful dog transplants using that drug, Calne returned to London in 1961 and combined Azathioprine with steroid treatments, which had been described just one year in advance by Willard Goodwin (1915-1998) at UCLA. This was the peri-transplant pharmacological regimen of immunosuppression that remained in use for nearly 20 years and it furthermore allowed for abandonment of total body irradiation before transplantation.
Calne later, in the 1970s, pioneered the use of another immunosuppressant called Cyclosporine. Cyclosporine also works by preventing T-cell activity, but due to a much more selective mechanism than the drugs mentioned before; it only acts on T-cells which have been stimulated to react to foreign tissue.

1.1.4. The first cadaveric kidney transplantation

In April 1962, Joseph Murray performed the first successful kidney transplantation with a graft from an unrelated and cadaveric donor. Under pharmacological immunosuppression, the recipient survived for one year. After this ground-breaking news, a wave of kidney transplants took place and a small international conference to discuss results took place in Washington DC in 1963. The results were very unsatisfying and showed survival rates of only 19% for unrelated and cadaveric transplants, compared to 48% for related transplants. The reasons for the poor outcomes, were, of course based on the underlying immunology, and opened a whole new chapter in the history of transplantation. Our understanding of Immunology improved further with a landmark publication in the New England Journal of Medicine in 1968 in which the benefits of HLA matching of donors and recipients were described. The underlying scheme was an early form of the HLA classification scheme which is used up until now[4]. Since the 1980s, a great pharmacological development has taken place with multiple agents to prevent rejection. These drugs can be adapted to the individual patient's needs and are quite well tolerated so that survival rates after transplantation improve steadily.

1.1.5. Availability of Organs

Another problem, which the pioneers of transplantation were facing, was the definition of death and therefore, the point of time at which organs become available for retrieval. Until the mid 1960s, death was defined as the time point at which the heart stopped beating. Hence, throughout the 1950s and early 1960s, once a patient had entered a state of irreversible coma, he had to be taken off mechanical ventilation and from that time, it sometimes took hours for the heart to stop beating. Furthermore, sometimes some more hours passed before the patient was legally declared as deceased and the retrieval process could start. This of course resulted in incredibly long times of warm ischemia to the organs that were meant to be transplanted. The definition of brain death had not been formed yet, when in 1963, the Belgian surgeon named Guy Alexandre performed the first ever transplantation using a kidney from a donor in ‚irreversible coma' without stopping mechanical ventilation. As a result, the donor organs were supplied with blood and oxygen up until the time of clamping, resulting in the shortest interval between that time, the retrieval and the replantation. By 1966, Dr. Alexandre had performed 9 kidney transplantations applying that new technique and when he announced his results together with tests, which were used to define ‚brain death' at a symposium in London, the audience was very suspicious and critical about the news. Sir Roy Calne commented, „Although Dr. Alexandre's criteria are medically persuasive, according to traditional definitions of death, he is in fact removing kidneys from live donors. I feel that if a patient has a heart beat, he cannot be regarded as a cadaver."[5] Less than two years later, the Ad Hoc Committee of the Harvard Medical School published a report on the 'Definition of Brain Death', largely agreeing with Dr. Alexandre's opinion and criteria[6]. After the release of that report, over the following years, laws were changed in Europe and the USA to reflect this new medical thinking and finally, the use of heart-beating but brain dead donors was legal and an acknowledged practice. There are still some subtle differences between countries worldwide in the diagnosis of brain death, for example in the UK diagnosis of brain death requires only brain-stem death, whereas in other countries, e.g. the USA global or whole brain death is needed for the permission to go ahead with the donation process, but altogether, the definitions are clear and the distinction between different types of donors is agreed on by medical societies all over the world.

As another contributor to the success of organ transplantation, the NHS Donor Register was launched in 1994 and as of 2017, there are approximately 21 million people registered as donors. This represents nearly a third of the adult population in the UK. Whereas people need to sign up actively as donors in the UK, other countries have gone further and made registration compulsory, with an ‚opt-out' register rather than an ‚opt-in' method. This remains a controversial subject in the UK and there is an aim to change towards the ‚opt-out' method in the near future. This could expand the donor pool, as many people are not aware and informed enough, even though willing to help and donate their organs. Improvement of awareness and information of the public about transplantation is urgently necessary to improve organ donation rates. Furthermore, in the UK, contrary to many other countries, altruistic living kidney donation is possible and this source of organs continues to grow and hopefully will further grow with increasing knowledge of the patients and the public.

1.2. The History of Organ Preservation

1.2.1. Cold Static Storage (CSS)

Methods to preserve donor kidneys outside the donor's body in the best possible physiological way has been a major subject for surgeons to study since the start of transplantation in the early 1960s.

However, the idea of keeping organs preserved ex vivo was not new back then and the earliest experiments on this topic date back to perfusion experiments on frog hearts, conducted by Carl Friedrich Wilhelm Ludwig (1816 – 1895) in the 1860s. Ludwig was one of the earliest pioneers of modern physiology, studying gas exchange in muscles, stimulation of nerves and recording the blood pressure within living vessels, whilst also caring for the pain and suffering of his experimental animals. Following up on these first footsteps of organ perfusion, Sydney Ringer (1835 – 1910) discovered in 1883 that specific concentrations of calcium, potassium and sodium ions were necessary for the prolonged function of isolated organs, when apparently by accident, his laboratory technician swapped his normal distilled water for simple London tap water.

In 1937, Alexis Carrel and Charles Lindbergh (1902 – 1974) developed the first device using artificial pumps and heat exchange loops, the so-called Carrell-

Lindbergh pump (***Fig. 1.1***). Using that pump, they were able to support entire isolated organs, in their case, the thyroid glands of cats for up to 21 days by mimicking normal physiology[7]. Also in 1937, in a quite unrelated study of isolated dog kidneys to investigate the production of urine from blood plasma, Reginald G. Bickford (1913 – 1998) and Frank Winton (1894 – 1985) came to the conclusion, that these kidneys survived many times longer when cooled below a critical temperature. Furthermore, they were more resistant to metabolic insults such as cyanide poisoning and reversal when kept at these lower temperatures – this resembled an early 'ischemia reperfusion experiment.[8] At the early stages of transplantation however, clamping times were minimal and the only transit of organs took place in between neighbouring operating theatres, so the issue of long times of organ storage was not present. Nevertheless, in 1960, a soviet surgeon, Dr. Lapchinsky, performed research on that topic and reported of the successful storage of canine kidneys at 2°C-4°C for up to 28 hours. This was followed up by a demonstration by Sir Roy Calne, that it was possible to retrieve kidneys from dogs, preserve them on chipped ice for 7-12 hours and then subsequently re-implant them with a good return of function. His work also covered one of the key facts for cold static storage of kidneys, namely that the renal oxygen consumption as well as the active tubular transport of water and salts within the kidney dropped to less than 5% of the normal rate at 5°C. Also, flushing the organs with cooled heparinised blood was shown to render more effective cooling of the tissue than simple surface cooling[9]. Whilst this method of cold preservation would soon emerge as the way forward in transplantation, the use of blood as a perfusate would not, and instead acellular solutions would predominate the field. This however, was the beginning of the era of cold static storage (CSS) for extracorporeal renal preservation and it was now possible to maintain kidneys outside the body for up to 24 hours with good return of function after reimplantation- long enough for centre-to-centre transportation. As a refinement of the protocol, cold flushing of the freshly explanted kidney with preservation solution became a standard and due to constant development of perfusion solutions, CSS became the gold standard for extracorporeal preservation of organs for transplantation and still is, up until this time.

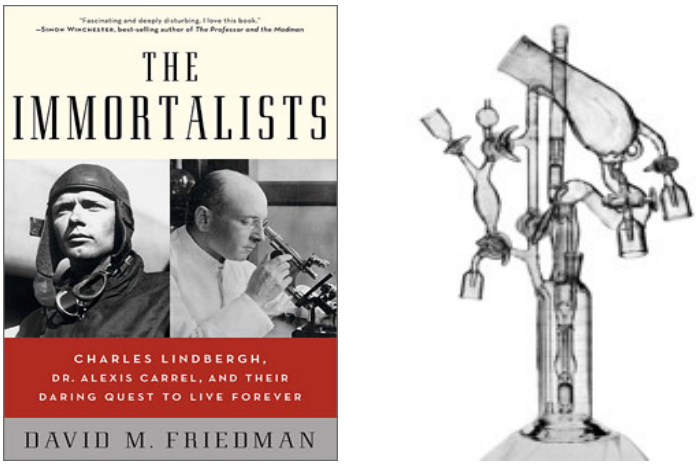


Figure 1.1. Carrel-Lindbergh pump

Fig. 1.1. *Shows two of the pioneers of ex-vivo machine perfusion, Charles Lindbergh and Dr. Alexis Carrel (left) and their first artificial perfusion pump, the Carell-Lindbergh pump on the right (pictures: Courtesy of the Rockefeller Archive Center)*

1.2.2. Pathophysiology during Cold Static Storage

Simple cold static storage (CSS) is still the gold standard in many centres around the world. It starts with a rapid vascular washout which allows a rapid cool down of the entire organ, a washout of all the blood components and the equilibration of the perfusion solution with the tissue[10,11](***Fig.1.2***). There are different pathomechanisms within the cells of an organ, which all together contribute to ischemia-reperfusion injury (IRI). To understand these mechanisms is crucial for the development of better strategies of organ preservation. Looking purely at the gold standard of organ preservation in transplantation and excluding the modern attempts of preserving organs at physiologic temperatures, the following important impacts to the organs have to be mentioned to better understand principles of organ preservation.

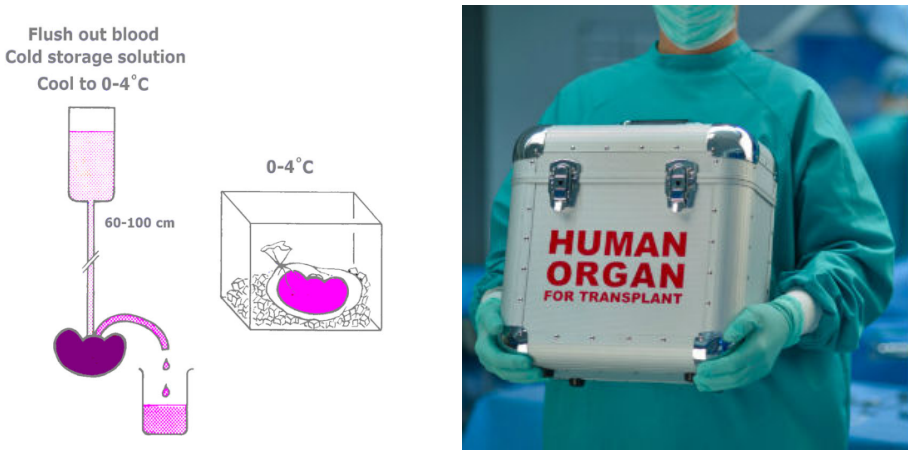


Figure 1.2. Cold Static Storage

Fig. 1.2. *Flushing of an organ after retrieval allows the cooling of an organ to 0-4°C rapidly, as well as a flushout of donor blood (left). Static Storage in an ice box still is the gold standard for organ preservation in solid organ transplantation as it is cheap, easy and efficient (right).*

1.2.2.1. Hypothermia – The temperature effect

As mentioned briefly in Chapter 1.2.1., the principle of CSS is based on the suppression of the metabolism of cells at lower temperatures- the metabolic rate is halved by a 10°C drop in temperature, which results in a remaining 10% metabolism rate at 4°C[12]. To further extend the cold ischemia time (CIT) and to counteract the side effects of hypothermia as well as the anaerobic conditions, special preservation solutions are necessary.

1.2.2.2. Cell swelling

One of the major side effects of hypothermia is the formation of oedema within the cells[13]. It is caused by an impaired activity of the Na^+/K^+ ATPase's. This allows for sodium to diffuse into the cell and due to osmolarity, an influx of water is the effect. To prevent this, impermeants and colloids are important contents of preservation solutions. Effective impermeants are saccharides and nonsaccharide-anions, with the ones of higher molecular weight being more effective than smaller ones[14–16]. In most of the modern preservation solutions, Mannitol, Sucrose or Raffinose (a combination) can be found to serve as such impermeants. As these larger saccharides are predominantly active at the cell membrane level and the interstitial compartment, colloids are added to act in the intravascular space. Colloid osmotic pressure is built

up by these macromolecules, which were originally added to hypothermic machine preservation solutions with the purpose of preventing hydrostatic pressure. Belzer et al. first used cryoprecipitated plasma, then albumin and finally hydroxyethyl starch (HES) as they were aiming to produce a preservation solution that could be used for CSS as well as for hypothermic machine perfusion (HMP)[17]. HES prevents interstitial oedema but increases viscosity and also leads to an increased red blood cell aggregability, so it was extensively debated whether it should be added to University of Wisconsin solution (UW; for detailed description see Chapter 1.2.3.3.) for cold static storage (UW-CSS) as both these factors could contribute to a slower washout of red cells and hence, to an initial patchy reperfusion of the organs[18–20]. Dextran and polyethylene glycol were other colloids used after that debate and for short cold ischemia times (CITs), Histidine-tryptophane-ketoglutarate (HTK; for detailed description see Chapter 1.2.3.4.) and Celsior (see Chapter 1.2.3.5.) solutions were shown to have similar outcomes for kidney and liver preservation without containing any colloids. For CIT above 24h, colloids seem to be necessary to maintain organ viability[17,21,22].

1.2.2.3. Energy and acidosis

At hypothermic temperatures (0-4°C), adenosine triphosphate (ATP) depletion within the cells takes place and within 4 hours, approximately 95% of the ATP disappears and adenosine monophosphate (AMP) remains as the predominant nucleotide. Also, during anaerobic glycolysis, only tiny amounts of ATP can be formed with lactate as a side product causing acidosis[10,22]. The effects of acidosis in ischemic damage are pH dependent: whilst severe acidosis causes lysosomal damage and cell death, mild acidosis (pH 6.9-7.0) has been suggested to have a protective effect by inhibiting glycolysis[23,24]. As a logic consequence, adequate buffering of the pH is an important function of a preservation solution. Celsior and HTK solutions both contain histidine (HTK in a higher concentration than Celsior), whereas UW-CSS contains phosphate as a buffer[25].

1.2.2.4. Reactive oxygen species

During cold static storage, reactive oxygen species (ROS) are produced and it is widely recognized that ROS play a crucial negative role when it comes to IRI. Most

probably, damage to mitochondria and an activation of xanthine oxidase promotes the formation of hydrogen peroxide (H_2O_2) and the superoxide anion (O_2). A downstream reduction of H_2O_2, which is catalysed by iron, leads to hydroxyl radical formation (•OH). Hence, free or chelatable iron is a catalyst to ROS formation and it furthermore contributes to hypothermia induced injury by promoting damage and apoptosis to mitochondria[26–29]. Apoptosis caused by ROS seems to be dependent on the presence of ATP. An absence of ATP, caused by ATP depletion, leads to cell necrosis[30,31]. Free radical-mediated injury is correlated with reduced graft function in renal transplantation[32]. Preservation solutions are therefore designed to counteract ROS mediated injury through added compounds like Allopurinol, Tryptophan and Glutathione (GSH) which inhibit xanthine oxidase[33]. GSH seems to be particularly important for long-term liver preservation. In absence of GSH, more lactate dehydrogenase (LDH) was released into the perfusate in an isolated perfused rabbit liver model[34]. GSH is used in UW-CSS and in Celsior solution whereas in HTK, tryptophan is contained to protect the organ against oxidative damage. Tryptophan is a controversial antioxidant because it can possibly even act as a pro-oxidant, and studies demonstrated superiority of UW-CSS above HTK when it comes to the antioxidant capacity[35–37].

1.2.2.5. Electrolyte composition

In the early years of the development of preservation solutions, a high potassium/low sodium ratio was assumed to be necessary to prevent cell swelling due to the inactivity of the Na^+/K^+ ATPase[38]. UW-CSS for example falls into that group which is also referred to as ‚intracellular' type of solution.

Recent work, however, demonstrated that the sodium/potassium ratio is not as important to prevent oedema as initially assumed and that results are equal or even better with the use of so called ‚extracellular' type of solution, such as Celsior and HTK, with a low potassium/high sodium ratio[39–44]. A low potassium content prevents potassium induced vasospasm during organ procurement and thereby facilitates the washout of blood[41,45].

To summarize, the essential components of effective preservation solutions are impermeants or colloids, buffers and anti-oxidants.

1.2.3. Development of preservation solutions

Research efforts have been made to counterbalance the metabolic need of cells within an organ whilst being cut off from oxygen- and nutrient supply. Whilst kept outside of the body an aim is to prevent the build up of toxic side products of anaerobic metabolism. One common philosophy in the development of storage solutions is, as mentioned above, to mimic the intracellular K^+ concentration and to neutralise the activity of the Na^+/K^+-ATPase, thereby reducing the ATP consumption and thus the O_2 demand of the cells further than by hypothermia alone. For non-oxidative ATP regeneration, ATP precursors can be added. Another aim is to prevent cellular oedema by adding osmotic agents such as raffinose and to scavenge free radicals by adding antioxidants, for example lactobionate, to the solution. The most commonly used preservation solutions throughout history, with their respective advantages and disadvantages will be highlighted within the next few paragraphs:

1.2.3.1. Collins Solution

One of the first preservation solutions was the Collins solution, developed in 1969 by GM Collins et al. at UCLA in the US. The solution was designed to replicate the intracellular electrolyte balance of mammalian cells with a high glucose and potassium - an attempt to prevent the damaging consequences of ischemia, as far as understood back at the time[46]. Due to glucose accumulation in anaerobic glycolysis, the maximal storage time was limited and soon a number of versions of the prototype followed, in which glucose was replaced by mannitol or sucrose. Amongst a number of different solutions named Collins solution, the most widely used version was modified for use in the Euro-Transplant network in Europe in 1976[47] and was termed 'Euro-Collins' solution. It was subsequently used in transplant centres worldwide for nearly two decades and allowed for the time of renal preservation to be extended to up to 24-36 hours for the first time. This was long enough for tissue matching and transport of organs between centres, even back in those days.

1.2.3.2. Marshall's Citrate

Marshall's citrate is a hyperkalaemic crystalloid solution with high levels of Na^+ and Mg^{2+}; offering improved intracellular buffering with citrate, which chelates calcium, thereby preventing intracellular build up. Mannitol is used as the impermeant to limit cellular swelling and hence, with Marshall's citrate, kidneys can be safely preserved for 24-40h. This effect could not be observed for the liver, the application of Marshall's citrate is limited with its main use for multi-abdominal organ retrievals[48].

1.2.3.3. University of Wisconsin Solution

Overlapping the Collins solution, the next ‚generation' of preservation solutions came up: the University of Wisconsin Solution (UW), developed by Belzer and colleagues[49]. Used since the late 1980s, the UW solution is still the most commonly used preservation solution and is acknowledged as the gold standard solution for abdominal organ transplantation today. The major effects of UW on the preserved organs can be summarized as: 1) prevention of oedema (raffinose, lactobionate); 2) delivery of ATP precursors (adenosine); and 3) ROS defence and delivery of antioxidants to the cells (allopurinol and reduced glutathione)[12]. Of course, research is on-going and in recent years, several modified perfusion solutions were developed, all with the aim to improve the outcomes after SCS: Bretschneider's (Custodiol) Solution, Celsior, Kyoto University Solution, and Institute George Lopez-1 solution, just to mention the best known solutions. However, numerous comparative studies so far have shown the superiority of UW for pancreas, heart, kidney and lung preservation and therefore, it is the current gold standard solution[17]

1.2.3.4. Bretschneider's (Custodiol) Solution

Another preservation solution commonly used throughout the history of transplantation is Histidine-tryptophan-ketoglutarate (HTK) otherwise known as Custodiol or Bretschneider's solution. It was first developed in 1970 by H.J. Bretschneider as a solution for cardioplegia during cardiac bypass surgery in 1970s and consisted of three main components: Histidine (as a buffer), Tryptophan (as

membrane stabilizer) and Ketoglutarate (as substrate for anaerobic glycolysis). Mannitol was added as an osmotic agent[50]. HTK has a lower viscosity than UW which makes larger volumes at lower flow rates necessary in order to achieve complete tissue equilibration, but early studies demonstrated both preservation solutions to be equally effective in preservation of kidneys and livers[51,52]. Due to the lower costs of HTK compared to UW, it became extremely popular over the last 20 years but despite it being considered as safe and effective, there have been studies suggesting it to be inferior to UW in DCD liver preservation when the CITs were longer than 8 hours[53,54].

1.2.3.5 Celsior Solution

Celsior solution is a relatively newly developed solution with a high Na^+ concentration. It was developed to be mainly used for cardiac allograft preservation and to combine benefits of various components of UW solution (lactobionate and mannitol) and from HTK (histidine as a buffering agent). Glutathione was added as a ROS scavenger. The solution has been demonstrated to successfully preserve not only cardiac allografts but also the lungs, pancreas and kidneys[55–57].

1.2.3.6. New Preservation Solutions

Due to the increasing awareness that in transplantation, the severity of IRI determines to a significant degree the post-transplant outcome, research on the development of new preservation solutions and methods to alleviate IRI have been stimulated.

One of the newer preservation solutions has been developed in Japan at Kyoto University and is therefore named Kyoto solution. The main idea behind the development of this solution, besides all the well-known and already previously applied principles, was to keep in mind the importance of saccharide- and electrolyte balance during hypothermic preservation. By adding trehalose, which is an alpha linked disaccharide, gluconate and HES, the osmotic strength of the solution is augmented, thereby cellular oedema will be further limited[58].

Another relatively new preservation solution has been developed at the University of Amsterdam and was named Polysol. Polysol is a classic preservation solution containing amino acids, vitamins and antioxidants and it was tested in experiments

for both, Static Cold Storage and Hypothermic Machine Perfusion. Its composition is based on the fact that at temperatures of 4°C, there is still metabolism present within the cells of an organ[59,60]. Many components in Polysol however, have not yet been tested separately but based on its ‚metabolic support' design, it is considered as a promising new preservation solution[17].

Another new and now clinically available preservation solution is IGL-1. Developed by the Institute Georges Lopez (IGL) in France, this preservation solution combines the extracellular composition of Celsior with the colloidal support of UW-CSS with the exception of using polyethylene glycol (PEG) instead of HES as a colloid[61]. In a porcine kidney auto transplant model, this was found to limit macrophage influx by 50%[62]. The underlying mechanism for this is that polymers such as PEG can bind to the surfaces of cells and tissues and thereby block them from interactions with other components. The main advantage of this effect is that it can directly modify the immunogenicity of the donor tissue[63,64]. Furthermore, PEG does not have any aggregatory effects on red blood cells and therefore, washout of blood from the donor organ should be superior to UW-CSS[19,65]. The first clinical results using IGL-1 in renal transplantation showed decreased rates of DGF and less apoptosis when compared to kidneys preserved with UW-CSS[66]. The numbers are, however, still too small to draw any clinically relevant conclusions and further randomized trials will be necessary to confirm these promising results.

1.2.4. Development of modern Machine Perfusion in solid organ transplantation

The first concepts for machines for organ perfusion date back to 1935, when the Carrel-Lindbergh machine was designed, not especially for organ preservation, but as a normothermic, oxygenated pulsatile perfusion system[67] (***Fig. 1.1***). Later on, in the 1970s, only one decade after the first successful organ transplantations, the significant influence of HLA-matching on the transplant survival rates became clear. Organs had to be transported in between transplant centres and especially with the high numbers of organs from deceased donors, preservation of the grafts on the so called Gambro machine with a continuous perfusion with a 4,5% albumin solution at 5°C (hypothermic) was shown to be superior to static cold storage and became the gold standard[68]. Even the idea of assessing and identifying irreversibly damaged organs before transplantation was already present at that time[68] (***Fig. 1.3***).

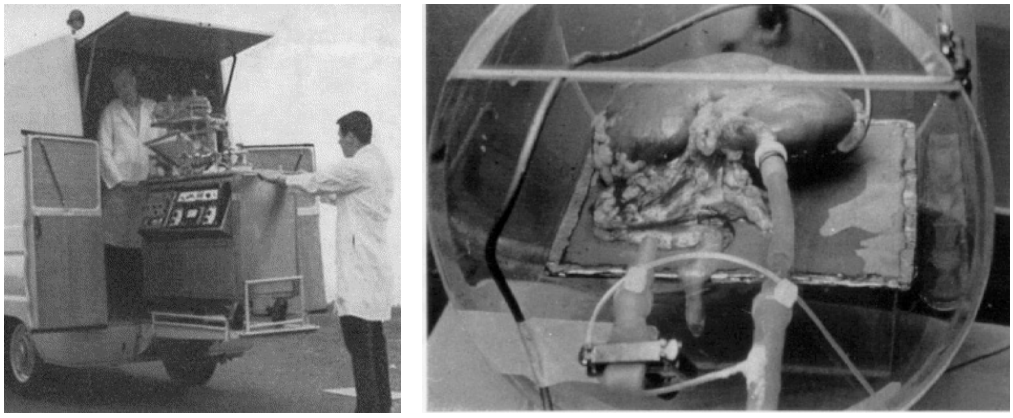


Figure 1.3. The Gambro machine

Fig. 1.3. *shows the Gambro machine, a machine perfusion device in the 1970s on the left (organs had to be transported over long distances) and a kidney attached to it on the right side.*

With the development of modern preservation solutions, in the 1980s, cold static storage (CSS) became superior to machine perfusion regarding the outcomes after transplantation, and hence, that time was the turning point in organ preservation towards CSS as the new gold standard[69]. Until now, CSS has been the most commonly used method of organ preservation[70].

However, at the present time, we are facing a problem in solid organ transplantation, which requires new strategies for organ preservation. We are currently experiencing an omnipresent shift from ‚ideal' donors, e.g. young and healthy brain dead donors, towards an increasing number of expanded criteria donors (ECD). The reasons for this are a) an increase of the older population and b) increase of the population with risk factors and comorbidities. Due to a constant shortage of donor organs, we have to consider these marginal organs for transplantation but as a logical consequence, we also need to rethink and adapt our strategies to preserve these organs. CSS with conventional preservation solutions has reached its limits once again and with modern technology in place, ex-vivo machine perfusion has been reintroduced as a preservation method in transplantation. CSS has been shown to be inferior to modern hypothermic machine perfusion (MP) in kidney transplantation[71] with works regarding its use in other organ transplants ongoing[70].

Machine perfusion is a tool whereby organs are continuously and dynamically perfused with preservation solution instead of being statically stored on ice. Continuous perfusion permits delivery of oxygen and nutrients to the cells as well as the removal of toxic metabolites[70]. Different methods have been established,

hypothermic machine perfusion at temperatures of 4°C and normothermic perfusion at physiological temperatures. The former strategy intends to preserve the organs in a low metabolic state while the latter will keep the organ in an active state. Machine perfusion allows for ex-vivo assessment of organ quality by measurement of biomarker levels in the perfusate (or even in secretions like urine, bile etc.) and, via intravascular administration, allows for organ preconditioning in regards of ischemia-reperfusion injury and immunomodulation to take place prior to transplantation[72]. ***Fig. 1.4*** pictures different fully automated devices for HMP and NMP, which are currently in use in clinical transplantation around the world. For HMP, a range of devices exists. The Waters RM3 pulsatile machine perfusion device was one of the first modern and fully self-contained devices (***Fig. 1.4 a***). Waters Medical Systems is fully owned by the Institute George Lopez (IGL), the provider of the WAVES device (***Fig. 1.4 b***). WAVES, as well as another similar device called the Life Port® (***Fig. 1.4 c***), are transportable, self-contained renal preservation devices which provide controlled pulsatile kidney perfusion using oxygenated hypothermic physiologic solutions. For normothermic machine perfusion, so far only one fully automated machine is available for liver perfusion, called Metra (***Fig. 1.4 d***). Metra was developed by OrganOx® and is in use by centres around the world to preserve and preassess liver grafts for transplantation. For normothermic kidney perfusion, circuits are used which are based on an extracorporeal membrane oxygenation (ECMO) principle, using centrifugal or pulsatile pumps, heating devices and oxygenators to perfuse the kidneys but no self contained and automated device is available so far.

Figure 1.4. Current ex-vivo machine perfusion devices

Fig. 1.4 *shows currently available automated machine perfusion devices. For HMP of the kidney, the Waters RM3 (**a**), Waves (**b**) and the Life Port (**c**) are the most commonly used. For NMP, so far only the Metra (**d**), a liver perfusion machine (OrganOx) is on the market.*

1.2.5. Current Issues around organ preservation in renal transplantation

In the last 50 years, solid organ transplantation was one of the major key advances in medicine, saving thousands of patient's lives every year around the world as the only curative treatment to end-stage failure of organs like the kidneys, the liver or the pancreas. The limiting factor to making this ‚cure' available to every patient is however the supply of suitable organs. A lot of logistic, ethical and societal factors influence the daily success of this treatment and without cadaveric and living donors; solid organ transplantation would not be possible. The quality and efficacy of treatments is therefore dependent on an overall multidisciplinary approach, which is in use by many countries with successful organ transplant programmes in place.

The successful transplantation of an organ starts with the allocation of a suitable organ by organisations like UK-Transplant or Eurotransplant, and ends with physicians and surgeons working closely and effectively together to keep the cold ischemic time of an organ as short as possible. In between there is the donor, who in some systems has to actively come forward, and in some countries passively agrees

to be an organ donor by not signing a declaration against the use of their organs for transplantation. In any case, somebody's death means a chance for a curative treatment for someone on the waiting list. As many people are not aware of their countries transplant programmes and the underlying regulations, a first step towards an expansion of the organ pool is to create more awareness and to keep the public informed about organ transplantation and all the available options, including living donation. For example in the UK, as one of the only countries in Europe, it is possible to altruistically donate a kidney to a stranger or to a friend. Many patients suffering from End Stage Renal Failure as well as their family and friends are not aware of that option just because information is not widely enough spread yet.
With many options, like television, the Internet and social media, one goal should be to create as much awareness about the options of solid organ transplantation as possible.

Once the cascade of transplantation in a particular case is running, an important factor to ensure the best possible outcome post transplantation lies in the period between retrieval and implantation of an organ, namely in organ preservation. As mentioned above, since the 1980s and still up to this time, Static Cold Storage of organs is the gold standard, with many clinical studies in place to investigate the superiority of different methods of ex-vivo machine perfusion (EVMP) and the ideal combination of the different methods with the most suitable preservation solution. The ultimate common aim is to preserve the organ ex-vivo and to protect it from mounting ischaemic- and later reperfusion injury, which is initiated upon revascularisation. The premise of IR injury is that in tissues exposed to ischemic stress, a proportion of cells will suffer from irreparable damage, which ultimately leads to cell death. Another proportion of the cells within that same organ will sustain damage but still be viable at the time of reperfusion, with a chance of undergoing self-repair and to return to normal function.
As the cells suffering from irreparable damage will definitely undergo cell death, the severity and degree of IRI will be dependent on the number of cells that are salvageable after reperfusion[73]. The cellular damage occurring is based directly on the lack and subsequent return of oxygen supply, which leads to the development of intracellular oedema, acidosis, calcium overload and the formation of reactive free radicals, as discussed above[74]. As a logical consequence, with all the above

mentioned developments of different perfusion solutions and preservation strategies, the ultimate and common goal is to minimize the number of cells that fall into the irreparably damaged group during IRI and to optimise the circumstances for the salvageable cells to survive and take up their normal function post IRI. During the last 30 years, developments in preservation solutions, as explained in detail above, made it possible to preserve organs in an efficient and cost effective way, so that up until now, Static Cold Storage is the gold standard for organ preservation in transplantation. However, earlier on in the days of solid organ transplantation, donors were far more ‚ideal' than now. They were brain dead, young and healthy and therefore, organs were more resilient. Due to an ageing population and a growing demand for organs, in an attempt to improve the balance between request and offer, organs from extended criteria donors are being introduce into transplantation and therefore, the strategies for ideal preservation of these organs also need to change. Learning from the history of transplantation but using modern perfusion solutions as well as modern technology led to the idea of reintroducing ex-vivo machine perfusion as a preservation method in transplantation.
Hence, there are currently three predominant methods of organ preservation present: Static Cold Storage, Hypothermic Machine Perfusion (HMP) and Normothermic Machine Perfusion (NMP).

1.2.6. Static Cold Storage

Whilst SCS dramatically improved the condition and the availability of organs for transplantation and therefore has been the most widely used method up to this day, there are drawbacks to it, which have to be considered. Kidneys, maintained on ice, statically surrounded by preservation solution can only remain viable for a relatively short period of time (around 24 hours), with a dramatic fall in function beyond that time. Compared to an average lifetime of around 80 years over which a human body is able to maintain good renal function, this is a very short time.
Cooling down an organ to low temperatures not only slows down the cellular metabolism, it can also cause changes to cellular biochemistry that lead to damage of the cells. For example, whilst the oxygen consumption of an organ can be dramatically reduced to around 5% of the usual at a temperature of 5°C, it still can't be reduced to 0%, which means that a remaining oxygen requirement remains

unsatisfied. The only way for the cells to survive that situation is through diffusion of oxygen from the preservation solution and from the atmosphere through the parenchyma of the kidneys[75]. Taking the estimation that the physiological oxygen demand of a healthy kidney equals approximately 10mL O_2/minute (measured via intravascular catheterisation in vivo), this means that at 5% of the metabolic rate, a kidney on SCS remains with an unsupplied demand for 0.5mL O_2/minute[76,77]. CSS starts with vascular washout at hydrostatic pressure to initiate cooling, washout of blood components, and equilibration of the preservative solution with the surrounding tissue. However, recent evidence indicates that the cooling during retrieval is not quick enough to avoid ischemic injury at that stage already[78]**.** Once cooled down to hypothermic temperatures, cold ischemia damage has its onset. At the low temperatures during CSS, membrane bound Na^+ pumps are suppressed whereas Na^+/K^+-ATPase's continue to function, which leads to consumption of ATP and an ionic as well as osmotic imbalance across the cell membrane, resulting in cellular oedema[79]. Also, at low temperatures, the cell membrane experiences structural alterations which affects the functioning of several enzymes within the cell and cell structures that are interacting with the cell membrane[75].

At the time of reperfusion with the recipient's blood, further damage to the cells is initiated, a phenomenon which is called reperfusion injury[78]. The already fragile cells, experience further cellular oedema and tissue damage through a rapid influx of sodium and calcium when the cytoskeletal structure and mitochondrial integrity is weak and ATP dependent ion pumps are malfunctioning. This leads to more accumulation of toxic metabolites and to further formation of ROS and free radicals once the reperfusion phase is fully established within the organ. An influx and activation of the recipient's leucocytes follows and alterations in endothelial cell structure lead to formation of a pro-inflammatory phenotype of endothelial cells. Subsequently, these interstitial endothelial cells stimulate cytokine release, which further potentiates inflammatory processes and activates innate immune responses. The coagulation cascade is triggered, leading to micro vascular injury, perfusion failure and prolongation of IR damage. Clinically, these factors together can lead to delayed function of the transplanted organ[78].

1.3. Ex vivo Machine Perfusion in Solid Organ Transplantation

Although static cold storage of organs for transplantation with a preservation solution has been the gold standard in donor organ preservation over the past decades, in an attempt to further limit reperfusion injury and also to address the problem of organ shortage, efforts are being made to further improve the quality of organs for transplantation. Instead of excluding and discarding marginal organs for transplantation straight away, trends lead towards graft assessment and preconditioning on perfusion machines with different approaches concerning the temperature and the composition of perfusates. ***Fig. 1.5*** [80] summarizes the potential advantages of ex-vivo machine perfusion of organs for transplantation. Highlighted in red are the two factors which are most relevant for the work within this thesis: Pharmacologic manipulation of grafts during ex-vivo perfusion, by infusing drugs or cells, like Mesenchymal Stem Cells into the organ. This could also be used to manipulate the genome of cells by delivering vectors (e.g. cells) for gene therapy into an organ[80]. These advantages could be more or less present in Hypothermic or Normothermic Machine perfusion, which has to be determined in further investigations, some of which are conducted within this thesis.

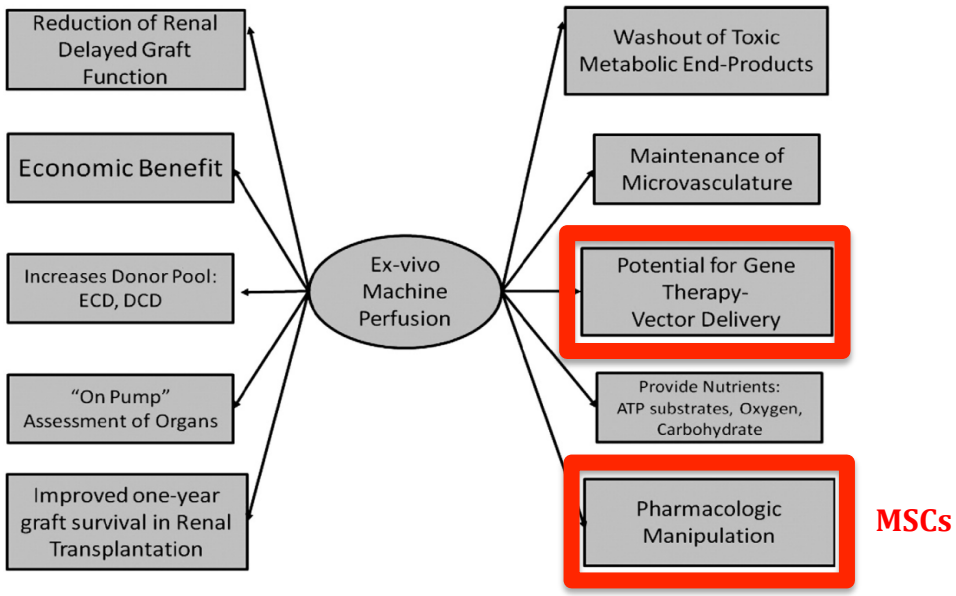


Figure 1.5. Advantages of ex-vivo organ perfusion

Fig. 1.5. *illustrates the many potential advantages of ex-vivo machine perfusion. Highlighted in red are the most relevant points for potential treatment of grafts with Mesenchymal Stem Cells (MSCs) during ex-vivo machine perfusion of solid organs.*
ECD= extended criteria donor; DCD= donation after cardiac death

1.3.1. Ex vivo Hypothermic Machine Perfusion (EVHMP)

Up to date, the exact mechanisms behind the beneficial effects of HMP have not been revealed but experts agree that perfusion of an organ most likely helps to maintain a healthy endothelium and to replenish ATP. Furthermore, it might even alter the organ's immunogenicity[81–83]. In HMP-preserved porcine DCD kidneys including 1h of warm ischemia time and either 24h of MP or CSS, MP resulted in an increased nitric oxide-dependent vasodilation and thus, in an improved cortical microcirculation at reperfusion. This effect was caused by an improved endothelial nitric oxide synthase phosphorylation in kidneys on MP, suggesting that MP protects the NO signalling pathway and confirming the value of MP for marginal kidney preservation[84]. Also, a degree of vascular shear stress is maintained when an organ is exposed to MP. This could activate flow-dependent anti-inflammatory genes and thereby, have a protective effect on the organ's vasculature[85,86].

Another idea to further modify HMP conditions towards supporting metabolism is to add oxygen during HMP to restore the ATP content in the kidney[87,88]. In preclinical studies on oxygenated HMP, particularly in DCD kidneys, an improvement of graft function has been demonstrated[87,89] and this effect is currently being investigated in two randomized clinical trials, one in DCD (ISRCTN32967929), the other in ECD kidneys (ISRCTN63852508), which will finish in 2018[90].

1.3.1.1. Animal studies on EVHMP

In a DCD kidney transplant model in the rabbit, Zhang et al. found that hypothermic machine perfusion decreased the renal cell apoptosis linked to IRI via the ezcrin/AKT pathway[91].

In an ex vivo model of pig heart preservation, it was possible to reliably assess the myocardial function of hearts subjected to 24 hours of ex vivo hypothermic perfusion. These organs showed little statistical difference in haemodynamic measurements from organs which were freshly excised, suggesting good preservation [92]. Even back at that time and reported by the same group, a portable hypothermic perfusion system allowed the storage of porcine hearts for up to 24 hours showing not much difference on functional testing to freshly excised hearts. Hearts which were only on static cold storage on the other hand, performed less well [93].

In a porcine heart transplant model, pulsatile hypothermic machine perfusion was demonstrated to better preserve cellular structures in hearts with prolonged cold ischemic times, when compared to hearts on static cold storage, suggesting that HMP could enable longer preservation times for hearts and thereby lead to an increase of transplantable organs[94]. In another study using a porcine DCD liver donor model, hypothermic oxygenated machine perfusion was shown to lead to a significant improvement in the prevention of hepatocellular damage when compared to static cold storage[95].

Most recently, our group was able to demonstrate in a translational HMP model using porcine kidneys and human kidneys discarded for clinical transplantation, that graft pre-treatment with cytotopic anticoagulants is feasible and ameliorates perfusion deficits locally, thereby improving the quality of the grafts[96]. Furthermore, Hamaoui et al. were able to demonstrate, that a modified adenosine and lidocaine solution (AL), which had been shown to be beneficial in cardiac perfusion, showed superiority in the quality of perfusion of hypothermically perfused porcine organs, compared to perfusion with UW. AL perfused kidneys had lower lactate-levels at warm reperfusion compared to UW perfused grafts, leading to the conclusion that by further modification of perfusion solutions, an improved graft viability assessment and hence, an expansion of the donor pools could be possible[97]. Our group is also making efforts, to translate the application of HMP into the pancreas, which is challenging because of its susceptibility to oedema. In a porcine model of ex-vivo HMP of the pancreas, we could however demonstrate that it is possible and feasible and that also for pancreas transplantation, this could be an important step towards organ preconditioning and pre-assessment[98].

1.3.1.2. Clinical studies on EVHMP

In 1978, Stoney et al. reported 24 patients in which a repair of extended lesions of the renal artery had to be done. Doing this ex-vivo whilst the kidneys were continuously and hypothermically perfused, prevented postoperative acute tubular necrosis, and 95% of these patients showed satisfying outcomes [99]. Two years later, the case of a 14-year-old boy was reported, who was suffering from severe bilateral nephrolithiasis and in whom a successful nephrolithotomy could be performed whilst the kidney was hypothermically perfused in situ. Thereby, the procedure could be performed safely, without much blood loss and without a significant reduction of

renal function [100]. In 1981, six cases have been reported, in which ex vivo reconstructive surgery to the renal arteries of the patients was successful whilst preserving the kidneys by hypothermically perfusing them [101].

In the more recent past, data from randomised controlled trials and several meta-analyses, looking at HMP compared to SCS have demonstrated good evidence for a reduction of delayed graft function (DGF) using HMP for kidneys from brain dead donors[102–106]. For kidneys donated after circulatory death, findings are not as straight forward. Although meta-analyses seem to confirm the above mentioned results also for DCD kidneys[104], the two largest randomized controlled trials focussing on this subgroup of organs, contradict each other. One shows a reduction of DGF from 69.5% to 53.7% (p=0.025) and a higher creatinine clearance up to 1 month after transplantation (P=0.027) in the HMP group[107], whereas in the other study, there was no difference in the occurrence of DGF between kidneys assigned to HMP or CSS and the renal function at 3 and 12 months was similar between the gorups[108]. On-going clinical trials might provide additional evidence as more data are needed looking at the effect of HMP on long-term graft function and survival. So far, improved 1 and 3-year graft survival rates after HMP have been shown for kidneys coming from expanded criteria donors (ECD)[109,110], but this effect is not present in DCD kidneys[102,107,108]. Furthermore, a clear benefit of HMP on other, less frequently occurring outcomes, such as primary nonfunction, has not been shown[111].

1.3.2. Ex vivo Normothermic Machine Perfusion (EVNMP)

1.3.2.1. Animal studies on EVNMP

As mentioned above, the idea of ex vivo normothermically perfusing organs was already present in the late 20th century and later again in the early 1980s, when lots of experiments on ex vivo hypothermic machine perfusion for preservation were going on. Rijkmans et al. could demonstrate, that canine kidneys which were ex vivo hypothermically preserved on a Gambro machine for up to 6 days, showed much better functional outcomes when intermittently ex vivo normothermically perfused by cannulation and connection of the renal arteries to the femoral arteries of the same donor dogs [112,113].

During the last two years, the Toronto group of Selzner *et* al. have set up a porcine ex-vivo normothermic kidney perfusion (NEVKP) model using Yorkshire pigs and they have been the pioneers in that field[114]. The aim of their first study was to minimise the duration of the cold ischemia time and to try out a period of 10 hours of NEVKP, thereby providing the sufficient oxygen and nutrition for the perfused kidney. After retrieval of the kidneys in a heart-beating model, the organs were stored on ice for 3 hours and then perfused normothermically with a combined solution containing washed erythrocytes, Steen solution and Ringer's Lactate. The albumin rich Steen solution served the purpose of regulating the oncotic pressure. The circuit was based on neonatal cardiopulmonary bypass technology with a centrifugal pump and an oxygenator to deliver a mix of 95% O_2 and 5% CO_2 to the perfusate. During NEVKP, the perfusion pressure was kept in between 60 and 80 mmHg and in this setup, the group reported that the quantity of urine production was dependent on the composition of the perfusion solution: the higher the oncotic pressure and the osmolarity of the perfusate was, the lower was the urinary output[115]. As a next step, they then directly compared kidneys after SCS and after NEVKP in a heterotopic heart-beating auto transplantation model. The pigs were followed-up for 10 days and interestingly, post transplant serum Creatinine and urea nitrogen levels were similar to the baseline (before transplantation) values in the NEVKP group, whilst being significantly inferior in the SCS group[116].

The same group reached another important milestone in research on NMP, when they compared different durations of NEVKP and SCS in DCD auto transplant models with a WIT of 30 minutes, again in the pig. They demonstrated, that the kidneys which underwent NEVKP for the whole preservation period of 16 hours had the lowest serum creatinine peak, the best 24-hour serum creatinine at day 3 and the best histological outcome in regards to apoptosis at the end of the follow-up period of 8 days[117]. These results were then underlined by a follow-up study where they looked at different durations of NEVKP, namely 1h, 8h and 16h after a clinically relevant period of 8h on SCS. Again, kidneys on either 8h or 16h of NEVKP showed the best results in regards to serum creatinine levels and histological evaluation of tubular injury[118].

Another group, based in Cleveland, have compared three different normothermic preservation methods in a porcine DCD kidney model. Kidneys were preserved for 24 hours either normothermically with whole blood alone or Steen solution plus whole

blood, or subnormothermically at 21°C using Steen solution alone. In this experiment, kidneys undergoing subnormothermic perfusion with Steen solution alone showed the best results in terms of vascular resistance and histological results[119]. They then went on to a study where they perfused porcine DCD kidneys with an average WIT of 45 min and after a CIT of 5 hours either hypothermically or normothermically for 8 hours followed by a 2 hours reperfusion phase with Ringer's Lactate and autologous whole blood. The solution used for NMP was a modification of the perfusion solution used by Kaths *et* al. and in order to reduce the risk of extracellular oedema due to the low osmotic pressure, they reduced the mean arterial perfusion pressure to 40 mmHg. Oxygenation was provided by delivering 95% O_2 and 5% CO_2 to the perfusate. During the reperfusion phase they did not observe any significant differences in oxygen consumption, urine production, creatinine clearance and proteinuria. Histologically, outcomes in the HMP group were slightly better with vacuolization and inferior tubular status in the NMP group but in summary, the group concluded that there was no remarkable difference between the two groups in terms of preservation[120].

1.3.2.2. Clinical studies on EVNMP

NMP is designed to resuscitate the kidney after SCS and is usually carried out for 1h whilst the patient is being prepared for transplantation. After NMP, the kidney is flushed with cold preservation solution and placed back on ice until transplantation[121]. Nicholson *et* al. were the first to transplant a kidney after a 60 minute period of EVNMP in 2011[122]. They then reported another case, where a pair of kidneys which had been declined for transplantation by every centre in the UK due to patchy perfusion, underwent 60 minutes of NEVKP and could then be transplanted without complications and with both recipients showing immediate graft function[123]. Since then, a score was developed to assess the quality of kidneys during EVNMP and to help deciding on whether or not a kidney should be transplanted or not. The score consists of three main parameters: the macroscopic appearance of the kidney (1 point for excellent perfusion, 3 points for poor perfusion), the renal blood flow in ml/min/100g (at least 50= 0 points, <50=1 point) and the total urinary output during the hour of perfusion in mls (>43 = 0 points, <43 = 1 point) . This results in an end score of 1-5 and kidneys with a score of 1-3 being kidneys that can be transplanted without concerns[124].

The first and largest clinical study on NMP to date included a series of 18 ECD kidneys. The outcome of these kidneys was compared to a control group of 47 ECD kidneys that underwent cold static storage (CSS). The delayed graft function rate (DGF) was 5.6% in the EVNP group vs. 36.2% in the CSS group (p=0.014) but there was no difference in graft- or patient survival at 12 months[125]. There are no survival data available on NMP.

As a result of their first encouraging reports, Nicholson and Hosgood are now in the process of recruiting a total of 400 patients for a randomised controlled trial where kidneys from controlled DCD donors in the category III/IV will either undergo 1h of NMP or remain on SCS before transplantation. The primary endpoint will be the rate of DGF and the first results will be expected by 2020[126].

As NEVKP is still a young discipline, the optimal perfusion settings in terms of temperature, perfusion pressure and duration of perfusion are not defined yet and further studies are needed to determine these settings. So far, a temperature of 37°C seems to be superior to a subnormothermic temperature of 32°C in regards to renal function and furthermore, gradual and slower rewarming of the grafts seems to be beneficial [127,128]. Also for the perfusion pressure, there is no defined standard yet and in both HMP and NMP, definition of an ideal perfusion pressure, as well as the choice between pulsatile or non-pulsatile perfusion, is a ‚hot potato' in this area of research. In NMP, a mean arterial pressure of 75mmHg seems to be superior than a lower mean arterial pressure of 55mmHg when it comes to preservation of the tubular structure, however, not many studies have been conducted to investigate that[129]. Also, the ideal duration of NEVKP will have to be determined in further studies.

1.3.3. Comparison between EVHMP and EVNMP in kidney transplantation

Many groups around the world are investigating either EVHMP or EVNMP in renal transplantation, compared to the current gold standard, CSS. Outcomes show advantages for both methods, as described above. However, data on a direct comparison between the two methods is scarce, with only two papers attempting to directly compare the two methods published so far. In the most recent, by Blum et al. in 2017, 10 porcine kidneys after a WIT of 45 minutes and a CIT of 5 hours either underwent NMP (n=5) or HMP (n=5) for 8 hours before undergoing a simulated reperfusion phase. Upon reperfusion, no differences between groups could be

detected in terms of oxygen consumption, rates of urine production, creatinine clearance or fractional sodium excretion. Furthermore, the resistance indices were similar after 30 minutes of simulated reperfusion. However, histologically, in NMP kidneys, increased vacuolization after preservation and greater loss of tubular integrity after reperfusion was demonstrated, compared to the HMP group. Interestingly, perfusate levels of alkaline phosphatase (AP) and gamma glutamyltransferase (GGT) were higher in NMP kidneys during preservation, however, during simulated reperfusion, peak AP and GGT values were over 14 times higher in HMP kidneys than peak AP and GGT during preservation of NMP kidneys. Their conclusions were, that through NMP, preservation of renal function was provided and comparable to HMP, and that furthermore, AP and GGT release upon reperfusion could be minimized when compared to HMP[130]. The limitations to this study were however, that kidneys were not investigated as pairs from the same donor pig. Kidneys were retrieved, randomized into the groups and the respective other kidney was discarded.

Another comparative study using porcine kidneys was performed by Metcalfe et al. in 2002, which showed an advantage for NMP, but again, pairs of kidneys were perfused on different machines and for different periods of time[131].

Therefore, a great deal of research is still needed to figure out which method for ex-vivo preconditioning is the best for which kind of organ. Furthermore, hypothermic and normothermic perfusion technologies will need to be optimized in terms of trained staff and logistics to be able to offer the technologies in all transplant centres.

1.4. Renal transplantation today

1.4.1. End Stage Renal Failure

Chronic kidney disease (CKD) is defined as the combination of reduced glomerular filtration rate and increased urinary albumin excretion and is a major health problem, which in its progression, leads to end stage renal failure (ESRF)[132]. CKD is an increasing public health issue with an estimated prevalence of 8-16% worldwide[132]. Complications include a number of serious health problems i.e. cardiovascular disease, hyperlipidaemia, anaemia and metabolic bone disease. Furthermore, as a

consequence of the growing elderly population, an increase in the prevalence of the most common underlying diseases for the development of CKD, Diabetes and Hypertension, can be observed [133]. ESRF is irreversible and by the time somebody has lost 85-90% of their kidney function, they are no longer able to balance the water and salts within their metabolism required for normal homeostasis. Essential hormones, usually produced by the kidneys, need to be delivered in the form of drugs, and the capacity of the kidney as filter needs to be replaced by dialysis. Dialysis helps to remove waste products (e.g. urea, creatinine), salt and extra water to prevent them from building up in the body and it restores safe levels of electrolytes such as potassium, sodium and bicarbonate, thereby helping to control the blood pressure.

Currently, we distinguish between two types of dialysis:

- **Peritoneal dialysis:** Approximately one third of patients with ESRF undergo peritoneal dialysis. This method is the milder form of dialysis and is often applied in younger patients or children. In principle, a specially prepared fluid is instilled into the patient's abdominal cavity via a peritoneal catheter and exchanged a number of times every day, or is perfused continuously into the abdomen by a bedside device over night. The bodies' own internal membranes are used to provide the large surface area and selectivity required for dialysis. Most patients are medically suitable for peritoneal dialysis. The exclusion factors from this modality of RRT are mainly the patient's age, anatomical concerns (adhesions, hernias) and obesity[134]. Another factor is the patient's confidence to handle the dialysis machine and the ability to connect themselves to the machine. It requires certain personality traits of the patient for the successful performance of PD dialysis at home: ideally, the patient is optimistic and resourceful, with good problem solving skills, and driven by the concepts of freedom which this modality of RRT provides in contrast to hospital based haemodialysis[135]. The patients are monitored by regional dialysis centres or by nurses in order to identify problems before they arise.

- **Haemodialysis:** The remaining two-thirds of ESRF patients must undergo haemodialysis 2-3 times a week, depending on the severity of the renal failure, either at a specialist centre, or at home. On average, every session of

haemodialysis takes approximately 3-4 hours at a time, so it is imaginable, that this is not only a time-consuming, but also a costly process. Therefore, whenever patients are fit enough, willing to learn how to needle themselves or connect themselves to the dialysis machine, and have a care partner to help, the patients can be offered home haemodialysis. To set it up, the patient needs to get a home haemodialysis machine and dialysis nurses need to visit the patient's home to train them and their caretaker to perform the haemodialysis. Home haemodialysis can then either be undertaken in the normal setting of 3-4 hours 3 times a week or over shorter periods 5 times a week or even over night. The patient is still monitored by the dialysis centre but is much more independent in that way. Another benefit of home haemodialysis is, that it is more cost effective.

For all modalities of renal replacement therapy, patients sometimes need to undergo a lot of interventions and/or surgeries in order to create a permanent access for dialysis. In case of complications from peritoneal dialysis, e.g. peritonitis, insufficient dialysis, conversion to haemodialysis may become necessary. Usually, when a patient is approaching the need for RRT, a plan for access is made in advance and either a PD catheter is inserted or an arterio-venous fistula is created in order to avoid the need for a temporary or permanent central venous dialysis line.

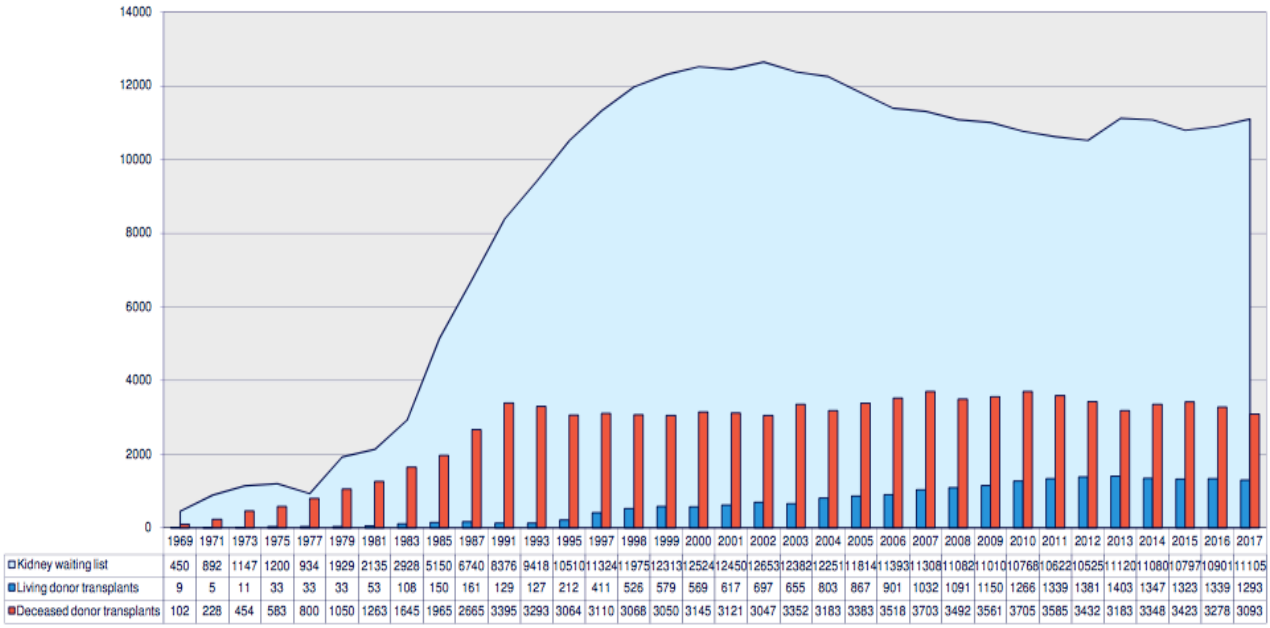
In recent times, efforts have been made by the NHS to encourage the promotion and support of home dialysis, mainly due to the improvements for the patient's quality of life, but also because of the cost savings. For many patients on haemodialysis, the main goal is to break free and to be able to do something as simple as taking an overseas holiday without weeks of planning their dialysis sessions in advance. Also, alongside the continuous appointments at the dialysis unit, the numerous drugs, vascular access procedures, injections and the strictly controlled diet and fluid intake, comes the awareness of all the potential complications of being on dialysis.
The survival rates for patients on renal replacement therapy (RRT) lie around 79% after one year and are also age dependent, with 5 year survival rates around 58% for the age group between 18-34 and around 12% for patients over 75 years[136]. This mortality rate mainly results from cardiac or infection related complications on dialysis and is worse than for some types of cancer.

Comparatively the most recent 1 and 5 year patient survival rates for deceased kidney transplant recipients in the UK are around 96% and 85%, respectively[137].

Besides the burden of RRT for the individual, the financial burden for the society has to be mentioned. The cost of chronic kidney disease (CKD) to the English NHS in 2009-10 was estimated at £1.45 billion, which was equal to 1.3% of all NHS spendings in that year. More than half this sum was spent on RRT, which was provided for 2% of the CKD population [138].

In the UK, alongside 33,500 patients on RRT live some 35,800 patients with functioning kidney transplants (as of March 2017). However, there were also 5081 patients active on the waiting list for a kidney transplant in between April 1st 2016 and March 31st 2017 and 261 of these patients died. The median waiting time for somebody on the list for a kidney transplant is 864 days[137]. Compared to the 5-year survival rate of people on RRT, the number of deaths on the waiting list is not surprising. Knowing these facts, the need for kidney transplantation as the only curative therapy for ESRF seems logic and despite from the costs and the risks of the operation itself, it saves many lives, improves the quality of those lives immensely and is far more cost effective than remaining on dialysis. However, the full potential of the mentioned benefits of transplantation cannot be obtained. Reason for this is at the same time the biggest problem in solid organ transplantation (SOT) in Europe as well as in the UK: the critical shortage of donor organs[137]. Figures 1 and 2 demonstrate the gap between the demand and the availability of organs in the Eurotransplant area (***Fig. 1.6***) as well as similarly for UK-transplant area (***Fig. 1.7***).

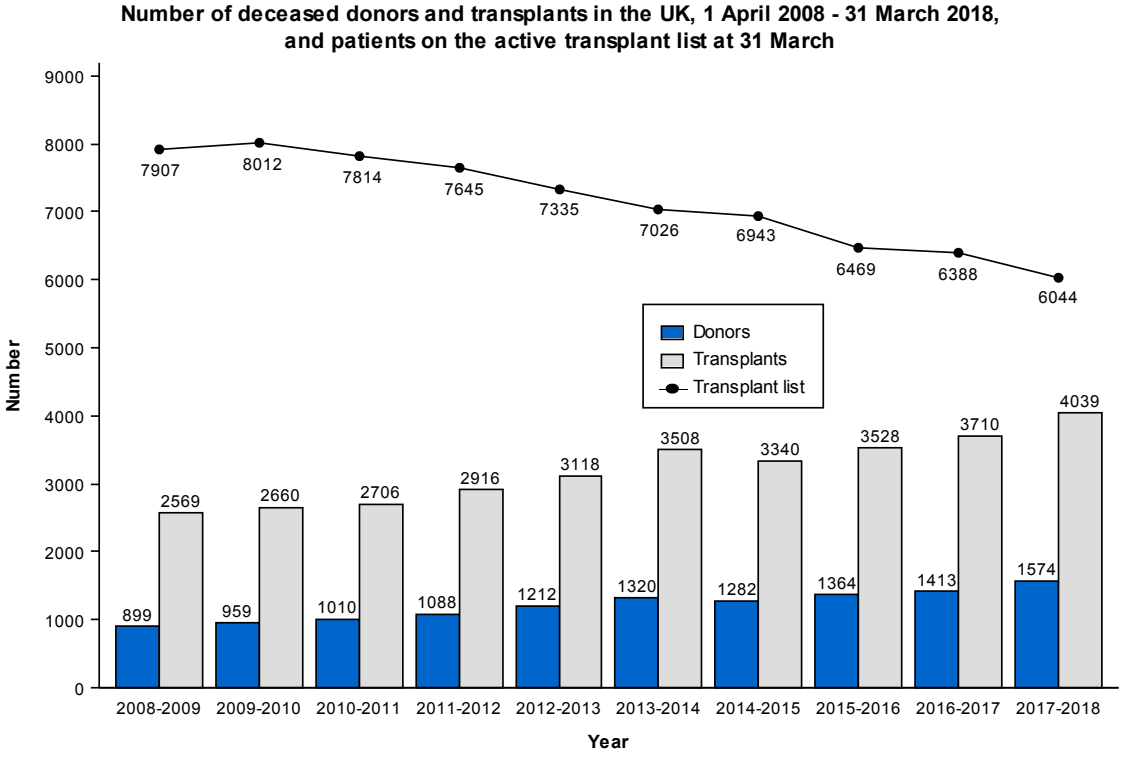
The common ultimate goal is to approximate or even close that gap and to make kidney transplantation as the only curative therapy for end stage renal failure available to every patient.



	1969	1971	1973	1975	1977	1979	1981	1983	1985	1987	1991	1993	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Kidney waiting list	450	892	1147	1200	934	1929	2135	2928	5150	6740	8376	9418	10510	11324	11975	12313	12524	12450	12653	12382	12251	11814	11393	11308	11082	11010	10768	10622	10525	11120	11080	10797	10901	11105
Living donor transplants	9	5	11	33	33	33	53	108	150	161	129	127	212	411	526	579	569	617	697	655	803	867	901	1032	1091	1150	1266	1339	1381	1403	1347	1323	1339	1293
Deceased donor transplants	102	228	454	583	800	1050	1263	1645	1965	2665	3395	3293	3064	3110	3068	3050	3145	3121	3047	3352	3183	3383	3518	3703	3492	3561	3705	3585	3432	3183	3348	3423	3278	3093

Figure 1.6. Dynamics of the kidney transplant waiting list Eurotransplant

Fig. 1.6. *illustrates the dynamics of the kidney transplant waiting list and performed transplantations in the Eurotransplant region, between 1969 and 2017*[352]***.*** *The number of living donor transplants is slowly increasing, whereas the number of deceased donor transplants remained quite stable over the last 20 years. The light blue area highlights the gap between the number of people on the transplant waiting list versus the number of performed transplants.*



Source: Transplant activity in the UK, 2017-2018, NHS Blood and Transplant

Figure 1.7. Kidney transplant activity United Kingdom

Fig. 1.7. *shows the dynamics of the transplant waiting list and performed transplantations in the UK between 2008 and 2018. There is also a gap between the number of people on the waiting list for organs and the acutal available number of organs but it seems like in the UK, there is a steady approximation visible between the two figures.*

1.4.2. Living- and Deceased donor kidney transplantation

In order to overcome the shortage of organs in kidney transplantation, introducing the living organ donation scheme has expanded the organ pool. Not only is it possible in the UK as one of the only countries in Europe, to altruistically donate a kidney to strangers, but also friends, cousins, siblings and spouses can donate a kidney to their loved ones. Due to a precise preoperative evaluation, these donated kidneys come from healthy individuals. This, in combination with a short cold ischemia time (CIT) in a planned surgical setting, leads to superior outcomes over transplantation of kidneys from deceased donors. The number of living donor kidney transplants has increased from around 300 per year in 1999 to around 1243 in 2014- a threefold increase which hopefully continues to rise due to better awareness and information of

the public, with newer media (internet, social media) of information in place.

The alternative to living donation kidney transplantation, which was also the conventional way before living donation, was possible, is the use of allografts from deceased donors. In deceased donation two types can be distinguished: donation after brain death (DBD) and donation after circulatory death (DCD). DBD means that the donor is suffering from primary brain death due to trauma or other causes and their circulation and respiration are maintained by medical intervention. The retrieval of the organs can be carefully planned with all the included retrieval teams and cold perfusion of the organs immediately succeeds the clamping of the aorta, which results in absence of any warm ischemia time (WIT) to the respective organs. Conversely, DCD refers to donors in which the criteria of brain death is not met, but in which survival is unexpected. Hence, cardiac death has to occur before organ procurement. This can either occur under unexpected and uncontrolled circumstances (uncontrolled DCD, uDCD) or under deliberate and controlled circumstances (controlled DCD, cDCD), whereby supportive intervention is systematically withdrawn after consent from the family[139]. The problem with this form of donation is the warm ischemia time, which is the interval between the cardiac arrest of the donor and the start of cold perfusion of the organs. The duration of this time lies in between 10 and 45 minutes. During that time, the organ is not perfused and hence, not provided with oxygen whilst being exposed to physiologic body temperature. Despite this, outcomes for DBD and DCD kidney transplants are similar in regards to allograft- and recipient survival, but DCD kidneys are at higher risk of delayed graft function (DGF) with 20-80% vs. 20-30% for DBD kidneys[140]. Delayed graft function is defined as the necessity of at least one further dialysis during the first postoperative week and thus, is also associated with an increased risk of acute rejection and poorer long term function[141,142]. In addition to these differences, DBD or DCD donors can furthermore be classified as standard criteria donors (SCD), or as extended criteria donors (ECD).

1.4.3. Extended criteria donors

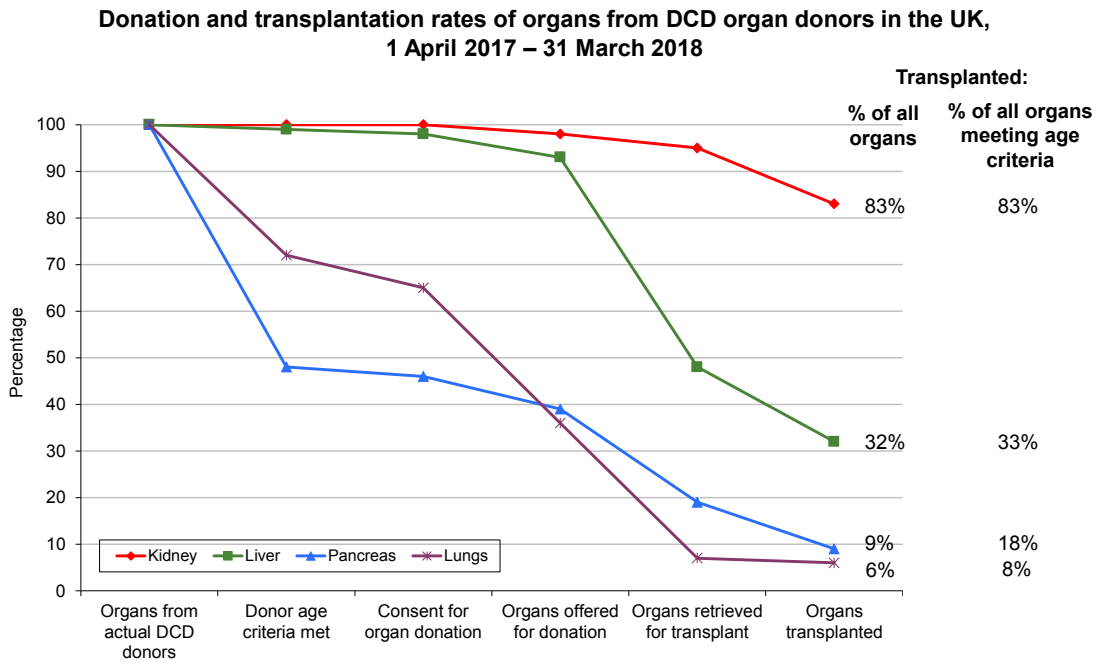
Extended criteria donors (ECD) are defined by the age > or = 60 years, or 50-59 years plus two of the following: cerebrovascular accident as the cause of death, pre-existing hypertension, or terminal serum creatinine greater than 1.5 mg/dl.

Furthermore, over time, donor characteristics have changed in general: donors are older, more obese and as an effect of that, are suffering from more comorbidities which means that they are also less likely to have suffered a trauma- related death. All these characteristics have their own adverse outcomes on transplantation.

Contrary to standard criteria donors (SCD), which refers to donors <50 years of age with no history of hypertension (HTN) or diabetes whose cause of death is a road traffic accident, and where donation occurs after brain death (DBD), a growing number of organs for transplantation come from Extended criteria donors (ECD).

In 2001 approximately 38% of cadaveric kidney donors and recipients in the UK were older than 50 years of age, 14 years later this number has risen dramatically to 60%[143]. Of course, all of these factors that lead to the organs being defined as ECD kidneys, are associated with inferior renal function, a reduced nephron reserve and decreased graft viability when compared to kidneys coming from a SCD. This subsequently leads to a 77% increased risk of graft failure[144] and an average graft survival expectancy of 5.1 years, compared to 10 years for a younger non-ECD kidney[145]. In addition ECD kidneys are more susceptible to ischaemic injury and display higher rates of DGF (33%) compared to an SCD kidney (21%), as well as primary non-function and acute rejection.

To justify the use of kidneys from ECD donors it is to mention, that recipients of such kidneys still have better survival rates compared to patients with ESRD remaining on RRT and on the waiting list[146,147]. Strategies need to be developed to improve the quality of organs coming from ECD donors before the potential transplantation and in an ideal case, even organs which are deemed untransplantable could be turned into transplantable ones, to finally narrow the huge gap between demand and offers for organs in transplantation. ***Figure 1.8.*** illustrates the percentage rates of utilization of offered organs. Even for kidneys, which, out of all the included organs are the least sensitive to ischemia times, there is still room for improvement. In an ideal case scenario, research to improve these rates for kidney transplantation could be translated to other organs. Especially for pancreas transplantation, a solution to make more grafts available needs to be found urgently.



Source: Transplant activity in the UK, 2017-2018, NHS Blood and Transplant

Figure 1.8. Utilization of DCD organs in the United Kingdom

Fig. 1.8. *shows the utilization rates in transplantation for organs from donors after cardiac death (DCD). The results for kidney transplantation are best with 84% of all offered organs being transplanted but there is still room for improvement. Research into optimization of kidney grafts could then be translated into improvment of the utilization rates for other organs.*

1.4.4. Kidney transplant activity in the UK

With an upwards trend in the number of organ donors over the past five years, a total number of 3348 kidney transplants was performed in the financial year 2016/2017[148]. One third of these transplants were living donation kidney transplants, one third were kidneys coming from donors after brain death (DBD) and the remaining cases, a bit less than one third, were kidneys from donors after cardiac death (DCD). Looking at the overall statistics of organ donation, there has been a 4% increase in the number of DCD donors, a 6% increase in DBD donors and a 3% decrease in the number of living organ donors compared to the previous year[148].

The overall numbers of donors are increasing but as mentioned above, it is important to be aware of the significant changes over time in regards to donor characteristics.

The percentage of donors aged 60 or above has increased from 21% in 2007-2008 to 36% in 2016-2017 and also the proportion of donors with a BMI above 30 has increased from 19% to 25%. Furthermore, the proportion of all deceased donors after a trauma death has decreased from 14% to 3% (Organ Donation and Transplantation Activity Report 2016/17). All of these changes point to the fact that the times of ‚ideal' donors are over and that we will have to deal with organs from ECD donors as a new standard in the future.

1.4.5. Delayed graft function

Delayed graft function (DGF) itself is defined as the need for dialysis in the immediate post-operative period[141], indicating a temporary failure of the transplanted kidney to resume adequate function. This occurs in approximately 20%-80% of all kidney transplants to different extents, depending on a number of variables, with the type of donor allograft being a predominant factor. This is supported by the fact that DGF is rarely experienced in living donor transplantation.

Dialysed delayed graft function and non dialysed slow graft function (SGF) can both contribute to a negative patient- and graft survival, to early acute rejection episodes and to tubular fibrosis or atrophy. DGF leads to a more complex post-operative period for the patient, not only as a result of the required dialysis but also due to an increased risk for acute rejection. This phenomenon is most likely triggered by an ‚unmasking' of hidden donor foreign antigens to the recipient and it is this increased immunogenicity that then contributes to instances of interstitial nephritis and tubular atrophy which negatively affects long-term graft and patient survival[149]. DGF increases the risk of acute rejection by 38%, and of graft loss by 41%, with worse graft function at 3.5 years compared to patients without DGF[142]. The five key contributors to this DGF/SGF complex intervene in chronologically related stages within the first 24 hours before and after the transplantation: donor tissue quality, donor brain death and related stress, preservation characteristics, immune factors and recipient variables[149]. As a result of this, DGF is the clinical consequence of ischaemic stress and injury sustained during organ retrieval and preservation, which is accentuated during reperfusion when the so called ischemia-reperfusion injury (IRI) becomes evident[142].

As a logic consequence, DGF is associated with prolonged periods of hospitalisation,

higher treatment costs and adverse effects on transplant patients' general rehabilitation[150,151].

Therapeutic interventions or preventive measures could be capable of partially reversing the factors contributing to the risk for DGF/SGF and hence, the expected dismal outcomes[149]. This underlines the necessity for organ preconditioning in times where ECD transplantation becomes routine with an aim to overcome organ shortage.

1.5. Mesenchymal stem cells in Machine Perfusion- a good combination to attenuate Ischemia- Reperfusion Injury in solid organ transplantation?

As discussed above in detail, CSS has reached its limits in transplantation and with organs from ECD rising in numbers, we need to develop strategies to improve the quality of these organs before transplantation. One possible option for organ preconditioning in a setting of machine perfusion could be the application of mesenchymal stem cells (MSC). MSCs are multipotent, self renewing cells which can be isolated from various types of tissue such as bone marrow, adipose tissue and many others[152]. In addition to their capacity to differentiate into osteoblasts, adipocytes and chondrocytes[153], MSCs also possess immunomodulatory capacities and therefore have been used as an experimental therapeutic agent in graft-versus-host disease (GVHD)[154] and Crohn's disease[155]. Furthermore, the safety and the properties of MSCs in preliminary data from clinical trials in this field have initiated interest in MSC therapy in the field of solid organ transplantation (SOT). In SOT, MSCs could potentially be used to treat ischemia-reperfusion injury, acute rejection and even to precondition organs and recipients in order to attenuate or even avoid those unwanted side effects and thus, optimize the long term outcomes.

1.5.1. Classification of Stem cells

Stem cells are cells with the capability to renew themselves through cell division as well as to differentiate into multilineage cells to a certain extent, depending on which category of stem cells they belong to[156,157]:

- **Embryonic Stem Cells (ESC)** are cells, which have been isolated from the inner cell mass (ICM) of mouse early pre-implantation blastocyst. Later on, ESCs have also been isolated from the ICM of human blastocysts[158], but until now ESCs have only been investigated in depth in the mouse[156]. ESCs are pluripotent cells with a capacity of self-renewal and genomic stability[159] which, as a consequence, are able to differentiate into any mature cell of the three germ lines[160]. Ever since the very first isolation of human ESCs, scientists have been very interested in the use of ESCs for regenerative medicine as well as drug development and immunotherapy, but their use is restricted due to ethical issues[156].

- **Induced pluripotent Stem Cells (iPSC)** are differentiated cells, which are reprogrammed to an embryonic-like state by transfer of nuclear contents into oocytes or by fusion with ESCs. These cells are almost similar to ESCs as they are able to self-renew, possess differentiation capacity and are able to produce germ line competent-chimeras[161]. However, their genomic stability is still questionable[156].

- **Mesenchymal Stem Cells (MSCs)** are self-renewable, multipotent and easy accessible cells, which were named after their ability to differentiate into specialized cells developing from the mesoderm. MSCs are extractable from a variety of tissues in humans as well as in rodents and are expandable in vitro with exceptional genomic stability. Therefore, the culture of MSCs goes along with only a few ethical issues, marking their importance in cell therapy, tissue repair and regenerative medicine[156,162]. Attention has to be paid in terms of the nomenclature as mesenchymal stem cells formally do not meet the generally accepted criteria for stem cell activity. The inconsistency between the nomenclature and the biologic properties of MSCs led to the suggestion that the fibroblast like, plastic adherent cells, regardless of the tissue from which they originated, were termed **multipotent Mesenchymal Stromal Cells**, while the term mesenchymal stem cells should only be used for cells that meet specified stem cell criteria. Nonetheless, according to the guidelines of the International Society for Cellular Therapy, the widely recognized acronym, MSC, may be used for both cell populations, as in the current practice, but

investigators should clearly define the scientifically correct designation in their reports[162]. Also, MSCs will be named Mesenchymal Stem Cells in this thesis to simplify the term and also due to the fact that in literature, both, the terms Mesenchymal Stromal- as well as Stem Cells are used for the same cell population. MSCs can furthermore be named after the source of which they have been extracted from. For example MSCs extracted from the bone marrow can be named BM-MSC whereas in the same way, MSCs extracted from adipose tissue can be named A-MSCs. Whether these cells are different remains to be determined. For this article, as long as fulfilling the minimal criteria for being MSCs, they will be the same cell type.

1.5.2. Mesenchymal Stem Cells

Mesenchymal stem cells were first described in the 1960s and 1970s, when Friedenstein and colleagues discovered the presence of a subpopulation of bone forming stromal cells within the bone marrow of the mouse. These cells were characterized by rapid adherence to tissue culture plastic, their fibroblast-like appearance and their capacity to form colony units[163]. Following that discovery, in the late 1980s, Owen and Caplan elaborated on this work and introduced the idea of a nonhematopoietic adult stem cell in the bone marrow[164,165] and the term mesenchymal stem cell was coined by Caplan in 1991[165]. His group was the first to isolate MSC from human bone marrow[166]. Since then, MSCs have also been isolated from a number of other sources, including umbilical cord blood, muscle, liver and adipose tissue[167,168]. Initially, MSC were thought to mediate tissue repair by replacing damaged cells due to their multilineage differentiation capacity[169,170]. However, subsequent findings suggested that this is unlikely[171,172] and it is now widely believed, that in response to tissue injury, MSC home to the site of damage where they engraft transiently to encourage repair through the production of anti- apoptotic, pro-mitogenic and vasculotropic factors, such as growth factors, cytokines, and antioxidants[173–177].

As a conclusion to this, MSC are considered to have therapeutic potential in a wide variety of medical disciplines and hence, a lot of studies have been conducted on the use of MSCs as therapeutic agents. However, as specific markers for MSCs are lacking, using different methods of isolation and expansion, and also distinct

approaches to characterize the cells, a reasonable comparison of the study outcomes used to be difficult. Therefore, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define MSCs: first, MSC must be plastic-adherent when maintained in standard culture conditions; second, MSC should possess a specific set of cell surface markers, i.e. cluster of differentiation (CD)44, CD105, CD73, CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules; and third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro[153]. Another definition by Maurer et al. suggests that MSCs do not express CD34, CD45, CD117 (cKit), HLA class I, and HLA-DR antigens, whereas they are positive for CD13, CD29, CD44, CD73, CD90, CD105, and CD166. These markers are located in the cell membrane of the MSCs, and antibodies are readily available for FACS analysis and sorting[178].These criteria will probably require modification depending on new knowledge in the future but, for the moment, this set of criteria helps to define a more uniform characterization of MSC and therefore, facilitates the exchange among investigators.

1.5.3. Cluster of differentiation

The cluster of differentiation (CD) is a protocol used for the identification and investigation of cell surface molecules providing targets for immunophenotyping of cells[179]. These cell surface molecules can have numerous functions, often acting as receptors or ligands initiating signal cascades within the cell. Some CD proteins do not play a role in cell signalling but function as adhesion molecules[180]. For humans, as of November 2014, CD is numbered up to 364. In order to better understand the function of mesenchymal stem cells, it is necessary to have a closer look at the function of expressed surface markers.

- **CD44:** The CD44 antigen is a cell surface glycoprotein which plays a role in cell-cell-interactions, cell adhesion and cell migration. In humans, the CD44 antigen is encoded by the CD44 gene on chromosome 11[181]. CD44 is expressed in a large number of mammalian cell types and it mainly functions as a receptor for hyaluronic acid, which is a polysaccharide found in mammalian extracellular matrix (ECM)[182]. Furthermore, CD44 has many more

functions, dependent on the extracellular structure of the protein, which can vary due to alternative splicing and post-translational modification[182]. Hence, this protein participates in a wide variety of cellular functions, including lymphocyte activation, hematopopoiesis, tumour metastasis and most importantly for MSCs, it functions as a ‚bone homing receptor', directing migration of MSCs to the bone marrow[183]. It is widely expressed in multiple cell types, including hematopoietic and cancer stem cells[184]. Although the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed the expression of CD44 to be one of the criteria for the characterization of MSCs, there is phenotypic, functional and molecular evidence, that bone marrow derived mesenchymal stem and progenitor cells physiologically do not express CD44 (in humans and mice). However, *in vitro* culture could result in acquisition of this cell surface marker, as well as in changes of expression patterns of cytokines, growth factors and other signalling molecules[185].

- **CD90:** cluster of differentiation 90 (CD90) or Thymocyte differentiation antigen (Thy-1) is a 25-37kDa heavily N-glycosylated, glycophosphatidylinositol (GPI), which was the first T-cell receptor to be identified by Reif and Allen in 1964 in the mouse. It was demonstrated by them to be present on murine thymocytes, on T-lymphocytes and on neuronal cells[186]. The human homolog was isolated in 1980 as a 25kDa protein[187] and further homologs have been described in many species i.e. rats, dogs, chickens. Thy-1 is expressed on fibroblasts, neurons, blood stem cells, i.e. MSCs and endothelial cells and it has a wide range of functions, including T-cell activation[188–191]. Thy-1 has been conserved throughout evolution suggesting an important function for this molecule[192]. In humans, it is encoded on chromosome 11, whereas in mice, it is encoded on chromosome 9[193,194]. Although the exact biological role(s) of Thy-1 has remained controversial and so far, investigations on its role in T-cell activation are hindered by the fact that a ligand or counter receptor for T cell associated Thy-1 has not been identified, evidence suggests Thy-1 to be more than a cell surface marker and to play a crucial role in immunomodulation[192]. As CD90 or Thy-1 is also expressed by MSCs, it has to be taken into account considering the immunomodulatory properties of MSCs. Further studies will have to clarify

the exact functions of Thy-1 as well as the mechanisms leading to T-cell modulation.

- **CD73:** cluster of differentiation 73, also known as 5' nucleotidase (5'NT) is an enzyme which converts adenosine monophosphate (AMP) to adenosine and in humans, is encoded by the NT5E gene[195]. CD73 generated adenosine functions in cell signalling in many physiologic systems and could also be important for T-cell development as CD73 is suggested to serve as a costimulatory molecule in T-cell activation[196]. Again, this supports the hypothesis, that MSCs have immunomodulatory functions.

- **CD105:** cluster of differentiation 105 is also known as endoglin and is a type I glycoprotein which is part of the TGF beta-receptor complex and located on cell surfaces. The human endoglin gene (END) is encoded on human chromosome 9 and primarily, the protein is associated with human vascular endothelium[197]. Besides TGF signalling, endoglin is suggested to be involved in cytoskeletal organization affecting cell morphology and migration. Furthermore, endoglin seems to be a crucial modulator for development of the cardiovascular system, as mice without the endoglin gene die due to cardiovascular abnormalities[198]. The fact that this cell surface marker plays a role in angiogenesis supports the thesis about regenerative capacities of the MSCs, and TGF signalling of endoglin possibly contributes to their immunomodulatory properties.

- **CD29:** cluster of differentiation 29 is also called integrin beta-1 and is encoded by the ITGB1 gene on chromosome 10 in humans[199]. It exists in 6 different isoforms via alternative splicing. Integrins are membrane receptors which are involved in cell adhesion and recognition in a variety of processes, i.e. haemostasis, embryogenesis, immune response and tissue repair[200]. In terms of MSCs and their function in solid organ transplantation, of course, tissue repair seems to be the most interesting involvement of CD29.

- **CD13:** cluster of differentiation 13 is also called alanyl (membrane) amino peptidase (ANPEP). It is located in the small intestinal and renal microvillar

membrane as well as in other plasma membranes. The gene for ANPEP is encoded on human chromosome 15 and in the small intestine it plays a role in digestion of peptides. Its role in proximal tubular epithelial cells and other cells has yet to be investigated[201].

- **CD166:** cluster of differentiation 166 is encoded by the ALCAM gene on human chromosome 3 and is therefore also named ALCAM (activated leukocyte cell adhesion molecule)[202]. It is a member of an immunoglobulin receptor subfamily and it binds to T-cell differentiation antigen CD6, thereby influencing processes of cell adhesion and migration. It is expressed on activated T-cells, activated monocytes and epithelial cells[203].

1.5.4. MSC in solid organ transplantation

In addition to their regenerative capacity, MSC also possess an immunomodulatory capacity in vitro and in vivo[152,154,155]. Therefore, these cells have been successfully used as an experimental therapeutic agent in immunological diseases, such as steroid resistant graft-versus-host disease (GVHD) following allogeneic transplantation of hematopoietic stem cells. In the underlying study (phase II) an intravenous infusion of mesenchymal stem cells (0,4-9x10e6 cells), irrespective to the donor HLA-match (HLA-identical sibling donors, haploidentical donors, and third-party HLA-mismatched donors), led to 30 cases of complete response and 9 cases of improvement out of 55 treated patients. There were no observed side effects during or after the treatment with the MSCs[154]. In another phase I study, the feasibility and safety of the application of MSCs could be shown in patients with Crohn's disease in an experimental setting[155]. These very preliminary data on efficacy and safety of MSCs initiated interest on using these cells as therapeutic agent for the treatment of transplantation- related complications in solid organ transplantation (SOT), such as ischemia- reperfusion injury, acute rejection and even long- term allograft pathology[152].

1.5.4.1. Preclinical data on mesenchymal stem cells in solid organ transplantation

The potential of MSC to prolong allograft survival in SOT has been investigated in a number of pre- clinical transplant models in-vitro as well as in vivo. Most recent findings will be discussed by category for small animal models, big animal models and preclinical data in human organs in the following paragraphs:

1.5.4.1.1. Small animal models:

In two different studies of rodent heart transplant models, it could be demonstrated, that infusion of donor-derived MSCs into the recipient after transplantation, especially in combination with a low-dose treatment with Rapamycin, led to long-term heart graft survival (>100 days) with normal histology[204]. In both studies, engraftment of infused MSCs within the recipient's lymphoid organs and allograft appeared to be instrumental for the induction of allograft specific tolerance and high frequencies of both tolerogenic dendritic cells (Tol-DCs) and CD4(+)CD25(+)Foxp3(+)T regulatory cells in the spleens of the recipients were observed. This, as well as the fact that MSCs expressed indoleamine 2,3-dioxygenase (IDO), a tryptophan catabolizing enzyme that is known to suppress T-cell responses, seemed to be the key factors for achieving tolerance[204,205].

Also in rodent kidney transplant models, allograft tolerance after MSC infusion, with high expression of CD4(+)CD25(+)Foxp3(+)T regulatory cells could be observed. Using IDO-/- knockout-mice as MSC donors, the dependency of an elevated expression of Tregs on the presence of IDO could be confirmed[206]. More recently, in rat kidney transplant models using expanded criteria organs (prolonged cold ischemia time, marginal donors), administered MSCs both intravenously and when administered via the renal artery were shown to ameliorate intragraft inflammation by reducing the gene expression of different pro-inflammatory cytokines, chemokines, and intercellular adhesion molecule-1. Also, the cellular inflammatory infiltration was reduced, suggesting MSCs could be used to treat acute rejection[207,208].

In contrast to the above findings, Eggenhofer et al. could not see any beneficial effects on graft survival after administration of MSCs alone in a mouse heart

transplant model. They could only see amelioration of the outcomes when MSC infusion was combined with immunosuppressive medication, of which the combination with mycophenolate mofetil was the most potent[209].

The above described data suggest, that MSC do not act by differentiating into functional cells[210], but target other cells via the secretion of cytokines, growth factors and prostaglandins with immunomodulatory and regenerative function. MSCs have been shown to secrete a range of anti-inflammatory factors when administered in a model of rat kidney injury, including IL-10, TGF- b, hepatocyte growth factor (HGF), nitric oxide (NO), HLA-G and PGE2, that are all indicated to play some role in their regenerative effect [204,211,212].

In a systematic meta-analysis of MSC therapy for impaired kidney function in small animal models, a total of 21 studies were analysed and again, an improvement of impaired kidney function after administration of MSCs could be confirmed. Here, the most effective path of delivery for the MSCs seemed to be intra-arterial, followed by intra-renal and intravenous application[213].

Most recently, in a rat heart transplant model, repeated infusion of human umbilical chord (UC-) MSCs reduced MHC class II expression on vascular endothelia of transplanted hearts and increased survival time of allograft. The UC-MSCs were shown to increase the regulatory cytokines IL10 and transforming growth factor (TGF)-β1, and suppressed pro inflammatory cytokines IL-2 and IFN-γ in vivo. The UC-MSC culture supernatant had similar effects on cytokine expression, and decreased lymphocyte proliferation in vitro[214].

This confirms the suggestion of MSCs acting in a paracrine manner but still more investigations are needed to unravel the effects of MSCs in detail.

1.5.4.1.2. Large animal models:

In a baboon model, Bartholomew et al. could demonstrate prolonged skin graft survival after intravenous administration of donor MSCs to MHC-mismatched recipient baboons prior to placement of autologous, donor and third-party skin grafts. This was accompanied by demonstration of a 50% reduction of lymphocyte proliferative activity after adding MSCs to a mixed lymphocyte reaction in vitro[215].

Further evidence for a potential role for MSCs in SOT comes from organ injury models. Studies demonstrated, for instance, improved cardiac function in acute

myocardial infarction in swine after intracoronary administration of MSCs[216] and in addition, MSCs have been shown to be beneficial in a sheep emphysema model[217], suggesting that MSCs have a regenerating effect on injured organs.

As solid organ transplantation is the only cure for some end-stage diseases but is still limited by organ shortage, organ retrieval from non-heart beating donors (NHBD) to expand the donor pool is an important issue. However, finding mechanisms to improve the quality of these ‚marginal' organs will be the key to transform them from discarded organs into transplantable organs.

Recently, in a porcine non-heart beating donor (NHBD) lung transplantation model, pre-ischaemic deposition of recipient-specific bone-marrow-(BM)-derived hMSC into donor lungs was demonstrated to be feasible and effective, leading to a significantly better dynamic lung compliance (DLC) when compared to controls[218]. Most recently, in a porcine model of renal auto transplantation, injection of autologous amniotic fluid-derived MSCs (af-MSCs) into the renal artery 6 days after transplantation resulted in improved glomerular and tubular function, leading to full renal function recovery and abrogated fibrosis development at 3 months[219], indicating the importance of MSC treatment after organ injury in solid organ transplantation.

Even in a more complex model using composite tissue allografts (CTA), namely a porcine model of hemi face transplantation, the significant effects of MSCs on allograft survival could be demonstrated. In that study, treatment of the recipients with a combination of MSCs (intravenously) and Cyclosporine (CyA) for three weeks postoperatively led to a significantly prolonged allograft survival compared to controls. Immunohistochemical staining showed a significantly decreased level of CD45 and IL-1 expression and, furthermore, the number of CD4+/CD25+ regulatory T-cells and IL-10 expression in the circulating blood were significantly increased in the MSC-CsA group. In summary, that study indicated that MSCs, especially in combination with CyA, not only suppressed inflammation and acute rejection of CTA, but also modulated T-cell regulation and related cytokine expression[220].

As there is a strong need for translational studies in order to be able to translate observations into clinical studies, the question arises, whether MSCs function across species barriers. This could be of importance when it comes to the requirement for large numbers of MSCs, where pigs as a source could be of interest. Also, when it comes to the idea of genetically modified MSCs as therapeutical target, porcine MSCs could be a good source. In order to give an answer to that question, Li et al.

performed a literature search where they could identify 94 reports about in-vivo cross species administration of MSCs. Their search strengthened the hypothesis that pig MSC would function satisfactorily in a different species, for example, humans[221].

1.5.4.2. MSCs in human organs/ clinical data

Not much preclinical data on the use of MSC in human organs is available yet. One recent study in human lungs rejected for transplantation revealed a positive effect of ex-vivo intravenous application of human MSCs on the quality of the graft. In this case, MSCs were effective in restoring the capacity of the alveolar epithelium to remove alveolar fluid at a normal rate, suggesting that this therapy may be effective in enhancing the resolution of pulmonary oedema in human lungs deemed clinically unsuitable for transplantation[222]. However, as the opportunity to get human organs rejected from transplantation for research is rare, not much is known about the effects of MSCs in an ex-vivo application.

Nevertheless, as preclinical data provides a vast amount of evidence that suggests that MSC therapy could have a beneficial effect on SOT, a number of research groups have taken the lead in setting up the first clinical trials. Worldwide, many studies on MSC infusion around the time of transplantation are on-going and an up to date summary of on-going trials and their status can be found on www.clinicaltrials.gov. ***Table 1.1.*** lists currently on-going clinical studies on MSC infusion in organ transplantation around the world. So far, the application of MSCs has been declared as safe and there have been no adverse effects described. Another observation is, that systemic MSC infusion has been shown to lead to a reduction in CD8+ T-cell activity and to an expansion of CD4+ Fox P3 T-regulatory cells.

Status	Study title	Conditions	Location
terminated	Properties of MSCs in lung transplantation	Lung transplantation	Emory University, Atlanta, Georgia, USA
unknown	Mesenchymal Stem Cell transplantation in the Treatment of chronic allograft nephropathy	Kidney transplantation	Fouzhou General Hospital, Fouzhou, Fujian, China
recruiting	Safety and tolerance of Immunomodulating therapy with donor specific MSCs in paediatric living-donor liver transplantation	Paediatric liver transplantation	University Children's Hospital, Tuebingen, Germany
completed	Mesenchymal Stem Cells and subclinical rejection	Organ transplantation	Leiden University Medical Centre, Leiden, Netherlands
Active, not recruiting	To elucidate the effect of Mesenchymal Stem Cells on the T-cell repertoire of kidney transplant patients	Renal transplantation	Translational and Regenerative Medicine, Chandigarh, India
unknown	Effect of BM-MSCs in DCD kidney transplantation	Renal transplantation	The first affiliated hospital, Sun Yat-sen University, Guangzhou, China
unknown	Human MSCs induce Liver Transplant tolerance	Liver transplantation	Beijing 302 Hospital, Beijing, China
unknown	Effect of BM-MSCs on chronic AMR after kidney transplantation	Renal transplantation	The first affiliated hospital, Sun Yat-sen University, Guangzhou, China
completed[223]	Induction therapy with autologous mesenchymal stem cells for kidney allografts	Renal transplantation	Stem cell therapy centre, Fuzhou General Hospital, Fujian China
completed	Mesenchymal Stem Cell therapy for lung rejection	Lung transplantation	Mayo Clinic Jacksonville, Florida, USA
recruiting	Mesenchymal Stem Cells in living donor kidney transplantation	Renal transplantation	Houston Methodist Hospital System, Houston, Texas, USA
unknown	Therapeutic strategy and the role of MSCs for ABO incompatible liver transplantation	Liver transplantation	The first affiliated hospital, Sun Yat-sen University, Guangzhou, China
unknown	Mesenchymal Stem Cells after renal or liver transplantation	Renal/liver transplantation	University Hospital Liege, Belgium
unknown	Induction with SVF derived MSCs in living related kidney transplantation	Renal transplantation	Stem cell therapy centre, Fuzhou General Hospital, Fujian China

Table 1.1. Clinical trials using Mesenchymal Stem Cells in transplantation

Table 1.1. *summarizes clinical trials on MSCs in transplantation around the world. www.clinicaltrials.gov.*

In kidney transplantation, Perico et al. were the first to report results of clinical application of MSCs when they treated two patients with culture expanded autologous MSCs at day 7 after living donation kidney transplantation. In that safety and clinical feasibility study, patients were given T cell-depleting induction therapy and maintenance immunosuppression with cyclosporine and mycophenolate mofetil. On day 7 post transplant, $1.7x10^6$ and $2.0x10^6$ MSCs/kg body weight, respectively, were administered intravenously. The authors observed an increase in serum creatinine 7-14 days after MSC infusion in both patients, however, kidney function was stable after 1 year of infusion, respectively. They also observed elevated numbers of T regulatory cells (Tregs) in the peripheral blood of the patients. Findings from that study show that MSC infusion in kidney transplant recipients is feasible, allows enlargement of Treg in the peripheral blood, but that timing and concurrent immunosuppressive therapy may need to be adapted for follow-up trials[224].

In a study conducted in the Netherlands, patients showing signs of subclinical rejection in protocol biopsies at 4 weeks and 6 months after kidney transplantation, received two doses of 1–2x10^6 cells per kilogram of body weight of MSCs, 7 days apart. In total, six patients received MSC infusions. They were followed up clinically and immune monitoring was performed up to 24 weeks afterwards. The results of this phase I/II trial show that treatment of subclinical rejection with autologous MSCs is feasible and well tolerated and the findings were suggestive of systemic immunosupression[225]. Finally, in kidney transplantation, two studies are registered by the Fuzhou institute in China. One study is completed and has evaluated the use of MSCs as an induction therapy after kidney transplantation, with results showing a superiority of autologous MSCs to anti IL-2-receptor antibodies as an induction, looking at the incidence of acute rejection, the occurrence of opportunistic infections and the renal function after one year[223].

Another study from Belgium, which is on going (status unknown), will examine the safety of MSC therapy in patients receiving either a liver or a kidney transplant. Patients will receive 1.5–3 x10^6 third party MSC/kg body weight accompanied with a standard immunosuppressive regimen (www.clinicaltrial.gov).

In liver transplantation, protocol for a planned study in which commercially available allogeneic mesenchymal progenitor cells will be administrated during and 3 days after liver transplantation combined with a bottom-up immunosuppressive regimen has been published[226]. Furthermore, in a study in Beijing (status unknown), patients will receive conventional immunosuppressive treatment and once per 4 weeks, a dose of 1×10^6 umbilical chord mesenchymal stem cells (UC-MSC)/kg body weight for 12 weeks to investigate the effect of MSCs on liver function recovery after transplantation. (Human Mesenchymal Stem Cells Induce Liver Transplant Tolerance, www.clinicaltrial.gov).

1.5.5. MSC and ischemia-reperfusion injury

Ischemia reperfusion injury (IRI) is an inevitable clinical consequence in solid organ transplantation which contributes to the occurrence of acute and chronic rejection and thus, also increases long-term morbidity and mortality of patients post transplant[227,228]. Better comprehension of the molecular mechanisms underlying that complex type of injury could help to find innovative starting points for therapeutic

interventions. Therefore, current research focuses on the identification of these mechanisms in order to be able to prevent or attenuate IRI- induced damage as well as to accelerate processes of tissue repair.

1.5.5.1. Key mechanisms of ischemia-reperfusion injury

Ischemia reperfusion injury occurs whenever the blood supply to a certain organ is disrupted for a certain time causing tissue hypoxemia, to be later restored and thus, followed by concomitant re-oxygenation. Solid organ transplantation is inseparable from ischemia-reperfusion injury and so, the lack of blood supply during the harvest and sometimes transport of the organ, known as the ischemic phase, causes an imbalanced metabolic supply as well as micro vascular dysfunction within the organ to be followed by an increase of the organ damage through innate and adaptive immune responses, triggered by the sudden reperfusion and re-oxygenation of the organ[229].

Despite the differences described between warm and cold ischemic damage[230], consistent pathophysiological features of IRI could be identified. For example, the lack of oxygen which leads to hypoxia during the ischemic period is associated with damage to the endothelial cell barrier due to decreases of intracellular cAMP levels which leads to vascular permeability and leakage[231]. In addition, ischemia and reperfusion lead to the activation of different cell death mechanisms such as apoptosis and necrosis[232]. If you split the process up into its two parts, the ischemic period in particular leads to alterations in the transcriptional code of gene expressions, for example, the stability of transcription factors like heat-inducible factor (HIF) and nuclear factor- kappa B (NF-kappa B) is influenced negatively[233]. Reperfusion injury on the other side is mostly characterized by autoimmune responses, such as natural antibody recognition of neoantigens and subsequent activation of the complement system[234].

With a few exceptions, ischemia-reperfusion injury takes place in a sterile environment and therefore, is also called ‚sterile inflammation'. Nevertheless, its pathomechanisms show many similarities to a host immune response towards invading microorganisms[235]. This sterile immune response includes downstream activation of different signalling cascades through pattern-recognition molecules such as Toll-like receptors (TLRs), as well as the activation of immune cells of the innate and the adaptive immune system and activation of the complement system. Of

course, these responses have different consequences, which can lead to tissue damage, hence, targeting immune activation is an emerging therapeutic concept in the treatment of IRI. In contrast to this, some aspects of the adaptive immune response may even be beneficial when it comes to alleviation of IRI, in particular the recruitment and expansion of regulatory T cells (Treg cells)[236]. In order to even better understand the potential beneficial effects of MSC treatment on IRI, we have to split up the immune response contributing to IRI into two parts: the innate immune response and the adaptive immune response.

- **Innate immune response:**

Tissue damage and cell death as it occurs during ischemia and reperfusion lead to release of damage associated molecular patterns (DAMPS), e.g. high-motility group box 1 (HMGB1) or ATP. These molecules usually are sequestered intracellularly, but upon tissue damage, get into the extracellular matrix, where they are able to activate toll-like receptors (TLRs)[236–238]. Activation of TLRs then leads to an activation of downstream signalling pathways, including NF-kappa B, mitogen activated protein kinase (MAPK) and type 1 interferon (IF1) pathways, resulting in an induction of pro inflammatory cytokines and chemokines[236].

One of the most studied pattern recognition receptors for DAMPs is toll-like receptor 4 (TLR-4). It is known to mediate inflammatory responses to gram-negative bacteria through its activation by lipopolysaccharide and furthermore, TLR-4 activation is suggested to be enhanced by oxidative stress as it occurs during IRI[45]. Remarkably, studies in mice suggested, that kidney intrinsic TLR-4 signalling has a predominant role in kidney injury[239], and moreover, studies on TLR-4 in human kidney transplantation showed that kidneys from individuals with a loss of functional allele of TLR-4 contained lower levels of pro inflammatory cytokines in association with better immediate graft function after kidney transplantation as compared to kidneys from individuals with active TLR-4 allels[240].

Also other TLRs, like TLR-2 and TLR-3, seem to contribute to the extent of IRI[45,236], therefore, taken together, inhibition of TLR signalling could be effective for the treatment of the ‚sterile inflammation' induced by IRI and currently, development of TLR inhibitors is on-going.

Another characteristic of IRI is an accumulation of inflammatory cells at the site of injury. Particularly during the early phase of reperfusion, innate immune cells

dominate the cellular composition of the infiltrate. However, the role of these cells in that stadium of sterile inflammation is not yet clear; they may either contribute to an activation of inflammation and promote collateral tissue damage or they may contribute to a faster resolution of the injury[241]. For example, studies in mice showed that monocytes recruited from a splenic reservoir could help in the healing of tissue injury after IRI in the heart[241], and also that depletion of conventional dendritic cells led to an increase of tissue injury in the context of hepatic IRI[242].

The beneficial effect of dendritic cells on IRI depends on their production of the anti-inflammatory cytokine interleukin-10 (IL-10) which attenuates levels of TNF-alpha, IL-6 and reactive oxygen species (ROS). ROS are toxic molecules that are implicated in the tissue damage during IRI and alter the cellular metabolism in a way which ends in cell dysfunction or cell death[236].

- **Adaptive immune response:**

Ischemia and reperfusion not only activate the innate immune system but also initiate a response of the adaptive immune system, which involves, amongst other cell types, T-lymphocytes. The mechanisms by which antigen-specific T-cells are activated during sterile inflammation are yet to be determined, but evidence indicates a contribution of both, antigen-specific and antigen independent mechanisms of activation[243,244].

Several studies have shown that T-cells accumulate during ischemia and reperfusion[245]. Studies of mouse lines deficient in specific lymphocyte populations showed that both CD4+ and CD8+ T-cells have a detrimental role in ischemia and reperfusion of the brain[246], the heart[247] and the kidneys[248]. In contrast to this, T-regulatory cells (Tregs) seem to have a protective role in ischemia and reperfusion, as for example in an experimental stroke model, where depletion of Tregs resulted in an increase of the delayed brain damage, and a deterioration in the functional outcome[249]. Furthermore, based on results that only wild-type Tregs but not IL-10 deficient Treg cells were able to attenuate ischemic brain injury, the authors of this study proposed, that Treg cell dependent IL-10 production leads to decreased levels of TNF-alpha at early time points and also delays interferon gamma accumulation at sites of inflammation[249]. In a model of transplant atherosclerosis, administration of ex-vivo expanded human Tregs led to beneficial outcomes. Therefore, MSC

application might be an approach to directly and also indirectly influence the number of Tregs at sites of inflammation.

1.5.5.2. Impact of IRI on graft function

Despite certain differences between warm and cold ischemic injury in transplanted organs[230], the main pathological features of IRI can be summarized as: impairment of the endothelial barrier, metabolic disturbances in the cells due to decreases in cAMP and adenosine triphosphate (ATP), induction of cell death programs, transcriptional reprogramming, the no-reflow phenomenon and (as described more in detail above) the induction of autoimmune and innate and adaptive immune mechanisms[229]. It is now well acknowledged, that IRI in transplantation influences graft function and graft survival as a non-allogeneic factor and hence also influences morbidity and mortality[250]. Ischemic damage to the organ, especially prolonged cold ischemia time, was shown to enhance organ immunogenity by unregulated expression of heat-shock proteins, adhesion molecules, chemokines and immunoproteasomes[251]. As a consequence to this, the survival of kidney transplant patients who immediately receive an organ from a related living donor is better than in patients receiving their kidney from brain-dead or deceased donors, where cold storage and transportation of the organ are often required[252]. There is clear experimental evidence that kidneys from brain dead donors might locally up regulate TLR4 and its ligands which leads to enhanced organ injury[253]. In kidney transplantation, acute kidney injury (AKI) caused by prolonged cold ischemia time often progresses to a clinical diagnosis of delayed graft function (DGF), which then might be followed by chronic transplant dysfunction as a long term result of IRI[254,255]. Interestingly, also liver grafts from donors who died from heart failure showed a higher degree of ischemic damage and this led to an increase of renal pathologies as well[256]. Taken together, these findings show that ischemia-reperfusion injury is a major contributor to acute graft failure, delayed graft function and graft loss and therefore, finding new therapies to ameliorate IRI in solid organ transplantation is a top priority in this field.

1.5.5.3. Mesenchymal stem cells for the treatment of IRI

Mesenchymal stem cells are multi potent cells present in the bone marrow which can in vitro differentiate into adipocytes, chondrocytes and osteocyte lineages[257], and which have been shown to be able to regenerate tissues of mesenchymal lineages[258–260] as well as to differentiate into neurons[261] and epithelial cells in vivo[262–265]. As consequence of this and due to encouraging observations from both preclinical studies and clinical trials, suggesting MSCs to have immunomodulatory, anti-inflammatory and regenerative properties, the administration of mesenchymal stromal cells at the time of solid organ transplantation might be beneficial in terms of alleviating IRI as well as immune responses triggering rejection[266]. In the following paragraphs, an overview about potential modes of action of MSCs and their application against IRI in solid organ transplantation will be given.

Ischemia- reperfusion injury results in a sterile inflammation which goes along with the production of damage associated molecular patterns (DAMPs) such as necrotic cells, cellular debris, heat shock proteins (HSP) and high mobility group protein box-1 (HMGB-1)[229]. DAMPs activate pathogen recognition receptors (PRR) such as TLRs and signalling through these receptors results in activation of the inflammasome[267] and the complement system followed by an up regulation of genes which are involved in the inflammatory process[229]. **Fig 1.9.** shows in a simplified way, the processes during IRI within the kidney, and the potential interaction of MSCs to alleviate the damage to a graft.

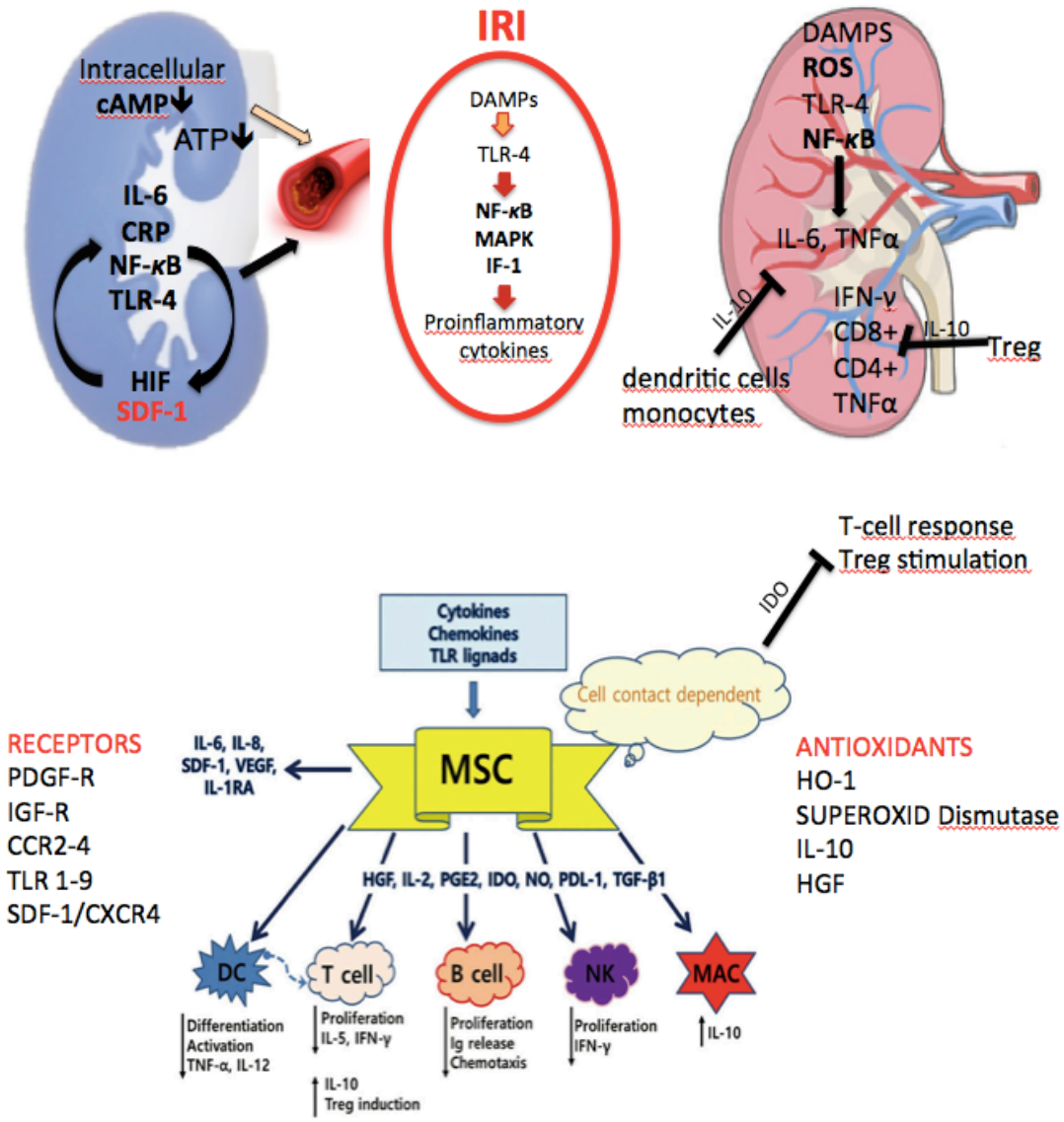


Figure 1.9. Pathophysiology of Ischemia Reperfusion Injury

Fig. 1.9. *illustrates the pathophysiology of ischemia-reperfusion injury (IRI), showing in a simplified way the signalling cascades within kidneys during the cold ischemic time, as well as during the reperfusion phase. The figure highlights schematically, how Mesenchymal Stem Cells (MSCs) could have a positive impact on IRI to protect the organs.*

MSCs express a number of PRRs including TLR 1-9[268–271], nucleotide-binding oligomerization domain (NOD) receptors[272,273] and also receptors for advanced glycation end products (RAGE)[274]. Once stimulated, these active receptors on the MSCs trigger different functions of the cells, for example stimulation of NOD like receptors on the MSCs leads to production of IL-8 and vascular endothelial growth factor[272,273]. TLR-3 and TLR-4 stimulation of MSCs was shown to result in differential effects, with TLR-4 inducing a proinflammatory state of the cell with secretion of IL-6, IL-8 and transforming growth factor-b (TGF-b), whereas TLR-3 stimulation resulted in

an anti-inflammatory state of the cell and production of IDO, prostaglandin E-2 (PGE-2), IL-4 and IL-1RA[275]. In regard to MSC immunosuppressive functions, TLR-3 and TLR-4 were shown by Opitz et al., to enhance MSC immunosuppression in vitro through IDO induction via IFN-b and protein kinase R signalling[270] but on the other hand, in a different study conducted by Liotta et al., TLR-3 and TLR-4 binding attenuated immunosuppressive effects of the MSCs[276].

Since the complement system is activated wherever there is tissue damage, it is also important to mention that complement activation products like C3a and C5a were shown to be chemo attractants for MSCs, which express both the C3a-receptor (C3aR) and the C5a-receptor (C5aR)[277]. Additionally, MSCs express CD59, a complement regulatory protein and are able to secrete complement factor H which protects them from complement lysis[278,279]. Importantly, MSCs also produce a number of antioxidants, including hemeoxygenase-1 (HO-1) and superoxide dismutase[280,281] and so far, have been shown in IRI models in vivo, to suppress oxidative stress and inflammation[251,282–284]. This was a result of increased expression of HO-1, IL-10 and hepatocyte growth factor and a decreased expression of the proinflammatory cytokines IL-1β, TNFα, IFN-γ as well as of reduced apoptosis, number of activated T-cells and immune cells[207,282–284]. Taken together, these findings show clearly, that MSCs are susceptible to environmental changes, as they may be present during ischemia-reperfusion injury, however, so far it is unclear what determines their effect in these circumstances to function in either a pro- or an anti-inflammatory way, and if this could be of use as a therapeutic target against IRI.

A protective effect of MSCs in ischemia- reperfusion transplant models has been reported. Whether this effect results from a direct cell-cell interaction in sense of an MSC trans differentiation at sites of damage or via paracrine mechanisms remains, as mentioned before, unclear. In an experimental model of kidney IRI in the rat, MSC derived micro vesicles together with soluble factors have been shown to protect against ischemia reperfusion induced acute and chronic kidney injury. This paracrine effect resulted from a horizontal transfer of messenger RNA and micro RNA by these micro vesicles and resulted in inhibition of apoptosis and stimulation of tubular epithelial cell proliferation[285]. Furthermore, in a rat kidney transplant model with prolonged cold ischemia time, in vivo administration of MSCs led to a reduced intragraft gene expression of different pro-inflammatory cytokines, chemokines and

ICAM-1. This effect could furthermore be confirmed in vitro by the same group[207]. Ischemia reperfusion injury itself plays a crucial role as a chemo attractant of MSCs to the transplanted organ[286]. This led to the observation by Casiraghi et al., that post transplant infused MSCs localized mainly into the graft, associated with neutrophils and complement C3 deposition, which resulted in premature graft dysfunction, whereas pretransplant infused MSCs induced a significant prolongation of kidney graft survival by homing to lymphoid tissues and promoting an early expansion of T regulatory cells (Tregs)[286].

In experimental models of kidney transplantation, intravenously administered MSCs could be detected within an hour of cell infusion and were shown to home to ischemic tubular sites[176,287]. However, after an hour, less than 1% of the injected stem cells could be detected in the injured kidneys and these cells were mainly trapped in lungs and liver. Interestingly, in the same study, direct administration of the MSCs into the renal parenchyma yielded significantly higher cell counts in the kidney than after intravenous application with 12,7% of the cells being found in the kidney after one hour. Nevertheless, these cells also disappeared and did not repopulate the regenerating organ. Although intraparenchymal injection of MSCs was followed by significantly reduced creatinine levels at day 1 and 2 when compared to other treatment groups, there was no difference in long term outcomes between groups, and taken together, these results suggest that paracrine effects of MSCs are the basis for an earlier recovery of MSC treated animals, not substantial grafting and repopulation of injured tubular epithelium by MSCs[287]. Moreover, in the same study, an important feature of MSCs could be proved, namely their tropism for injured tissues, as a significant higher number of MSCs in the first two days could be discovered in the kidneys with IRI compared to kidneys in animals undergoing sham operations[176].

The migration and homing of MSCs to sites of injuries is not fully understood yet but in vitro, hyaluronic acid was detected to be the major ligand for CD44 and as a result, MSCs injected into mice migrated to injured kidneys where the expression of hyaluronic acid was increased. Also, in the same study, the presence of MSCs in these kidneys correlated with their morphological and functional recovery, so that as a result, Herrera et al. could postulate that CD44 and hyaluronic acid recruit exogenous MSCs to injured tissue and enhance regeneration[288]. Furthermore, cell recruitment is also thought to be caused by migration of MSCs towards SDF-1

(stromal derived factor 1), which is over expressed in post ischemic kidneys and which binds to CXCR4 and CXCR7 on the MSCs. Both these receptors were shown to be required for paracrine actions of MSCs in vitro as well as in vivo[289]. In this context, transforming growth factor-β1 (TGF-β1) has been demonstrated to promote the homing of MSCs in renal ischemia-reperfusion injury in an in vitro as well as an in vivo model in the rat. The hypothesis behind these findings is based on the SDF-1/CXCR-4 signalling pathway, a pathway also important in neo- angiogenesis. CXCR-4 is a chemokine receptor, which is mainly localized in the cytoplasm of cells, but a variety of cytokines can stimulate the expression of membrane CXCR4. Cell surface levels of CXCR4 on MSCs could be enhanced by stimulation with insulin-like growth factor 1, hypoxia inducible factor 1 and TGF-β1[290]. In the same study, a significant alleviation of renal tubular damage was observed after systemic administration of $4x10^6$ MSCs in a bilateral IRI model in the rat. Teo et. al were able to show, that MSCs are not only able to pass the endothelial layer to reach sites of inflammation but are also partially integrated into TNF-alpha activated endothelium in a vascular cell adhesion molecule-1 (VCAM-1) and G-protein-coupled receptor signalling-dependent manner[291]. This supports the findings by Xing et al., who describe improved renal function, enhanced survival and a reduction of macrophage infiltration in a murine renal IRI model after i.v. infusion of $2x10^6$ MSCs, but interestingly, not after infusion of conditioned media alone[292].

Nevertheless, intravenously applied MSCs do not show high rates of engraftment and replacement of damaged tissues in models of organ damage like acute kidney injury (AKI) and myocardial infarction and therefore, it is suggested that MSC beneficial effects could be mediated through paracrine and endocrine modes of action[177,293,294]. For example, in a rat model of acute kidney injury, the effect of MSC treatment was shown to be highly dependent on the ability of the stem cells to secrete the growth factor vascular endothelial growth factor (VEGF) and animals treated with VEGF-knockdown MSCs showed less renal micro vessel density compared to those treated with fully functioning MSCs[295]. Also, insulin like growth factor (IGF-1) seems to play a crucial paracrine role in sustaining MSC mediated renal repair as this growth factor was shown to be highly expressed as mRNA and protein in co-cultures of murine MSCs with cisplatin damaged proximal tubular epithelial cells, and contributed significantly to stimulation of tubular cell proliferation, which was not seen after blocking the function of IGF-1 with a specific antibody[296].

In addition to these paracrine actions in regeneration of damaged tissues, immunologic responses have been observed to be controlled by paracrine actions of the MSCs, for example their expression of prostaglandin E2 (PGE2) can act immunosuppressive because of this cytokine being suspected to be one of the major soluble factors with an ability to influence T-cell activity[297]. Yet, the mentioned mediators are only some examples among many other growth factors and cytokines that MSCs have been described to secrete, such as hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), monocyte chemo attractant protein-1 (MCP-1), fibroblast growth factor (FGF) and cell derived factor-1 (SDF) on the side of the growth factors[295,298–301], and the mediators IL-10, IL-6, TGF-beta, NO and indoleamine-dioxygenase (IDO) on the side of chemokines which locally generate an anti-inflammatory pro reparative cellular state[296,297]. Additionally, MSCs were reported to promote the expansion of regulatory T cells in animal models of heart transplantation[204,302] and also, to promote a shift of macrophages towards the more anti-inflammatory M2 phenotype, which was defined as macrophages with high secretion of IL-10 and IL-6 and low secretion of IL-12 and TNF-alpha[303]. This could influence allograft rejection and therefore, also might contribute to an alleviation of IRI[304].

In an IRI study in the mouse, it could be demonstrated that infusion of MSCs but not conditioned media only, led to a significant higher survival rate ($p=0.02$) at day 7 of the study and furthermore, MSC treated mice recovered from surgery faster than mice treated with DMEM or conditioned media only. Additionally, the prominent infiltration of CD68-positive macrophages observed in the kidneys at day 3 post AKI was dramatically reduced in the MSC infusion group but not in the conditioned media (CM) treatment group[292]. In this study, not even consecutive treatment with a high dose of CM was effective in ameliorating I/R injury. Therefore, it contradicts theories that only paracrine mechanisms would be responsible for effects of MSC infusions in models of kidney injury.

Overall, it seems that MSCs can exert protective effects in organs exposed to ischemia reperfusion injury through anti- inflammatory and paracrine factors, however there is still a lot to learn with regard to the effect of the respective microenvironment on MSC function and how this might impact MSC application in solid organ transplantation.

1.5.5.4. Route of application for MSCs

As the exact mechanisms of action of MSCs in their described immunomodulatory actions in the context of IRI remain unclear, also understanding of their delivery and subsequent bio distribution is lacking. In a study about the time-dependent migration of systemically delivered MSCs in rat model of myocardial infarction (MI), MSCs were labelled with (99m)Tc-HMPAO and injected via the tail vein 7 days after MI. Cell migration and localization was traced by gamma camera imaging at 5, 15, 30 and 60 minutes after cell inoculation. The cells migrated mainly to the lungs (70%) in both the control as well as in the treatment group, but in the MI group, a significantly higher amount of MSCs could be observed in the heart, compared to the amount of cells in the untreated hearts. Xing et al. labelled systemically administered MSCs with the green fluorescent tracer CMFDA and infused them via the tail veins of mice on day 1 after unilateral IR injury to their kidneys. They observed that MSCs were recruited to the injured kidneys but that the number of cells decreased over time and they furthermore found many MSCs in the lungs and spleens, but not in the hearts of the animals[292]. In summary, these studies suggest that systemically delivered MSCs home to sites of injury in a time dependent manner but that systemic delivery is limited by entrapment of the cells in the lungs[294].

This was confirmed in a recently conducted study by Iwai et al., who compared the effects of MSCs delivered ex-vivo via the renal artery to systemic treatment of the recipient with the same concentration of MSCs in a DCD kidney transplant model in the rat. They found that systemic infusion of MSCs did not improve recipient survival and that most of the MSCs were trapped in the recipient's lungs. Contrary to that, ex vivo injection of MSCs via the renal artery showed a benefit regarding survival of the recipient as well as graft function without adverse effects[305]. Also, in an ex vivo perfusion model of human lungs deemed unsuitable for transplantation, MSC administration into that circuit led to a restoration of alveolar fluid clearance to a normal level as opposed to perfusion alone[306]. In another study of lung transplantation conducted in a porcine model, endobronchial nebulization of MSCs prior to preservation and subsequent transplantation showed superior results in terms of the outcomes than trans vascular application of the MSCs, but together suggested that MSC therapy could be useful preventing the bronchiolitis obliterans syndrome seen in human lung transplantation[218].

Another study, performed by Freyman et al. aimed to compare three methods of delivery of MSCs in a randomized design into a porcine model of myocardial infarction. After induction of MI, MSCs were delivered intravenously, intracoronarily or endocardially. Intracoronary and endocardial injection of MSCs post MI resulted in increased engraftment within infarcted tissue when compared to intravenous infusion, and intracoronary application was more efficient than endocardial application. However, intracoronary delivery was also associated with a higher incidence of decreased coronary blood flow[293]. In kidney injury models, a comparative randomized study about the ideal ways of application of MSCs as a therapeutic drug is outstanding and also the ideal number of used stem cells has to be determined in the future.

Chapter 2: Objectives:

2.1. Project 1: Development of a Normothermic Machine Perfusion circuit for ex-vivo kidney preconditioning on the RM3 perfusion machine and testing of a portable micro dialysis device.

The first aim of this PhD project was to develop a normothermic kidney perfusion circuit using the RM3 perfusion machine. The reason for this was that in order to investigate the potential effects of the introduction of MSCs on kidney grafts in ex-vivo machine perfusion, we had to be aware of the potential effect of different temperatures on the viability of Mesenchymal Stem Cells. Therefore, conducting our studies in both, a hypothermic as well as a normothermic machine perfusion model using the machines present in our lab was the logical consequence.
So far, research in our group had been exclusively focused on hypothermic machine perfusion of organs as a preconditioning method. The Waters RM3 perfusion device was built for hypothermic kidney perfusion. Up until now, there is only one commercially available device for NMP of kidneys available (Kidney Assist from Organ Assist, Netherlands). Costs to buy such a device would have exceeded our budget and therefore, objective 1 for us was to establish such a circuit in the laboratory using the RM3 perfusion device.

2.2. Project 2: Investigation and comparison of the effects of current methods of ex-vivo organ preservation on porcine and human kidney grafts after prolonged cold ischemia times.

After establishing a circuit for NMP on the RM3 perfusion machine and before infusing MSCs into the grafts, we were interested in the impact of the different ex-vivo preservation methods on porcine and human kidneys. Many studies have been published on the advantages of either hypothermic- or normothermic machine perfusion of kidneys when compared to static cold storage of the organs, but data on a direct comparison of both methods were scarce.
Therefore, objective 2 was to conduct a direct comparison of HMP and NMP, with reperfusion on one machine and kidneys coming from the same donor, to rule out as many influencing factors as possible.

2.3. Project 3: Investigation of the introduction of MSCs into ex-vivo organ preservation systems as a preconditioning method for porcine and human kidney grafts (translational model).

Objective 3 was the main interest of the conducted research: to investigate the possibility of delivering Mesenchymal Stem Cells into kidney grafts directly, using ex-vivo machine perfusion models. Furthermore, investigation in a translational model, using porcine kidneys in a first instance, and to then try to translate findings into a model using human organs rejected from clinical transplantation, was the goal.
Objectives were to investigate whether Mesenchymal Stem Cells can be delivered into kidney grafts during ex-vivo machine perfusion as a preconditioning method and if so, whether the cells could be traced within the organs. Also, safety and feasibility of the introduction of MSCs in ex-vivo machine perfusion models were to be investigated by comparing physiological parameters of treated and untreated organs directly in a phase of reperfusion.

2.4. Project 4: Investigation of the effects of ex-vivo delivered Mesenchymal Stem Cells on Ischemia-Reperfusion Injury in a rodent autologous kidney transplantation model.

Objective 4 was to investigate whether Mesenchymal Stem Cells, which were delivered to rodent kidneys using an ex-vivo hypothermic machine perfusion model, would have an effect on outcomes after transplantation in terms of renal function. MSCs were extracted from transgenic rats, ubiquitously expressing green fluorescent protein. Another aim was to investigate whether the cells were detectable within the kidneys after ex-vivo perfusion of the organs, as well as after reperfusion up to a time of 24 hours post transplantation. The initial objective was to conduct a bigger controlled in-vivo study with a time of investigation of the renal function for up to one week post transplantation; however, due to the lack of a project licence for animal work for over 2 years of my time as a PhD student, the objectives had to be changed and therefore, more focus was laid on work in the porcine ex-vivo model for which no project licence was necessary, as the animals were sacrificed in a method according to schedule 1 by the technicians in an abattoir.

2.5. Project 5: To investigate mechanisms of action of MSCs and potential differences of GFP+ and WT- MSCs on rodent macrophages in vitro

In order to better understand the mechanisms of action of MSCs in general as well as their potential paracrine influence at sites of inflammation, objective 5 was to conduct an in vitro study on the effects of supernatants from different types of MSCs on macrophages with or without prior inflammatory stimuli. A transgenic WKY rat, positive for an ubiquitous expression of green fluorescent protein (GFP) had been introduced by our group prior to the start of this project. However, it had not been clear to that time, whether the the GFP would also be expressed in bone marrow cells after extraction and whether the expression of GFP would be maintained throughout passaging of such bone-marrow derived cells. Hence, we wanted to find out more about MSCs coming from those animals before using them for in-vivo experiments. As MSCs had been described to act differently, depending on the genetic background of the donor animal, our aim was to investigate whether culture supernatants from GFP+ MSCs had different effects on wild-type bone marrow derived macrophages when compared to supernatants coming from WT-MSCs. Furthermore, we were curious about the effects of donor age on extracted MSCs, which is why we also aimed to include supernatants of different MSC passages from younger and older donors, respectively, into this study. The hypothesis was that results could get us closer to finding out about the exact mechanisms behind anti-inflammatory effects of MSCs in vitro.

Chapter 3: Materials and Methods

3.1. Project 1: Development of a NMP circuit for ex-vivo kidney preconditioning on the RM3 perfusion machine and testing of a portable micro dialysis device.

Our research for this PhD project started with the development of a normothermic kidney perfusion circuit using the RM3 perfusion machine as we did not know what effect the cold conditions in HMP would have on the MSCs and if it would be possible to deliver the cells to the kidney grafts in a viable state using either method. For previous work conducted by our group, the Waters RM3 perfusion machine had been used. The machine was constructed to support ex-vivo HMP of kidney grafts and therefore, a solution had to be found to use the same machine for ex-vivo NMP. The two crucial parts to be integrated into this circuit to make NMP possible were a heater for the right temperature of the perfusate and an oxygenator to deliver oxygen to a red cell based perfusate. With the help of perfusionists from the Department of Cardiothoracic Surgery at the Hammersmith Hospital in London, it was possible for me to learn about Extracorporeal Membrane Oxygenation (ECMO) and to include unused membrane oxygenators (Eos adult or Maquet Qadrox-i adult, ***Fig. 3.1.***) into the circuit. By applying the protocol for NMP, which had been published by Prof. Nicholson et al.[307], we were able to establish an NMP circuit, which was tested on 4 pairs of porcine kidneys to certify the reproducibility. As a continuation to projects of our group in the past, we were working in collaboration with the Department of Bioengineering at Imperial College. The colleagues there are developing the worldwide first portable micro dialysis device to measure lactate and glucose levels from the extracellular space of the kidney in real time (measurements every 30 seconds). The following paragraphs describe the materials and methods used for Hypothermic- and Normothermic ex-vivo kidney perfusion using the RM3 device.

3.1.1. The RM3 hypothermic perfusion machine

The following paragraphs describe the function and details of the Waters RM3 perfusion device, which have been extracted and modified from the RM3 device manual **(Waters Medical Systems RM3 Instruction Manual. Lissieu, France: Institute George Lopez; 2013).** In order to build a circuit for NMP using this machine

perfusion device, it was first essential to understand all the single parts and functions for HMP.

The RM3 Renal Preservation System was built by Waters to maintain kidneys for transplantation. It was one of the first modern and portable self-contained systems for ex-vivo kidney perfusion. The RM3 is a two-part system consisting of (**A**) a control unit (the RM3) and (**B**) a sterile, single- use cassette, in which up to two kidneys at a time can be perfused and monitored (**Fig. 3.1.**). The system displays trends and saves important perfusion parameters, including perfusate flow rates, temperature, perfusion pressure and renal resistance.

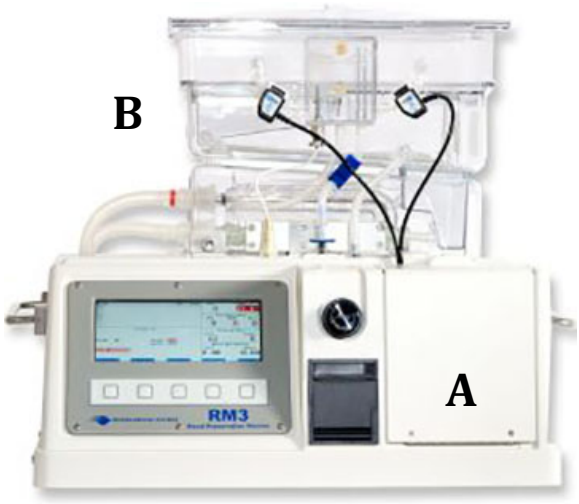


Figure 3.1. The Waters RM3 perfusion machine

Fig. 3.1. *shows the Waters RM3 perfusion device, consisting of (A) the control unit and (B) the sterile single use cassette.*

3.1.1.1. The RM3 control unit

The RM3 control unit regulates and monitors in real-time the pulsatile output of the perfusate. The measured parameters are real-time pressure, -flow and the temperature of the perfusate. A variable stroke volume control allows the operator to mechanically adjust the occlusion grade of the pulsatile pump arm. By turning the stroke volume knob clockwise, the volume of perfusate from the pump head to the outlet (or when connected into the kidney(s)) is increasing. In this way, when an

organ is connected to one or both of the arterial outlets, a corresponding increase of perfusion pressure would be the consequence of the higher volume of perfusate.
On the display screen, during normal operation, in three different sections, the real-time analogue waveform as well as trend-graphs and most importantly, the numerical perfusion parameters, can be read. These include the manually set systolic perfusion pressure as well as the resulting mean and diastolic pressures, and respective flow rates from both the right and the left kidney. Furthermore, the temperature of the perfusate, the renal resistance indices (RRI) of both the kidneys, and the pulse pump rate can be read from the display at all time points of perfusion (see ***Figure 3.2.***). A user can also select alarm conditions for which the system will signal an audio as well as a visual alarm.

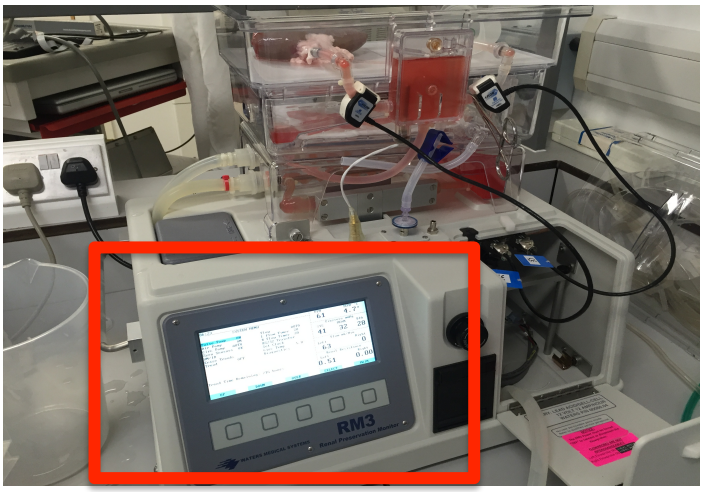


Figure 3.2. The Waters RM3 machine with a kidney attached

Fig. 3.2. *shows the Waters RM3 machine with a kidney attached. Marked in the red square, the display is shown on which continuous measurements are displayed.*

3.1.1.2. The cassette

The MOX DCM-100 cassette is a gravity flow circulation system consisting of an organ chamber, venous and arterial reservoirs, bubble trap, oxygenator, heat exchanger and pulsatile pump head. The perfusate is pumped from the arterial reservoir through the heat exchanger to the bubble trap from where it is delivered to the cannulated kidney(s). The perfusate then recirculates into the arterial reservoir by gravity and thereby passes the right and left venous reservoirs containing a

membrane oxygenator. Via tubing, which is connected to the membrane oxygenator, by switching on an ambient air pump in the control unit, 500ml/min of ambient air can be delivered to further oxygenate the perfusate coming through the venous reservoirs. The cassette is easily attachable onto the RM3 control unit by using the mounting brackets on the left front and right rear connectors on the cassette and can provide circulation of up to one litre of perfusate to one or two kidneys, attached either singly or en bloc. After the cassette is placed and attached onto the control unit, the pulsatile pump head is placed in between the pump arms of the RM3. The pressure transducer line, the inlet tubing for the ambient air pump, the temperature probe cable and the flow transducers have to be attached for the full setup for measurement of all parameters concerning the organ(s). The heat exchange lines are attached, either coming from the ice water bath within the RM3 control unit, or from an external water-heating device. The temperature probe is located in the bubble trap and is controlling a circulation pump located in the ice water bath to thermostatically maintain the temperature of the water bath. In case of use of the external heater, the temperature of the water to keep the perfusate warm is thermostatically regulated by the external heater itself and the temperature is only measured by the temperature probe and displayed on the screen. Unplugging the pump in that case, disrupts the connection to the circulatory pump in the ice water bath. The ice water bath maintains the hypothermic perfusate temperature for approximately three hours at 7°C at an ambient temperature of 22°C. After that time or at different ambient temperatures, the excess water in the water bath needs to be drained and ice needs to be replaced frequently.

The bubble trap prevents air bubbles from reaching the perfused organ(s) and also provides the mounting positions for the perfusate temperature probe and the pressure transducer line. Furthermore, by levelling the fluid within the bubble trap, the pressure gradient for production of the perfusion pressure is being produced, therefore, it is critical to ensure that there are no air leaks in the pressure system. All tubing connectors are tie-strapped for airtight connections. Via two syringe access sample ports, which are located above the fluid level and below the fluid level, respectively, the operator can regulate the proper perfusate level by either infusing or withdrawing air in the former case, or infuse products into the perfusate or take perfusate samples in the latter case. A persistently rising fluid level in the bubble trap indicates a leak in the pressure system. For accurate pressure measurements, the

perfusate level has to be at the indicator line at all time. As perfusate migrating down the pressure transducer line would damage the transducer, a hydrophobic filter is put in between the line and the transducer (WPN 3180003.005) during each operation of the RM3.

The pulse pump arm of the RM3 control unit and the pump head in the cassette together produce a pulsatile flow. Within the pump head, a pair of occlusive valves placed in series directs the perfusate whilst it is being compressed and released by the pump arm. Therefore, the pump head has to be placed correctly into the pump arm assembly with the holding brackets positioned directly at the base of each valve in the pump head. During the initial priming of the cassette, trapped air can be removed by raising the discharge end of the pump arm and holding it at an upward angle whilst manually squeezing the pump head to fill the system up to the indicator line of the bubble trap.

The degree of occlusion of the pulsatile pump arm and hence, the volume of perfusate being pumped from the pump head to the kidneys can be manually adjusted by turning the stroke volume knob on the RM3 control unit clockwise. The increased volume of perfusate goes along with an increased pressure in case an organ is attached to the respective outlet. The venous outflow from each organ is collected in the respective venous reservoirs. The flow from the right half of the cassette flows into the left venous reservoir and vice versa. The device measures the flow rate by timing the flow from each kidney into the venous reservoir.

3.1.1.3. Parameter measurements in organ perfusion using the RM3

3.1.1.3.1. Measuring the flow

The RM3 measures flow in two different ways. The first method uses Transonic ultrasonic clamp-on flow probes, which can be attached on each inlet tube coming from the bubble trap and going into the organ chamber. These flow-probes measure real-time flow values by ultrasound signal method. The sterile perfusate does not come in contact with the flow probe and therefore, the flow probe can be reused.

- **How the Flow probes work:**

  The Transonic flow probes in the RM3 work with transit time ultrasound technology to accurately measure the perfusate flow. Each flow probe contains two transducers that generate ultrasonic beams, which alternately intersect the perfusate. The volume flow is calculated as the difference between the upstream and downstream ultrasonic beam transit times. The flow probes are pre-calibrated for the use with the RM3 and the perfusate.

  The second method for measuring the flow is the Direct Timer Method. This method is based on the filling time of the venous reservoirs. This method, however was not used for our experiments.

The values for the flow rates are automatically placed in the appropriate display areas on the screen in the control unit of the RM3.

3.1.1.3.2. Pressure Measurements

A replaceable Honeywell transducer measures the perfusion pressure of the organ(s). The control unit displays digitally as well as in analogue waveform the systolic, mean and diastolic pressures and the values are continuously updated. The accuracy of these measurements can be checked with a sphygmomanometer and to ensure correct values, the fluid level in the bubble trap should be at the perfusate level mark. The perfusate level can be controlled by insufflation of air into the fluid level adjust port by the use of a 30cc syringe with a 22 gauge or smaller needle.
The mean pressure is calculated by the formula:

$$(2 \times \text{Diastolic} + \text{Systolic})/ 3$$

3.1.1.4. Circulation (CIRC) Pump and Reservoir

The circulator pump (CIRC PUMP) is a submersible pump located in the 5-litre coolant reservoir. It circulates the water from the reservoir through the cassette's

heat exchanger at a rate of 3.5 litres per minute and thereby, cools the perfusate. The circulating water is kept cold by frequently adding ice cubes to the reservoir. Hence, periodic drainage of coolant water is necessary as more ice is added. The electrical connector between the pump and the machine is located inside and at the upper front of the reservoir and it is important to keep that part dry, hence, the condensed water needs to be emptied via the outlet tubing located inside the battery compartment of the RM3 control unit when ice is added. After each use, the coolant tank is being emptied completely.
Control of the hypothermic cooling time also depends on the ambient conditions and the use of a cassette cover helps to maintain the temperature for a longer time. An alternative cooling or heating method can be used and in that case, the circulator pump has to be turned from AUTO to OFF in the System Menu.

3.1.1.5. Air Pump

When switched on in the System Menu, the air pump circulates approximately 500ml/min of ambient air over the oxygenator membrane into the cassette. This allows more air to circulate across the oxygenator membrane. Also removing the air tubing from the air pump can use gas mixtures other than ambient air and connecting a flow limited, pressure regulated gas mixture. The maximum flow should be set no higher than 1 litre per minute (l/m) and 2 psi (100mmHg).

3.1.1.6. Temperature Sensor

The RM3 will report temperature readings from 0 to 45°C. For values outside this range the RM3 will display - - (for readings less than 0°) and + + (for readings greater than 45°C).

3.1.1.7. RM3 operational Checklist

Before each experiment, the RM3 machine was primed and the cassette was filled with the respective fluid for each preservation method or for the reperfusion phase. In case of normothermic perfusion, an oxygenator was attached in between the venous reservoir and the bubble trap. In that case, the circuit was primed with Ringers solution to make sure there were no air bubbles trapped in the circuit.

The following things were checked before an organ was attached to the circuit for HMP:

1) Perfusion cassette attached to the RM3 Machine via the top mounted screw clamps.
2) 1.5L of water and ice added to the coolant reservoir.
3) Connection of the cassette gas inlet tubing to the RM3 machine.
4) Connection of the temperature probe to the cassette and RM3 machine.
5) Attachment of the Transonic flow probes to the arterial tubing at the correct sides.
6) Attachment of the tubing for the coolant fluid to the cassette.
7) Priming of the cassette with the desired volume of perfusate (UW solution, KHB, packed red cell bases perfusate).
8) De-bubbling of the pump head once the machine is switched on and perfusate flowing through the cassette.
9) Adjusting the fluid level in the bubble trap to correspond to the centre line.
10) And once the perfusate is at the desired temperature – (4-8˚C or normothermic), the pressure sensor and flow probes are zeroed.

3.1.2. Ex vivo normothermic machine perfusion using the Waters RM3 kidney perfusion device for human and porcine organs

3.1.2.1. The circuit for NMP using the RM3 machine

So far, there is no commercially available machine for ex-vivo Normothermic Kidney Perfusion (EVNKP) on the market. The Oxford group is currently working on a machine similar to the Metra (OrganOx) Normothermic Liver perfusion device, but meanwhile, most normothermic perfusion systems have been built by individual groups in slightly different ways by putting together centrifugal pumps, oxygenators and heating devices to create circuits based on the extracorporeal membrane oxygenation principle (ECMO), used in cardiothoracic surgery, when patients are connected to a heart-lung-machine. In order to perform Normothermic ex-vivo Kidney Perfusion at our lab, the Waters RM3 perfusion units were modified by incorporation of a few essential components of normothermic perfusion. Both the Waters control

unit as well as the DCM-100 cassettes (***Fig 3.1.***) were used as the basic central pieces of equipment. For NMP, in order to reach the required perfusate temperature, an automated temperature controlled water heating pump (Grant, ***Fig. 3.3.***) had to be incorporated. For an adequate and controlled oxygenation of the perfusate, an oxygenator was implemented into the circuit. Oxygenators, usually used in extracorporeal cardiopulmonary bypass technology (Eos, Maquet, ***Fig. 3.5.***), were used. The oxygenators had been opened in operating theatres at the Department of Cardiothoracic Surgery at the Hammersmith Hospital, but had not been used, so they were new and clean. By delivering a mixture of 95% O_2 and 5% CO_2 to the oxygenator by attachment of tubings at the correct size, the perfusate could be enriched with oxygen. Carbon dioxide was included in the mixture to supplement acid buffering within the perfusate during reperfusion. In order to control the flow rate of the delivered O_2/CO_2 mix, a flow meter was interconnected. The circuit was primed with Ringer's solution and bubbles were removed before adding the perfusate to the circuit.

Once the machine was activated, the perfusate was coming out of both the arterial outlets with the flow probes attached, collected in the venous reservoirs, pumped through the heating valves, through the oxygenator and back into the arterial reservoir with the bubble trap and the temperature probe to ensure the presence of the correct temperature. This circuit was suitable to be used for NMP of both porcine and human organs, as they are similar in size and anatomy and besides this, perfusion was conducted pressure controlled for each organ individually, depending on its weight and condition. For preconditioning of organs, one of the two arterial outlet tubings was usually clamped to enable perfusion of a single organ. ***Fig. 3.4.*** shows the circuit for HMP on the left side (***A***), and the modified circuit for NMP with a built in membrane oxygenator which is connected to the O_2/CO_2 on the right side (***B***).

Figure 3.3. The Grant water-heating pump

Fig. 3.3. *illustrates the automated temperature controlled water pump (Grant)*

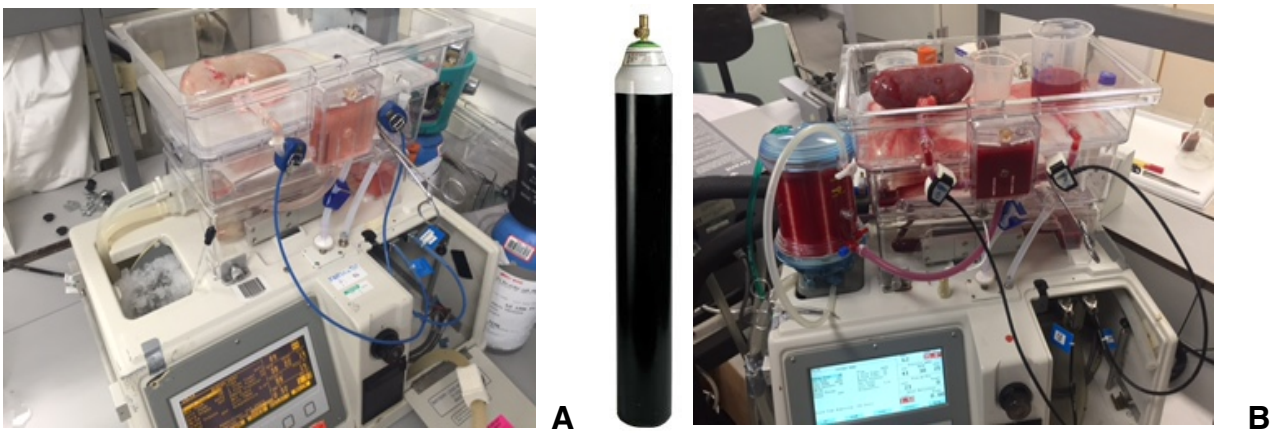


Figure 3.4. Circuits for Hypothermic and Normothermic machine perfusion using the RM3 machine

Fig. 3.4. *shows the circuit for Hypothermic Machine Perfusion (HMP) on the left* ***(A),*** *and the modified circuit for Normothermic Machine Perfusion (NMP) of kidneys in comparison on the right* ***(B)****. Instead of cooling the perfusate down with ice, the fluid was kept at physiological temperatures by connecting the Grant temperature controlled water pump (****Fig. 3.3.****) to the machine instead oft the tubings attached to the cooling reservoir. A membrane oxygenator was delivering O_2/CO_2 to the perfusate. On both pictures, one kidney is attached to the respective circuit and the other perfusate outlet (for a potential second kidney) is clamped.*

3.1.2.2. Membrane Oxygenation for NMP

As mentioned above, 2 types of membrane oxygenators were built into the machine perfusion circuit, which had been used for HMP, in order to be able to convert to NMP with a red cell based perfusate. The oxygenators were kindly provided from the Department of Cardiothoracic Surgery at the Hammersmith Hospital after being allocated to a patient but then not needed during surgery.

The **EOS ECMO** (***Fig. 3.5. A***) is equipped with a plasma tight Polymethylpentene (PMP) hollow fibre, which provides stable performance in extended cardiac-respiratory support. The design has been focused on a low priming volume (150 ml), a reduced membrane surface area (1.2 m^2) and the entire blood contacting surface is coated with the biocompatible Phosphorylcholine PHISIO coating. It supports a blood flow of up to 5 litres/min.

The **Maquet QUADROX-i** Oxygenators (***Fig. 3.5. B***) are equipped with a micro porous membrane. Oxygenators contain two chambers. In the first chamber gas fibre mats made of micro porous polypropylene are alternated with heat exchange mats made of polyurethane. The blood is temperature-adjusted, oxygenated and de-carboxylized. The priming volume is 215 ml, the membrane surface area is 1.8 m^2 and this oxygenator supports a blood flow of up to 7 litres/min.

Figure 3.5. Types of oxygenators

***Fig. 3.5.**: Oxygenators which had been opened but not needed for cardiothoracic surgery are shown in this picture. The Eos ECMO (**A**) or the Maquet Quadrox-i adult (**B**) were used for membrane oxygenation of the red cell containing perfusate during Normothermic Machine Perfusion (NMP).*

3.1.2.3. The perfusate

3.1.2.3.1. Perfusate for Porcine NMP experiments

The perfusate for ex-vivo Normothermic Kidney Perfusion of porcine organs mainly consisted of leukocyte depleted autologous blood and 0.9% Sodium Chloride. ***Table 3.1.*** shows the exact components of the perfusates used for NMP experiments, as published by Nicholson and Hosgood[307]. After arrival at the laboratory with the porcine kidneys and the heparinized autologous porcine whole blood, approximately 500mls of blood were depleted of leukocytes by using leukocyte filters, provided by Macopharma (***Fig. 3.6. A***)**.** 500mls of whole blood resulted in 250-300ml of leukocyte depleted blood (1 unit). The blood, as well as the remaining whole blood, was then transferred into CPDA-1 bags, also provided by Macopharma (***Fig. 3.6. B***), for overnight storage at 4°C. The amount of added sodium bicarbonate 8.4% was dependent on the pH of the perfusate. Every time, several arterial blood gas measurements had to be undertaken whilst titrating the adequate amount to the perfusate prior to starting experiments, until the pH was within a physiological range (7.35-7.45).

Components	
Perfusate	
Leukocyte depleted autologous blood	250 mL
Compound sodium chloride (Baxter Healthcare, Thetford, UK)	250 mL
Mannitol 10% (Sigma-Aldrich)	25 mL
Dexamethasone 10 mg (Organon Laboratories, Cambridge, UK)	2 mL
Cefuroxime 750 mcg (Stragen, Reigate, UK)	5 mL
Sodium bicarbonate 8.4% (Fresenius Kabi, Cheshire, UK)	12 mL
Heparin 1000 iu/mL (CP Pharmaceuticals, Wrexham, UK)	2 mL
Creatinine (Sigma-Aldrich, Steinheim, Germany)	1500 µmol/L

Table 3.1. Components for the perfusate for Normothermic Machine perfusion of kidneys

Table 3.1. *shows the components used for the NMP perfusate[322]. Published by Hosgood et al. this table was used as a guide to produce NMP perfusate, however sodium bicarbonate volumes were varied according to the pH.*

Figure 3.6. Leukocyte filtration and blood storage system

Fig 3.6. *illustrates the leukocyte filtration system (Macopharma,* ***A****) and the CPDA-1 blood bags (Macopharma,* ***B****) used to filter and store the porcine blood over night after collection at the abattoir.*

3.1.2.3.2. Perfusate for NMP experiments on human organs

For NMP experiments on human organs discarded from transplantation, either Krebs-Henselleit buffer (Sigma-Aldrich, Product Number K3753) was used or, whenever possible, expired units of blood group compatible packed red cells coming from the blood bank at Chelsea Westminster Hospital, were used to produce the same perfusate as explained above in detail for porcine experiments (***Table 3.1.***).
Hans Krebs and Kurt Henseleit developed krebs-Henseleit buffer in the early 1930's. This modification of Ringer's solution was used to maintain liver tissue during experiments that led Krebs to postulate the urea cycle. The formula offered by Sigma has been modified by the addition of 2 g/L of glucose as an energy source for cell maintenance, and by the omission of calcium chloride.

Components:	**g/L**
D-Glucose	2.0
Magnesium Sulphate	0.141
Potassium Phosphate Monobasic	0.16
Potassium Chloride	0.35
Sodium Chloride	6.9

The solution was prepared according to manufacturer's instructions:

1. 90% of final required volume of water was measured out.
2. While gently stirring the water, the powdered medium was added and stirred to dissolve.
3. 0.373 g of calcium were added to the solution.
4. 2.1 g sodium bicarbonate or 28 ml of sodium bicarbonate solution (7.5% w/v) for each litre were added and stirred until dissolved.
6. Whilst stirring, the pH of the medium was adjusted by titration of HCl or NaOH.

3.1.2.4. Ex-vivo normothermic perfusion of porcine and human kidneys

Once the circuit was primed and pre-filled with perfusate (600ml/kidney) at a temperature in between 35°C and 37°C, the respective kidney was attached to the circuit. The mean perfusate pressure was adjusted to 50mmHg. The perfusate was pumped through the circuit with the heat exchanger (grant water bath) maintaining a perfusate temperature of 35°C-37°C, and a gas exchange unit (oxygenator) maintaining a pO2 of 40 to 60 kPa. The gas for exchange was 95% O2 and 5% CO2, the latter for pH homeostasis. The perfusate was supplemented by infusions of colloid containing nutrients to replace urinary losses as described above (***Tab. 3.1.***). To support the best possible microcirculation, 0.05 mg epoprostenol were administered per hour and also 5ml of 5% glucose solution were infused to maintain the glucose concentration in the perfusate. To maintain the physiological pH, 8.4% sodium bicarbonate (Fresenius) was added to the perfusate in varying amounts (depending on the pH). 750 mg Cefuroxime were added to the perfusate already after putting the venous porcine blood into the storage bags to minimize micro bacterial contamination and furthermore, before start of the perfusion period, Creatinine was added. The warm oxygenated blood perfused the kidney through the cannulated renal artery and drained back into a venous reservoir. The ureter was cannulated and urine drained away from the circuit.

3.1.3. Application and testing of a portable Rapid Sampling Micro dialysis device for Organ viability assessment

3.1.3.1. Development of a portable rsMD device for monitoring during transport

During our experiments on the porcine DCD model, the feasibility of using a portable rapid sampling micro dialysis (rsMD) system for tissue viability assessment was tested. During transportation of the organs from the abattoir to the laboratory, the micro dialysis probes were in place and the influences like movement of the boxes containing the organs on the readings on the iPads were carefully observed.

RsMD assessment of tissue metabolism was conducted in collaboration with the Department of Bioengineering, Imperial College London. The micro dialysis device was custom made and consists of a fine tubular dual lumen micro dialysis probe, with a semi-permeable membrane. The probe was inserted into the tissue of interest by pre-forming a tiny tunnel with a 21G needle. Once inserted, a physiological isotonic solution is delivered at a constant flow rate through the inlet tubing of the probe, past a semi-permeable membrane tip. This creates a concentration gradient across the membrane, causing low molecular weight compounds to diffuse across the membrane, whilst larger molecules such as proteins, or those bound to proteins are excluded by the inherent pore size of the membrane. The dialysate is sampled at the outlet port and analysed for compounds of interest.

One micro dialysis probe (MAB11.35.4, Microbiotech, Stockholm, Sweden) was inserted superficially into the cortex of each kidney (***Figure 3.7.***) immediately after the intraabdominal organ package was retrieved and before the kidneys were mobilised and dissected, so that also the period of warm ischemia time could be observed.

The probes were analysed in real-time for levels of glucose and lactate, using a tablet. RsMD devices were initially developed for other fields in medicine, for example to monitor brain injury[308], bowel ischemia[309], or free flap surgery[310] and some changes had to be made to make them suitable for monitoring a pair of kidneys. The probe inlet tubing is connected to a syringe pump (CMA 400 pump, CMA Microdialysis, Stockholm, Sweden) and perfused with T1 physiological solution (2.3 millimolar [mM] calcium chloride, 147 mM sodium chloride, 4 mM potassium chloride) at a rate of 2 µL/min. This flow rate was chosen to minimise the delay in the dialysate reaching the analyser whilst maximising the recovery of the probe. The

outlet tubing of the probe was connected to the 200 Nano litres (nL) sample loop of a custom-made 6-port internal loop valve (Valco Instruments, Schenkon, Switzerland). A high-pressure liquid chromatography pump (Rheos 2000, Flux Instruments, Basel, Switzerland) pushes a filtered analysis buffer (0.1 mM sodium citrate, 150 mM sodium chloride, 1.0 mM ethylenediaminetetraacetic acid, 1.5mM ferrocene monocarboxylic acid, filtered through 100 nm, then 20 nm anodisc membranes) at 100 µL/min into an analysis loop within the valve. The outlet tubing of two probes is connected to two separate sample loops of the valve, either side of the analysis loop, allowing two kidneys to be monitored simultaneously. The stream accelerates the dialysate through an enzyme reactor containing lactate oxidase (LOx) and horseradish peroxidase (HRP) (Genzyme Diagnostics, Kent, UK). The LOx recognises the analyte, producing hydrogen peroxide, which reacts with the HRP. The HRP is regenerated by oxidation of the ferrocene mediator species, producing ferrocinium ions, which are detected at the downstream electrode (BASi, West Lafayette, IN) by reduction. Due to the small sample size, the current produced is directly proportional to the concentration of lactate in the sample, even above the free solution Km of the lactate oxidase enzyme (0.7 mM). Data was collected using a PowerLab data acquisition unit (8/30, ADInstruments, New South Wales, Australia) and LabChart software (ADInstruments, New South Wales, Australia) running on a MacbookPro portable computer (Apple Computers, Cupertino, CA).

Fig. 3.8. shows, as an example, the readings obtained from one of the kidneys from the point of retrieval until arrival at the lab, when the calibration process of the probes started for measurements during the actual perfusion experiments on the next day.

The recorded peaks were separated in Matlab (R2011b, MathWorks, US), to produce results for each kidney individually, and were converted into concentrations based on the calibration (using different concentrations of lactate) carried out before, during, and after each experiment. The results were adjusted to correct for the transit time between the micro dialysis probe and the analysis system.

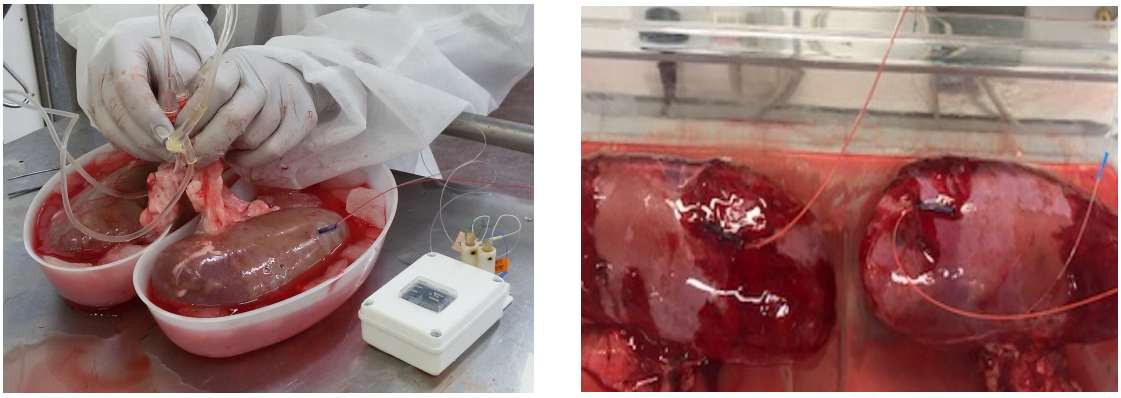


Figure 3.7. Insertion of micro dialysis probes

Fig 3.7. *The micro dialysis probes were inserted superficially into the cortex straight after retrieval of the intraabdominal organ package and before starting the cold flush. Sampling every 30s allowed for monitoring of lactate –and glucose concentrations in the extra cellular fluid with a high temporal resolution (Jones et al. 2000).*

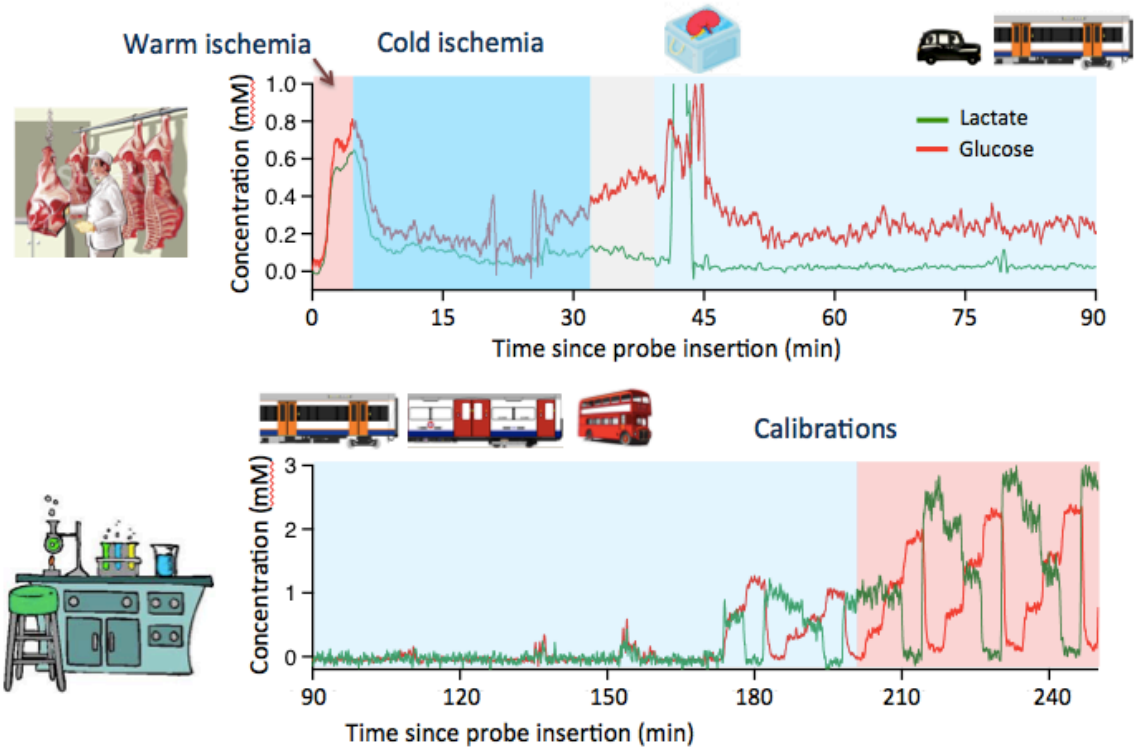


Figure 3.8. Schematic illustration of micro dialysis readings during organ retrieval

Fig 3.8. *Schematic illustration of the glucose- and lactate measurements from the time of insertion of the probes until reaching the laboratory. On a timeline of events including the phase of warm ischemia time (arrow) and different methods of transport, the green line shows the continuous lactate concentrations and the red line shows the glucose concentrations. A peak in both values was observed during warm ischemia time (arrow).*

3.2. Project 2: Investigation and comparison of the effects of current methods of ex-vivo organ preservation on porcine and human kidney grafts after prolonged cold ischemia times.

3.2.1. Graft- and blood retrieval for the porcine ex-vivo perfusion model

Porcine kidneys were obtained from adult landrace pigs of approximately 70 – 90kg at an abattoir. After sacrifice by a gunshot to the head and exsanguination, the intra abdominal organ package was retrieved. This equals a model of controlled DCD kidney donation with a warm ischemia time between 20 and 30 minutes. Approximately 1l of venous blood was collected into a plastic flask containing 20000IU of Heparin and 750mg Cefuroxime. During transportation to the lab, the blood was kept at room temperature. In the lab, 500ml of the blood were transferred into CPDA-1 blood collection bags (Macopharma, ***Fig. 3.6. A***). For the NMP protocol, the other 500ml of whole blood were filtered using a white cell filter (Macopharma, ***Fig. 3.6. B***), in order to produce 1 unit of leukocyte- depleted blood. All the bags containing either whole blood or leukocyte- depleted blood were then stored in the fridge over night at 4°C.

Both kidneys from one donor pig were harvested immediately after the intraabdominal organ package was removed from the cadaver and put onto a dissection table (***Fig. 3.9. A***). The kidneys with their ureters and renal arteries were identified and freed from surrounding tissue. The aorta was cut open and aortic patches were left for the renal arteries, respectively. The kidneys were carefully harvested, separated and the ureters were cut approximately 10 cm distal to the pyelum. Thereafter, each kidney was immediately placed into a dish containing ice and perfusion fluid was flushed into the kidneys using a 14G cannula, respectively. The kidneys were flushed with 500ml of Soltran kidney preservation solution (Baxter) of 4°C at hydrostatic pressure (100cm H_20) until the venous effluent was clear (***Fig. 3.9. B***). The warm ischemia time for all the kidneys was kept in between 20 and 30 minutes, only for a group of kidneys for a separate experiment on the use of the portable micro dialysis device, it was deliberately kept at 45 minutes (as explained below). After flushing, the kidneys were stored in a plastic bag filled with Soltran on ice until arrival at the laboratory, where the bags containing the kidneys were transferred into the fridge for over night storage at a temperature of 4° C for 24 hours of Cold Static Storage (CSS).

Figure 3.9. Retrieval process for porcine organs

Fig 3.9. *The retrieval at the abattoir: after retrieval of the intra abdominal organ package from the pig, the two t0 core biopsies were taken, respectively (**A**)*, before the kidneys were dissected from surrounding tissue and the renal arteries were cannulised to start the cold perfusion on ice with Soltran perfusion solution (Baxter Healthcare Ltd.) in two separate kidney dishes (***B***).

3.2.1.1. Short (20 minutes) ischemia time

Pigs were sacrificed as described above. The time between death and flushing was maintained at 20 minutes. During this time, organs were dissected and prepared for flushing. After 20 minutes, the kidneys were flushed with 500ml of Soltran solution, respectively, at a pressure of 100cm H_2O. Organs were flushed until effluent via the venous drainage was clear and then packed in the same flush preservation solution for the transport back to the laboratory. Transport time was approximately 3 hours.

3.2.1.2. Long (45 minutes) ischemia time

Pigs were sacrificed as described above. The time between death and provision of the organs was kept at 45 minutes. During this time, the kidneys were dissected and prepared for flushing. The cold flush with 500ml of Soltran at a pressure of 100cm H_2O was initiated and again, kidneys were flushed until effluent via the venous drainage was clear. The transport time to the laboratory was approximately 3 hours.

3.2.1.3. Graft Anatomy and bench work: porcine vs. human kidneys

The size and the anatomy of porcine kidneys are similar to human kidneys. The renal artery and vein were dissected and freed from surrounding tissue in the lab and ties

were placed to ensure there were no leaks. Porcine kidneys have one main renal artery coming from the aorta and going into the kidney as well as one vein in most of the cases. In human kidneys, it is much more often the case that there are additional arteries, either coming directly from the aorta or splitting proximally from the main renal artery. Also, more than one vein or ureter can be present in human kidneys quite frequently, so the bench work for human organs usually consumed more time than the bench work for porcine kidneys. For all organs, an 18-French urinary catheter was placed into the ureter for urine collection during the experiments. During all times of the benching, the kidneys were placed into a kidney dish containing ice and soltran solution. The kidney grafts were weighed using electronic scales and the renal artery was cannulised with a perfusion cannula used for attachment to the RM3 arterial tubing. The vein was left to drain freely.

3.2.2. Normothermic perfusion of porcine kidneys

After 24 hours of cold static storage (CSS), kidneys for NMP were taken out and benched. 9 pairs of kidneys coming from the same donor pig were used for a direct comparison of HMP vs. NMP. Additionally to that, 6 pairs of porcine kidneys were used for a comparison between NMP alone or NMP with infusion of 1-5x10^6 MSCs into the circuit. Another group of kidneys (n=3) underwent NMP after an extended warm ischemic time of 45 minutes, with a respective kidney from the same donor pig undergoing HMP for a direct comparison and with a special interest on micro dialysis readings during prolonged warm ischemic times.

After removal of the organs from CSS, two skin punch core biopsies of the upper pole were taken, respectively (t24h biopsy; 1 sample for histology, 1 sample for RNA processing). A steel cannula of suitable size was inserted into the renal artery and secured with a silk tie. For collection of urine, a Foley catheter was placed into the ureter and secured by expansion of the balloon. The oxygenator (Eos ECMO, Sorin group, CA or Maquet Quadrox-i) for the NMP perfusion setup was primed with the respective recommended volume of Ringer's Lactate solution and attached to the RM3 pulsatile perfusion machine (Waters Medical Systems, Rochester, MN USA). The perfusate for NMP was prepared according to the protocol published by Hosgood et al., which can be found in ***Table 3.1.***[4] A total of 0.1 g of creatinine was added to the perfusate, resulting in an end concentration of approximately 0.2mg/ml

(depending on the creatinine concentration in the pig's blood). After warming up the perfusion circuit to a minimum of 35°C, a perfusate sample was taken and analysed with a blood gas (ABG) analyser (GEM Premier 4000). The pH was corrected to a physiological range by adding Sodium Bicarbonate 8.4% (Fresenius Kabi, Cheshire, UK). As soon as the target temperature was reached and the pH of the perfusate was within range (7.35-7.45), the kidney was attached to the machine. The temperature of the perfusate was kept at 35°C- 38°C. The mean arterial blood pressure was kept in between 40mmHg and 50mmHg by manually controlling the machine.

During 4 hours of NMP, the temperature, arterial blood pressure, intra-renal resistance index (RRI), perfusate flow, urine production and pulses-per-minute (PPM) were carefully noted at 0, 0.25, 0.5, 1, 2, 3 and 4 hours of perfusion. Furthermore, at 0, 0.25, 1, 2, 3 and 4 hours of perfusion arterial blood, venous blood and urinary samples were collected into sterile Eppenorf cryovials. At each time point, further arterial and venous perfusate samples were collected and analysed with the Arterial Blood Gas machine. During perfusion, prostacyclin (Epoprostenol, Flolan), Mannitol 10% solution (Baxter) and Glucose 5% solution (Fresenius) were added to the perfusate in units of 5mL per hour[1]. Urine volume was replaced with the same volume of Ringer's lactate. After four hours of perfusion, the kidney was detached from the machine and flushed with 500mL of cold Soltran solution.

3.2.3. Normothermic perfusion of human kidneys

Human kidneys undergoing NMP (n=7) were received at De Wardener ward of the Hammersmith Hospital after being declined for clinical transplantation for different reasons. At the time of acceptance of these organs, cold ischemic times were different as this was influenced by the cold ischemic time present at the time of rejection as well as by the organ location. Upon arrival at the laboratory, human organs for Normothermic machine perfusion were benched and a steel cannula of a suitable size was tied into the artery to be attached to the machine. Two skin punch biopsies were taken, one for histological evaluation and one for RNA processing, respectively. The kidneys were weighed and as soon as the circuit for NMP was ready, the kidneys were attached to undergo NMP for 4 hours. The ureter was cannulised with a Foley catheter for the collection of urine. The perfusate used was

either 600ml of Krebs-Henselleit buffer at 37-38°C (n=6) or, when available, 1 unit of blood group compatible packed red cells to produce a perfusate as described for porcine NMP experiments (n=1) (***Tbl. 3.1.***). A mean arterial pressure of 50mmHg was maintained throughout the perfusion period and a total of 0.1g of creatinine was added to the perfusate, resulting in an end concentration of approximately 0.2mg/ml. During 4 hours of NMP, the temperature, arterial blood pressure, intra-renal resistance index (RRI), perfusate flow, urine production and pulses-per-minute (PPM) were carefully noted at 0, 0.25, 0.5, 1, 2, 3 and 4 hours of perfusion. Furthermore, at 0, 0.25, 1, 2, 3 and 4 hours of perfusion arterial blood, venous blood and urinary samples were collected into sterile Eppenorf cry vials. At each time point, further arterial and venous perfusate samples were collected and analysed with the Arterial Blood Gas machine. During perfusion, prostacyclin (Epoprostenol, Flolan), Mannitol 10% solution (Baxter) and Glucose 5% solution (Fresenius) were added to the perfusate in units of 5mL per hour[1]. Urine volume was replaced with the same volume of Krebs-Henselleit buffer or Ringer's lactate in case of packed red cell based perfusate. After four hours of perfusion, the kidney was detached from the machine and flushed with 500 mL of cold Soltran solution. Thereafter, the organs were reperfused for 2 hours with Krebs-Henseleit Buffer at 37°C and a mean arterial perfusion pressure of 80 mmHg.

3.2.4. Hypothermic perfusion of porcine kidneys

After the period of cold static storage (CSS), kidneys undergoing HMP were taken out and benched in the same way as kidneys undergoing NMP. In 9 cases (n=9), pairs of kidneys coming from the same donor pig were used for a direct comparison of HMP vs. NMP, meaning that these pairs were thereafter reperfused on one machine as a pair. In 6 other cases (n=6), kidneys underwent HMP as a control group for kidneys undergoing HMP with infusion of MSCs into the circuit. These kidneys were analysed as a separate group as well as part of a pool of all kidneys undergoing HMP (n=15) only, after the reperfusion period. A third group of kidneys (n=3) underwent HMP after an extended warm ischemic time of 45 minutes. This group was also analysed separately, with a special interest on results of the micro dialysis measurements. After removal of the kidneys from CSS, two skin punch biopsies were taken as it was described for kidneys of other treatment groups. The renal artery was cannulised and a Foley catheter was placed into the ureter as

described earlier for the NMP protocol. The RM3 pulsatile perfusion machine was filled with 500mL of modified UW solution (Belzer) and the circuit was prepared according to manufacturer's instructions. The preservation solution was cooled down to 4°C within the machine by filling the reservoir for the cooling fluid with ice. Whilst the kidneys for HMP were perfused for 4 hours, the temperature of the perfusate was being kept in between 4°-6°C. The mean arterial pressure was kept at 40mmHg and the pump frequency was approximately 60PPM. The temperature, arterial blood pressure, intra-renal resistance index, perfusate flow, urine production and pulses-per-minute were carefully noted at 0, 0.25, 0.5, 1, 2, 3 and 4 hours of perfusion.

3.2.5. Hypothermic perfusion of human kidneys

13 human kidneys underwent ex-vivo hypothermic machine perfusion, either as part of one pair in a direct comparison, or as control samples, or as part of the study on MSCs in hypothermic machine perfusion. After removal of the kidneys from CSS, two skin punch biopsies were taken as it was described for kidneys of other treatment groups. The renal artery was cannulised and a Foley catheter was placed into the ureter. The RM3 pulsatile perfusion machine was filled with 500mL of modified UW solution (Belzer) and the circuit was prepared according to manufacturer's instructions. The preservation solution was cooled down to 4°C within the machine by filling the reservoir for the cooling fluid with ice. Whilst the kidneys for HMP were perfused for 4 hours, the temperature of the perfusate was being kept in between 4°-6°C. The mean arterial pressure was kept at 40mmHg and the pump frequency was approximately 60PPM. The temperature, arterial blood pressure, intra-renal resistance index (RRI), perfusate flow, urine production and pulses-per-minute were carefully noted at 0, 0.25, 0.5, 1, 2, 3 and 4 hours of perfusion.

3.2.6. Application of rapid sampling micro dialysis measurements during the experiments

RsMD was instituted during these experiments in line with the methodology described in Project 1. Micro dialysis probes (MAB11.35.4, Microbiotech, Stockholm, Sweden) were inserted superficially into the parenchyma of the lateral cortex of some of the kidneys at the start of HMP or NMP. These probes were connected to an in-

house built rapid-sampling micro dialysis analyser. Every 30 seconds a 200nL dialysis sample is automatically injected into the analysis flow stream, alternating between each kidney. The stream accelerates the dialysate through an enzyme reactor containing the enzymes lipoxygenase (Lox) and horseradish peroxidase (HRP, Genzyme Diagnostics, Kent, UK). The LOx recognises the analyte, producing hydrogen peroxide, which reacts with the HRP. The HRP is regenerated by oxidation of the ferrocene mediator species, producing ferrocinium ions, which are detected at the downstream electrode (BASi, West Lafayette, IN) by reduction. Due to the small sample size, the current produced is directly proportional to the concentration of lactate in the sample. Data were collected using a PowerLab data acquisition unit (8/30, ADInstruments, New South Wales, Australia) and LabChart software (ADInstruments, New South Wales, Australia) running on a Macbook Pro portable computer (Apple Computers, Cupertino, CA). Lactate levels were analysed in real-time to reveal information on interstitial lactate concentration during the 4 hours of preconditioning as well as during the 2 hours of reperfusion, as an indication of tissue viability and metabolism.

3.2.7. Provision of Oxygen during Hypothermic Machine Perfusion

For organs on HMP, oxygen was administered by inserting a thin hollow probe through which an oxygen / carbon- dioxide mixture (95% O2, 5% CO2) was passed into the arterial reservoir to allow effective oxygenation of the perfusate. Carbon dioxide was included in the mixture to supplement acid buffering within the perfusate during reperfusion.

3.2.8. Cold Static Storage of porcine and human kidneys

All kidneys in this group, of porcine as well as human origin, were stored at 4°C in a plastic bag filled with Soltran for 28 hours. These organs underwent reperfusion thereafter and served as control groups for all the other experiments in order to have an idea of what results kidneys without any pretreatment but after prolonged cold ischemic time would show. After SCS, the kidneys were benched and weighted. Two skin punch biopsies from the upper pole were taken as described for the other groups, respectively. A cannula was inserted into the renal artery and a Foley catheter was placed into the ureter as previously described for the other groups.

3.2.9. Reperfusion of porcine organs after preconditioning

After NMP, HMP or CSS, all porcine kidneys were reperfused for two hours on the RM3 pulsatile perfusion machine. The reperfusion perfusate consisted of 600mL pig's whole blood, which had been stored in CPDA-1 bags overnight after the organ retrieval. The whole blood was diluted 1:1 with 600mL of 0.9% Saline (Baxter) and spiked with a total of 0.2g Creatinine, resulting in a concentration of 0.4mg/ml (depending on the creatinine concentration of the blood). 5000IU of Heparin were added for anticoagulation. Oxygen was delivered to the perfusate via an oxygenator at a flow rate of approximately 0.5l/h. Reperfusion was mimicked by keeping a temperature of 36.5°C and a systolic arterial blood pressure of approximately 100mmHg – the maximum possible using the RM3 machine. Temperature, arterial blood pressure, intra-renal resistance index, perfusate flow, urine production and pulses-per-minute were documented at 0, 0.25, 0.5, 1 and 2 hours of reperfusion. Furthermore, at 0, 0.25, 1, and 2 hours of reperfusion, arterial blood, venous blood, and urine samples were collected for each kidney. Samples were also analysed using the ABG machine for electrolyte levels, pH, lactate levels and oxygen consumption measurements. Creatinine-, Na^{2+}-, K^{+}-, and Cl^{-}- concentrations in all samples were determined by the clinical pathology laboratory at Hammersmith Hospital.

3.3. Project 3: Investigation of the introduction of MSCs into ex-vivo organ preservation as a preconditioning method for porcine and human kidney grafts (translational model).

3.3.1. Human Mesenchymal Stem Cells

In order to investigate the effect of Mesenchymal Stem Cells on porcine and human kidney grafts, perfusion experiments for Hypothermic and Normothermic machine perfusion were repeated as described in detail in Chapter 3.2. With the difference, that both kidneys of a pair were perfused in the same way, adding MSCs (1-7x10^6) to the perfusate for one kidney, but not the other one. One cry vial containing 5x10^6 human bone marrow derived Mesenchymal Stem Cells in passage 2 was kindly given to us by Prof. Francesco Dazzi's research group at King's College. The cells were confirmed to be Mesenchymal Stem Cells in their laboratory by quality testing and once in our possession, the MSCs were expanded in our lab using the following protocols (provided by Prof. Dazzi et al.):

3.3.1.1. Human MSC Expansion

The cry vial containing MSCs in passage 2 was thawed slowly, spun for 5 minutes at 1500 RPM for 5 minutes and the freezing medium was carefully taken away, leaving the pellet of cells behind. Using fresh growth medium (described below), the pellet was resuspended and the cells were transferred into flasks to be expanded.
The recommended seeding numbers for expansion of human MSCs were used:

75 cm2 flask:	3x10^5-5x10^6 cells in 10-15 ml
175 cm2 falsks:	8x10^5-1.2x10^6 cells per flask in 20-25 ml

Cells were split when a confluence of 80% was observed. At times where no MSCs were needed for experiments, the cells were frozen in freezing medium and stored at -80°C until needed.

- **Medium for MSC expansion:**

  PLT lysate 5%+MEMalfa

  - aMEM, GlutaMAX™, no Nucleosides, Invitrogen, Cat number: 32561-094

- PLT lysate, Supplier: Cook, Product name: PL-S-100, Cat number: G3521

- **Protocol for 5% PLT lysate preparation:**

  - 500 µl of Heparin were added to 500ml of aMEM
  - The PLT lysate was spun at 4000 RPM for 15 minutes
  - The supernatant and flow through were filtered using a cell strainer to get rid of any remaining debris
  - The PLT was then added to the aMEM containing the heparin
  - The medium was aliquoted in 50 ml tubes and stored at -80C°
  - After thawing, the medium could be kept in the fridge for up to 1 week

- **Freezing medium:**
  - 4.5 ml Foetal Calf Serum
  - 0.5 ml dimethylsulfoxide (DMSO)

3.3.1.2. Perfusion experiments including MSCs

For the experiments on MSCs in ex-vivo machine perfusion, the same protocols for NMP and HMP were used as described in detail in Chapter 3.2 with the difference, that for experiments including MSCs, both kidneys from one donor pig underwent either HMP or NMP, respectively, with MSCs (1-5x 10^6) being added to one of the two perfusion circuits during the preconditioning phase. Perfusate samples were analysed for levels of NGAL and IL-β by ELISA and biopsy samples were analysed for expression of inflammatory cytokines using RT-PCR. Functional parameters including urinary output, oxygen consumption, creatinine clearance and fractional sodium excretion were compared upon reperfusion. Furthermore, four consecutive single human kidneys declined for transplantation were treated with HMP (n=2) or NMP (using a packed red cell based perfusate, n=2) and an infusion of $1x10^6$ and $5x10^6$ MSCs, respectively. Thereafter, they were reperfused with KHB for 2 hours at physiological temperatures. Histology samples were taken as for experiments explained in Chapter 3.2.
In summary, following experimental groups were used for the study:

- ***PORCINE KIDNEYS:***

Group 1: 24h of CSS, 4h HMP, 2h REP (n=6)
Group 2: 24h of CSS, 4h HMP+1-5x10^6 MSCs, 2h REP (n=6)
Group 3: 24h of CSS, 4h NMP+ 2h REP (n=6)
Group 4: 24h of CSS, 4h NMP, 1-5x10^6 MSCs, 2h REP (n=6)

- ***HUMAN KIDNEYS***

Group 1: 24h of CSS, 4h HMP+ 1x10^6 MSCs (n=1), +5x10^6 MSCs (n=1), 2h REP
Group 2: 24h of CSS, 4h NMP+ 1x10^6 MSCs (n=1), + 5x10^6 MSCs (n=1), 2h REP

One of the questions this study was supposed to answer was whether MSCs could be delivered into kidney grafts by the application of ex-vivo machine perfusion. Therefore, to distinguish between pre-existing and delivered cells and to trace delivered MSCs for their localization, MSCs were double-labelled with fluorescent cell dye as follows:

3.3.1.3. Double labelling of human MSCs

In order to trace the human MSCs in histology samples after perfusion experiments, the cells were double- labelled with fluorescent cell dye before use. The required number of human MSCs were double labelled with PKH67 fluorescent cell dye (Sigma-Aldrich) for green fluorescence (excitation at 490nm, emission at 502nm) and Qdot® (molecular probes®, life technologies™) Nano crystals with a red signal (excitation at 405-615nm, emission at 655nm). First, the red Qtracker dye was applied to the cells according to the manufacturer's instructions, before the PKH67 green fluorescent dye was applied.
Cells were trypsinized and counted using a haemocytometer to determine the exact cell count per ml. Thereafter, the needed cell count (1-7x10^6 cells) was washed once with HBSS. For 1x10^6 MSCs, a 10nM labelling solution was prepared by pre-mixing 1µl Qtracker® component A and 1µl Qtracker® component B in a 1.5mL micro centrifuge tube. This was incubated for 5 minutes before 0.2 ml of fresh growth medium were added and the mix was vortexed for 30 seconds. 1x10^6 cells from a cell suspension of 1x10^7 cells/ml in growth medium were then added to the tube containing the labelling solution. In case of higher numbers of the cells, the volumes

were adapted accordingly. The sample was incubated for 3 hours at 37°C. Then, the cells were washed twice with complete growth medium.

3.3.2. Fluorescence microscopy

To visualize the cells after labelling them, the Zeiss Axio Observer wide field inverted microscope was used, which is based at the FILM facility at Imperial College. It is linked to the Zen acquisition software and using LED light sources, fluorescent signals from UV to far-red can be detected and visualized. To be able to use the microscope as well as the software, training was necessary. At this point a special thanks has to be expressed to Stephen Rothery who is the most experienced researcher in the field of imaging and who was the most helpful and patient trainer, helping me to take pictures of the double-labelled MSCs.

Fluorescence results from the absorption and release of light energy by electrons in a molecule. When absorbing light of a particular wavelength, electrons rise from a lower to a higher energy level, thereby becoming ‚excited'. Excited electrons are highly unstable and tend to return quickly to their initial state. When doing so, they release the energy of the absorbed wavelength as visible light, fluorescence, and as heat. Because some of the energy is lost as heat, the fluorescence light emitted always has a longer wavelength and containing less energy than the excitation light. By the use of appropriate filters, the fluorescent light of the longer wavelength alone can be detected, so that a fluorescent object can be made to appear bright against a dark background. Fluorescence can be an intrinsic property of a biological molecule, like for example chlorophyll, which therefore shows auto-fluorescence. Most biological specimens however, are not auto fluorescent and so they usually have to be stained with a fluorochrome, or an immunofluorescent detection method has to be applied. To visualize the double-stained MSCs, some cells were spun onto an objective, covered with DAPI and then looked at under the fluorescent microscope. Pictures were taken and saved onto a hard drive.

3.3.3. Immunohistochemical Staining against human MSCs in porcine samples

3.3.3.1. Anti Vimentin staining of porcine histology sections

Vimentins are class-III intermediate filaments found in various non epithelial cells, especially mesenchymal cells. Vimentin is attached to the nucleus, endoplasmic reticulum, and mitochondria, either laterally or terminally.

Anti-Vimentin staining using an anti-human-Vimentin antibody (Abcam, Cat.Nr. ab92547) was conducted on porcine histology slides in order to stain the human Mesenchymal Stem Cells which were delivered to the organs during ex-vivo machine perfusion. The same protocol was followed as for anti-MHC-I stainings, following the manufacturer's instructions as well as published literature to find the optimal antibody concentration. Concentrations starting at a dilution of 1:100 in 20% human serum up to a dilution of 1:1000 were used. Human breast adenocarcinoma slides were ran with each batch of staining as a positive control, a sample without cells served as negative control and another sample for which PBS was used instead of the antibody, served as an internal negative control.

Unfortunately for the anti-vimentin staining, we saw a cross-reaction with porcine tissue so that a non specific staining was present in all the slides. Therefore, staining against MHC-I was conducted and the protocol will be described in detail below.

3.3.3.2. Anti MHC-I staining of porcine histology sections

After the unsuccessful staining against Vimentin, immunohistochemical staining against human MHC class I was performed to identify the human cells within porcine tissue samples. The following antibody used was ordered from 2BScientific:

Mouse anti Human HLA Class I Heavy Chain (Restricted expression), Clone - HC10 – 0.1mg, Cat. Nr. MUB2037P. HC10 is a mouse monoclonal IgG2a antibody derived by fusion of SP2/0-Ag14 mouse myeloma cells with spleen cells from BALB/c mice immunized with HLA-B7 and -B40 heavy chains. It was raised against free class I heavy chains of HLA antigens to obtain antibodies that would still react with denatured class I antigens. The optimal antibody dilutions were recommended to be determined by titration, the recommended range was 1:100-1:200 with avidin-biotinylated horseradish peroxidase complex (ABC) as detection reagent for immunohistochemistry. The following protocol was used for immunohistochemical staining using the DAKO rabbit envision kit:

Paraffin embedded slides were dewaxed by placing them through xylene and a series of graded alcohols to water. Antigen retrieval was performed by placing the slides in pre-warmed sodium citrate buffer which was placed into a heated water bat hat 95°C for 20 minutes. Thereafter, slides were placed into PBS. Previous steps were not necessary for frozen slides, which were thawed, fixed in acetone for 5 minutes to be then placed into PBS. Using a paraffin pen, sections were marked by drawing a circle around them. Peroxide block was performed by putting 2 drops of peroxide from the envision kit onto the tissues to incubate them for 10 minutes at room temperature. Another rinse in PBS was performed before 20% goat serum block was conducted by putting 200µl on each slide and incubate for 15 minutes at room temperature. Thereafter, goat serum was tapped off and the anti-vimentin antibody in a dilution of 1:200 was applied. Slides were incubated for 1 hour in a humidified chamber for 1 hour at room temperature. Slides were then again washed in PBS. 2 drops of the polymer from the envision kit were applied for 30 minutes at room temperature, which was followed by another wash in PBS. 50µl DAB from the envision kit (2µl DAB chromogen in 100µl DAB substrate buffer), as recommended by manufacturer's instructions were added until brown staining was detected. All slides were then placed into PBS before counterstaining, dehydration and mounting in pertex.

- **Materials and Reagents:**
  - Anti-human-Vimentin antibody (Abcam, Cat.Nr. ab92547)
  - Mouse anti Human HLA Class I Heavy Chain antibody, (2BScientific, Cat. Nr. MUB2037P)
  - PBS (Sigma Aldrich)
  - Sodium Citrate buffer (1.47g sodium citrate in 500ml distilled water, pH 6.0)
  - 20% human serum (diluted 1:5 from 100% human serum)
  - DAKO mouse EnVision™kit (Cat. Nr. K4006)
  - Pertex mounting medium (VWR)

3.3.4. TUNEL staining for human/porcine kidney biopsies

The abbreviation TUNEL stands for TDT (Terminal Deoxynucleotidyl Transferase)-Mediated dUTP (2'-Deoxyuridine 5'-Triphosphate) Nick-End Labelling (biochemistry). The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. This allows discrimination of apoptosis from necrosis and from primary DNA strand breaks induced by cytostatic drugs or irradiation.

TUNEL staining was performed using the In Situ Cell Death Detection kit (Roche 11684795910) according to the following protocol:

Paraffin-embedded histology sections were dewaxed by incubation in rising concentrations of xylene and ethanol followed by dH_2O. Proteinase K digestion was performed by adding 2 drops of Proteinase K (Sigma Aldrich, 20µg/ml working concentration) to each section and incubation at 37°C for 30 minutes. The slides were rinsed in PBS. 100µl of the label solution (vial 2) were spared for negative controls before 50µl of of enzyme solution (vial 1) were added to the remaining 450µl of label solution to obtain 500µl of TUNEL reaction mixture. 50µl of TUNEL reaction mix or label solution only (for negative controls) were added to each slide before they were incubated for 60 minutes at 37°C in a dark and humidified atmosphere. The slides were then rinsed in PBS before being mounted with Faramount (DAKO, S3025). Analysis was performed by counting the TUNEL positive cells under a fluorescence microscope.

3.3.5. Quantification of protein expression in porcine and human perfusate- and urine samples

3.3.5.1. Porcine IL-1β/IL-1F2 Duo Set ELISA (R&D Systems, Cat. Nr. DY681)

For detection and quantification of the expression of IL-1β in perfusate- and urine samples after ex-vivo perfusion of the porcine organs, the DuoSet® ELISA kit by R&D Systems, Catalogue Number: DY681, was used to perform sandwich ELISA assays.

The perfusate and urine samples were spun for 5 minutes at 12000 rpm, respectively and the supernatants were used for the assays.

Sandwich ELISA is a highly sensitive variant of ELISA, which quantifies antigens between two layers of antibodies, usually the capture and the detection antibody. The

antigen to be measured must contain at least two antigenic epitopes capable of binding to at least two antibodies acting in the sandwich. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in an antigen. A polyclonal antibody is often used as the capture antibody to pull down as much of the antigen as possible. To summarize the principle of the assay, the target protein binds to the capture antibodies present in the wells of the plate, whilst unbound material is removed by washing. A monoclonal detection antibody is added to each test well and it attaches to bound target protein. Again, unbound detection antibody is removed by washing. When HRP-conjugated streptavidin is added to each well, a complex with the bound biotinylated antibody is formed. Unbound conjugate is removed by washing. A colour forming peroxidase substrate containing tetramethylbenzidine (TMB) is added and the bound HRP-streptavidin reacts with the substrate to generate a blue colour. The enzymatic reaction is then stopped by adding dilute sulphuric acid (Stop Solution), which changes the colour of the wells to yellow. The yellow colour intensity is read photo metrically at 450nm in an ELISA reader.

The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to 2 to 5 times more sensitive than direct or indirect ELISA). Sandwich ELISA assays were performed according to the following protocol, provided by the supplier of the kit:

The capture antibody was diluted to the working concentration of 2µg/ml in PBS without carrier protein. It was immediately used to coat a 96-well micro plate with 100µl per well. The plate was sealed and incubated over night. On the next day, the wells were drained and washed twice with 400µl Wash Buffer per well, using a squirt bottle. The plate was blotted against clean paper towels to remove the remaining Wash Buffer, before 300µl Block Buffer were added per well. Block Buffer was incubated for a minimum of 1 hour. Within this hour, the standards were prepared according to manufacturer's instructions. The provided vial with IL-1β standard was reconstituted with 500µl Reagent Diluent to result in a 125ng/ml porcine IL-1β standard stock solution. The stock solution was further diluted with Reagent Buffer to a concentration of 4000pg/ml as a high standard. A seven point standard curve was obtained by using 2-fold serial dilutions (***Fig. 3.10.***)

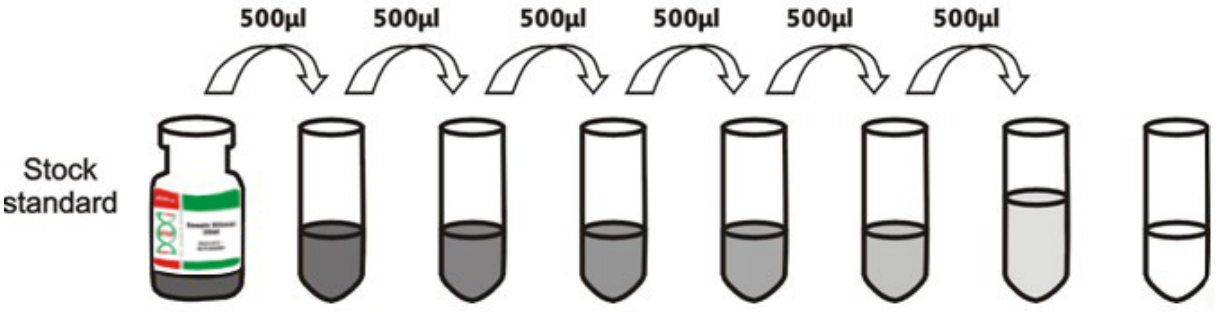


Figure 3.10. The principle of standard dilution for ELISA

Fig 3.10. *The principal of standard dilution as taken from the manufacturer's instructions is illustrated.*

The wash step as described above was repeated and 100µl of either the standards, or the samples were added per well, in duplicates. Urine- and perfusate samples were used without any dilution in this case. The plate was incubated for 2 hours, then the washing step was repeated. 100µl of Detection antibody in a working concentration of 50ng/ml (diluted in Reagent Diluent) were added to each well and again, the plate was incubated for 2 hours.

The washing step was repeated and 100µl of Streptavidin- HRP in working concentration, diluted with Reagent Diluent were added per well and incubated in the dark for 20 minutes. After another washing step, 100µl of Substrate Solution (1:1 mixture of Colour reagent A (H_2O_2 and Colour Reagent B (Tetramethylbenzidine)) were added and depending on the intensity of the change of colour to blue, incubated for a maximum of 20 minutes before stopping the reaction by adding 50µl of Stop Solution (2 NH_2SO_4) to each well.

The optical density of each well at 450nm was determined immediately, using a micro plate reader and concentrations were calculated using the standard curve by means of an ELISA reader software program incorporating curve fitting procedures. The procedure of choice is to use linear x- and y axes with a logarithmic curve fit.

3.3.5.2. Porcine Lipocalin-2 ELISA (Abcam, Cat. Nr. ab207924)

For detection and quantification of the expression of Lipocalin-2 in perfusate- and urine samples after ex-vivo perfusion of porcine organs, the Abcam Lipocalin-2 ELISA Kit (Cat. No. 207924) was used. This ELISA assay is an in-vitro enzyme linked immunosorbent assay for the quantitative measurement of Lipocalin-2 levels in urine and plasma samples. The perfusate and urine samples were spun for 5 minutes at 12000 rpm, respectively and the supernatants were used for the assays.

The assay is a sandwich ELISA performed in micro wells coated with a mouse monoclonal antibody against pig NGAL. Bound NGAL is then detected with another mouse monoclonal antibody labelled with biotin and the assay is developed with horseradish peroxidase (HRP) conjugated streptavidin and a colour-forming substrate.

In contrast to the ELISA kit for porcine IL-1β, the plates in this assay are pre-coated with the primary monoclonal capture antibody.

The samples were diluted 1:2000 after spinning and all the provided solutions were prepared according to manufacturer's instructions. The pig NGAL standards were provided ready to use in concentrations from 400pg/ml to 0pg/ml (blank). 100µl of each sample were used in duplicates. The protocol for the NGAL ELISA assay is similar to the protocol for the porcine IL-1β assay, with the only difference that the included plate is precoated and the incubation time for samples as well as for the Biotinylated secondary pig-NGAL antibody is only 60 minutes instead of 2 hours.

Absorbance was measured at 450nm as for the pig IL-1β ELISA assay, and concentrations were calculated using the standard curve by means of an ELISA reader software program incorporating curve fitting procedures. The procedure of choice is to use linear x- and y axes with a logarithmic curve fit. See ***Fig. 3.11.*** for a typical standard curve for the pig-NGAL ELISA kit. Standard curves for different ELISA kits vary but in general, look similar.

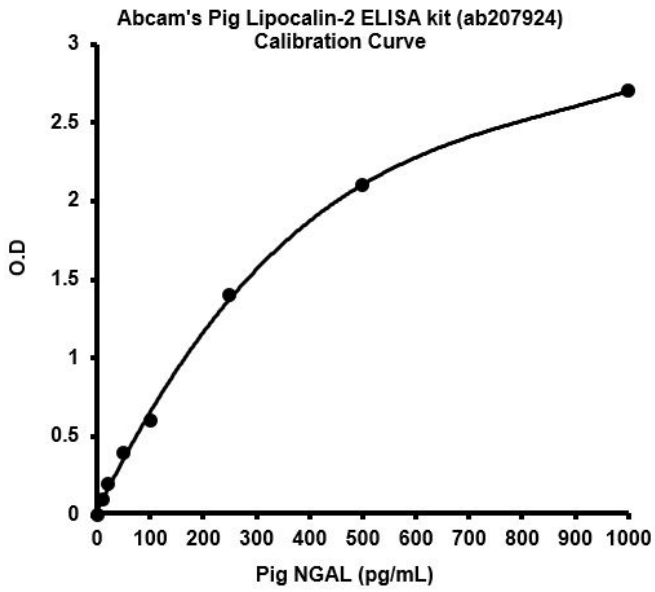


Figure 3.11. Standard curve ELISA

Fig 3.11. *The standard curve as resulting from the pig NGAL ELISA kit, when the standards are applied correctly, is shown. The optic density of the samples after reaction is drawn at the y-axis and the concentrations of the standard dilutions are drawn on to the x-axis. NGAL= Neutrophil gelatinase-associated lipocalin.*

3.3.5.3. Human TIM-1/KIM-1/HAVCR Immunoassay (R&D Systems, Cat. Nr. DSKM100)

For serum- and urine samples of the human kidneys after ex-vivo perfusion, the expression of kidney injury molecule1 (KIM-1) was determined by ELISA, using the human TIM-1/KIM-1/HAVCR Immunoassay by R&D Systems, Cat. Nr. DSKM100.

KIM-1, also known as T-cell immunoglobulin and mucin domain (TIM-1) or Hepatitis A virus cellular receptor 1 (HAVrc), is involved in the regulation of adaptive and innate immune responses[311,312]. KIM-1 is a type I trans membrane protein that contains an N-terminal immunoglobulin-like domain, a mucin domain with O- and N-linked glycosylation, a trans membrane segment and a cytoplasmic signalling domain[313,314].

In vivo, KIM-1 is expressed on splenic B cells and it is a marker for the identification of IL-10^{+} regulatory B cells. KIM-1 is also expressed on CD4^{+} T cells, mast cells, invariant NKT (iNKT) cells, dendritic cells, kidney epithelium and a broad range of mucosal epithelium. The expression of KIM-1 is up regulated on activated Th2 cells after dendritic cell maturation, and on kidney tubular epithelial cells after injury.

Metalloproteinase-mediated cleavage of KIM-1 at the membrane-proximal region results in the release of a soluble form of KIM-1 which is detectable in the urine and circulation. Urinary KIM-1 is highly elevated in nephropathy and may be a useful biomarker for renal damage which is why determination of its expression levels in serum- and urine samples could give information about the tubular health in kidneys after IRI and ex-vivo preconditioning.

The Quantikine® KIM-1 Immunoassay is a 4.5 hour solid phase ELISA which contains NS0-expressed recombinant human KIM-1 and antibodies raised against the recombinant factor. This assay also employs the sandwich enzyme immunoassay technique as mentioned above. The plate is pre-coated with a monoclonal antibody specific for human KIM-1. The steps are equal to the steps for the porcine Lipocalin-2 assay. Briefly summarised, standards and samples were pipetted into the wells and any KIM-1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human KIM-1 was added to the wells. Following that, unbound antibody-enzyme reagent was removed and a substrate solution was added to the wells. Colour developed in proportion to the amount of KIM-1 bound in the initial step. The colour development was stopped by adding stop solution and the intensity of the colour was measured at 450nm using the ELISA plate reader.

3.3.5.4. Human MMP-9/ NGAL Complex Duo Set ELISA (R&D Systems)

Materials and Reagents not included in the kits:

- **ELISA plate reader**
- **Adjustable micropipettes** covering the range 1-1000µl
- **Deionized or distilled water**
- **96 well micro plate** (R&D Systems, Cat. No. DY990)
- **Plate sealers** (R&D Systems, Cat. No. DY992)
- **PBS** (R&D Systems, Cat. No. DY006)
- **Tween® 20**
- **Wash Buffer:** 0.05% Tween® 20 in PBS, pH 7.2-7.4
- **BSA**

- **Block Buffer:** 1% BSA in PBS, pH 7.2-7.4, 0.2µm filtered
- **Tris Buffered Saline** (20mM Trizma base, 150mM NaCl), pH 7.2-7,4, 0.2µm filtered
- **Reagent Diluent:** 0.1% BSA, 0.05% Tween® 20 in Tris Buffered Saline
- **Substrate Solution:** R&D Systems, Cat. No. DY999
- **Stop Solution:** 2N H_2SO_4

3.4. Project 4: Investigation of the effects of ex-vivo delivered Mesenchymal Stem Cells on Ischemia-Reperfusion Injury in a rodent autologous kidney transplantation model.

3.4.1. Extraction of bone marrow derived Mesenchymal Stem Cells from the rat

For the extraction of bone marrow derived Mesenchymal Stem Cells (BM-MSCs) male Wystar Kyoto wild type (WKY-wt) and WKY rats transgenic for the expression of green fluorescent protein (WKY-GFP) were used as donors. The WKY-GFP rat came from an ubiquitous GFP reporter transgenic rat line and was generated by Garcia-Diaz et al. The donors for ‚old' MSCs were 6 months old and the donors for ‚young' MSCs were 6 weeks old.

Cold and sterile PBS was prepared and put into a 50ml Falcon tube on ice. The instruments for the retrieval of femurs and tibias of the rat were autoclaved. In the animal facility the rat was culled according to the Schedule 1 protocol. The femurs and the tibias of the rat were dissected, retrieved and put into the Falcon tube on ice containing sterile PBS. In a laminar flow cabinet, a petri dish containing sterile, cold Hank's Balanced Salt Solution (HBSS; Gibco) and a 10ml syringe with a 21G needle were prepared. The bones were transferred from the cold PBS into the Petri dish and whilst holding each bone firmly with a pair of sterile forceps, the ends were cut off using a sterile bone cutter. The bone marrow was flushed into a fresh 50ml Falcon tube using the syringe with a 21G needle. The Falcon tube was put into a centrifuge for 5 minutes at 1500 rpm and a temperature of 4°C. The supernatant was discarded and the pellet containing the cells was resuspended in 1 ml of MesenCult MSC Basal Medium (Stem cell) that contained MSC stimulatory supplement and 0.5% Penicillin/Streptomycin (Invitrogen). 250 ul of the cell suspension, respectively, were put into a T150 tissue culture flask in 25 ml of MesenCult MSC Basal Medium.

The cultures were maintained in an incubator providing 37°C and 5% CO_2 and their media was changed every 3-6 days. At a confluence of 80%, the cells were trypsinized, collected from the flask and counted in a total volume of 1ml HBSS using a haemocytometer. For each passage and each phenotype, $100x10^3$ cells were put into 3 wells of a 6 well plate, respectively, adding 2 ml of fresh medium which resulted in a density of $50x10^3$ cells/ml. Each time, 100ul of the cell suspension were taken, cells were spun down and after decanting the supernatant carefully, they were either lysed in RNA lysis buffer (Qiagen) according to manufacturer's instructions and

then frozen, or put into freezing media for later experiments. The cells in freezing medium were frozen using a Mr.Frosty at -80°C. The rest of the cells (2-3x10^6 cells) were split into two 175 cm^2 flasks, respectively, adding 25ml of fresh MSC growth medium. This was performed for passage 0 up to passage 10. The cell counts at each time of splitting were documented and the cells were photographed under the microscope.

For each passage, out of the 6 well plates with a cell count of 100x10^3 cells per well, after 24 hours in culture, 500ul of supernatant were taken, resulting in triplicates of supernatants for each passage. The supernatants were frozen for further experiments later.

- **Materials**

- Hank's balanced salt solution (HBSS; no calcium, no magnesium) Gibco - Cat. No. 14170-138
- Mesencult Proliferation kit (medium+serum) - Cat. No. 05511; Stem cell technology, prepared as described in user's instructions, filtered before use
- Penicillin/Streptomycin - Cat. No. 15070-063 - Invitrogen
- Trypsin/EDTA for MSCs- Cat. No. CC-3232- Lonza
- PBS: 10x in 1l aqua dist.: 80g NaCl, 2.01g KCl, 11.49g Sodium phosphate dibasic, 2.09g Potassium phosphate monobasic
- Foetal Calf Serum (FCS):
- T175 flaks – Cat. No. 660175 - VWR
- 2 x scissors, 2 x standard forceps, 1 x straight bone cutter, scalpel - **all tools autoclaved before use**
- Syringe (10 mL) and needle (21G)
- 50 mL Falcon tube
- Freezing medium: 500ul DMSO in 4,5ml Foetal calf serum

3.4.2. Sub culturing rat derived Mesenchymal Stem Cells

At a confluence of 80% of the cells (observed under a 20x focus of a light microscope), in the culture flask, the culture medium was removed and collected as supernatant sample for each passage (P1-P10). The cells were washed once with HBSS at 30°C. 2 ml of Trypsin were spread on the cells to cover the whole surface. After 2-5 minutes, the cells were scraped off the flask using a cell scraper. Immediately adding the same amount of MSC culture medium to the cell suspension neutralized the Trypsin. The cell suspension was put into a 50ml Falcon tube. The cells were spun in a centrifuge for 5 minutes at 1500 rpm at room temperature. The supernatant was decanted and discarded. The pellet was then resuspended in 1ml of Hank's balanced salt solution (HBSS). Of that cell suspension, 10ul were put into a well of a 96 well counting plate. 10ul of Tryptophan-blue were added and after pipetting up and down, 10ul of the mix were transferred onto a haemocytometer. Cells were counted and $50x10^3$ cells were put into each well of a triplicate on a 6 well plate, containing 2 mls of MSC growth medium (Stem Cell), respectively for GFP+ and wilt type cells. The rest of the cells were spread into 2 T175 flasks containing 25 ml of MSC growth medium (Stem Cell), respectively for GFP positive cells and wild type cells.

Each group of cells was passaged from passage 1 (P1) to passage 10 (P10) and for the cells in the 6 well plate, 500ul of supernatant were collected and put into cry vials respectively after 24 hours of incubation. For the cells in the T175 flasks, medium was changed regularly and supernatants were collected into 50ml Falcon tubes. Cells were split once a confluence of 80% was reached and cell counts for each passage were noted.

3.4.3. Differentiation of MSCs into adipocytes and osteocytes

After passage 5, MSCs were differentiated into adipocytes and osteocytes in separate wells for proof of the MSC phenotype. Therefore, in an additional 6 well plate, cells were again seeded at a density of $50x10^3$ cells/ml per well. For differentiation into adipocytes, rat MSC Adipogenic Bullet kit (Lonza) was used according to manufacturer's instructions. At a cell confluence of 100%, the MSC growth medium was replaced with adipogenic induction medium and cells were incubated at 37°C, 5%CO_2. Medium was changed every 2-3 days for a total of 8

days, followed by incubation in adipogenic maintenance medium. The phenotype of adipocytes was then confirmed by Oil-Red-O staining:

After removing the adipogenic maintenance medium, the cells were washed once with 2ml of PBS. PBS was removed and 2ml of 10% Formalin (room temperature) were added. The cells were incubated for 10 minutes at room temperature. In a next step, the Formalin was replaced by fresh Formalin and then the cells were incubated for one hour (the 6 well plate was wrapped in aluminium foil for the cells not to dry out during this time). Then the Formalin was removed and the wells were washed twice with 2ml of ddH_2O, respectively. In a next step, the cells were washed with 2ml of 60% Isopropanol for 5min at room temperature (RT). After removing the Isopropanol, the cells were left to dry completely at RT. 1ml of Oil red O working solution was added, and cells were incubated for 10 minutes. After that, the Oil red O solution was removed and the cells were washed 4 times with ddH_2O. Imaging under the microscope then showed adipocytes in red.

For osteogenic differentiation of the MSCs, rat Osteogenic Bullet kit (Lonza) was used according to manufacturer's instructions. Upon 100% confluence of the MSCs, the MSC growth medium was replaced with Osteogenic medium. The medium was changed every 2-3 days and the cells were observed under a microscope until bone matrix formation could be detected. As a proof for bone matrix formation, Alizarin Red staining was performed. Cells were washed with PBS and after that fixed with 10% paraformaldehyde for 15 min at room temperature. Thereafter, cells were again washed with PBS and then stained with Alizarin red solution at a pH of 4,2, incubation time of 10 minutes whilst gently shaking the dish. Before observation under the microscope, the cells were washed another 3 times with PBS.

- **Materials**

- Rat MSC Adipogenic Bullet kit (Lonza, PT-3102B, PT3102A)
- MesenCult Osteogenic Stimulatory kit (Mouse) (Stem cell, Cat.No. 05504)
- Oil Red O, certified by the Biological Stain Commission
- Alizarin Red S, certified by the Biological Stain Commission
- X10 16% Formaldehyde (W/V), Methanol free

3.4.4. Confirmation of MSC phenotype using Flow Cytometry

Preparation of Flow Cytometry experiment:

- Cells without staining served as a control
- FMO: fluorescent minus one: all antibodies apart from one were used for this group to ensure that there was no overlap of fluorescence in the channels

When MSCs were sub cultured from passage 2 to passage 3, after spinning the cells for 5 minutes at 4°C at 300-400g, a total of approximately $2x10^6$ cells were put aside from each group (WT and GFP positive cells) to be re dissolved in a total of 600ul of FACS staining buffer (50ul per well, each containing $2x10^5$ cells).

In a round bottom 96-well plate, cells were distributed according to a scheme resulting in duplicates for each staining with antibodies. With two different groups of cells and duplicates per antibody, the concentration for the antibody was calculated for a total number of approximately $8x10^5$ cells (4 wells a $2x10^5$). The following antibodies were used to characterise the MSCs:

- **CD29 antibody: PE anti-mouse/rat CD29, clone: HMβ1-1, Bio Legend Cat: 102207, Conc: 0.2mg/ml**

  Recommended concentration: <0.25ug/10^6 cells

  1ul antibody was dissolved in 199ul FACS staining buffer in which cells were dissolved to be then spread over 4 wells on the 96 well plate.
- **CD90 antibody: PerCP anti-rat CD90/mouse, CD90.1 (Thy-1.1) clone: OX-7, BioLegend Cat: 202512; Lot: B171081, Conc: 0.2mg/ml**

  Recommended concentration: <0.06ug/1x10^6 cells

  The antibody was pre diluted 1:10 and then 2.4ul of the dilution were dissolved in 197.6ul FACS buffer to obtain a concentration of 0.048ug/ $8x10^5$ cells.

  These were spread over 4 wells.
- **CD34 antibody: Alexa Fluor 647 anti-mouse CD34, clone: lCO115, Novus Biologicals Cat: NBP2-33076AF647; Conc: 0.75mg/ml**

  Recommended concentration: no recommendation, 2 dilutions pre-tested: 1:250 and 1:500, 1:250 was used for experiments

  0.8ul of the antibody were dissolved in 199.2ul FACS buffer.
- **CD45 antibody: Alexa Fluor 647 anti-rat CD45, clone: OX-1, BioLegend Cat: 202212; Conc: 0.5mg/ml**

Recommended concentration: <0.25ug/1x10^6 cells

The antibody was pre diluted 1:10 and then 4ul of the dilution were diluted in 196ul FACS staining buffer.

- **CD44 antibody: RPE mouse anti-rat CD44, clone OX-50, Bio Rad Cat: MCA643PE**

  Recommended concentration: 8ul of antibody in 192ul of FACS buffer, according to manufacturer's instructions (10ul for 1x10^6 cells)

Per well, 50ul of the cell suspension were mixed with 50ul of the respective antibody dilution to obtain 100ul per well. The cells were incubated with the antibody for 30 minutes on ice. Thereafter, as washing steps, the cells were spun down for 5 minutes at 300-400g and resuspended in FACS buffer twice. After the washing steps, the cells were transferred into FACS vials and measured one by one on the Flow Cytometer (BD Accuri C6). Appropriate settings were chosen and gating of the right channels for evaluation of results was performed with the help of Dr. Kevin Woollard and Dr. Ana Garcia-Diaz.

3.4.5. Autologous rat kidney transplantation

All experiments in the rat were conducted using WKY rats. The reason for this was that the Mesenchymal Stem Cells positive for green fluorescent protein (GFP) were coming from that strain and the aim was to focus on their effect on Ischemia Reperfusion Injury initially and to exclude immunological factors by using an autologous transplant model (WKY to WKY).

3.4.5.1. Preparation of the donor kidney in the rat

Adult rats were anaesthetised using Isoflurane at a flow rate appropriate to the species, the size and the individual needs but within the recommended rates provided by the animal facility (CBS, Imperial College). After induction of anaesthesia, the animals were shaved and placed onto the operating table providing a heating pad to maintain the animal's physiological temperature. Standard surgical aseptic technique was used to clean and cover the abdomen of the animals with sterile drapes. A midline abdominal incision was made and the left kidney with the left

renal artery and vein was visualised (***Fig. 3.12. b, c, d***). After placement of a self retaining retractor, the left adrenal vein was ligated with 4-0 vicryl and the kidney was freed from surrounding tissue. The left renal artery and vein were dissected and also freed from any adherent tissue. The ureter was dissected and ligated using 4-0 vicryl. Thereafter, the ureter was cut distally from the ligature. The aorta proximal to the renal arteries was dissected and slung with a strong tie (1-0 or 0 vicryl) and the same was done for the proximal vena cava and distally for aorta and vena cava together in one sling. The donor was injected with no more than 0.5ml anticoagulant intravenously before the vessels were tied and the kidney was removed. The animal was sacrificed by a schedule 1 method.

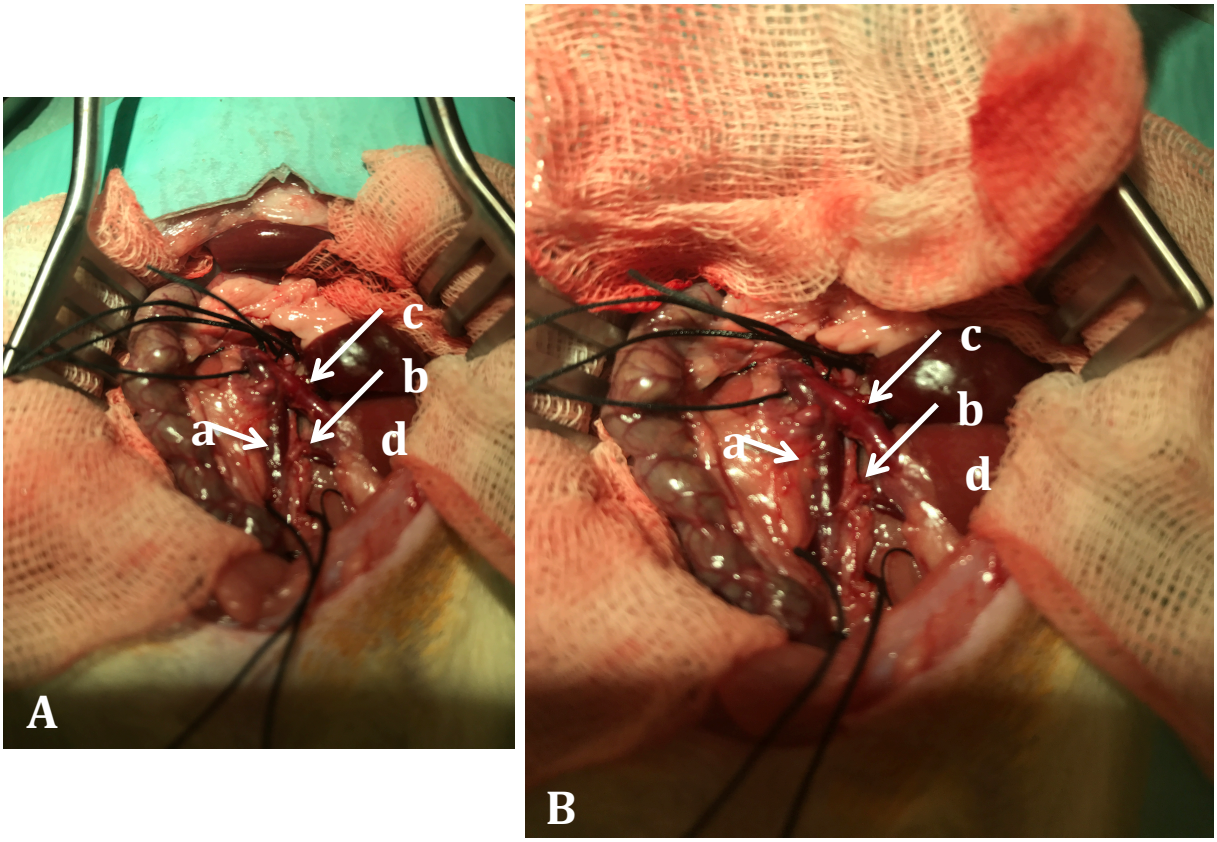


Figure 3.12. Donor preparation for rat kidney transplantation

Fig 3.12. *overview (**A**) and closeup (**B**) of the operating field during the operation of a donor rat for renal transplantation. The vena cava (**a**),* the left renal artery- (***b***) and vein (***c***) as well as the left kidney (***d***) were dissected , before the vena cava and the aorta were tied proximally and distally (black slings).

3.4.5.2. Ex-vivo hypothermic perfusion of rodent kidneys with infusion of green fluorescent MSCs

3 rodent kidneys were hypothermically perfused for 1h at a temperature between 4° and 6°C (the flask containing the fluid was kept on ice), of which 2 were perfused with $3x10^6$ green fluorescent MSCs in 50mls of Machine Perfusion Solution (modified UW; Bridge to Life). This was a prototype study to investigate whether MSCs could be detected later on within the kidneys and where exactly they would be localised. Following method was used to perform ex-vivo hypothermic perfusion of the kidneys: Ex-vivo, the aorta was identified, separated from the renal vein and a paediatric cannula of 24G (yellow) was inserted and held in place with a 4-0 vicryl tie. In case of ex vivo machine perfusion, the kidney was placed onto an ex- vivo machine perfusion circuit. The empty circuit is shown in ***Fig. 3.13. A.*** It consisted of a roller pump (Masterflex), silicone tubing with an inside diameter of 3mm, which provided a flow rate of 2ml/min of perfusate to the kidney via the yellow 24G cannula. The kidney was floating in a glass flask filled with 50ml of Machine Perfusion Solution (Bridge to Life) and the venous outflow was recirculated into the kidney- equivalent to the perfusion circuit for porcine and human organs. As we were purely focussing on delivery of MSCs into the kidneys, the circuit was kept as simple as possible and there was no oxygen delivered to the perfusate. Kidneys were either flushed hypothermically with Machine Perfusion Solution (Bridge to Life) only, or with Machine Perfusion Solution containing various numbers of Mesenchymal Stem Cells ($3\text{-}10x10^6$) positive for green fluorescent protein (GFP) which had been extracted before from the same strain of rat. ***Fig. 3.13. B*** and ***C*** illustrate a rat kidney attached to the circuit, with the aorta being tied onto the yellow 24G cannula (arrow). In ***Fig. 3.13. C***, some lighter areas can be seen within the kidney, which is due to the fact that the blood is flushed out from the kidney gently.

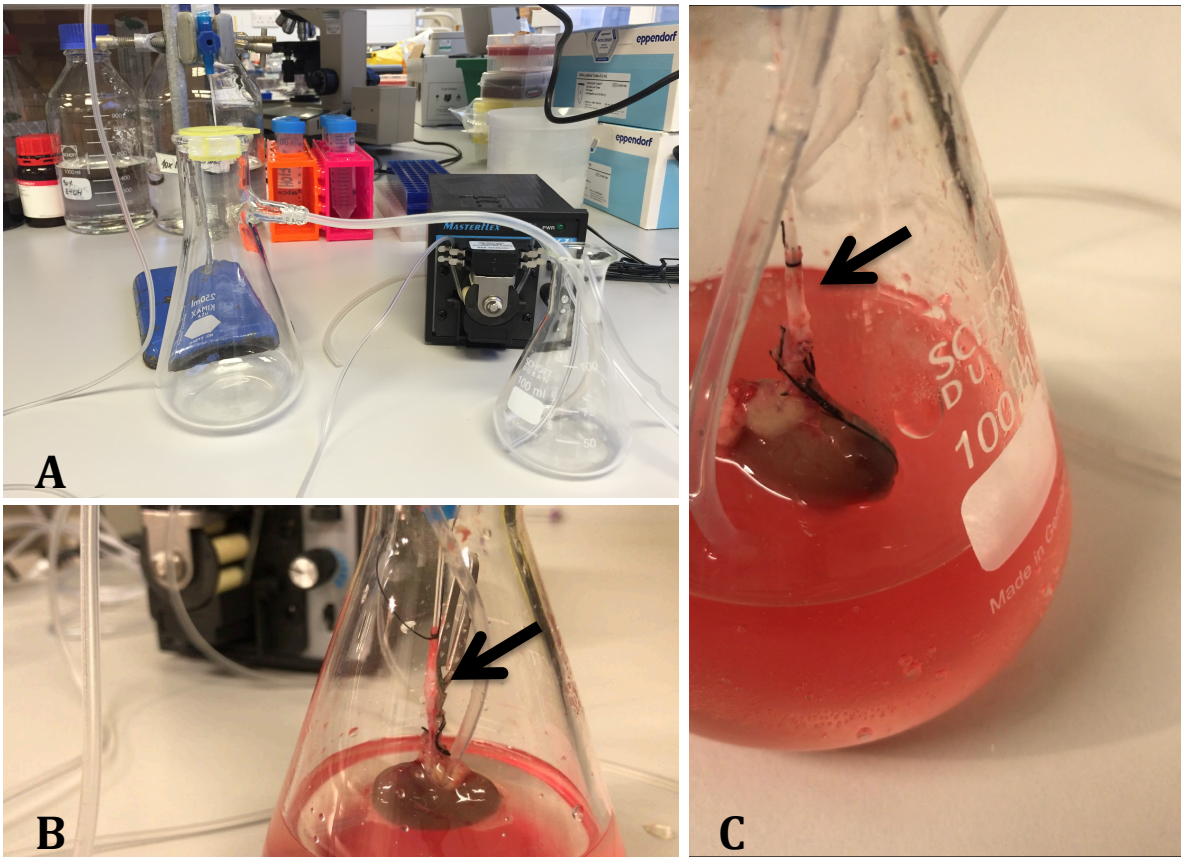


Figure 3.13. Ex-vivo perfusion circuit for rodent kidneys

Fig 3.13. *The circuit for the perfusion of the rodent kidneys (**A**) consisted of a Masterflex rollerpump, tubings and glasses for the circulating fluid.* ***B*** and **C** show the kidney during perfusion, with the arrow highlighting the aorta, which was tied onto a 24G cannula.

3.4.5.3. Rat kidney transplantation- recipient procedure

After several rat kidney transplantations (n=6), which were performed to learn the method but in which the procedure had to be interrupted due to an expected fatal outcome, another 4 recipients were transplanted in which the kidney was reperfused after finishing the anastomoses. Unfortunately, also in these cases, we were not able to recover the animal due to the judgement of the dedicated trainer of a fatal outcome. The following protocol was followed for the recipient operations: After retrieval, the donor kidney was either put into saline (n=1), treated by machine perfusion alone (n=1) for the purpose of learning the cannulation and technique of the perfusion, or treated by machine perfusion containing $3x10^6$ green fluorescent MSCs (n=2). After one hour of cold storage or perfusion of the kidneys, the recipient rat was anaesthetised as described above for the donor rats and the first dose of

Vetergesic (diluted 1 in 5 in 0.9% saline, 0.5ml) was given subcutaneously. After placing the recipient on the operating table, again standard aseptic technique was applied and a midline laparotomy was performed. The left kidney was freed from surrounding tissue and the left adrenal vein, the left ureter and the left renal artery were tied off using 4-0 vicryl. Before removing the left kidney, the left renal vein was cleared from surrounding tissue and a micro bulldog was placed so that the renal vein could be cut at an angle, leaving as much length to it as possible (***Fig. 3.14. A, a***). After removing the recipient's kidney, the corner stitches were placed to the recipient's renal vein with 7-0 silk. Then, the donor kidney was taken out and the equivalent corner stitches placed to the renal vein. The anastomosis was done with continuous stitching. The venous clamp was removed (***Fig. 3.14. B, a***) and the aortic patch of the donor kidney was anastomosed to the aorta (***Fig. 3.14. B, b***) of the animal in the same way, using continuous stitching with 7-0 silk (***Fig. 3.14. C, a***). After removing the clamps, the ureter was connected to the bladder (***Fig. 3.14. C, b***). This was performed by cutting a tiny whole into the front wall of the bladder and pulling the ureter through the back wall with a sewing needle, using a long end of the ligature which had been placed to tie the ureter off in the donor animal. Thereafter, the ureter was fixed onto the inside of the bladder's back wall with a stitch using 7-0 silk. As a last step, the hole in the front wall of the bladder was closed by continuous stitches with 7-0 silk. The animal's abdomen was closed in layers using 3-0 Vicryl continuously for the abdominal muscle layer, followed by interrupted closure of the skin with 4-0 Prolene.

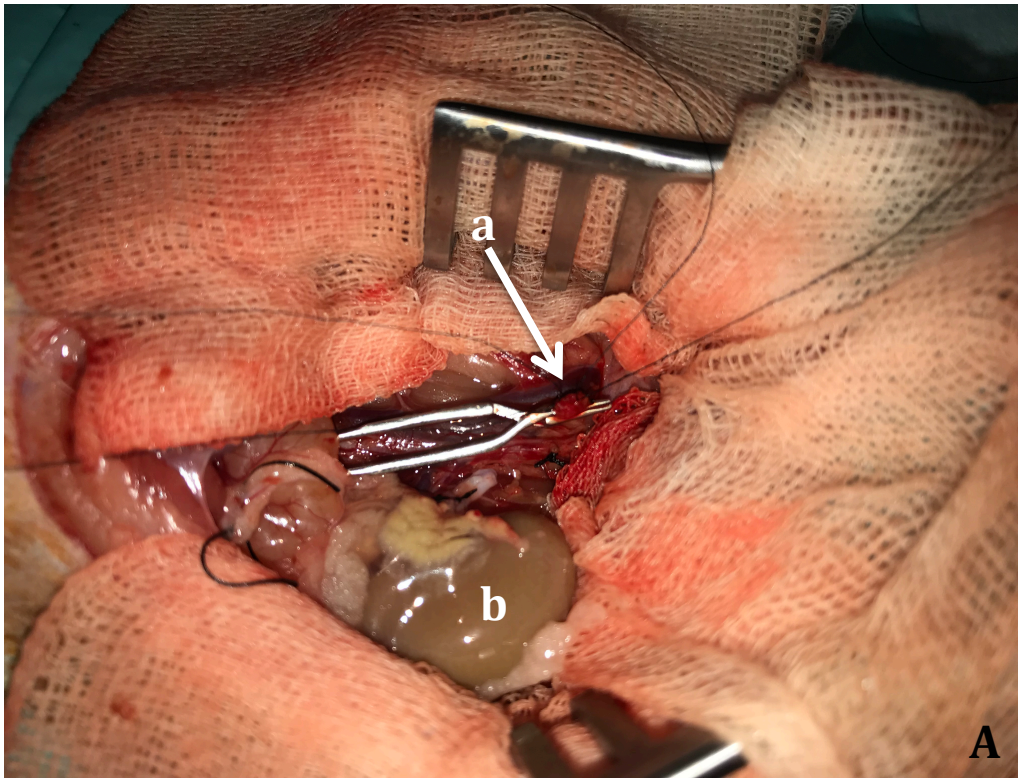


Figure 3.14. A: Preparation of the recipient animal during rat kidney transplantation

Fig 3.14. A: *The operating field for performance of the renal transplant into the recipient animal is shown after removal of its left kidney. At this point, the anastomosis of the renal vein was prepared. Arrow (**a**) points to the end of the recipient's renal vein, which was clamped with a micro- bulldog. The corner stitches were placed into the recipient's vein with 7-0 silk sutures and the next step was to anastomose it with the donor renal vein end-to-end by continuous stitching (**b**).*

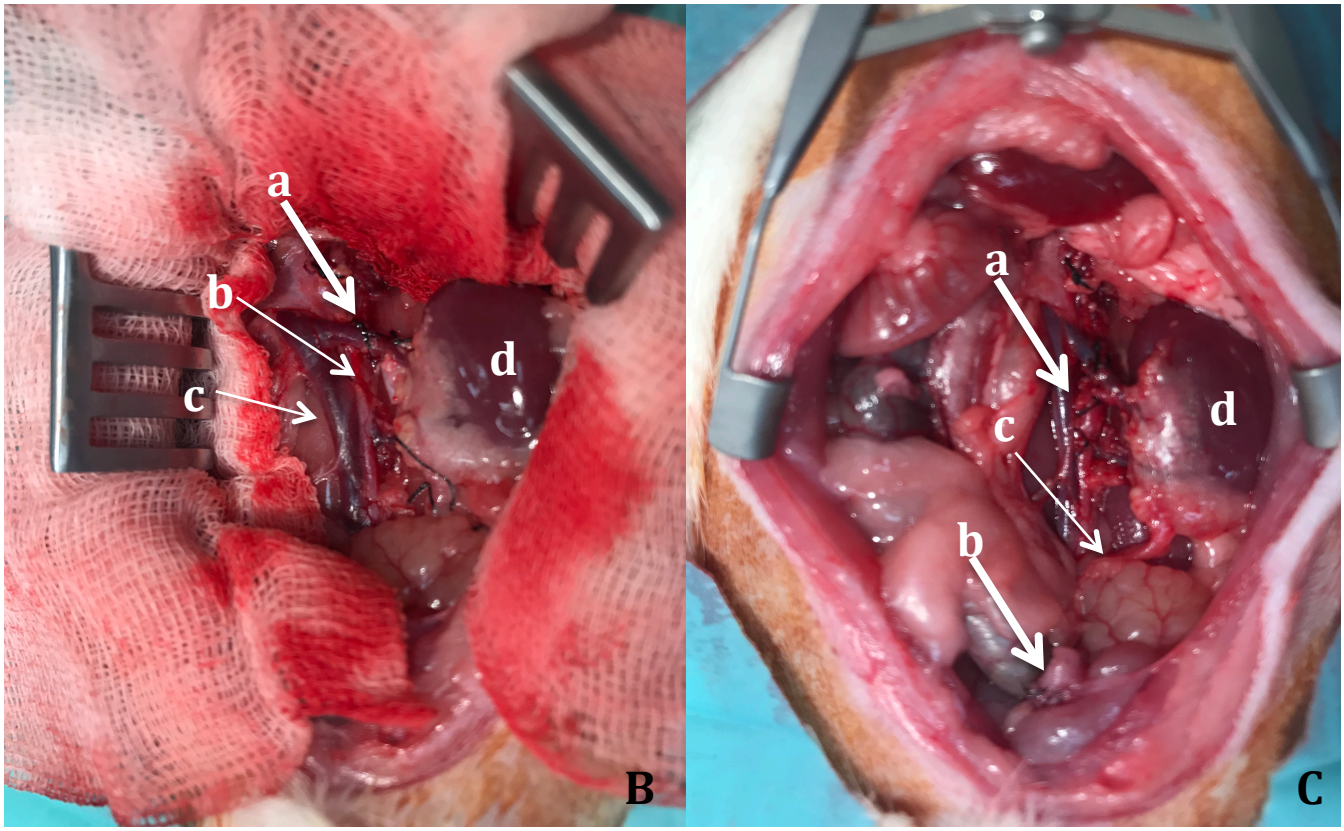


Figure 3.14. B and C: Implantation of kidney in rat kidney transplantation

Fig 3.14. B: *after end-to-end anastomosis of the renal vein (**a**), the donor kidney changed its colour to pink (**d**), indicating a patent renal vein. Arrow (**b**) points to the aorta and arrow (**c**) points towards the inferior vena cava. In **C**, the situs is shown after anastomosing the patch of the donor aorta including the renal artery onto the recipient's aorta (**a**). As a last step, the kidney's ureter (**c**) was anastomosed onto the recipient's bladder (**b**).*

3.5. Project 5: Investigation of the mechanisms of action of MSCs and potential differences of GFP+ and WT- MSCs on rodent macrophages in vitro

Rodent MSCs were extracted and sub cultured as explained in the previous chapter. Frozen samples from each passage (P1-P10) were stored in freezing medium at -80° until used for experiments. Supernatants from the same number of cells for each passage were collected 24 hours after cell seeding to make sure the samples were comparable.

3.5.1. Extraction of bone marrow derived Macrophages from the rat

Cold and sterile PBS was prepared and put into a 50ml Falcon tube on ice.
Instruments for retrieval of the femur and tibia of the rat were autoclaved.
The full culture medium for the macrophage culture was prepared and filtered with a sterile filter:

DMEM (+L-glu)	240ml
Hepes 25mM	3g
L929 conditioned media (25%)	125ml
Pen/Strep (100 U/ml, 100 ug/ml)	10ml

In the animal facility the rat was culled according to the Schedule 1 protocol. The femurs and the tibias of the rat were retrieved and put into the Falcon tube on ice containing the sterile PBS. In a laminar flow cabinet, a petri dish containing sterile, cold Hank's Balanced Salt Solution (HBSS; Gibco) and a 10ml syringe with a 21G needle were prepared. The bones were transferred from the cold PBS into the Petri dish and whilst holding each bone firmly with a pair of forceps, the ends were cut off using a sterile bone cutter. The bone marrow was flushed into a fresh 50ml Falcon tube using the syringe with a 21G needle. The Falcons were immediately kept on ice. The Falcon tube was put into a centrifuge for 5 minutes at 1500 rpm and a temperature of 4°C. The supernatant was discarded and the pellet containing the cells was resuspended in 10 ml of HBSS, then the tubes were placed into the 37°C and 5%CO_2 incubator for 10 minutes to lyse the red blood cells (during lysis, the tube

was inverted). The tube was taken out at placed on ice for a few minutes. Cells were centrifuged again for 5 min at 1500rpm, 4°C. The supernatant was aspirated and the cells were resuspended in 1ml of full culture media. The cells were dispensed equally into 3 large petri dishes containing 25ml full culture medium and cultured for 5 days in the 37°C, 5%CO_2 incubator.

3.5.2. Rat BMDM cell dissociation step (day 5)

In the laminar flow cabinet, the full culture medium was aspirated. 4ml of cell dissociation buffer were added (to cover the surface of the cells) and incubated for 10-20min at 37°C, 5%CO_2. The cells were collected into a 50ml Falcon tube using a cell scraper and adding 4ml of culture medium to neutralize the cell dissociation buffer. Cells were spun down for 5mins at 1500rpm.The supernatant was aspirated and 1ml of full culture medium was added to resuspend the cells. For counting, 10ul tryptan blue and 10ul of the cell suspension were mixed and 10ul of that were put onto a haemocytometer for counting of the cells. In the meanwhile, the cell suspension in the Falcon tube was kept on ice. The cells were counted by counting the 5 outer squares of the square in the middle of the haemocytometer.
The total number was then multiplied times 10 (dilution factor) in order to get the number of cells x10^4/1ml of cell suspension. 2,5x10^5 cells per well were seeded into 24 well plates and 400ul of full culture medium were added, respectively. Cells were incubated over night to adhere to the plastic again. According to the protocol, the experiment was started the next day.

3.5.3. Treatment of LPS stimulated and non stimulated rat BMDMs with supernatants of MSCs

On the next day, the macrophages were inspected under the microscope to make sure the cells looked viable and had the right density. All the supernatants were aspirated and the wells were washed with HBSS. After that, half of the cells within one plate were stimulated with LPS for 1 hour, respectively. The other half of the cells underwent the same procedure of media change but were given plain growth medium before being put back into the incubator for 1 hour. ***Fig. 3.15.*** shows a scheme of the experiment with the respective wells within each 24-well plate.

All the wells were again washed with HBSS before being treated with 250ul of respective supernatants from MSCs P1-P10 (***Fig 3.15.***). To make sure the macrophages had enough nutrients during the overnight treatment with the supernatants, each well contained 150ul DMEM based medium and 250ul of the respective supernatants. The samples ‚macrophage control (Mac ctrl)' were treated with 400ul of the DMEM based medium and the samples ‚MSC control (MSC ctrl) contained 150ul DMEM based medium+ 250ul plain MSC growth medium which was freshly prepared in order to rule out that any effect could come from any of the unknown ingredients of the MSC growth medium (MesenCult proliferation kit, Stem Cell Technologies).

The plates were left in an incubator at 37°C and 5%CO_2 over night for the treatment.

On the next day, the macrophages were washed with HBSS and the cells from each well were lysed using 250ul of Trizol reagent for RNA extraction.

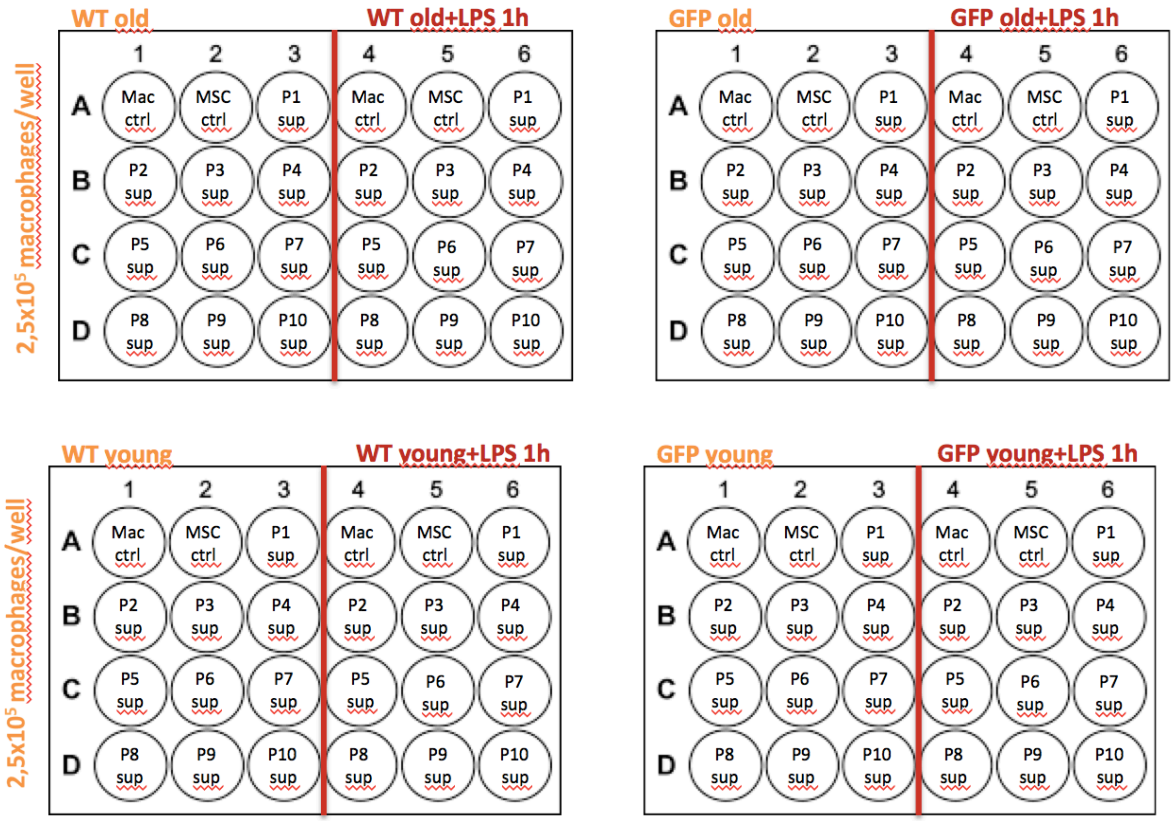


Figure 3.15.: Scheme of in vitro supernatant transfer experiment

Fig 3.15. *The experimental scheme of different in vitro treatments of BMDMs with different supernatants coming from MSCs is illustrated in the Figure.*

WT old= Wildtype Wistar Kyoto rat > 6 months of age as a Mesenchymal Stem Cell (MSC) donor.

WTyoung= Wildtype Wistar Kyoto rat < 6 months of age as MSC donor.

LPS= lipopolysacharid treatment

GFP old= transgenic Wistar Kyoto rat positive for ubiquituous expression of green fluorescent protein (GFP) > 6 months of age as MSC donor.

GFP young= transgenic Wistar Kyoto rat positive for ubiquituous expression of green fluorescent protein (GFP) < 6 months of age as MSC donor.

Mac ctrl= untreated macrophage as control

MSC ctrl= macrophage treated with blank MSC medium

P1-P10= passage 1-10; sup= supernatant

Materials:

- Full culture medium for macrophage culture, consisting of:

DMEM (+L-glu)	240ml
Hepes 25mM	3g
L929 conditioned media (25%)	125ml

Pen/Strep (100 U/ml, 100 ug/ml)	10ml

- Treatment medium without LPS, consisting of:

DMEM	24ml
Foetal calf serum (FCS)	250ul
Penicillin/Streptomycin	500ul
L-glutamine	250ul

- Treatment medium with LPS, containing a final LPS concentration of 100ng/ml:

DMEM	23.975ml
FCS	250ul
Penicillin/Streptomycin	500ul
L-glutamine	250ul
LPS	25ul of 1:100 of stock (conc.: 1mg/ml)

Reagents used:

DMEM
FCS
L929 conditioned media (25%)
LPS (E.coli, Sigma-Aldrich Cat.Nr.: L4524-5MG)
Penicillin Streptomycin
Hank's balanced salt solution (HBSS)
L-glutamine

3.5.4. MTT viability Assay

The MTT viability Assay was performed on the macrophages undergoing previously described experiment to investigate whether the treatments lead to cell death that could cause altered results.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was diluted 1:10 in media. In a 96-well plate, rat bone marrow derived macrophages (BMDM) underwent the same treatment as macrophages in 24-well plates for the previously described experiments. Tissue culture supernatant was removed from cells without disturbing the cell sheet and 100µl MTT solution per well were added. Cells were incubated overnight at 37°C in the incubator. On the next day an equal volume of SDS MTT solution (10% SDS, 0.01M HCl) was added and again, incubated at 37°C for at least 3 hours or overnight. The plate was then read on an ELISA plate reader at 592nm and ODs were compared. For an ideal result, the ODs should be around 1, which indicates no significant amount of cell death through the treatments.

3.5.5. RNA extraction from cells- Phenol Chloroform method

Cells were homogenised in TRIzol (ThermoFisherScientific), 250 µl per 2,5 x 10^5 cells. Homogenised cells were incubated at room temperature for 10 minutes. Chloroform at a ratio of 1: 5 (50 µl) was added and samples were shaken vigorously for 15 seconds. Samples were incubated for 3 minutes at room temperature to be then centrifuged at 12.000 RCF for 15 minutes at 4°C. The top aqueous layer containing the RNA was removed and precipitated with a 1:2 volume (125 µl) of 2-propanol to the volume of TRIzol used for homogenisation. This was incubated at room temperature for 10 minutes and then centrifuged for 10 minutes as 12.000 RCF at 4°C. The supernatant was removed and the remaining pellet, which was often very small or invisible, was washed with a 1:1 volume (250 µl) of 75% ethanol to the TRIzol used for homogenisation. This was then centrifuged at 7.500 RCF for 5 minutes and this step was repeated twice as wash steps. Thereafter, the pellet was air dried for 6 minutes at room temperature to remove all the ethanol. The dry pellet was then resuspended in 20 µl of RNAse free water. DNA was digested using DNase I (Sigma-Aldrich). Per 8µl of RNA, 1µl of DNAse and 1µl of 10x Reaction Buffer were added and the samples were incubated at room temperature for 15 minutes before 1µl of stop solution was added, respectively, and samples were incubated in a heat block at 70°C for 10 minutes before being placed on ice. The concentration and purity of RNA was assessed using the A_{260}:A_{280} ratio and A_{260}:A_{230} ratio, respectively on the Nano drop 2000c Spectrophotometer (Thermo Fisher Scientific).

3.6. Material and Methods used for all projects

3.6.1. Histology

2 biopsy samples from the upper pole of the kidneys were taken at the following times: at the start of the warm ischemia time (t=0); after 24h of cold static storage (t=24h); after the preconditioning period (t=28h) and after reperfusion (t=31h). One sample was placed in a tube filled with Allprotect and stored at -80°C. The other sample was fixed in Formalin to be transferred into 70% Ethanol 24 hours later and then handed over to a lab technician for processing into paraffin embedded histology slides. (4mm, stained with Haematoxylin and eosin as well as Periodic acid–Schiff–diastase, respectively). The slides were evaluated by two blinded and independent researchers and ranked for tubular injury: thereby, brush boarder, tubular dilation, tubular debris and epithelial flattening were used to determine the grade of damage.

3.6.1.1. RNA extraction from kidney biopsy samples

For RNA extraction from kidney biopsies, the samples stored in Allprotect were used. RNA extraction was performed using the AllPrep® DNA/RNA/Protein Mini Kit (Quiagen) according to manufacturer's instructions. Tissue samples of 15-20mg were disrupted and homogenized using the TissueLyser II (Qiagen). Thereby, tissues were disrupted by rapid agitation in the presence of steel beads (5mm, Qiagen) and lysis buffer. Lysis buffer consisted of RLT buffer provided in the kit and β-Mercaptoethanol (10µl per ml RLT). After homogenization, the lysates were centrifuged at full speed for 3 minutes. The supernatants were carefully removed and transferred to AllPrep DNA spin columns in 2ml collection tubes and spun for 30 s at 10.000 x g. The DNA columns were discarded and to the flow-trough's from the previous step, 250 µl of 96-100% Ethanol were added. After mixing by pipetting up and down, the samples were transferred onto the RNeasy spin columns placed in 2ml collection tubes and centrifuged for 15s at 10.000 x g. The flow-trough's were transferred into 2ml tubes for protein purification in the next step. RNeasy columns underwent wash steps with provided buffers and as a last step, RNAs were obtained by adding 30µl of RNAse-free water onto the RNeasy spin columns and spinning them for 1 min at 10.000 x g to elute the RNA. DNA was digested using DNase I (Sigma-Aldrich). Per 8µl of RNA, 1µl of DNAse and 1µl of 10x Reaction Buffer were added and the samples were

incubated at room temperature for 15 minutes before 1µl of stop solution was added, respectively, and samples were incubated in a heat block at 70°C for 10 minutes before being placed on ice. The concentration and purity of RNA was assessed using the A_{260}:A_{280} ratio and A_{260}:A_{230} ratio, respectively on the Nano drop 2000c Spectrophotometer (Thermo Fisher Scientific).

3.6.1.2. Interpreting Nano drop (Spectrophometric) Results

The foundation of Spectrophotometry is the Beer-Lamber Law:

$$A=\varepsilon cl$$

A= absorbance, ε= extinction coefficient, c= concentration and l=path length

The Beer-Lambert law draws a direct correlation between absorbance and concentration. Nucleic acids have a peak absorbance of UV light at 260nm. Thus, the amount of light absorbed in this region can be used to determine the concentration of RNA or DNA in solution by applying the Beer- Lambert law. Nucleic acids absorb UV light at 260 nm due to the aromatic base moieties within their structure and proteins and phenolic compounds have a strong absorbance at 280 nm. Therefore, the A260/280 ratio is generally used to determine protein contamination of a nucleic acid sample. For pure RNA and DNA, A260/280 ratios should be somewhere around 2.1 and 1.8, respectively. A lower ratio indicates the sample is protein contaminated, which may have an effect on downstream applications that use the nucleic acid samples.

When measuring the concentration of nucleic acids, Nano drop also shows a A260/230 ratio, which indicates the presence of organic contaminants, such as with phenol, TRIzol, chemotropic salts and other aromatic compounds. In a pure sample, the A260/230 should be close to 2.0.

3.6.2. Reverse transcription

Reverse transcription was performed from 100ng (for cells) to 1µg total RNA per sample using the iScript™ Reverse Transcription Supermix for RT-qPCR (BioRad,

Hertfordshire, UK) according to manufacturer's instructions. For all the experiments, the total amount of RNA used in one PCR run was the same so that an optimal calculation was possible.

3.6.3. qRT-PCR

Quantitative real time PCR (qRT-PCR) was performed using the iScript Sybr Green Supermix (BioRad, Hertfordshire, UK). Optimal Primer combinations, their concentrations and the necessary PCR programmes were tested before the actual qRT-PCR. The PCR samples were performed in duplicates in 96-well PCR plates (Thermo Fisher Scientific) on an Eppendorf Mastercycler® RealPlex. Melt curves for the resulting products were analysed for every reaction. The housekeeping genes β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as positive controls. Primer pair sequences for these genes as well as their product lengths and melting curves are shown in ***Tables 3.2. - 3.4.*** All primer pairs were designed using Primer blast (NCBI) and were synthesised by Sigma- Aldrich. The relative quantification of a target gene in the PCR reaction compared to the control was calculated using the comparative Ct method ΔΔCt. Thereby, the following formula was used for the calculation of the relative expression of the target genes:

∆Ct = Ct (gene of interest) – Ct (housekeeping gene)

and

Fold gene expression = 2^-(∆∆Ct)

The following species specific Primer pairs were used for the experiments:

- **RAT SPECIFIC PRIMER COMBINATIONS:**

GENE	SEQUENCE	Tm	PRODUCT LENGTH (bp)	concentration used in PCR
HPRT for	GCACGAGGGACTTACCTCAC	63.7°C	128 bp	100nM
HPRT rev	TAATCACGACGCTGGGACTG	66.2°C	128 bp	100nM
IL-10 for	TAAAAGCAAGGCAGTGGAGC	64.3°C	147 bp	100nM
IL-10 rev	TGCCGGGTGGTTCAATTTTTC	69.7°C	147 bp	100nM
TNFα for	ATGGGCTCCCTCTCATCAGT	65°C	106 bp	100nM
TNFα rev	GCTTGGTGGTTTGCTACGAC	64.5°C	106 bp	100nM
NOs-2 for	TCAGGCTTGGGTCTTGTTAGC	65.4°C	110 bp	100nM
NOs-2 rev	GAAGAGAAACTTCCAGGGGCA	66.5°C	110 bp	100nM
Mrc-1 for	TGATTCCGGTCGCTGTTCAA	68.9°C	99 bp	100nM
Mrc-1 rev	GAACGGAGATGGCGCTTAGA	66.5°C	99 bp	100nM

Table 3.2. Rat specific primer combinations

Table 3.2.: *Primer combinations for experiments in the rat are shown in Table 3.2. Combinations have been created using Primer-Blast.*

- **PIG SPECIFIC PRIMER COMBINATIONS**

GENE	SEQUENCE	Tm	PRODUCT LENGTH (bp)	concentration used in PCR
Actin for	AAGCTCAGTCGGGCTTCTCT	64.5°C	111 bp	300nM
Actin rev	CGTAGAGGTCCTTGCGGATG	66.9°C	111 bp	300nM
NGAL for	CATCATCTTCCCCGTCGCAA	70.4°C	108 bp	300nM
NGAL rev	ATGGGCAAAGGCTGAAGACA	66.9°C	108 bp	300nM
EDN-1 for	CCGCTTTCGGTTTAGTTCGC	64.5°C	108 bp	300nM
EDN-1 rev	GAGCTCCTTGGAAAGCCACA	65.3°C	108 bp	300nM
TNFα for	GCCCTTCCACCAACGTTTTC	69.8°C	97 bp	300nM
TNFα rev	CAAGGGCTCTTGATGGCAGA	64.1°C	97 bp	300nM
IL-1β for	CCTTGAAACGTGCAATGATGACT	62.3°C	100 bp	300nM
IL-1β rev	GCCAGCCAGCACTAGAGATT	67.4°C	100 bp	300nM

Table 3.3. Pig specific primer combinations

Table 3.3.: *Primer combinations for experiments in the pig are shown in Table 3.3. Combinations have been created using Primer-Blast.*

- **HUMAN SPECIFIC PRIMER COMBINATIONS**

GENE	SEQUENCE	Tm	PRODUCT LENGTH(bp)	concentration used in PCR
NGAL for	TCACCCTCTACGGGAGAACC	71.1°C	109bp	500nM
NGAL rev	GGGACAGGGAAGACGATGTG	68.5°C	109bp	500nM
KIM-1 for	ATGTGTGGGGAAGAAGCTGG	66.3°C	82bp	500nM
KIM-1 rev	CTGAGAGCTCTGTGCCTTCC	64.3°C	82bp	500nM
b-Act for	GTGGGGCGCCCCAGGCACCA	65.3°C	100bp	500nM
b-Act rev	GTCCTTAATGTCACGCACGATTTC	62.1°C	100bp	500nM
TNFa for	AGAGGGAAGAGTTCCCCAGGGAC	59.8°C	97bp	500nM
TNFa rev	TGAGTCGGTCACCCTTCTCCAG	64.5°C	97bp	500nM
EDN1 for	GCCCTCCAGAGAGCGTTATG	63.4°C	111bp	500nM
EDN1 rev	GATGGTTGGGGGAACTCCTT	65.1°C	111bp	500nM

Table 3.4. Human specific primer combinations

Table 3.4.: *Primer combinations for experiments in the human are shown in Table 3.4. Combinations have been created using Primer-Blast.*

PCRs were run on an Eppendorf EP 384 Mastercycler and following protocols were used:

- **RAT PCR PROTOCOL:**

  3 minutes at 95°C- hot start, followed by 40 cycles of the following:

  15 seconds at 95°C denaturation step
  20 seconds at 60.4°C primer annealing step
  40 seconds at 72°C elongation step, followed by

  Melting curve
  Hold at 4°C

- **PORCINE PCR PROTOCOL:**

  3 minutes at 95°C- hot start, followed by 35 cycles of the following:

  15 seconds at 95°C denaturation step
  20 seconds at 60°C primer annealing step
  40 seconds at 72°C elongation step, followed by

  Melting curve
  Hold at 4°C

- **HUMAN PCR PROTOCOL:**

  3 minutes at 95°C- hot start, followed by 40 cycles of the following:

  15 seconds at 95° denaturation step
  20 seconds at 60°C primer annealing step
  40 seconds at 72°C elongation step, followed by

  Melting curve
  Hold at 4°C

3.6.4. Agarose gel electrophoresis

In order to check whether the PCR products were specific for the selected primer pair, besides looking at the melting curves of the respective PCR products, agarose gel electrophoresis was performed. Agarose gel electrophoresis is a method used to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose. DNA and RNA fragments are separated by length by applying an electric field to move the negatively charged molecules from the negative pole to the positive pole through an agarose matrix. Ethidium bromide is added to the agarose solution in order to visualize the products. It intercalates into the major grooves of the DNA, and fluoresces under UV light.

For the preparation of the gel, 3g of agarose were added to 150ml of Tris-acetate-EDTA (TAE) buffer and the mixture was heated in the microwave until the powder was completely dissolved. 7µl of ethidium bromide were added and the hot fluid was poured into the apparature where it was left to cool off and harden. Combs were inserted to create little pockets to load the DNA products.
Into the respective last pocket of each side, 10µl of DNA ladder, marking 25bp steps, were added. In between the ladder, 20µl of PCR product with 4µl of a 6x loading dye were put into the pockets. The gel was ran at 120 Volts for approximately 40 minutes from the negative pole to the positive pole. Straight after that, a picture was taken under UV light and the size of the PCR products was compared to the size of the products, estimated by Primer Blast.

Materials:

- Agarose (Sigma-Aldrich)
- Tris Acetate EDTA (TEA) buffer, general lab supply
- Ethidium bromide

Chapter 4: Results

4.1. Project 1: Development of a Normothermic Machine Perfusion circuit for ex-vivo kidney preconditioning on the RM3-perfusion machine and testing of a portable micro dialysis device.

4.1.1. Development of an NMP perfusion circuit using the Waters RM3 perfusion machine

As mentioned in chapter 3.1., for the experiments on the introduction of Mesenchymal Stem Cells into an ex-vivo organ perfusion system, it was necessary to also think of the influence of temperature on the MSCs and their viability. Therefore, the development of a normothermic perfusion circuit for ex-vivo perfusion of porcine and human kidneys was necessary.

By studying the Waters RM3 circuit for Hypothermic Machine Perfusion, it was possible to figure out how to amend the circuit accordingly. Oxygenators, opened but not used for extracorporeal machine oxygenation (ECMO) support of patients undergoing cardiac surgery, were integrated in between the reservoir which collected the venous outflow and the arterial outlet of the machine, which delivered the oxygenated blood to the organ. The Grant water heating device sustained the temperature of the perfusate and via the built in silicone membrane within the bubble trap at the arterial outflow chamber, necessary drugs like glucose, prostavasin, heparin and vitamins, could be delivered according to protocol.

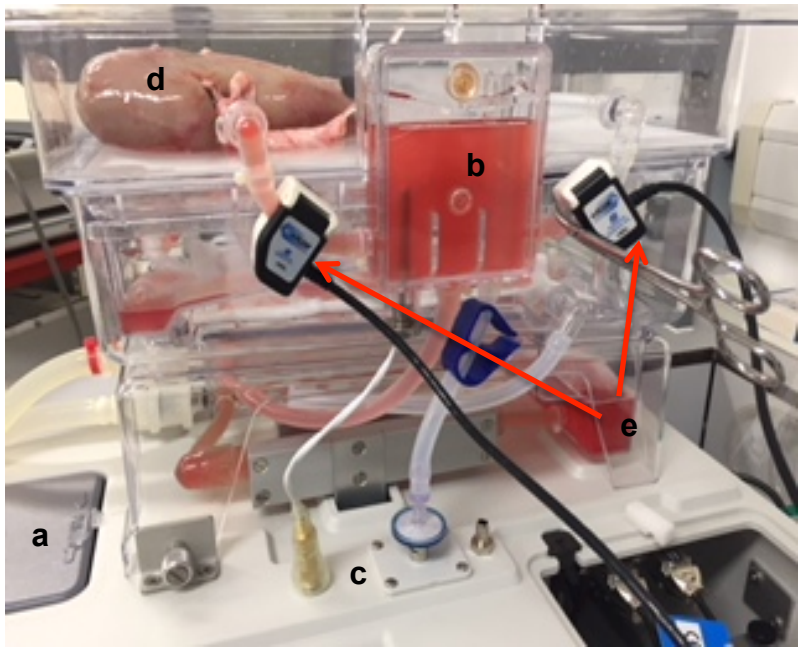


Figure 4.1.: Hypothermic machine perfusion circuit for porcine and human organs

Fig 4.1. *Shows the circuit for Hypothermic Machine Perfusion (HMP) of human and porcine kidneys using the RM3 pulsatile machine perfusion device.*

- ***a)*** *Cooling reservoir for water+ice*
- ***b)*** *Bubble trap and arterial reservoir with silicone membrane to deliver drugs and take perfusate samples with a syringe*
- ***c)*** *Temperature sensor*
- ***d)*** *Kidney, attached to the arterial outlet of the RM3 perfusion machine*
- ***e)*** *Flow probes (arrows) for real time measurement of perfusate flow rates. If only one kidney was perfused at a time, the other arterial outlet was clamped and the flow rate was 0 for that outlet.*

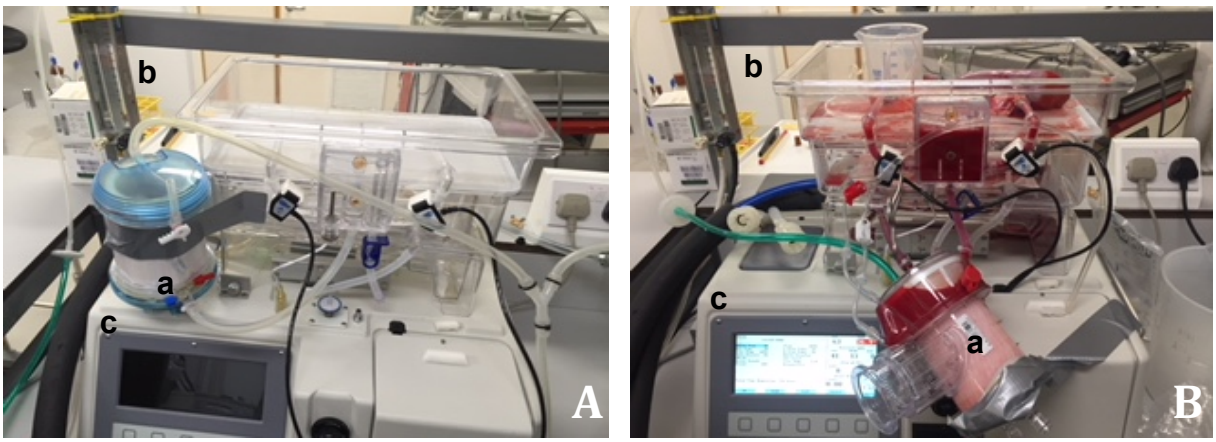


Figure 4.2.: Modified circuit for Normothermic machine perfusion using the RM3 machine

Fig 4.2. (A) *and* ***Fig. 4.2. B*** *both show the modified circuit for Normothermic Machine Perfusion (NMP) of human and porcine kidneys using the RM3 pulsatile machine perfusion, (**A**) shows the empty circuit and (**B**) shows the circuit with a kidney attached. Everything remained as described for the HMP circuit but the following instruments were added to the circuit for NMP:*

- ***a)*** *Oxygenator*
- ***b)*** *Flow meter for measurement of delivered oxygen*
- ***c)*** *Tubes coming from the Grant water heating device (not shown)*

The O_2/CO_2 containing bottle is not shown on this illustration.

Fig. 4.1. *and* ***Fig. 4.2.*** show how the circuit for HMP using the Waters RM3 perfusion device was modified. Unused membrane oxygenators (arrow) were used to deliver oxygen (95% O_2/ 5%CO_2) to the packed red cell based perfusion solution, which was kept at 36°C-37°C by connecting the tubes from the coolant reservoir to a Grant heated circulating water bath. 4 pairs of porcine kidneys were used to set up the Normothermic Machine Perfusion circuit and to establish a reproducible experimental protocol with timings for blood gas measurements and biopsies. 1 kidney per pair was thereby perfused hypothermically (data not shown) in order to prepare the logistics for the actual experiments. ***Fig. 4.3.*** summarizes the results for the first normothermically perfused kidneys (n=4). The intrarenal resistance index (RRI) (***Fig. 4.3. a***) was going up slightly after starting NMP to then come down after 30 minutes and to reach the lowest values at the time point of 120 minutes and to stabilize at values of around 1.2 after the preconditioning phase. The minimal value was 1.02 and the maximum was 1.7 in that cohort. For the perfusate flow-rate (***Fig. 4.3. b***), which is dependent on the RRI, interestingly, the highest value of 23.86ml/min/100g tissue was reached 1 hour after the RRI being at its lowest value, before then decreasing when the RRI went up. The perfusate flow rate was at its lowest at the start of NMP with a minimum value of 3.69 ml/min/100g tissue. This is unsurprising considering the organ was stored on ice and the perfusion is started abruptly. There was also some urine production during the NMP period, which was encouraging and indicating that the model worked. ***Fig. 4.3. c*** illustrates the urinary output in ml at the time points of measurements, with increasing intervals. For the oxygen consumption, a steady increase could be observed, with a minimal oxygen consumption of 0.77 ml/min/g of tissue at 30 minutes of perfusion, when also the RRI was highest and the perfusate flow rate was low, and a maximum of 14.72 ml/min/g of tissue. As only viable tissue is consuming oxygen, this was a further encouraging result for the reliability of the NMP perfusion circuit.

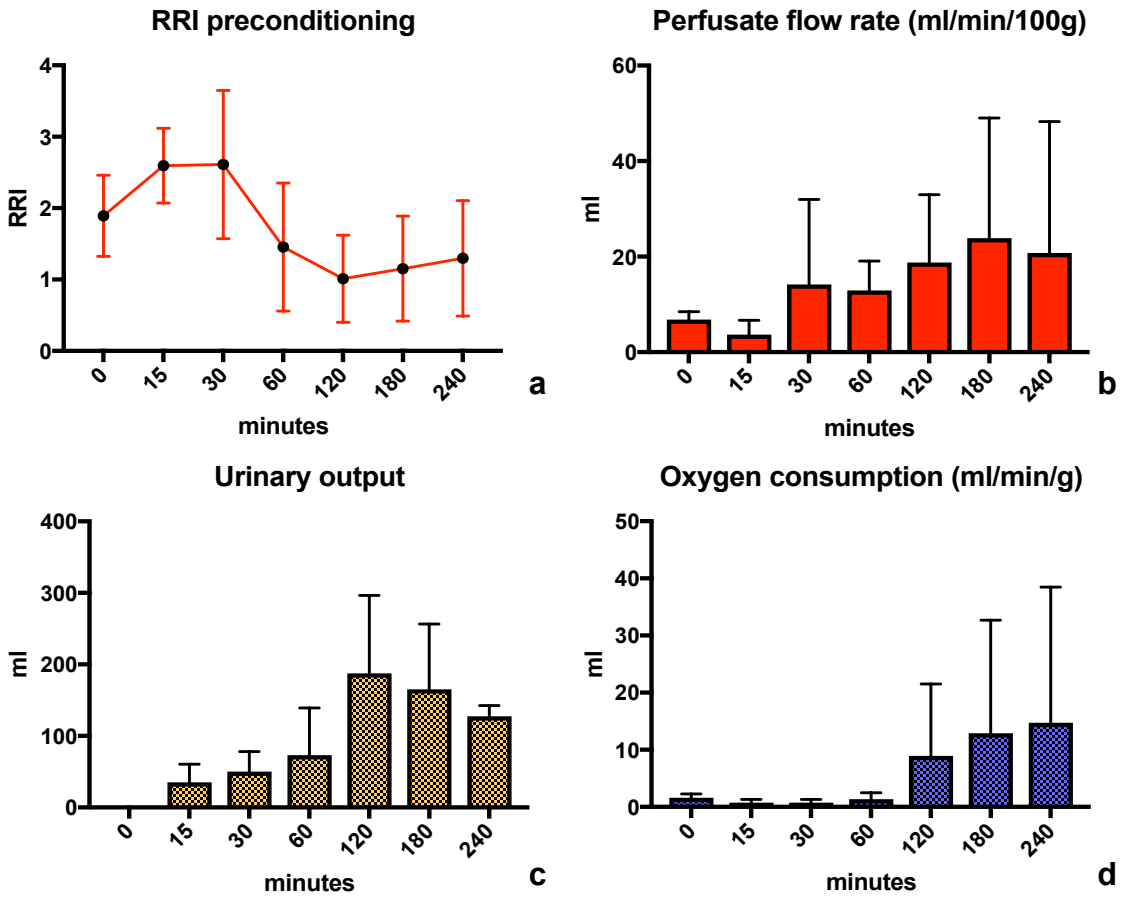


Figure 4.3.: Physiological parameters for the first kidneys undergoing NMP on the RM3 machine

Fig 4.3. a *illustrates the Renal Resistance Index of the first 4 kidneys undergoing Normothermic Machine Perfusion on the RM3 perfusion device. The perfusate flow rates at the measured time points in ml/min/100g tissue are shown in* ***b****.*
Graph ***c*** *shows the total urinary output at each time point of measurement from the previous time point in mls and graph* ***d*** *highlights the oxygen consumption at each time point in ml/min/g of tissue.*

4.1.2. Testing of a portable micro dialysis device

These first pairs of porcine kidneys also served as an important test group for the portable micro dialysis device, which is in the development phase at the Department of Bioengineeering at Imperial College London. The device would be the first of its kind and the technology is very new and the sensors are very sensitive to environmental influences like temperature and movement. Therefore, it was important to test the device on some kidneys in this phase before using data from the measurements during valid experiments. We were able to measure the glucose and lactate levels of 3 kidneys from the time of retrieving the intraabdominal organ package until we finished an experiment on the next day after reperfusion. Some of the data from that period was not usable due to calibration errors or problems with

the device itself. However, at the end of the test phase, we were able to draw one graph that demonstrated the influence of events during the retrieval of the organs, like warm ischemic time, the cooling process of the organs and also the time of CSS during transport. ***Fig. 4.4.*** shows (***a***) the inserted micro dialysis probe with the fluid pump during the time of cold perfusion of the organs and (***b***) the probes in place during the reperfusion period. ***Fig. 4.5.*** illustrates the results of one of the first successful mobile micro dialysis measurements from the time of retrieval until arrival at the laboratory, in a kidney with a long warm ischemic time of 45 minutes at retrieval. It shows high levels of both glucose and lactate in the extracellular space of the kidneys straight after insertion of the probe. The peaks during the period of WIT are artefacts, which occur during movement of the kidney whilst the dissection of surrounding tissue was performed and highlights how sensitive the probes are to environmental influences. The graph however highlights a steady decrease of both glucose- and lactate levels during the period of WIT, which was further enhanced by the cold perfusion and thereafter remained steady at levels close to 0 during the phase of transit to the laboratory. After these pilot experiments, we were able to record some further data during experiments comparing the effects of Hypothermic- and Normothermic Machine Perfusion with each other as well as the influence of the treatment with MSCs. Due to a delay of ten minutes for live measurements within the dialysate, only a small window of warm ischemia time at the time of harvesting the organs could be measured during these experiments. For this reason, after completion of experiments with the focus on different preservation modalities, a further 3 pairs of kidneys were exposed to a warm ischemia time of 45 minutes in order to obtain a longer window for observations using the micro dialysis instrument.

In summary, we were able to obtain micro dialysis data on the following experimental groups of porcine kidneys:

- HMP: 8 kidneys, including 3 kidneys with a longer WIT of 45minutes
- NMP: 8 kidneys, including 3 with a longer WIT of 45 minutes
- HMP + MSC : 2 kidneys
- NMP + MSC: 4 kidneys
- cold storage: 2 kidneys, including 2 with longer WIT of 45 minutes

In terms of pairs in direct comparison that was:

- HMP vs. NMP, no stem cells, short WIT: 3
- HMP vs. NMP, no stem cells, long WIT: 3
- stem cells vs. no cells, HMP, short WIT: 2
- stem cells vs. no cells, NMP, short WIT: 3
- NMP with stem cells vs. cold storage, short WIT: 1
- HMP (no stem cells) vs. cold storage, long WIT: 1

The overall results of the micro dialysis results will be summarized at the end of Chapter 4.3. An overview of the outcomes including kidneys from all study groups will be shown.

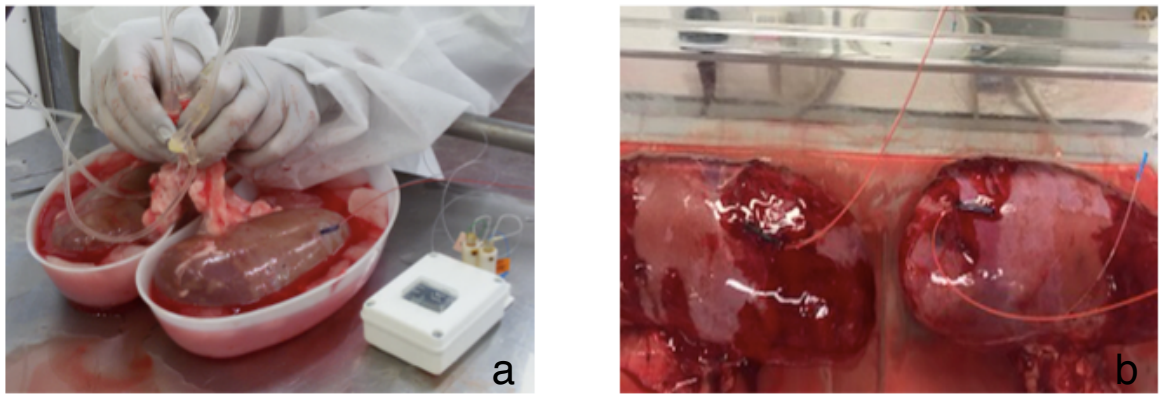


Figure 4.4.: Insertion of micro dialysis probes at the abattoir

Fig 4.4. a *shows a pair of porcine kidneys undergoing cold perfusion after retrieval at the abattoir. A microdialysis probe was inserted into the cortex of one kidney. Stitching around the probe using 5-0 Prolene sutures kept the probe in place during that time.* ***Fig. 4.5. b*** *shows the same pair of kidneys undergoing reperfusion at the laboratory, with microdialysis probes in place to measure lactate and glucose concentrations in real time.*

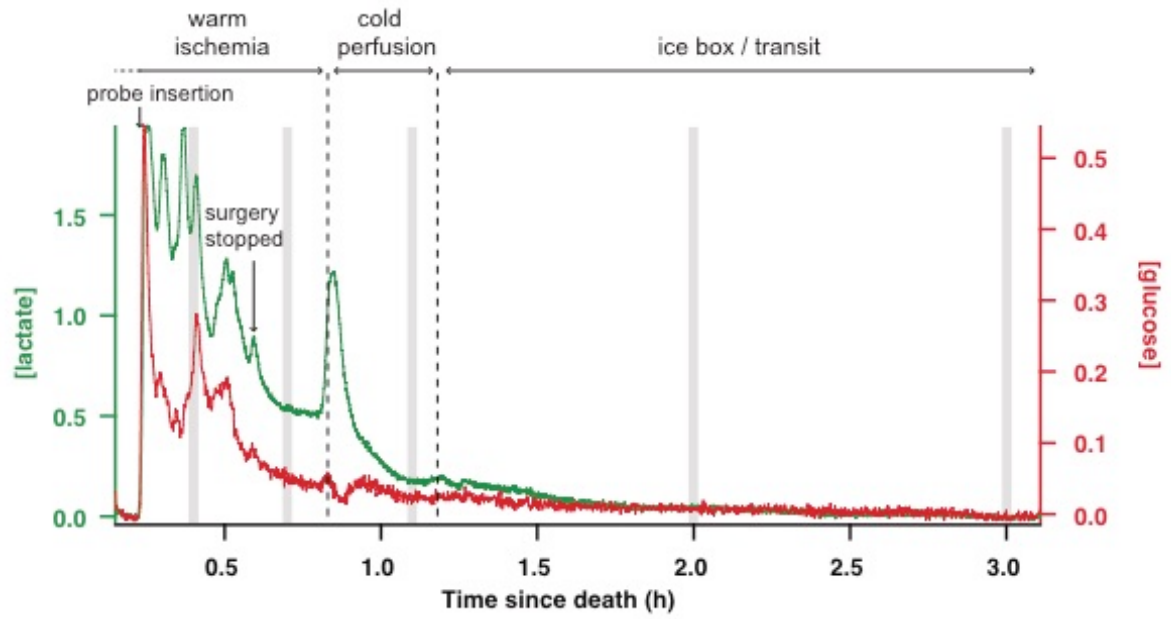


Figure 4.5.: Micro dialysis measurements during retrieval of porcine kidneys

Fig. 4.5. *Typical dialysate glucose and lactate levels of a kidney after prolonged warm ischemia time (WIT) immediately after retrieval during passive warm ischemia, cold perfusion and cold storage. After retrieval of the intra-abdominal organ package, the kidney was left at body temperature (37°C) for 45 min before being perfused with cold (4°C) Soltran solution and stored on ice while in transit to the laboratory. Each trace represents real-time data recorded at 10 Hz and smoothed with a Savitsky-Golay 101-point filter. (Figure created by Dr. Isabelle Samper from the Department of Bioengineering at Imperial College).*

In conclusion, we were able to successfully establish a Normothermic Machine perfusion circuit for our series of experiments, using the Waters RM3 perfusion device. The effect of it being a pulsatile perfusion circuit, contrary to other similar perfusion circuits, will be discussed in Chapter 5. Furthermore, it was possible to test the worldwide first mobile micro dialysis device which delivered live data on lactate- and glucose concentration from the extracellular space in kidneys used for our experiments. Once fully established, data from the micro dialysis device will give important information on what is happening within the graft during warm- and cold ischemia times and it will also help to estimate the condition of organs for transplantation in the future.

4.2. Project 2: Investigation and direct comparison of the effects of current methods of ex-vivo organ preservation on porcine and human kidney grafts after prolonged cold ischemia times

After successfully establishing a circuit for NMP using the RM3 pulsatile perfusion device, we were interested in the impact of the two most widely used ex-vivo preservation methods on porcine and human kidney grafts in a direct head to head comparison, as the data on such a direct comparison is scarce, with only two studies published so far. In the most recent, performed by Blum et al. in 2017, 10 porcine kidneys after a WIT of 45 minutes and a CIT of 5 hours either underwent NMP (n=5) or HMP (n=5) for 8 hours before undergoing a simulated reperfusion phase. Upon reperfusion, no differences between both groups could be detected in terms of oxygen consumptions, rates of urine production, creatinine clearance or fractional sodium excretion. Furthermore, the resistance indices were similar after 30 minutes of simulated reperfusion. However, histologically, in NMP kidneys, increased vacuolization after preservation and greater loss of tubular integrity after reperfusion, were demonstrated, compared to the HMP group. Interestingly, the perfusate levels of alkaline phosphatase (AP) and gamma glutamyltransferase (GGT) were higher in NMP kidneys during preservation, but during simulated reperfusion, peak AP and GGT values were over 14 times higher in the HMP group than peak AP and GGT during preservation of NMP kidneys. Their conclusions were that through NMP, preservation of renal function was provided and was comparable to HMP, and that furthermore, AP and GGT release upon reperfusion could be minimized by NMP when compared to HMP[120]. The limitations to this study were however, that kidneys were not investigated as pairs from the same donor pig. Kidneys were retrieved, randomized into the groups and the respective other kidney was discarded.

The other comparative study was performed by Metcalfe et al. in 2002[315]. Similar to our study, perfusion on a pulsatile organ perfusion system (POPS) at normothermia (37°C) and with a mean perfusate pressure of 50mmHg, was compared to hypothermic machine perfusion on the RM3 machine of the paired porcine kidneys was performed. The perfusion time in that study however was 16 hours and the number of pairs was six (n=6). After that period, organ function and quality was assessed by a reperfusion period of 2 hours, comparing functional parameters like urinary output, perfusate flow rate and pressure, and urine and perfusate concentrations of sodium, creatinine, protein, and glucose. From these, the intrarenal

vascular resistance and the creatinine clearance were calculated. In their study, The POPS-preserved kidneys performed better than the RM3-preserved organs, however only for creatinine and sodium concentration ratios, these differences were significant.

There are no data so far on a direct comparison of human kidneys in such an ex vivo perfusion model, comparing two human kidneys coming from the same donor and undergoing either HMP or NMP ex vivo using the exact same perfusion device.

4.2.1. Direct comparison of HMP and NMP in the porcine model

In our porcine study, 22 kidneys from 13 donor pigs were retrieved at an abattoir as described above and then stored at 4°C (CSS) for 24 hours, before either undergoing HMP (n=9) or NMP (n=9) for 4 hours, and thereafter being reperfused for 2 hours with whole blood. Experiments for the direct comparison between HMP and NMP were conducted on pairs coming from the same donor pig, respectively. 4 consecutive single kidneys were allocated to be stored on ice and then reperfused for 2 hours, to serve as a small internal control group for preservation on CSS. To summarize, the groups for this initial study were put together as follows:

Group 1*: 24 hours of CSS, followed by 4 hours of Normothermic machine perfusion (NMP) and 2 hours of reperfusion (n=9)*
Group 2*: 24 hours of CSS, followed by 4 hours of Hypothermic machine perfusion (HMP) and 2 hours of reperfusion (n=9)*
Group 3*: 28 hours of Cold static storage (CSS) and 2 hours of reperfusion (n=4)*

Kidneys after HMP showed significantly higher urinary output rates, both for continuous measurements in ml/min, with a mean of 4.8±1.7 ml/min vs. 2.0±1.2 ml/min (p=0.011, ***Fig.4.6. a***) and for the total volume over 2 hours of reperfusion, with a mean of 120.7±49.5ml vs. 55.5±13.8ml (p=0.041, ***Fig.4.6. b***). Also, the oxygen consumption of kidneys after HMP during the reperfusion period was significantly higher than for kidneys after NMP with a mean of 23.7±5.3ml/min vs. 11.1±3.2ml/min (p=0.006), suggesting better viability of the organs after HMP (***Fig.4.7.***).

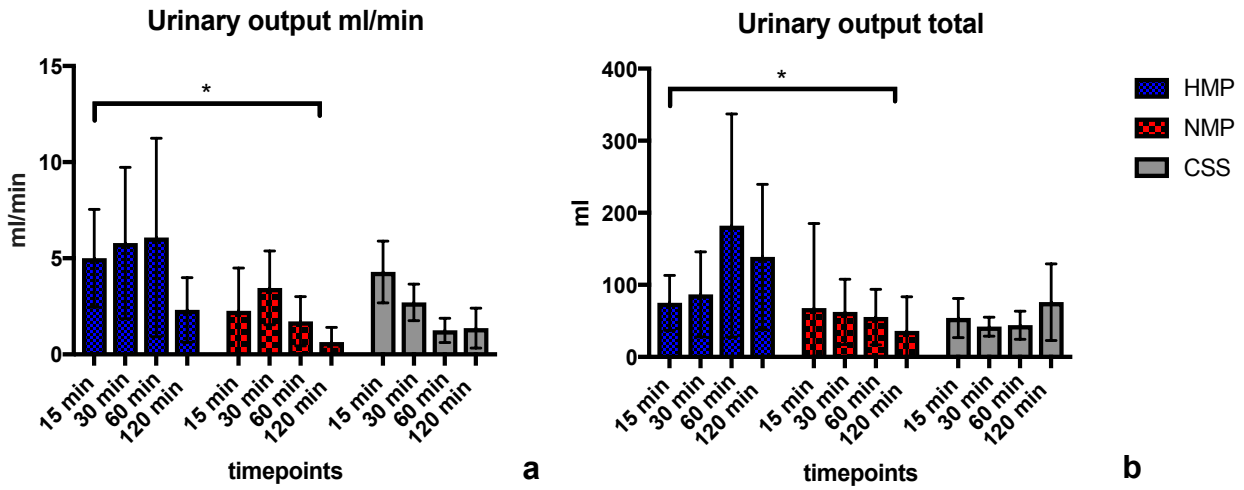


Figure 4.6.: Urinary output rates NMP vs. HMP in the porcine model

Fig. 4.6.: *urinary output rates during reperfusion in ml/min (**a**) and in total volumes (**b**) are shown for the different groups at timepoints of measurement.*
HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion; CSS= Cold Static Storage.
Statistical analyses carried out using ANOVA, statistical significance: p<0.05 (*); p<0.005 (**).

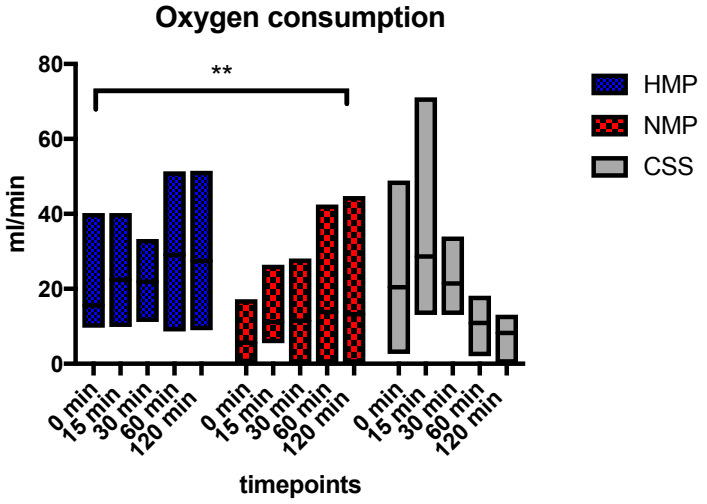


Figure 4.7.: Oxygen consumption rates HMP vs. NMP in the porcine model

Fig. 4.7.: *oxygen consumption rates during reperfusion in ml/min for the different groups at the timepoints of measurement, starting at t0 for the moment of onset of the reperfusion period.*
HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion; CSS= Cold Static Storage.
Statistical analyses carried out using ANOVA, statistical significance: p<0.05 (*); p<0.005 (**).

The intrarenal perfusate flow rates in ml/100g of tissue during the reperfusion period were significantly higher for kidneys in the HMP group compared to kidneys in the NMP group, with mean values of 46.5±14.9ml/min/100g vs. 26.1±7.8ml/min/100g (p=0.042, ***Fig. 4.8. b***), which is unsurprising considering the significantly lower

intrarenal resistance indices over time in the HMP group compared to the NMP group during the preconditioning period, with mean values of 0.71±0.27 vs. 1.49±0.19 (p=0.0075, ***Fig. 4.8. a***).

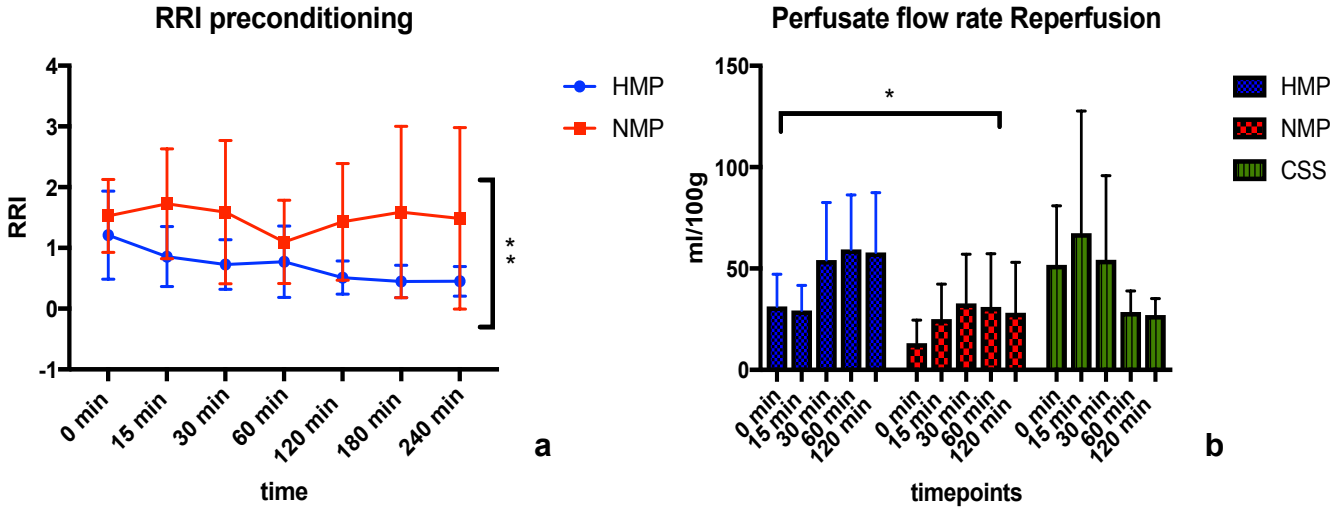


Figure 4.8.: Intrarenal Resistance index and perfusate flow rate HMP vs. NMP in the porcine model

Fig. 4.8.: *Intrarenal Resistance Indices over time for both groups during the preconditioning phase are shown in* ***a****. The resulting perfusate flow rates during reperfusion are shown in* ***b*** *for the different treatment groups.*
HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion; CSS= Cold Static Storage
Statistical analyses carried out using ANOVA, statistical significance: p< 0.05 (*); p<0.005 (**).

In regards to the Creatinine Clearance, kidneys after HMP showed higher values at all time points of reperfusion (***Fig.4.9. a***). There was a median sodium excretion of 5.91mmol/min (IQR: 5.51; 9.19) in the HMP group vs. 2.81mmol/min (IQR: 1.74; 4.41) in the NMP group (p= 0.26). The sodium excretion was almost identical in both groups during the reperfusion period (***Fig.4.9. b***). The fractional sodium excretion can be a useful tool in the evaluation of acute kidney failure. In the context of physiology of the kidney, low fractional excretion can point towards sodium retention suggesting pathophysiology extrinsic to the urinary system, e.g. low output heart failure, whereas higher values can suggest sodium wasting due to intrinsic kidney failure, e.g. acute tubular necrosis. Therefore, similar sodium excretion rates were a useful result in the setting of ex-vivo perfusion and let us exclude effects due to different extrinsic settings.

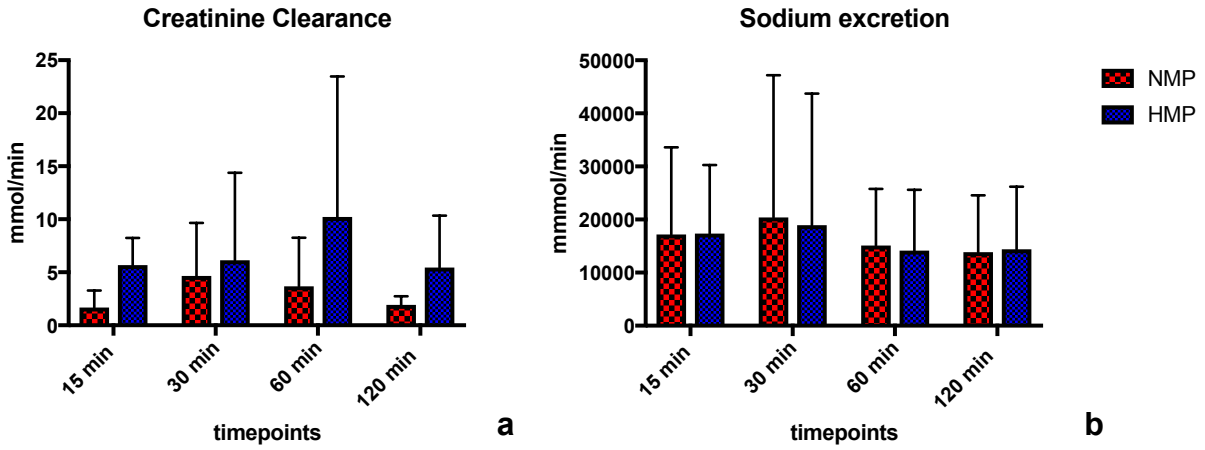


Figure 4.9.: Creatinine clearance and sodium excretion HMP vs. NMP in the porcine model

Fig. 4.9.: *Creatinine clearance rates in mmol/min during reperfusion for timepoints of measurements are shown in* ***a****. Fractional sodium excretion rates in mmol/min are shown in graph* ***b****.*

HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion.

Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk) p<0.05 (*); p<0.005 (**).

We were looking at perfusate samples collected from the venous end during the reperfusion period in regards to markers of kidney injury and inflammation. Levels of IL-1β as a marker of inflammation, and NGAL as a well known marker for kidney injury and DGF in renal transplantation, were analysed by ELISA. There was no significant difference between the two groups, neither for serum nor for urinary samples at time points of measurement (***Fig. 4.10. a-d***). Furthermore, lactate levels within the perfusates were analysed in order to estimate whether the organs were exposed to hypoxic damage. Interestingly, at the end of the reperfusion period of 240 minutes, lactate levels were highest for kidneys after SCS, with a mean level of 8.62mmol/l ± 1.69mmol/l, followed by kidneys after NMP, with a mean level of 5.26mmol/l ± 2.84mmol/l and lowest for kidneys after HMP with a mean value of 4.32mmol/l ± 2.11mmol/l (p=0.026). Comparing the lactate levels for HMP and NMP, over all time points, there was a trend observed towards lower levels for kidneys after HMP than for kidneys after NMP, with a mean of 5.91 ± 1.23mmol/l vs. 6.71 ± 0.78mmol/l (p= 0.1, ***Fig. 4.11***).

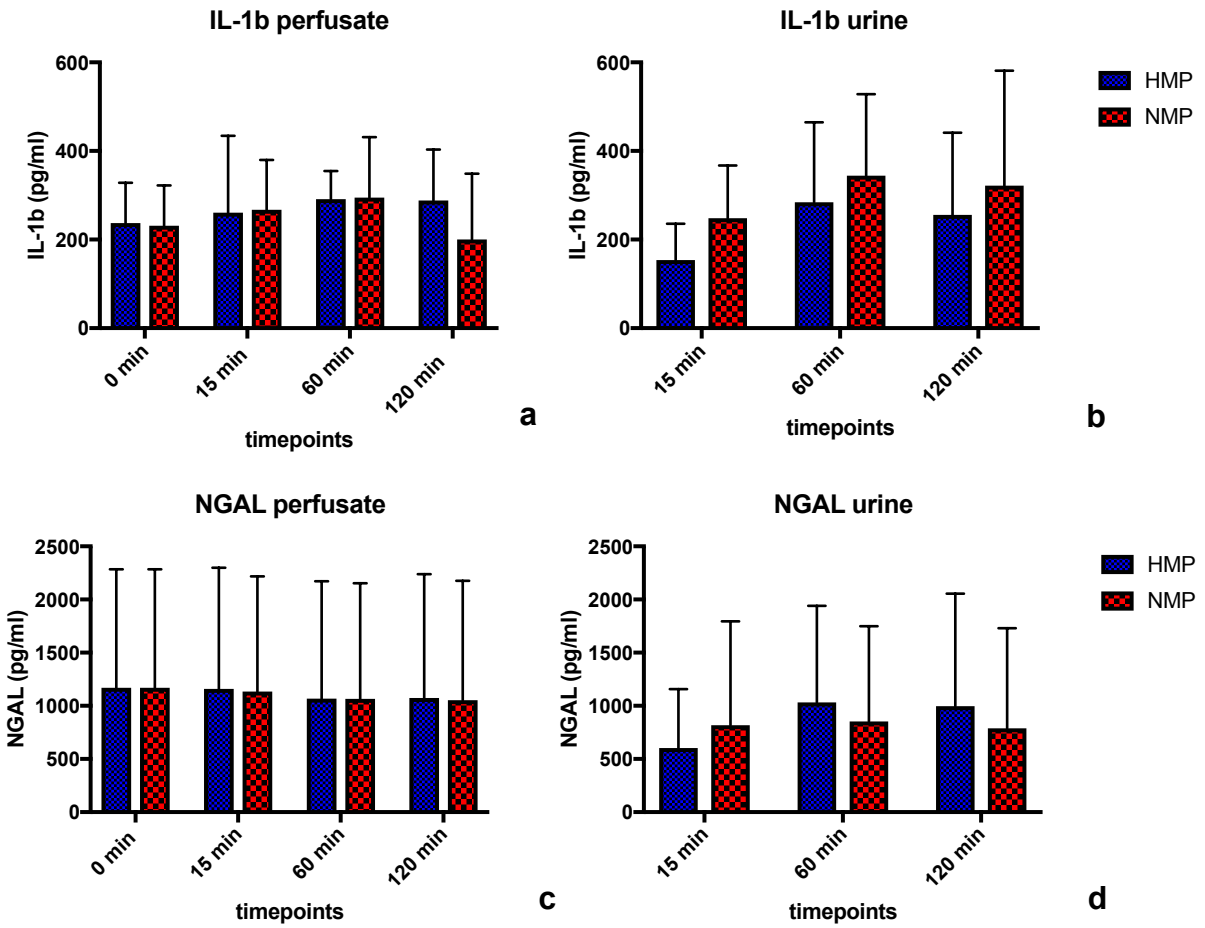


Figure 4.10.: Perfusate ELISA results HMP vs. NMP in the porcine model

Fig. 4.10.: *Levels of interleukin 1β (IL-1β) in perfusate- (**a**) and urine samples (**b**) during reperfusion, measured in pg/ml by ELISA for named timepoints of reperfusion. Same was performed for Neutrophil gelatinase-associated lipocalin (NGAL) -levels in perfusate- (**c**) and urine samples (**d**).*

HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion.

Statistical analyses carried out using ANOVA, no statistical significance (ns; no asterisk) p<0.05 (*); p<0.005 (**).

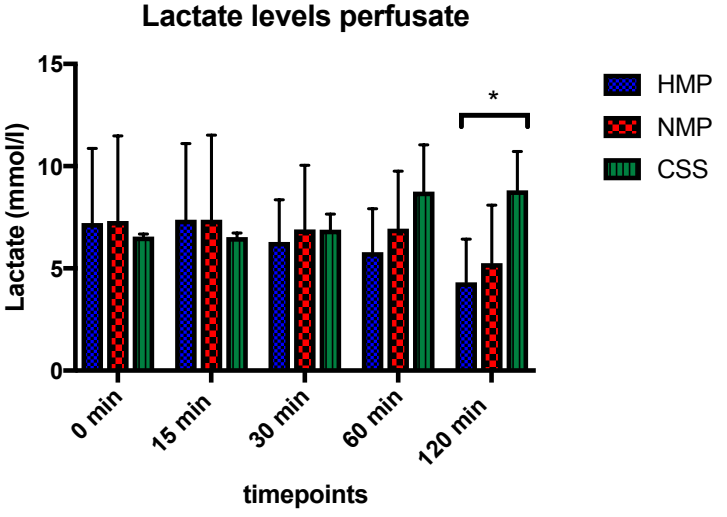


Figure 4.11.: Perfusate Lactate levels during reperfusion of porcine kidneys

Fig. 4.11.: *Lactate levels in perfusate samples during the reperfusion period, measured in mmol/l by an arterial blood gas analyser at defined timepoints. The difference between the groups reached statistical significance, with a p-value of 0.026. HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion.*
Statistical analyses carried out using ANOVA, no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).

Expression levels of the kidney injury markers NGAL, the endothelial injury marker EDN-1, as well as the inflammatory markers TNFα and IL-1β, were analysed at mRNA level using RT-PCR. We observed a significantly higher expression of EDN-1 for kidneys after HMP than for kidneys after NMP, after the reperfusion period of 2 hours (mean expression of 2.63 ± 1.72 in after NMP vs. 12.34 ± 7.09 after HMP, p=0.03, ***Fig. 4.12. a***). Interestingly, for the kidney injury marker NGAL, a trend towards a stronger down regulation after HMP than after NMP at reperfusion was observed, in comparison to the internal control, with mean expression levels of 0.23 ± 0.21 in the former, and 0.36 ± 0.34 in the latter group (p= 0.38). NGAL was not detectable in samples after Cold Static Storage (CSS), (***Fig. 4.12. b***).
Expression levels were calculated compared to the respective t0 biopsy sample, using the ΔΔCt method and with β-Actin as a housekeeping gene. For the proinflammatory markers TNFα and IL-1β, we could observe a higher up regulation in the HMP group, with mean expression levels of 7.64 ± 5.81 vs. 3.18 ± 2.71 for TNFα (p= 0.22) and 9.59 ± 2.82 vs. 6.06 ± 4.21 for IL-1β (p=0.21), which did not reach statistical significance (***Fig. 4.12 c, d***). The expression levels of samples after CSS shown in the graphs served as a guideline to where expression levels were after CSS, however, as the sample size was not as big for that group with a number of only 4 kidneys, the group was not included into statistical analyses. Interestingly however, the graphs show these samples to have a similar expression pattern as the ‚control'. This could be due to the short period of these kidneys being perfused, in contrary to the kidneys within the experimental groups undergoing perfusion for 4 hours before being reperfused.

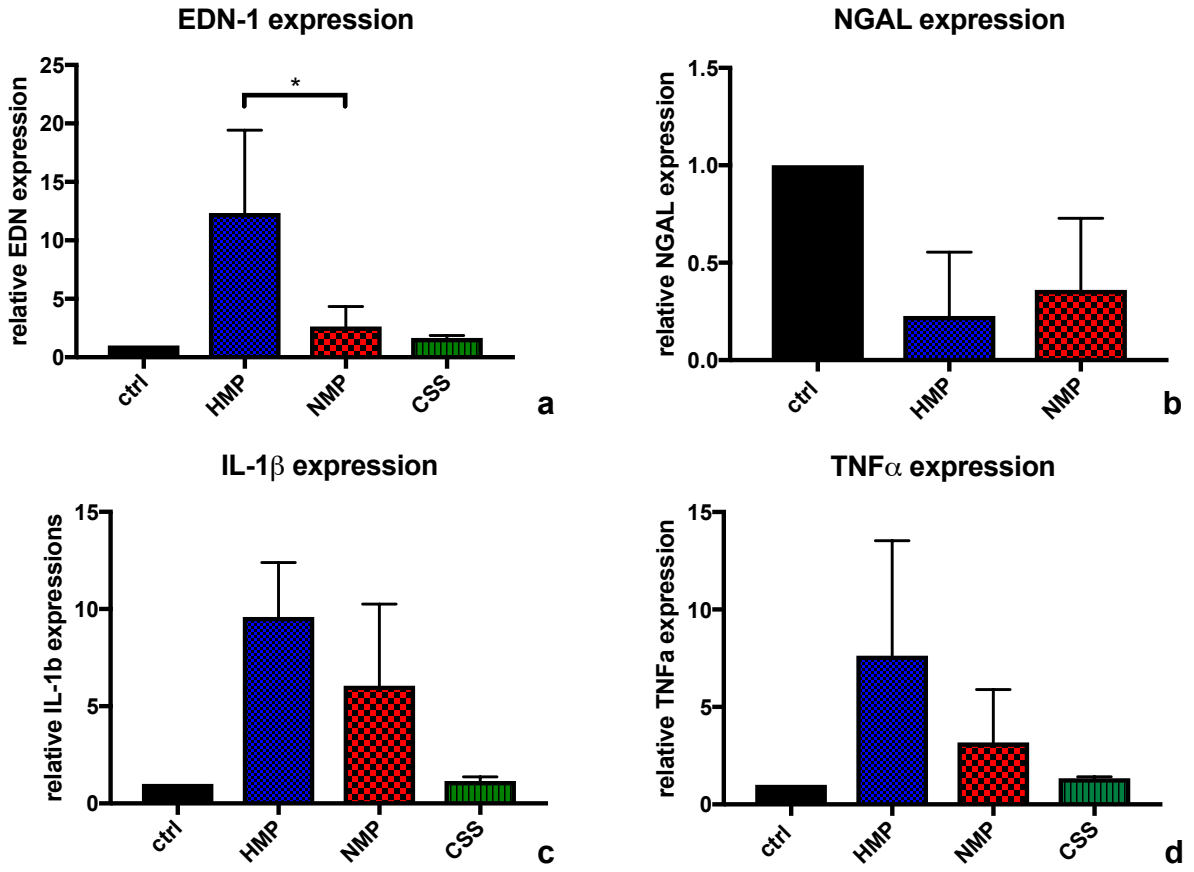


Figure 4.12.: mRNA expression levels of kidney injury markers and inflammation markers in the porcine model

Fig. 4.12.: *Relative mRNA expression levels of the kidney injury markers Endothelin-1 (EDN-1) (**a**), Neutrophil gelatinase-associated lipocalin (NGAL) (**b**) and the inflammatory markers IL-1β (**c**) and TNFα (**d**) are shown, determined by RT-PCR and calculated using the ΔΔCt method.*

HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion; ctrl= control.

Statistical analyses carried out using Mann-Whitney U test, statistical significance: no statistical significance (ns; no asterisk) p<0.05 (*); p<0.005 (**).

The randomised and blinded scoring of histology sections followed by a ranking, demonstrated better outcomes for kidneys after HMP. Especially looking at tubular injury, we observed less tubular dilatation and tubular epithelial flattening (***Fig.4.13. a, arrows***) in these samples when compared to samples from the NMP group (***Fig.4.13. b***). The statistical analysis of the ranking using Mann-Whitney-U test revealed no statistical difference between the groups, however, the graph indicates for a better histological outcome of porcine kidneys after HMP compared to NMP, but NMP showing better preservation of a healthy histological structure than CSS (***Fig. 4.13. c***).

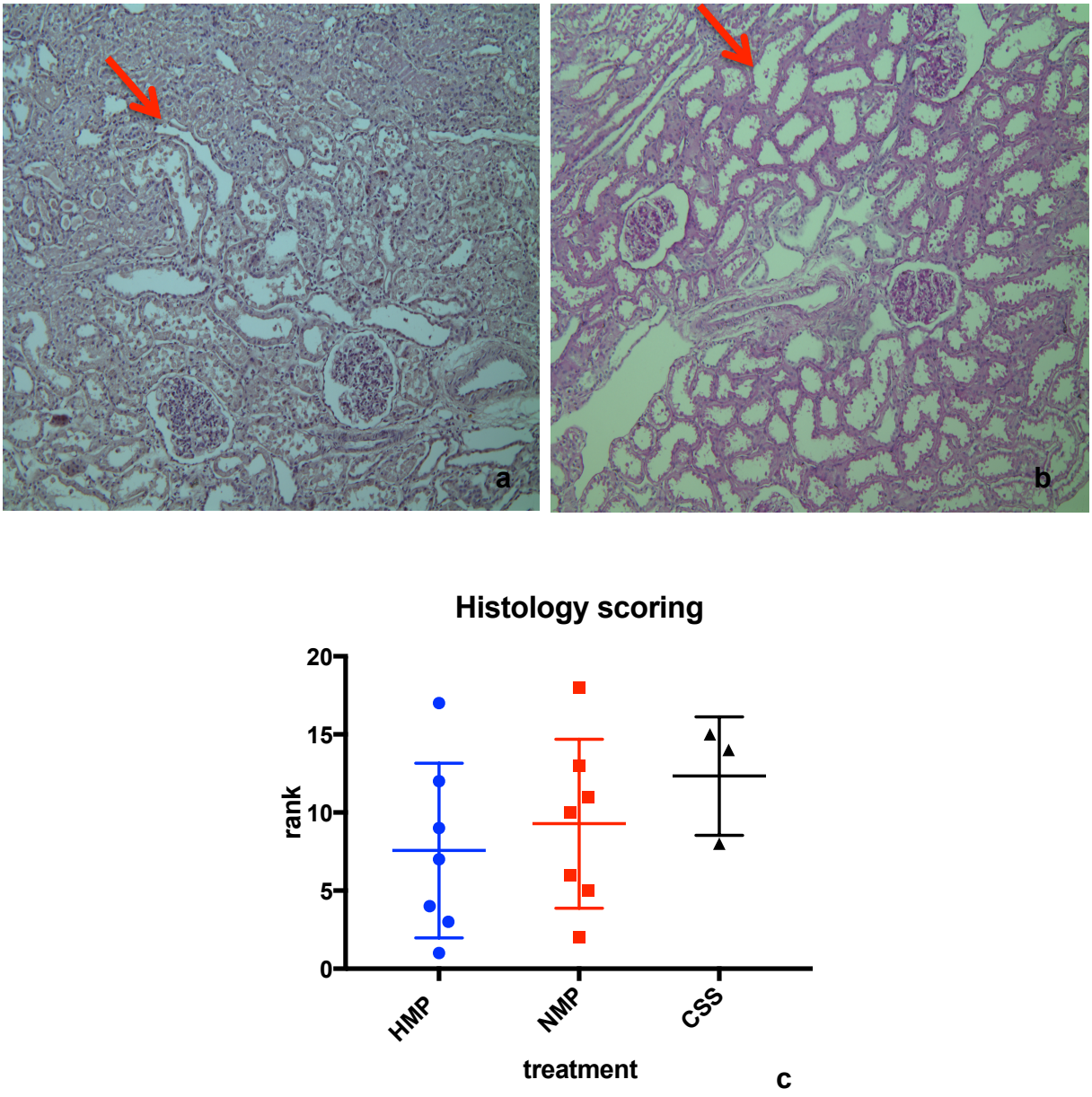


Figure 4.13.: Histological evaluation of HMP vs. NMP in the porcine model

Fig. 4.13.: *Examples of histological evaluation under the microscope (20x magnification) for samples taken after Hypothermic Machine Perfusion (HMP) (**a**) or Normothermic Machine Perfusion (NMP) (**b**) and reperfusion. All the slides were ranked blindly by two individual researchers and a Mann-Whitney-U test was performed (**c**). The sample size for kidneys after Cold Static Storage (CSS) was only n=3, one sample was lost during processing for histology.*

Tunnel staining of slides after reperfusion revealed a significantly higher number of apoptotic cells per 20x magnification field for kidneys in the NMP group when compared to the HMP group, with a mean number of 3.9 ± 6.17 vs.1 ± 3.05 (p= 0.03), which further highlights the above described results and is shown in ***Fig.4.14 a-c.***

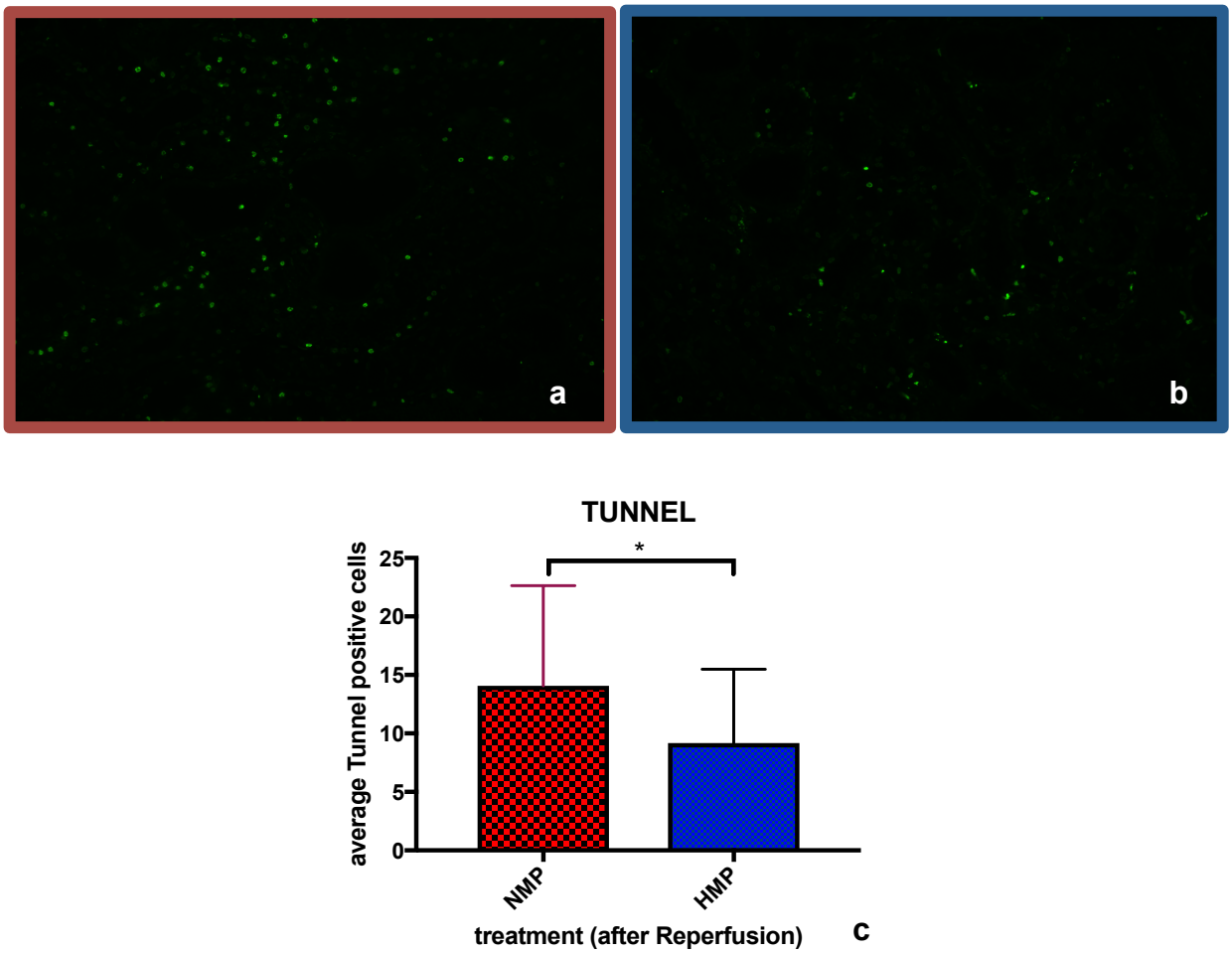


Figure 4.14.: TUNNEL staining for HMP vs. NMP in the porcine model

Fig. 4.14.: *Examples of TUNNEL-stained slides of samples after Normothermic Machine Perfusion (NMP) (**a**) or Hypothermic Machine Perfusion (HMP) (**b**) and reperfusion. The green fluorescent spots represent cells undergoing apoptosis. The graph (**c**) illustrates the result after counting 10 fields of 20x magnification per slide. A paired t-test for the two columns with the mean value for each slide revealed a significant difference in favour of HMP; p<0.05 (*).*

4.2.1.1. Expansion of the experimental groups in the porcine model

As mentioned in Chapter 3, Materials and Methods, further porcine kidneys underwent either HMP or NMP as controls for the kidneys undergoing treatment with MSCs during the respective preconditioning method (n=6). We performed calculations including the additional 6 pairs of kidneys in order to obtain a higher number of treated organs (n=15), conscious of the fact that 6 pairs out of the 15 were not from the same donor pigs. This gave important information about the reliability of the previously shown data, as it was interesting to see whether the data could be confirmed.

Including the additional data did not change the shape of the graphs but it increased the significance of the results, which is shown in ***Fig. 4.15 a-d***. The difference of the intrarenal resistance indices during the preconditioning phase became highly significant, with lower mean values in the HMP group: 0.47 ± 0.35 vs. 1.43 ± 0.23 (p= 0.0008), ***Fig. 4.15. a***. This again resulted in significantly higher perfusate flow rates within the HMP group during reperfusion when compared to the NMP group (mean of 46.24 ± 12.49ml/min/100g vs. 26.16 ± 4.57ml/min/100g, p= 0.0051), ***Fig. 4.15. b***. Furthermore, the results for higher oxygen consumption rates as well as a higher urinary output in kidneys after HMP were more significant with the higher number of kidneys, without altering the overall look of the graphs (***Fig. 4.15. c, d***). The mean oxygen consumption rates were 22.71 ± 6.27ml/min/100g in the HMP group vs. 11.83 ± 1.29ml/min/100g in the NMP group (p= 0.0016). As a comparison, for the 4 kidneys being kept on CSS, the mean oxygen consumption rate was 17.93 ± 8.32ml/min/100g. The 4 kidneys on CSS were not included into the statistical analysis because the number of the group was smaller and we wanted to focus on the comparison between NMP and HMP; however, it is a good indicator of where these values are compared to the current gold standard. The urinary output rates during reperfusion in this cohort were 5.31 ± 2.07ml/min in the HMP group vs. 2.45 ± 1.19ml/min (p= 0.002) and again as an orientation, the mean urinary output rates for kidneys after CSS were 2.35 ± 1.25ml/min, which is comparable to the outcomes after NMP for this study.

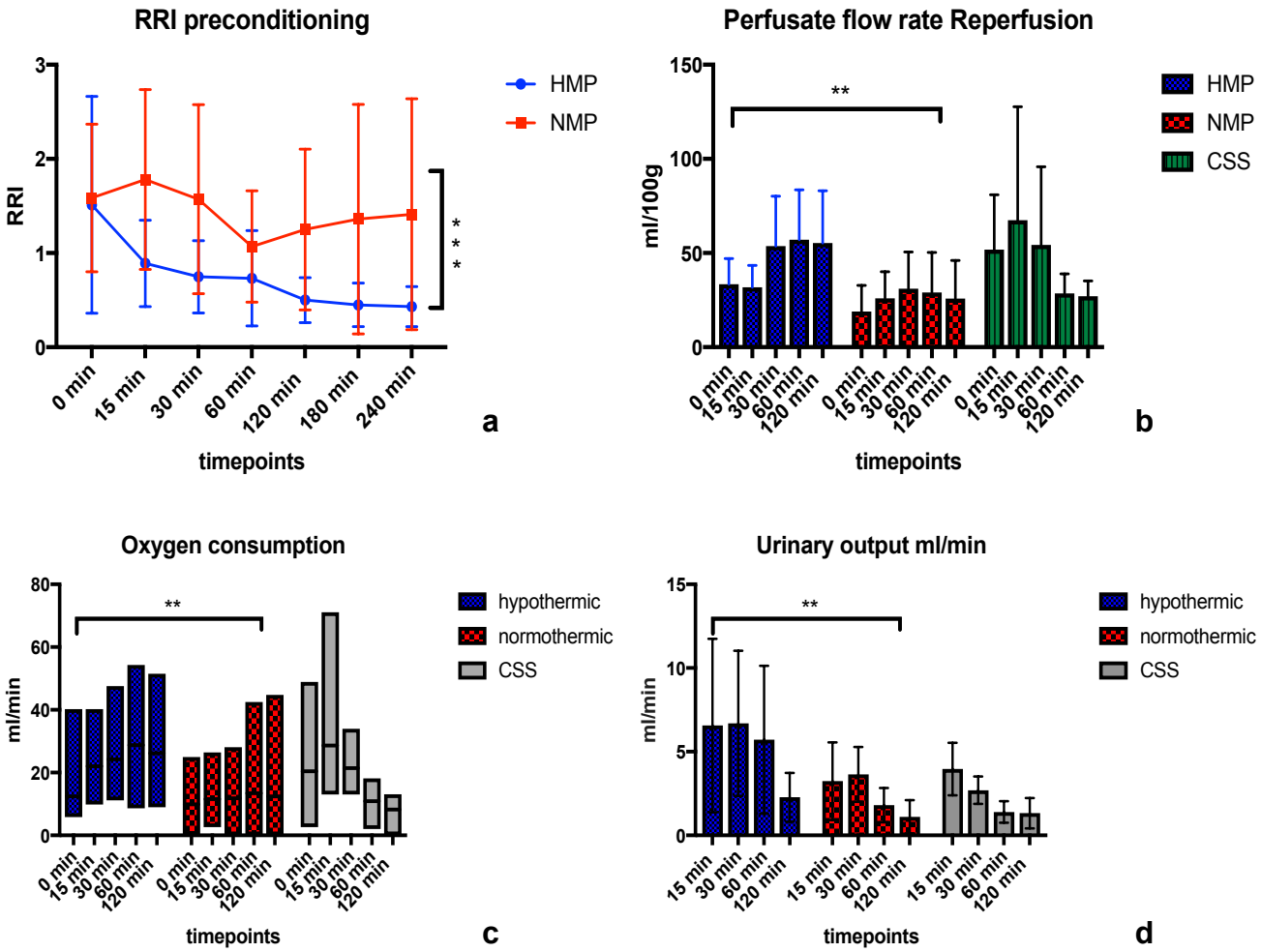


Figure 4.15.: Physiological parameters of HMP vs. NMP in the porcine model with an expanded number

Fig. 4.15.: *Graphs for results including unmatched kidneys undergoing Hypothermic Machine Perfusion (HMP) or Normothermic Machine Perfusion (NMP) with a total of n=15 kidneys per group are shown for the Renal Resistance Indices (RRI) (**a**), the perfusate flow rates in ml/min (**b**), oxygen consumption rates (**c**) and urinary output rates in ml/min (**d**). The cohort of kidneys after Cold Static Storage (CSS) is shown as an indicator for the current gold-standard and the number of organs in that group was n=4 kidneys.*

Statistical analyses carried out using ANOVA, statistical significance: p<0.05 (*); p<0.005 (**).

HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion; ctrl= control.

The results for the creatinine clearance, the sodium excretion rates as well as the lactate concentrations in the perfusate samples remained similar to previously described results, with no change in the statistical significance (data not shown).

For the expression levels of the kidney injury markers EDN-1 and NGAL, the same pattern could be observed as in the smaller study group, however the difference in the EDN-1 up regulation for kidneys after HMP was not statistically significant anymore. The mean expression levels for EDN-1 were 7.39 ± 7.76 in the HMP group vs. 3.66 ± 2.71 in the NMP group, with a p-value of 0.6 (***Fig. 4.16 a***). The NGAL

expression levels showed the same pattern as previously in the smaller study group, with a greater down regulation after HMP than after NMP, but still statistically not significant, with a p-value of 0.25 (***Fig. 4.16. b***).

The inflammatory parameters IL-1β and TNFα in the bigger cohort were still higher up regulated after HMP than after NMP when compared to the control, but to a less extent than in the smaller study group, with p-values of 0.44 for IL-1β and 0.73 for TNFα (***Fig. 4.16. c, d***).

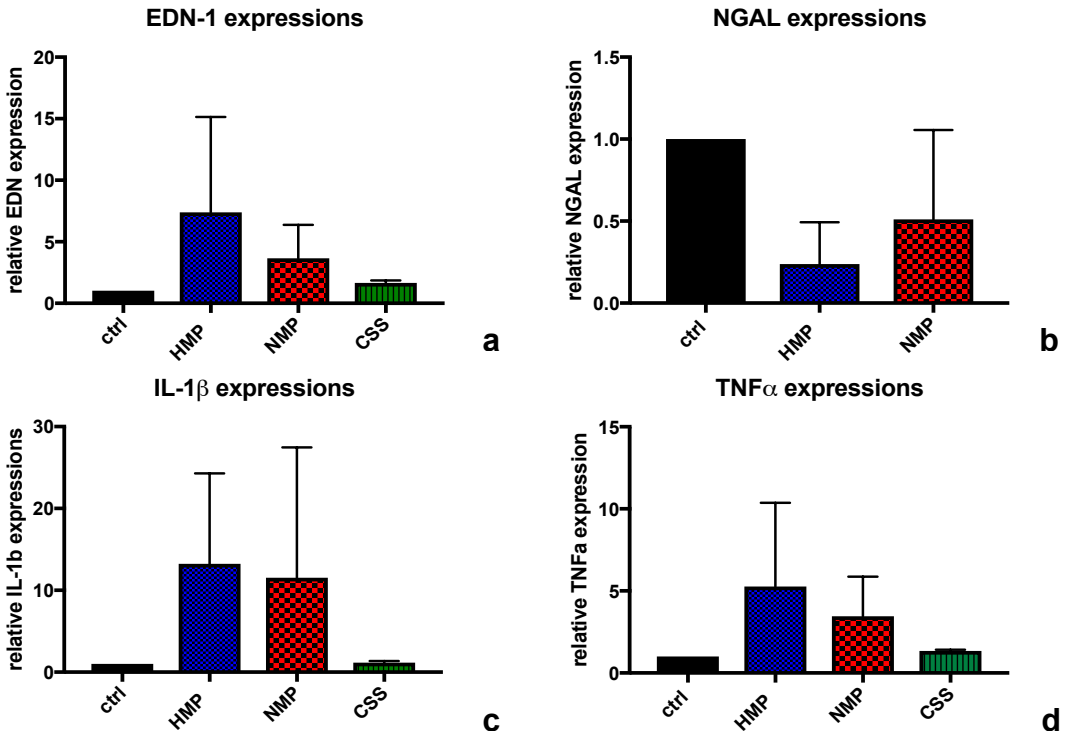


Figure 4.16.: mRNA expression levels of kidney injury markers and inflammatory markers HMP vs. NMP in the porcine model with an expanded number

Fig. 4.16.: *Relative Expression levels of Endothelin-1 (EDN-1) (**a**), Neutrophil gelatinase-associated lipocalin (NGAL) (**b**), IL-1β (**c**) and TNFα (**d**) determined using real time (RT)-PCR of biopsy samples after reperfusion of the respective treatment groups, compared to an internal control (t0 biopsies) and using β-Actin as a housekeeping gene.*

Statistical analyses carried out using Mann-Whitney U test, statistical significance: no statistical significance (ns; no asterisk) p<0.05 (*); p<0.005 (**).

Overall, we have observed similar results when we added non-paired kidneys undergoing HMP or NMP and reperfusion to the pool of kidneys coming from the same donor pig, which is encouraging for the results of our primary study to be reliable and valid, even when non- paired kidneys were included to the organ pool.

4.2.1.2. HMP vs. NMP after prolonged WIT in the porcine model

Mainly for the purpose of measurements using the micro dialysis device, a longer WIT was required to adequately assess that most interesting period. This was due to the reason that the measurements with the micro dialysis instrument are 10 minutes delayed and in order to have a good timeframe to obtain proper results, more time was needed. Therefore, further 3 pairs of kidneys underwent either 4 hours of HMP or NMP after a prolonged WIT of 45 minutes, followed by CSS for 24 hours. After the respective treatments, a reperfusion period of 2 hours followed. 1 kidney was kept on CSS when at the point of retrieval for a pair, the other kidney was damaged and no direct comparison was possible. The same studies were performed as for the previous study groups, with the following results:

After warm ischemia times of 45 minutes, during ex-vivo machine perfusion, RRIs remained quite high during the preconditioning period in both groups, with mean values of 1.1 ± 0.46 in the HMP group vs. 0.78 ± 0.18 in the NMP group (p=0.59). Interestingly, the RRIs remained on a steady level throughout NMP, whereas the RRIs during the starting period of HMP were higher than in the NMP group but slowly came down to slightly lower levels than in the NMP group at the end of perfusion (***Fig. 4.17. a***).

The perfusate flow rates during reperfusion were again higher in the HMP group than in the NMP group, with mean values of 48.49 ± 6.14 ml/min/100g vs. 25.25 ± 9.67 ml/min/100g, which is almost double the flow rate in the HMP group compared to the NMP group (p=0.14). As a comparison, the mean perfusate flow rate for the kidney after CSS was 42.54 ± 24.8ml/min/100g (***Fig. 4.17. b***). As a consequence, also the oxygen consumption rates, as well as the urinary output were higher in kidneys after HMP than after NMP, with a p-value of 0.11 for the former and 0.12 for the latter comparison (***Fig. 4.17. c, d***). In respect to the small number within the groups (n=3), these results deliver a clear message without reaching statistical significance and therefore, underline our previous data.

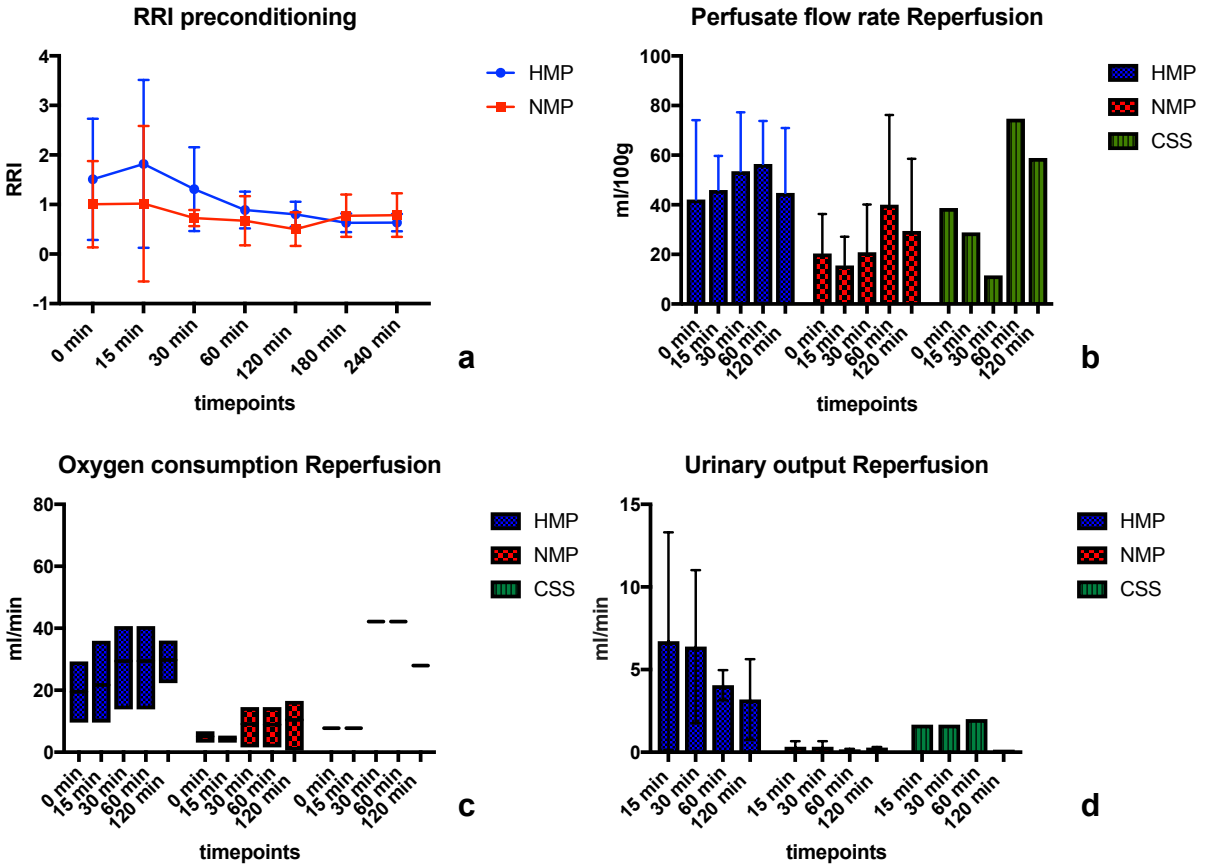


Figure 4.17.: Physiological parameters of porcine kidneys for HMP vs. NMP after prolonged warm ischemia time

Fig. 4.17.: *Physiological parameters of kidneys after a prolonged warm ischemia time (WIT) of 45 minutes are shown in this graph. (**a**) shows the intrarenal resistance indices (RRI) over time during the preconditioning phase, (**b**) shows the resulting perfusate flow rates during timepoints of reperfusion, (**c**) illustrates the oxygen consumption rates for the 3 different treatment groups and in (**d**) the urinary output rates in ml/min are shown fort he three groups.*
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).
HMP= Hypothermic Machine Perfusion; NMP= Normothermic Machine Perfusion; CSS= Cold Static Storage

Also for the creatinine clearance in kidneys exposed to a prolonged WIT, higher rates could be detected during the reperfusion period in kidneys after HMP at all measured time points when compared to kidneys after NMP. This again did not reach statistical significance (***Fig. 4.18. a***). For the fractional sodium excretion, kidneys after HMP showed higher rates at the beginning of the reperfusion phase, to then decrease to values similar to the kidneys after NMP (***Fig. 4.18. b***), which over all time points resulted in a statistically significant higher sodium excretion rate for the HMP group, with values of 16457 ± 3336mmol/min in the HMP vs. 11218 ± 3595mmol/min in the NMP group (p= 0.03). The mean values for the creatinine clearance over all time points were 5.45 ± 1.04 mmol/min in the HMP group vs. 1.19 ± 0.54 mmol/l in the NMP group (p=0.18).

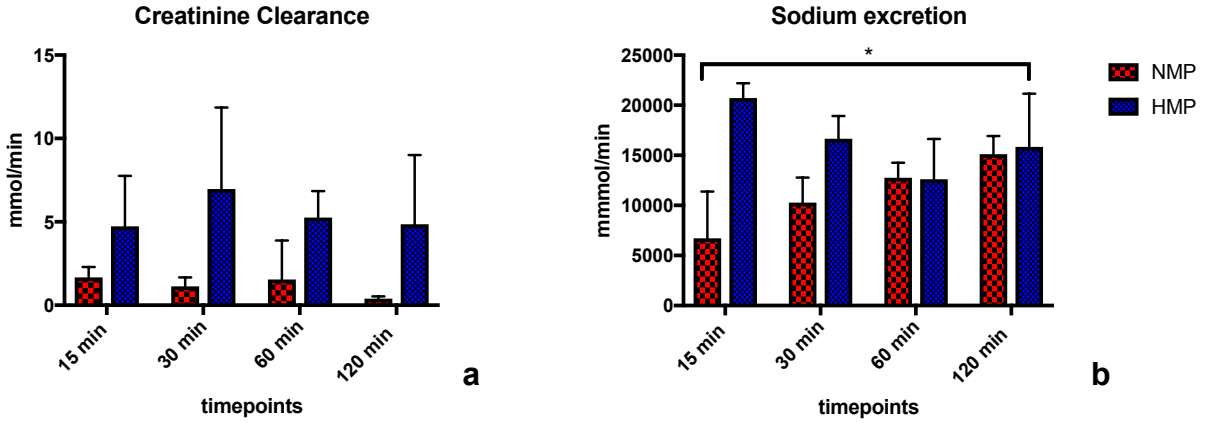


Figure 4.18.: Creatinine clearance and sodium excretion in HMP vs. NMP in the porcine model after a prolonged warm ischemia time

Fig. 4.18.: *Creatinine clearance (**a**) and sodium excretion rates (**b**) for kidneys after a warm ischemia time (WIT) of 45 minutes followed by 4 hours of Normothermic Machine Perfusion (NMP) or Hypothermic Machine Perfusion (HMP) at defined timepoints of reperfusion are shown. Overall sodium excretion rates were statistically significantly higher in the HMP group (p=0.037).*

Statistical analyses carried out using ANOVA, statistical significance: : no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).

We also analysed serum- and urinary samples for the levels of NGAL and IL-1β as performed for our previous studies. There was no difference in IL-1β levels in urinary samples and no difference in NGAL levels in either, perfusate- or urine samples (data and graphs not shown). The only interesting observation for the results of this group was higher levels of IL-1β in the perfusate samples, especially at the end of the reperfusion phase. With mean concentrations of 156.1 ± 70.9pg/ml in the HMP vs. 315.1 ± 259.1pg/ml in the NMP group during times of reperfusion, this did not reach statistical significance, with a p-value of 0.27. This is an interesting result as it contradicts the observation of lower IL-1β levels at the end of the reperfusion phase in the previous study with a higher number per group but with a shorter WIT (***Fig. 4.19. a, b***).

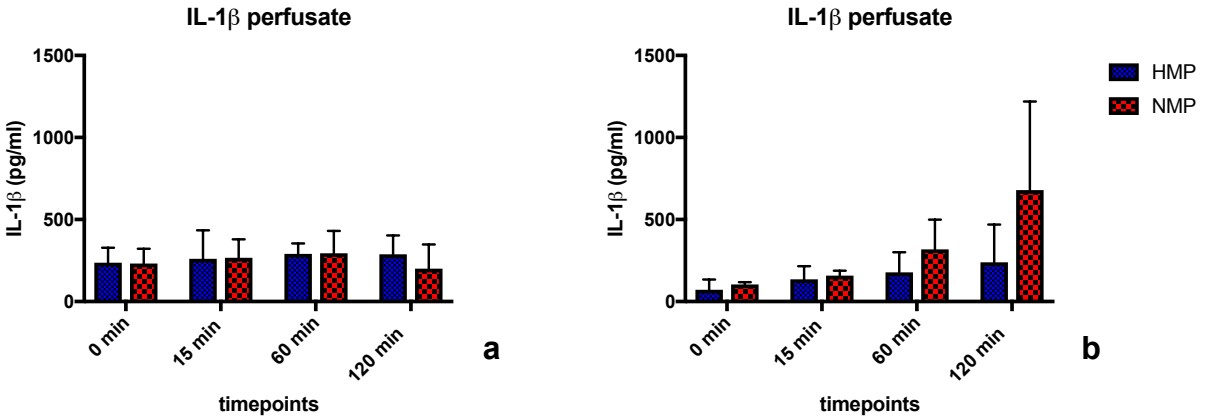


Figure 4.19.: Overview of IL-1β perfusate levels for kidneys after short and long warm ischemic times

Fig. 4.19.: *IL-1β levels of the previous experiments on a direct comparison between Normothermic Machine Perfusion (NMP) and Hypothermic Machine Perfusion (HMP) after a WIT of 20 minutes (n=9) are shown in (**a**). Results for the same experiments with a WIT of 45 minutes and a sample size of n=3 per group are shown in (**b**) for IL-1β levels in the perfusate.*
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).

The pattern of the lactate levels was the same as in the previous experiment, with the lactate values at all time points of reperfusion being highest for the kidney after CSS, followed by the kidneys after NMP and being lowest for kidneys after HMP, without reaching statistical significance (***Fig. 4.20. b***). As a comparison, the graph for the same measurements from the original experiment will be shown (***Fig. 4.20. a***).

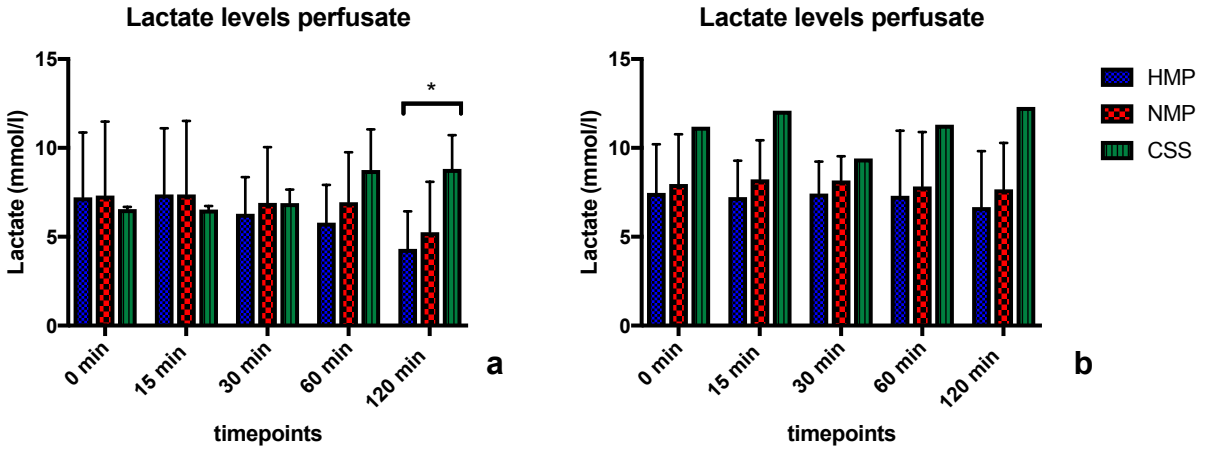


Figure 4.20.: Overview for perfusate lactate levels for porcine kidneys after short or long warm ischemic times

Fig. 4.20.: *Lactate levels at defined timepoints during reperfusion for the original experiment (**a**) with a cohort of n=9 per group for Normothermic Machine Perfusion (NMP) and Hypothermic Machine Perfusion (HMP) and n=4 for the Cold Static Storage (CSS) group are shown in direct comparison to lactate levels for the same experiment with kidneys after a warm ischemia time (WIT) of 45 minutes (**b**) and a cohort of n=3 kidneys for NMP and HMP groups and n=1 kidney for CSS.*
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).

To complete this experiment, we also analysed the mRNA expression levels of the kidney injury markers NGAL, EDN-1 as well as the inflammatory markers IL-1β and TNFα and again used the biopsy sample taken at the time point of retrieval as a control sample for each individual kidney (t0 biopsy).

Results are similar to previous results, with a higher up regulation of EDN-1 in samples after HMP and reperfusion than after NMP and reperfusion and mean values of 5 ± 3.05 in the former vs. 3.43 ± 3.35 in latter group (p=0.4). The expression for EDN-1 after CSS was similar to the control at 1.16 ± 0.21 (***Fig. 4.21. a***).

Interestingly, the relative NGAL expression was also higher for the HMP group within this cohort than for the NMP group, which was contrary to the results for the group with a shorter WIT. The mean expression levels for this kidney injury marker were 1.64 ± 1.72 for the HMP group vs. 0.66 ± 0.81 in the NMP group (p=0.4, ***Fig. 4.21. b***). The expression of the inflammatory marker IL-1β in this cohort was dramatically up regulated after reperfusion in both treatment groups, pointing towards an inflammatory response due to the long warm ischemia time and was similar in both groups, with slightly higher values in the NMP group: 83.26 ± 109.31 vs. 70.46 ± 84.65 in the HMP group (p=0.9). The expression level in the CSS kidney was 56.1, again pointing towards the inflammatory response after a prolonged WIT (***Fig. 4.21. c***). The expression level for TNFα was again highest in the HMP group with a mean level of 5.78 ± 5.4 vs. 4.07 ± 0.15 in the NMP group (p=0.7). The expression level for the kidney after CSS was 2.6.

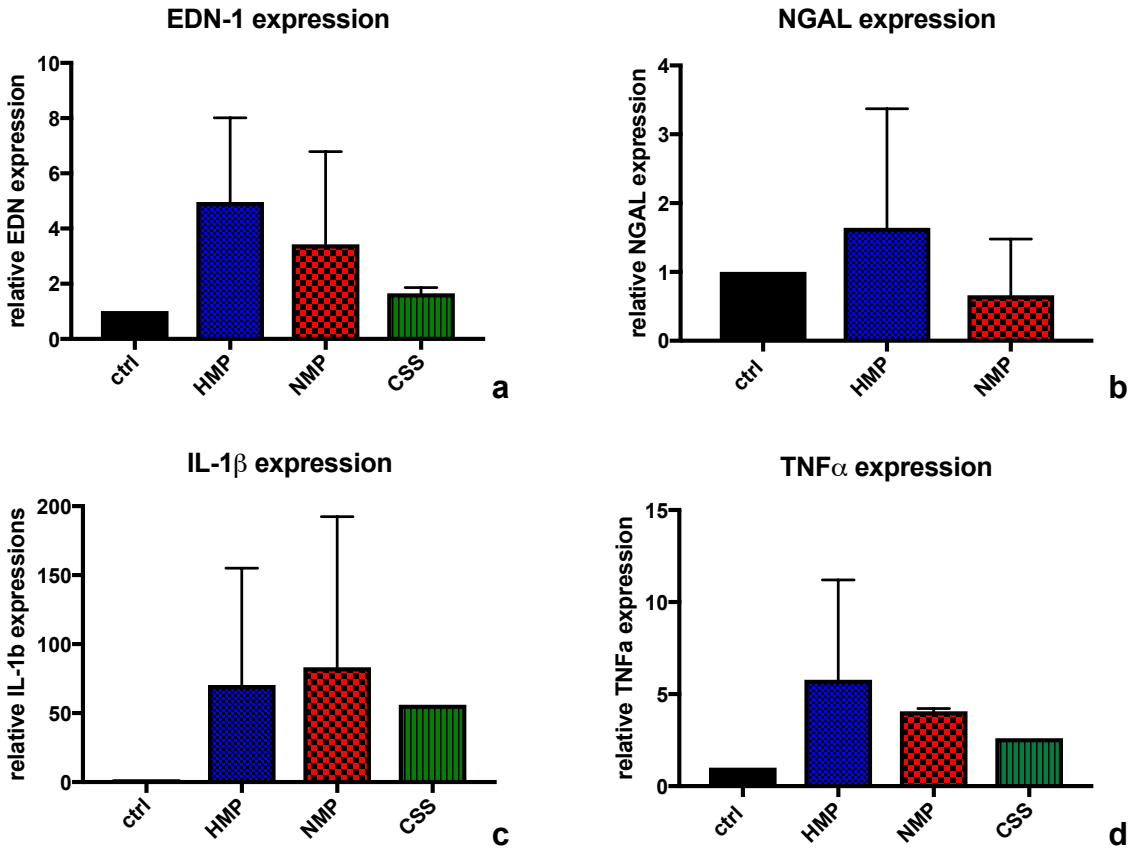


Figure 4.21.: mRNA expression levels of kidney injury markers and inflammatory markers for HMP vs. NMP in the porcine model after a prolonged warm ischemia time

Fig. 4.21.: *Relative mRNA expression levels for the kidney injury markers Endothelin-1 (EDN-1) (**a**), Neutrophil gelatinase-associated lipocalin (NGAL) (**b**) as well as the inflammatory markers Interluekin-1β (IL-1β) (c) and Tumor necrosis factor α (TNFα) (**d**) for kidneys after prolonged warm ischemia time (WIT) (45min), followed by 24h of cold static storage (CSS), 4h of normothermic machine perfusion (NMP) (n=3), hypothermic machine perfusion (HMP) (n=3) or cold static storage (CSS) (n=1) are shown. RT-PCR was used as a method and calculations were made using the ΔΔCt method. Statistical analysis was made using Mann- Whitney- U test, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).*

4.2.2. Direct comparison of HMP and NMP in the human model

So far, there are no data on a direct comparison of HMP versus NMP in a human ex vivo model. We used human kidneys rejected for clinical transplantation and aimed to reperform the same study as for the porcine ex-vivo kidney perfusion model. Due to the fact that for human organs, we were dependant on offers of declined organs and receiving kidneys as pairs from the same donor was rare, the number of kidneys for this study was lower (n=12). 6 pairs of human kidneys were included into this study. The cold ischemic times were different for the organ pairs depending on the time of being rejected from clinical transplantation and the distance of the organs to our centre.

Table 4.1. summarizes the demographics of human kidneys used for the project.

Pair	CIT	DBD/DCD	Age	Reason for decline
1	28h	DBD	87y	Arteriosclerosis
2	12h	DBD	61y	Malignancy (sigma carcinoma)
3	24h	DBD	40y	Malignancy
4	18h	DBD	77y	Infection
5	38h	DBD	50y	Arteriosclerosis
6	24h	DCD	46y	Infection

Table 4.1.: Demographics for human kidneys rejected from clinical transplantation

Table. 4.1.: *Demographics of pairs of kidneys rejected from clinical transplantation and used for a direct comparison on ex-vivo hypothermic machine perfusion (HMP) vs. normothermic machine perfusion (NMP).*
DCD= donation after cardiac death; DBD= donation after brain death

Kidneys were perfused hypothermically (n=6) or normothermically (n=6), using Krebs-Henselleit Buffer (KHB) as a normothermic perfusate. The perfusate was oxygenized as described above but could not be analysed using the ABG machine due to its different composition to blood. Hence, oxygen concentrations and oxygen consumption could not be evaluated adequately. Only physiological parameters like the intrarenal resistance indices and urinary output could be compared upon reperfusion. Reperfusion was also mimicked using KHB, with a mean systolic pressure of 80mmHg, as it was a big problem to obtain human packed red cells or whole blood of the respective same blood type at the time of receiving the organs from NHSBT. Anyhow, the results were very interesting:

Fig. 4.22. a and ***b*** illustrate the intrarenal resistance indices, in the first case for time points during the preconditioning phase (***a***) and in the second case for time points during the reperfusion period (***b***). As illustrated, in both groups during the preconditioning phase, a decline of the RRIs could be noted. However, whereas there was a steady decline in the HMP group towards an RRI of approximately 0.4, in the NMP group, an increase of the RRI was detected, from the time point 120 minutes to the end. This resulted in an RRI of approximately 0.5 in that group (***a***). Overall, the mean values for the RRI were higher in the HMP group over time, namely 0.42 ± 0.05 vs. 0.39 ± 0.05 (p=0.7). During the reperfusion phase, the RRI within the NMP group remained stable at around the same level that was reached after the preconditioning period, and overall, was statistically significantly higher in

the NMP group than in the HMP group, with a mean of 0.47 ± 0.01 vs. 0.28 ± 0.03 (p= 0.024, ***b***). This, unsurprisingly, resulted in higher thorough perfusate flow rates in the NMP group during the preconditioning period, with a mean of 38.6 ± 2.86 ml/min/100g vs. 33.26 ± 3.4 ml/min/100g (p=0.33, ***c***), but a lower thorough perfusate flow rate during reperfusion for that group, with a mean of 27.06 ± 0.9ml/min/100g vs. 38.58 ± 2.22ml/min/100g tissue (p=0.16, ***d***).

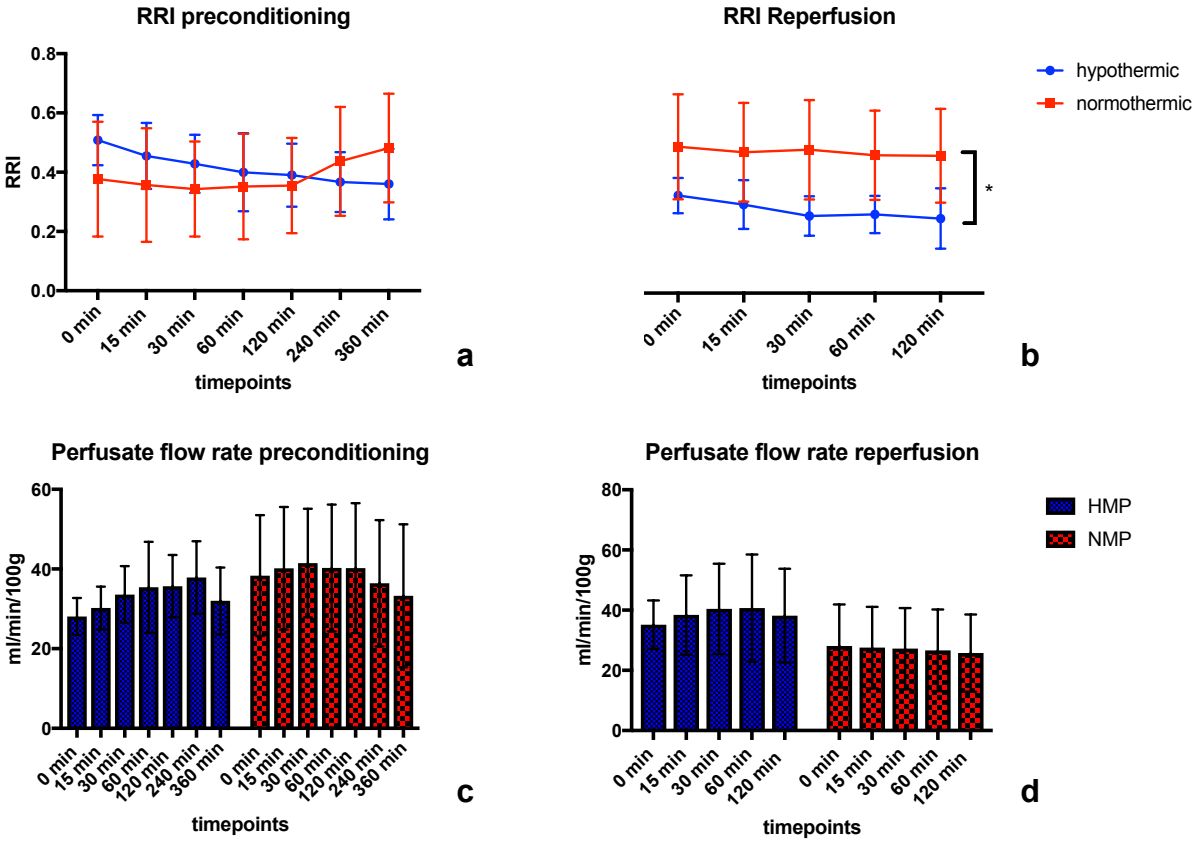


Figure 4.22.: Physiological parameters for human kidneys during/after HMP or NMP

Fig. 4.22.: *Intrarenal resistance indices for kidneys during normothermic machine perfusion (NMP) and hypothermic machine perfusion (HMP) are shown in (**a**), and during reperfusion in (**b**). The resulting perfusate flow rates during the preconditioning period (**c**), followed by the reperfusion period (**d**) for human kidney pairs are shown in ml/min/100g of tissue.*
Statistical analyses were carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).

The results during the reperfusion period confirmed our results obtained in the porcine study. The next step was however, to analyse the renal function for both groups at reperfusion. With ABG measurements not being an option during these experiments, we looked at the only and probably most important parameter for the renal function: the urinary output. The results for the 6 analysed pairs of kidneys were surprising at first sight, as they contradicted what we had observed in the porcine

model. As shown in ***Fig. 4.23. a***, the urinary output rates at reperfusion were higher in the NMP group, with a mean of 0.18 ± 0.07ml/min vs. 0.11 ± 0.02ml/min in the HMP pre-treated group (p=0.13). Looking for a potential explanation for this finding, we noticed the fact that the human kidneys were received after different periods of cold ischemia time and after a closer look at the data, we realized that exactly half of the pairs (n=3) were exposed to a cold ischemia time below 24 hours (12-24 hours) and the other half (n=3) was received after a cold ischemia time of more than 24 hours, with a maximum of 38 hours (24-38 hours). This is highlighted in ***Table 4.1.*** as well as in ***Fig. 4.23. b***.

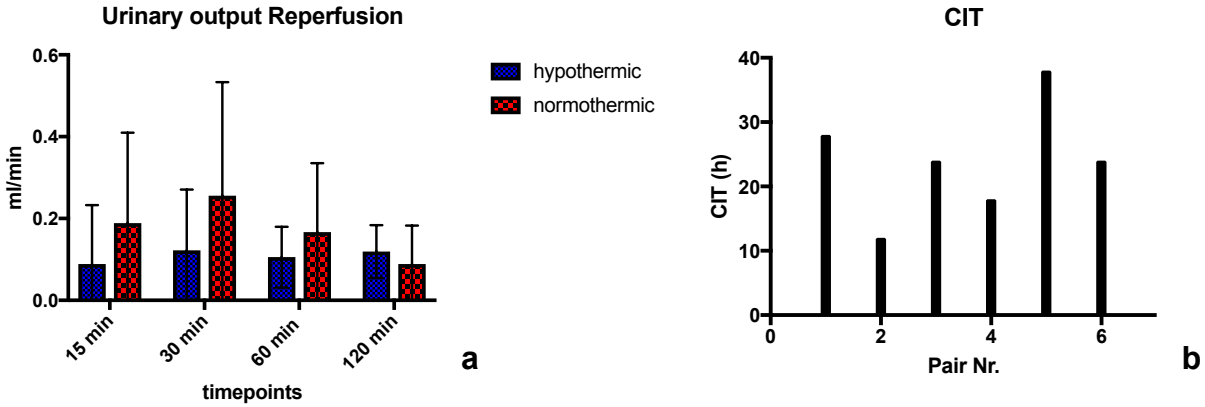


Figure 4.23.: Urinary output rates and cold ischemia times of human kidney pairs

Fig. 4.23.: *Urinary output rates at defined timepoints during reperfusion for human kidneys after undergoing 4h of hypothermic-(HMP) or 4h of normothermic machine perfusion (NMP), respecively, are shown in (**a**). Statistical analysis using ANOVA did not reveal any difference but contrary to findings in the porcine model, kidneys after NMP seemed to have better urinary output rates. The different cold ischemia times of the 6 organ pairs are illustrated in (**b**). Kidneys pairs 1,3 and 5 belonged to the group with a longer CIT (24-38h), the remaining kidneys built the group of a CIT< 24h.*

Due to the fact that in clinical transplantation, the maximally tolerated CIT for kidneys is around 24 hours, the idea was to subdivide the study groups into a group with kidneys after a CIT shorter than 24 hours and one with the pairs of kidneys after a CIT of longer than 24 hours and to look again at the urinary output within these groups.

Looking at the perfused kidneys within their respective treatment group, we discovered, that after undergoing HMP, kidneys with cold ischemia times < 24h produced more urine at during reperfusion than the kidneys with CITs > 24h (***Fig. 4.24. a***). The mean urinary output rates in this case were 0.15 ± 0.02ml/min in the CIT< 24h group vs. 0.07 ± 0.05ml/min in the CIT> 24h group (p=0.22). Interestingly,

the observations for kidneys after NMP were exactly the opposite, with kidneys after CIT> 24h producing more urine over the observed period of time than kidneys after CIT< 24h. This result was close to being statistically significant, with a p-value of 0.09 and mean urinary output rates of 0.3 ± 0.12ml/min in the former and 0.05 ± 0.01ml/min in the latter group (***Fig. 4.24. b***).

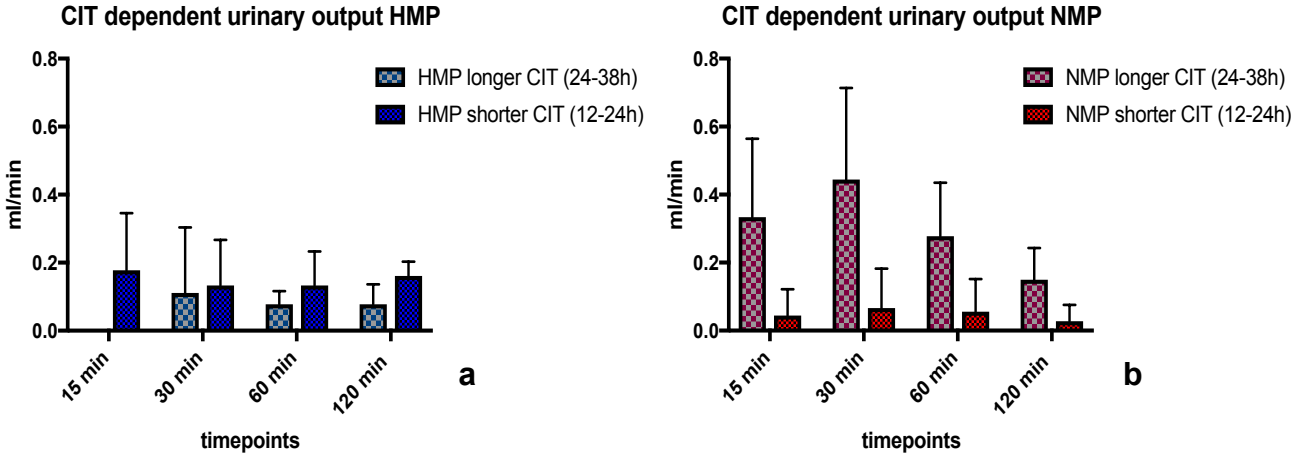


Figure 4.24.: Urinary output rates of human kidneys depending on cold ischemia times

Fig. 4.24.: *Urinary output rates at defined timepoints were compared within subgroups of the two treatment groups hypothermic- (HMP) and normothermich machine perfusion (NMP). Within the HMP group, kidneys after a shorter cold ischemia time (CIT; 12-24h) showed higher urinary output rates at all timepoints than kidneys after a longer CIT (24-38h), as illustrated in (**a**). The opposite result was demonstrated within the NMP group, where kidneys after a longer CIT (24-38h showed better results than kidneys after a shorter CIT (12-24h), as shown in (**b**).*
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).

With this observation in place, the next logical step was to compare the urinary output rates during reperfusion between NMP and HMP treated kidneys but under consideration of their previous cold ischemic times. As mentioned, two groups of 3 kidney pairs were divided, with a cut-off CIT of 24h and one group containing kidneys after CIT < 24h and the other group containing kidneys after CIT of > 24h.
Interestingly, for the group with CIT< 24h, our results from the porcine experiments could be confirmed, as we found higher urinary output rates at all time points of reperfusion for kidneys after HMP than after NMP, with a mean urinary output rate of 0.15 ± 0.02ml/min vs. 0.05 ± 0.02ml/min (p= 0.17), however the result did not reach statistical significance in this case (***Fig. 4.25. a***).
For the group of kidneys after a longer CIT (24-38h), we saw higher urinary output rates at all measured time points of reperfusion in the NMP treatment group than in

the HMP group. The mean urinary output rate was 0.3 ± 0.12ml/min in the former, vs. 0.07 ± 0.04ml/min in the latter group. Again, this did not reach statistical significance (p= 0.12, ***Fig. 4.25. b***).

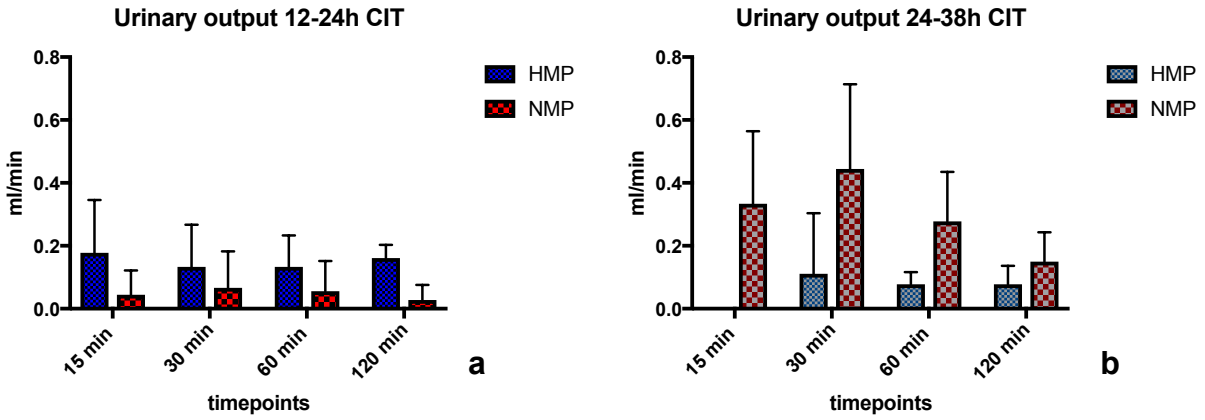


Figure 4.25.: Urinary output rates of human kidneys classified by duration of their cold ischemia times

Fig. 4.25.: *Comparison of urinary output rates after 4h of hypothermic machine perfusion (HMP) or normothermic machine perfusion (NMP) followed by 2h of reperfusion, at defined timepoints during reperfusion in ml/min, for kidneys exposed to a cold ischemia time (CIT) of 12-24h (**a**) or a longer CIT of 24-38h (**b**).*
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).

This encouraged us to also look at mRNA expressions of the kidney injury markers KIM-1, EDN-1, NGAL and the inflammatory markers IL-1β and TNFα more closely and to calculate the overall expression, followed by expression levels within the same subdivision of groups (***Fig. 4.26. a-o****).*

Overall, the expression of KIM-1 at mRNA-level was higher in kidneys after HMP than after NMP. The median expression levels were 0.72 (IQR: 0.22; 9.25) in the HMP group vs. 0.1 (0.03; 7.53) in the NMP group (p=0.87, ***Fig. 4.26. a***). However, looking at the subgroups, we could again detect opposite results for the expression of KIM-1, with a dramatic down regulation after pretreatment with NMP and an up regulation after HMP in the group with a CIT< 24h, and a higher up regulation after NMP than after HMP in the CIT> 24h group. Neither of the results reached statistical significance and the median expression levels for KIM-1 were 0.03 (IQR: 0.02; 0.04) in the NMP group vs. 10.53 (IQR: 0.72; 16) in the HMP group (p=0.18) in the <24h CIT group (***Fig. 4.26. b***), and 1.8 (IQR: 0.1; 13.27) in the NMP vs. 0.25 (IQR: 0.18; 2.11) in the >24h CIT group (p=0.45, ***Fig. 4.26. c***).

The kidney injury marker EDN-1 was overall higher up regulated in kidneys after NMP than after HMP, which contradicts our results for the equivalent study in the

porcine model. The median expression levels were 3.04 (IQR: 0.04; 7.53) in the former vs. 1.02 (IQR: 0.42; 2.36) in the latter group (p=0.46, ***Fig. 4.26. d***). However, looking at the named subgroups, we could again identify the exact opposite result depending on the CIT of the organs, namely a relative down regulation of EDN-1 in kidneys with a CIT< 24h after NMP and an up regulation after HMP, whereas in kidneys with a CIT> 24h, we observed an up regulation of EDN-1 after NMP and a relative down regulation after HMP. For the porcine experiments, our CIT was a maximum of 24 hours, so after all, our result for the EDN-1 expression was confirmed in kidneys <24h. The median expression levels were 0.03 (IQR: 0.02; 0.06) for the NMP group vs. 2.45 (IQR: 2.19; 2.53) for the HMP group in kidneys < 24h CIT, which was statistically significant (p=0.016, ***Fig. 4.26. e***), and a median of 7.26 (IQR: 0.08; 7.8) for the NMP group vs. 0.61 (IQR: 0.23; 0.8) for the HMP group (p= 0.22, ***Fig. 4.26. f***).

The kidney injury marker NGAL was slightly higher expressed in the HMP group than in the NMP group after reperfusion with median expression levels of 1.52 (IQR: 0.64; 2.72) in the former and 1.23 (IQR: 0.2; 2.8) in the latter group (p=0.71, ***Fig. 4.26. g)***. Also for NGAL, we saw a contrary result for the two CIT dependent subgroups, with a down regulation after NMP and an up regulation after HMP in the shorter CIT group (12-24h), which reached statistical significance (0.24, IQR: 0.02; 0.24 vs. 1.34, IQR: 0.88; 1.54, p= 0.007, ***Fig. 4.26. h***), whereas we observed an up regulation of NGAL after NMP in the group with the longer CIT (24-48h). The median values for this group were 2.55 (IQR: 0.02; 3.1) in the NMP group vs. 0.91 (IQR: 0.4; 3.86) in the HMP group (p= 0.9, ***Fig. 4.26. i***).

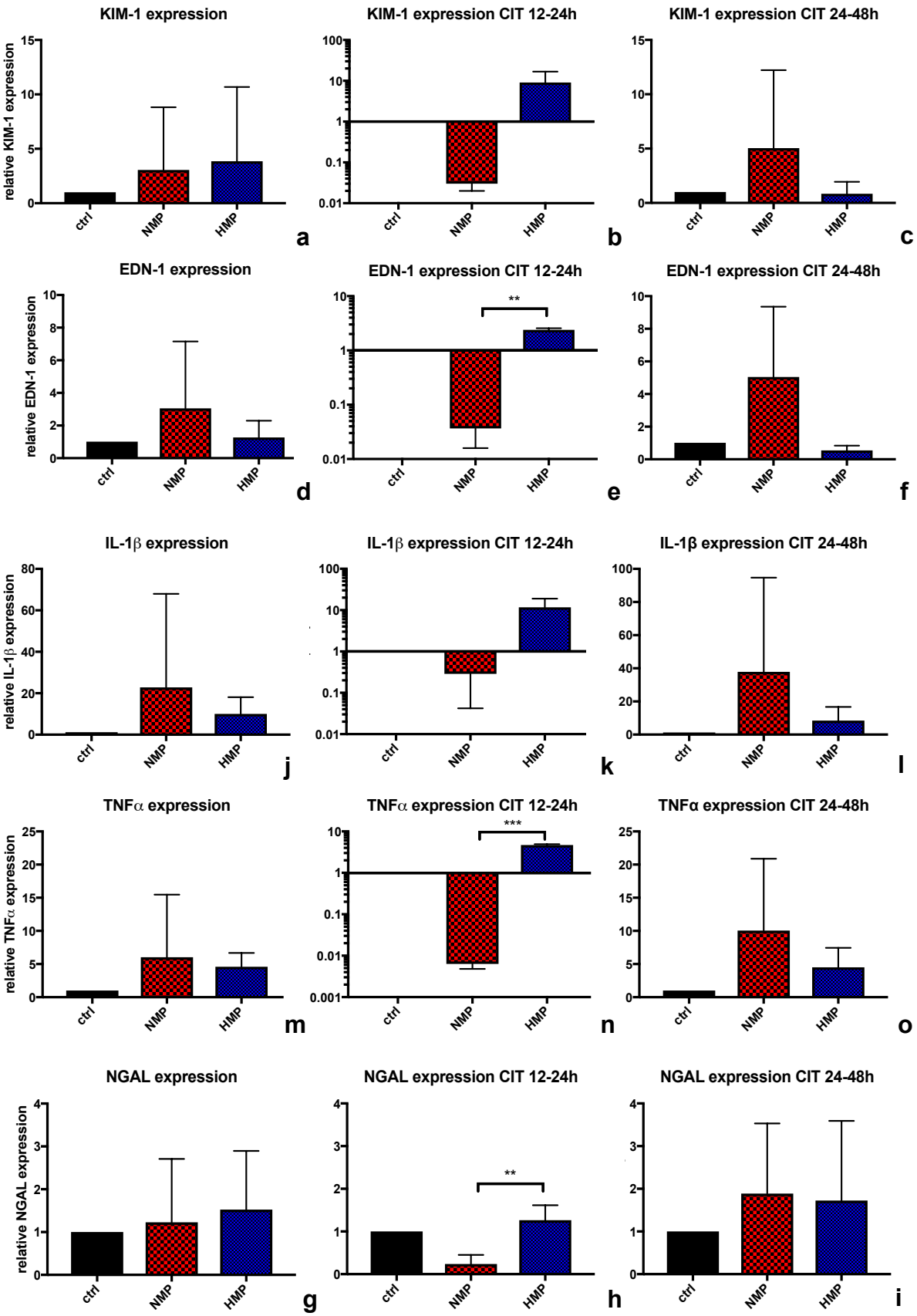


Figure 4.26.: mRNA expression levels of kidney injury markers and inflammatory markers in human kidneys after HMP or NMP

Fig. 4.26.: *Graphs for the relative mRNA expression levels of Kidney injury marker-1 (KIM-1) (**a-c**), Endothelin-1 (EDN-1) (**d-f**), Neutrophil gelatinase-associated lipocalin (NGAL) (**g-i**), Interluekin-β (IL-1β) (**j-l**) and Tumor necrosis factor α (TNFα) (**m-o**) are shown for an overall comparison between 6 pairs of human kidneys undergoing either hypothermic- (HMP) or normothermic machine perfusion (NMP) followed by reperfusion, respectively, as well as for their cold ischemia time (CIT)- dependent subgroups. Levels were determined using real time (RT)-PCR, with a t0 biopsy as a control for every respective organ and β-Actin as housekeeping gene. The calculations of the relative mRNA expressions were performed using the ΔΔCt method. Statistical analysis was performed using Mann-Whitney U test with statistical significance of p<0.05 (*); p<0.005 (**) and p<0.001 (***).*

Looking at the expression levels of the inflammatory markers IL-1β and TNFα, we could again make the interesting observation of opposite results for kidneys in the <24h CIT group in comparison to kidneys in the >24h CIT group, with the overall expression levels being slightly lower in the HMP group than in the NMP group after reperfusion (***Fig. 4.26. j, m***).

For IL-1β, the following median expression levels were observed: analysing all kidney pairs as one big group, we saw a median expression level of 22.83 (IQR: 0.26; 56.48) in the NMP vs. 10.5 (IQR: 2.9; 18.3) in the HMP group (p= 0.54). Split into subgroups, we saw a down regulation of IL-1β expression with a median of 0.34 (IQR: 0.02; 0.5) after NMP and an up regulation after HMP with a median of 10.2 (IQR: 5.31; 19.56), p= 0.12 (***Fig. 4.26. k***). A different result, in favour of HMP with less IL-1β expression levels was observed in kidneys after a CIT > 24h, with median values of 9.71 (IQR: 0.65; 103.3) vs. 7.84 (IQR: 0.5; 17.03, p= 0.41, ***Fig. 4.26. l***).

The same pattern of expression levels was observed for TNFα, which was overall slightly higher expressed after NMP than after HMP (6.04, IQR: 0.065; 14.21 vs. 4.59, IQR: 3.01; 6.17, p= 0.76), but statistically significantly lower expressed after NMP than after HMP in the CIT < 24h group, with a down regulation of TNFα to a median of 0.006 in the NMP group (IQR: 0.005; 0.008) vs. an up regulation to a median of 4.67 (IQR: 4.49; 4.92) in the HMP group (p= 0.0007, ***Fig. 4.26. n***). In the group with the longer CIT of 24h-48h, we observed an up regulation of TNFα after both treatment modalities, but higher in the NMP group, with a median of 6.11 (IQR: 1.77; 22.3) vs. 4.6 (IQR: 1.53; 7.41) in the HMP group, which was not statistically significant (p= 0.5, ***Fig. 4.26. o***).

We also looked at the perfusate- and urinary samples of the perfused kidneys, and analysed them for the expression of the kidney injury marker KIM-1. In both, perfusate- as well as urinary samples for the time points of analysis during

reperfusion, namely at 60 minutes and 120 minutes of reperfusion, respectively, we measured higher KIM-1 concentrations in the NMP group than in the HMP group. In both cases, however, there was no statistical significance. The mean KIM-1 perfusate level was 505 ± 48.88ng/ml in the NMP vs. 283 ±81.97ng/ml in the HMP group (p=0.28, ***Fig. 4.27. a***). For the urinary samples, the mean KIM-1 level was 536.7 ± 30.1ng/ml in the NMP vs. 324 ± 36.79ng/ml in the HMP group (p= 0.46, ***Fig 4.27. b***).

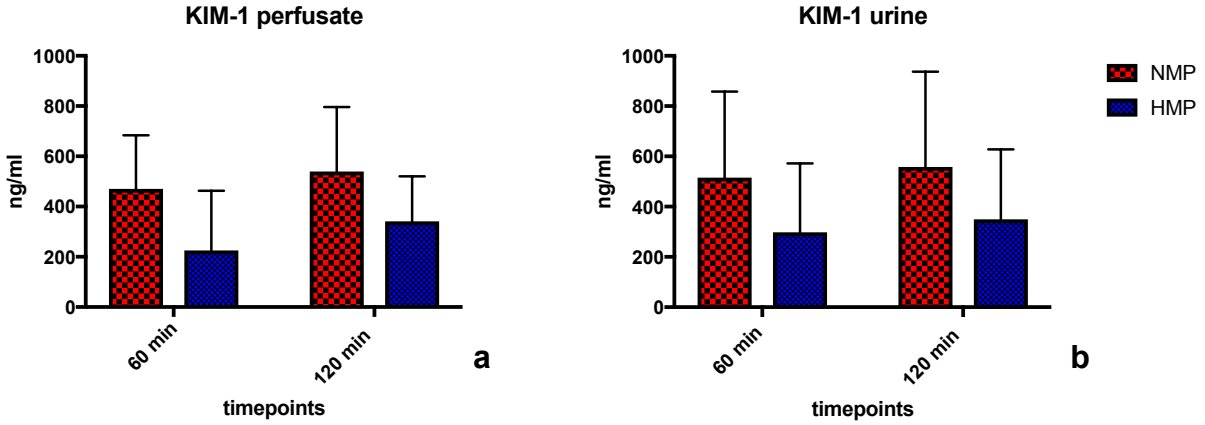


Figure 4.27.: Perfusate- and urinary levels of KIM-1 in human kidneys after HMP vs. NMP

Fig. 4.27.: *Analysis of the perfusate samples (**a**) as well as the urinary samples (**b**) at 1 hour and 2 hours of reperfusion was performed using ELISA for Kidney injury marker 1 (KIM-1). KIM-1 concentrations were measured in ng/ml.*
Statistical analysis was performed using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**).

We can conclude, that in the porcine experiments on a direct comparison of HMP and NMP, kidneys after HMP show better results in terms of physiological parameters, with higher urinary output rates as well as oxygen consumption rates, despite showing higher expression levels of inflammatory parameters. Also, the histological structure was better preserved in kidneys after HMP than after NMP, with a statistically significant higher amount of cells undergoing apoptosis in kidneys after NMP. In similar experiments on human kidneys, we could confirm our results for kidneys after a cold ischemia time of up to 24 hours. Interestingly, contrary results were found for kidneys of a longer cold ischemia time (>24 hours). These results will be discussed in details in Chapter 5.2.
Overall, we can conclude for this chapter, that porcine kidneys after a period of HMP performed better in terms of physiological parameters during mimicked reperfusion than porcine kidneys after the same period of time undergoing NMP. This was

despite the fact that on mRNA level it looked like kidneys after HMP showed a more ‘pro-inflammatory’ profile than kidneys after NMP. For human kidneys, the same observations were made when the cold ischemia times (CIT) the organs were exposed to were similar, but contrary results were observed when the CITs were longer. The reasons for this are unclear but the results indicate that different treatments for individual cases might be a way forward. More research is still needed to optimize and also logistically improve both, hypothermic and normothermic perfusion technologies, but there is a clear difference in outcomes for kidneys undergoing different preservation treatments. In the future, we might have to progress towards organ-tailored preservation methods, whereby high-risk kidneys can undergo assessment and repair before transplantation.

4.3. Project 3: Investigation of the introduction of MSCs into ex-vivo organ preservation as a preconditioning method for porcine and human kidney grafts (translational model).

After establishing circuits for both, NMP and HMP on human and porcine kidneys using the RM3 machine, we were interested in the potential effects of MSCs when being introduced into these circuits. The questions were, whether MSCs could be delivered to a perfused graft during the ex-vivo preconditioning phase and whether it was possible to trace them later within that graft. Thereby, one interest was the localization of delivered MSCs and of course, whether these MSCs have an effect on the function of an organ during a short period of reperfusion. Human MSCs were double-labelled with green and red fluorescent cell dye as explained in Chapter 3. Imaging showed successful labelling of the cells with the green cell membrane dye and some red staining in the cytosol of the cells. By mounting the cells on the objective using DAPI, the nuclei of the cells appeared as blue structures (***Fig. 4.28. a, b***). After retrieval and 24 hours of CIT, pairs of porcine and human kidneys underwent either HMP or NMP for 4 hours at the same time but on separate circuits. One perfusion circuit contained a number of double-labelled human MSCs (1-5x10^6) whereas the other circuit only contained the perfusate without MSCs and served as the control.

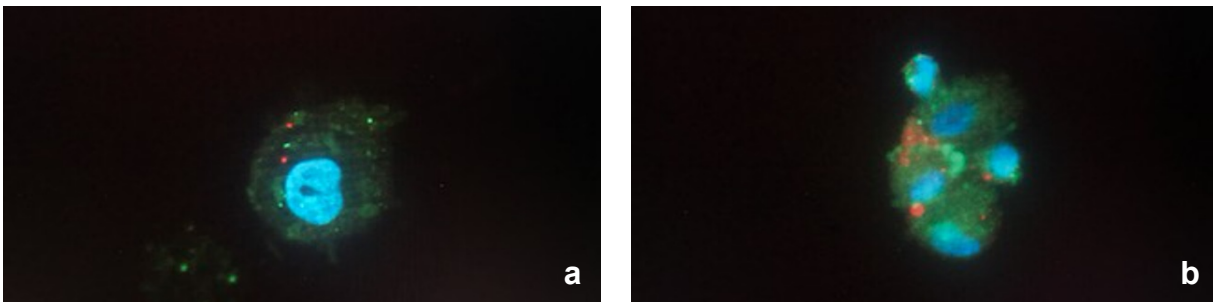


Figure 4.28.: double labelled Mesenchymal Stem Cells

Fig. 4.28. *(**a**) and (**b**) show double labelled Mesenchymal Stem Cells (MSCs), envisualized using wide field microscopy.* ***Fig. 4.17. a*** *shows one cell, with a blue nucleus, a green cell membrane and some red granulae in the cytosol.* ***Fig. 4.17. b,*** *showing a cluster of MSCs with multiple nuclei in blue.*

4.3.1. Investigation of the introduction of human Mesenchymal Stem Cells into porcine models of ex-vivo machine perfusion:

For this study, the following groups of porcine perfusion experiments were conducted:

***Group 1**: 24h of CSS, 4h HMP, 2h REP (n=6)*

***Group 2**: 24h of CSS, 4h HMP+1-5x10^6 MSCs, 2h REP (n=6)*

***Group 3**: 24h of CSS, 4h NMP+ 2h REP (n=6)*

***Group 4**: 24h of CSS, 4h NMP, 1-5x10^6 MSCs, 2h REP (n=6)*

4.3.1.1. Mesenchymal Stem Cells in a porcine HMP model

For the ex-vivo HMP organ perfusion experiments, the same protocols as for the experiments in Chapter 4.2. were followed. For each group containing MSCs, 2 kidneys per group were infused with 1x10^6 MSCs, 2 kidneys were perfused with 3x10^6 MSCs and 2 kidneys were perfused with a perfusate containing 5x10^6 MSCs. The MSCs were harvested by trypsinization in the lab and then infused into the HMP circuit via the arterial silicone reservoir of the cassette straight after starting the HMP preconditioning phase of 4 hours. The cells were infused in a volume of 10mls of MSC growth medium.

During the preconditioning phase of 4 hours of HMP, there was no difference of perfusate flow rates and the development of the RRIs between kidneys with and kidneys without MSCs in the perfusate. ***Fig. 4.29. a*** and ***b*** show the curves for the measured RRIs during the HMP period and the resulting perfusate flow rates during that time for both treatment groups. There was no significant difference between the RRIs at all time points (p=0.86) and no difference in the resulting perfusate flow rates between the two groups with median values of 19.27ml/min/100g in the HMP group vs. 17.36ml/min/100g in the HMP+ MSC group (p= 0.65).

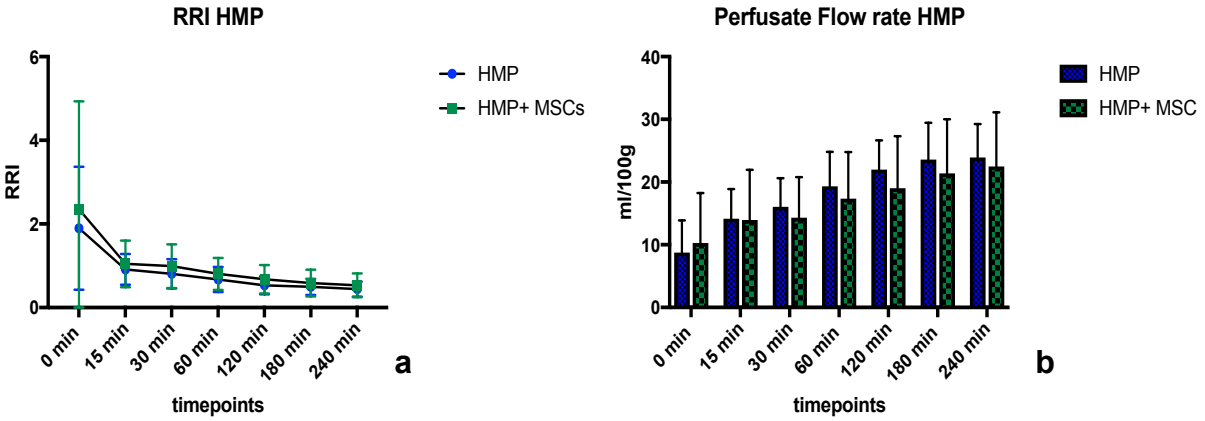


Figure 4.29.: RRI and perfusate flow rates in HMP+/- MSC treatment

Fig. 4.29 *(**a**)* and (***b***) show the intrarenal resistance index (RRI) and the resulting perfusate flow rates during the hypothermic machine perfusion (HMP) phase of 4 hours, with and without Mesenchymal Stem Cells (MSCs) in the perfusate.
Statistical analyses carried out using ANOVA, statistical significance: p<0.05 (*); p<0.005 (**); p<0.001 (***).
HMP=Hypothermic MAchine Perfusion, HMP+MSC= Hypothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the perfusate.

Also in the reperfusion phase, similar physiological parameters were observed in both groups, as demonstrated in ***Fig. 4.30. a-d***. For the RRI, the median values were 0.38 in kidneys without MSC infusion vs. 0.35 in kidneys with prior MSC treatment (p=0.9, ***Fig. 4.30. a***). Therefore, also the resulting perfusate flow rates were similar for both groups with median values of 58.03 ml/min/100g in the HMP only group vs. 56.24 ml/min/100g in the MSC group (p=0.9, ***Fig 4.30. b***). Also the oxygen consumption was similar between both group during the reperfusion phase, with median values of 30.08 ml/min/100g in the HMP group vs. 28.13 ml/min/100g in the HMP+MSC group (p=0.92, ***Fig. 4.30. c***), resulting in similar values for the urinary output rates at all time points between the two groups, with median values of 4.6 ml/min in the former and 4.0 ml/min in the latter of the groups (p= 0.77, ***Fig. 4.30. d***). Furthermore, the creatinine clearance and the sodium excretion were similar in both groups, with a median creatinine clearance of 4.44 mmol/min (IQR: 3.94 mmol/min; 5.33 mmol/min) in the HMP treated kidneys vs. 4.51 mmol/min (IQR: 3.44 mmol/min; 4.8 mmol/min) in the HMP+MSC group (p= 0.9) and a median sodium excretion of 14558 mmol/min (IQR: 11161 mmol/min; 15929 mmol/min) in the former vs. 16050 mmol/min (IQR: 10966 mmol/min; 18147 mmol/min) in the latter group (p= 0.66) (***Fig. 4.31. a, b***).

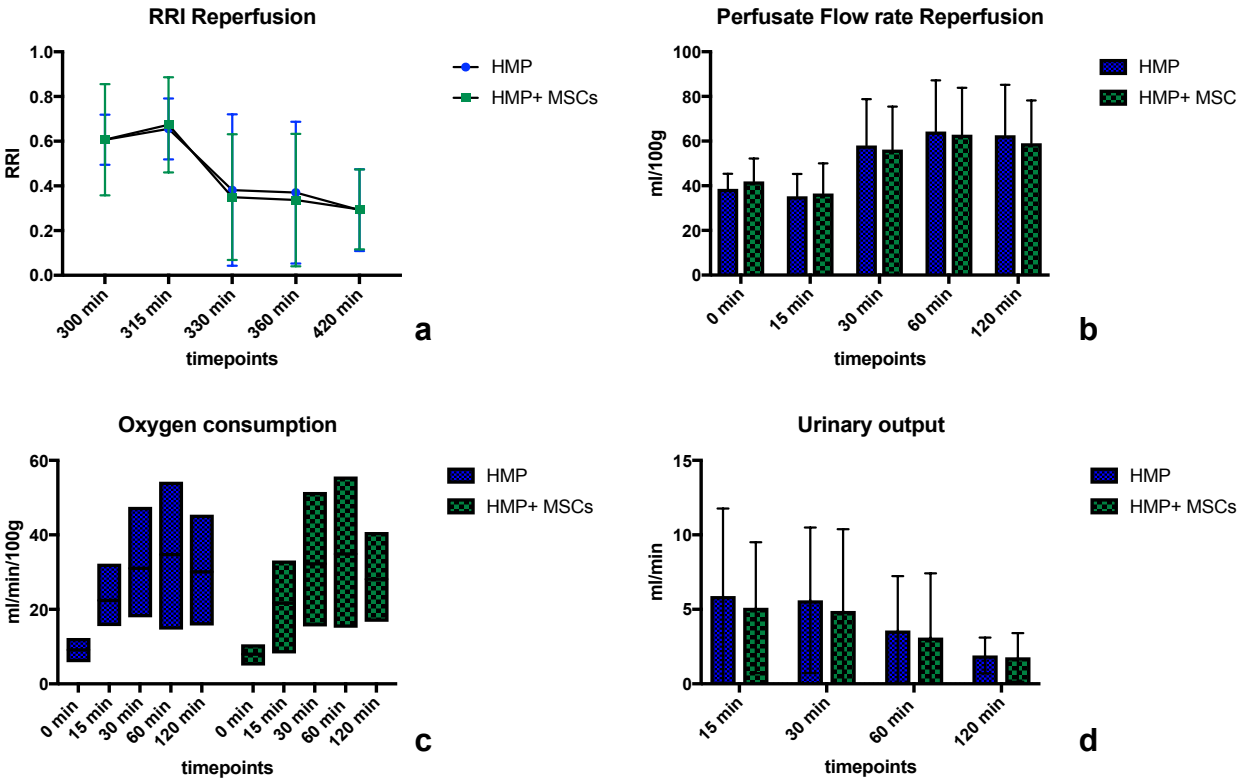


Figure 4.30.: Physiological parameters of kidneys undergoing HMP+/- MSCs

Fig. 4.30. : *physiological parameters of kidneys after hypothermic machine perfusion (HMP), with or without infusion of Mesenchymal stem cells (MSCs) during the 2h reperfusion period. No significant difference was observed for values of intrarenal restistance indices (RRI) (**a**), perfusate flow rates (**b**), oxygen consumption (**c**) and urinary output (**d**).*
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).
HMP=Hypothermic MAchine Perfusion, HMP+MSC= Hypothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the

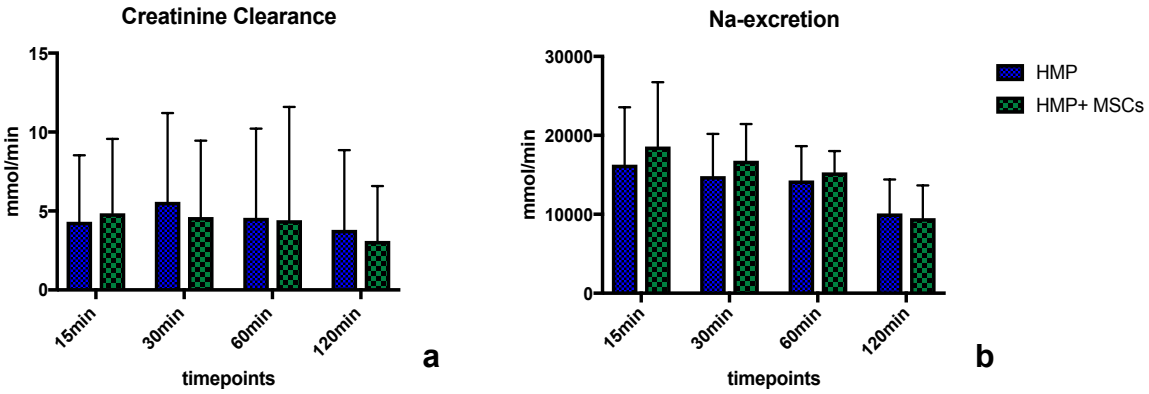


Figure 4.31.: Creatinine clearance and sodium excretion for porcine kidneys after HMP+/- MSCs

Fig. 4.31.: *Creatinine clearance (**a**) and fractional sodium excretion rates (**b**) were similar between the two groups at all time points during the 2 hours of reperfusion.*
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).
HMP=Hypothermic MAchine Perfusion, HMP+MSC= Hypothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the perfusate.

To further investigate a potential influence of MSCs on the porcine kidney grafts, an analysis of the perfusate- and urine samples for the protein levels of IL-1β and NGAL was performed. We wanted to know, whether MSCs were able to influence the inflammatory milieu at the time of reperfusion. Results of ELISA blots revealed higher concentrations of IL-1β in perfusate- and urine samples of MSC treated kidneys, with median concentrations of 208.2 pg/ml (IQR: 157pg/ml; 312pg/ml) vs. 152 pg/ml (IQR: 143.2 pg/ml; 159.2 pg/ml), p=0.51 for perfusate samples and 239.5 pg/ml (IQR: 176 pg/ml; 317.1 pg/ml) vs. 149.2 pg/ml (IQR: 77.11 pg/ml; 171.9 pg/ml), p=0.15 for urine samples. The median values of NGAL concentrations were similar for both groups in perfusate samples (130.1 pg/ml, IQR: 83.98 pg/ml; 139.1 pg/ml in the HMP group vs. 130.5 pg/ml, IQR: 93.56 pg/ml; 165.2 pg/ml, p=0.91), but again higher in the HMP+MSC group compared to the HMP only group at reperfusion, with median values of 217.2 (IQR: 77.27 pg/ml; 343.2 pg/ml) in former group vs. 128.5 pg/ml (IQR: 82.17 pg/ml; 201.9 pg/ml) in the latter group (p=0.23). The results are illustrated in ***Fig. 4.32. a-d***.

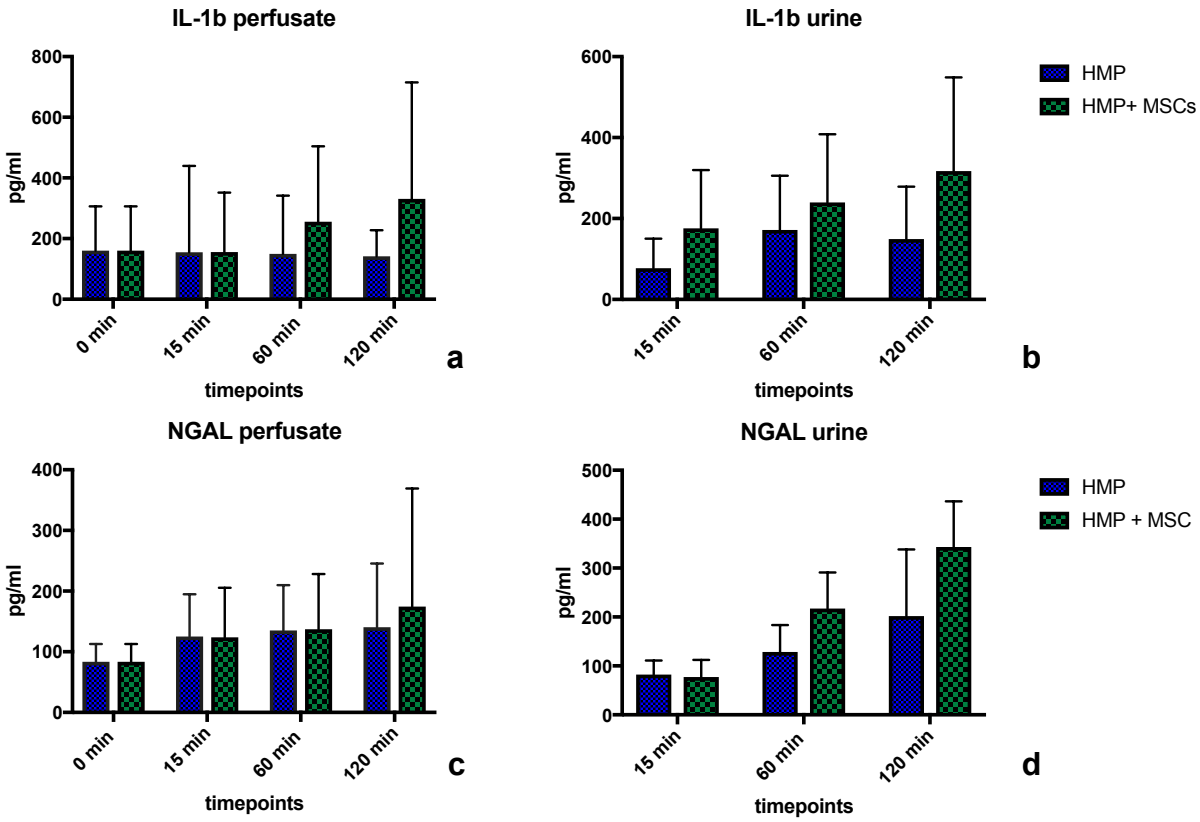
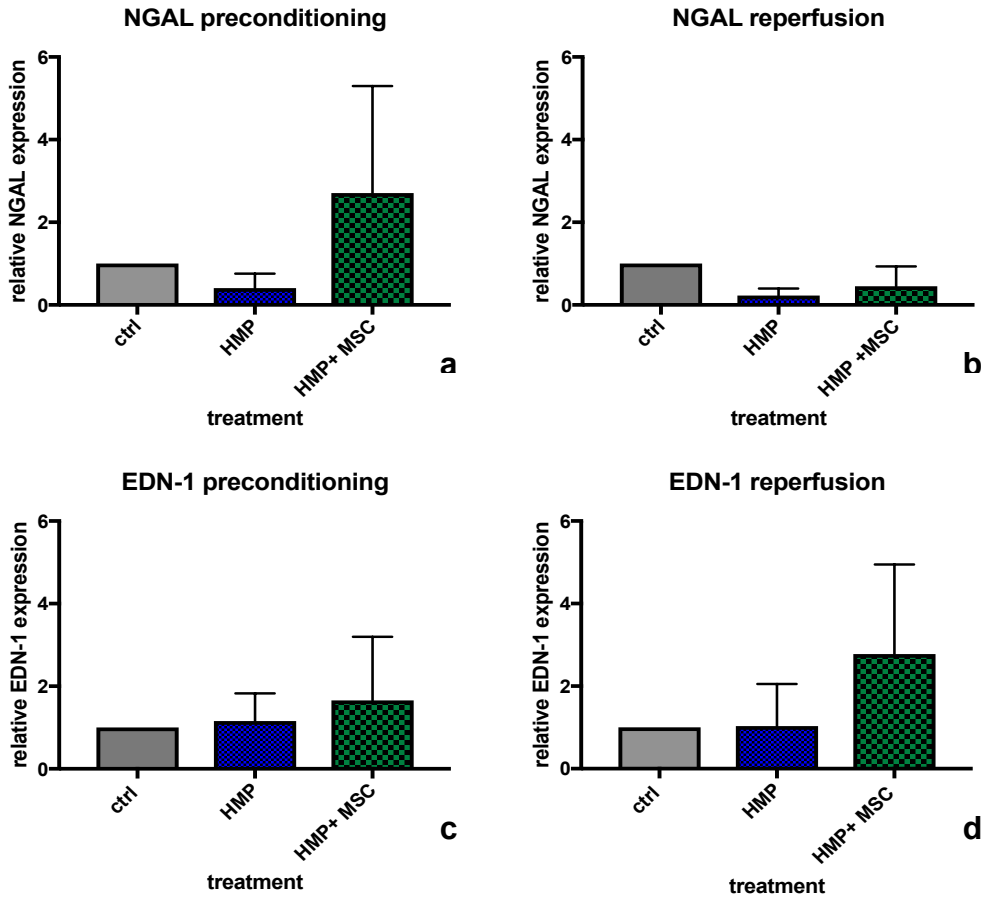


Figure 4.32.: IL-1β and NGAL levels in perfusate and urine samples of porcine kidneys after HMP+/- MSCs

Fig. 4.32.: *Concentrations of Interleukin-1β (IL-1β) in perfusate samples (**a**) and urine samples (**b**) for porcine kidneys after 4 hours of normothermic machine perfusion only (NMP), or 4 hours of normothermic machine perfusion + treatment with Mesenchymal stem cells (NMP+MSC), measured at defined time points during reperfusion. The same measurements were made and compared for NGAL concentrations in perfusate- (**c**) and urine samples (**d**).*
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).
HMP=Hypothermic MAchine Perfusion, HMP+MSC= Hypothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the

To further investigate the impact of MSC infusion on kidney grafts in a HMP model, we analysed biopsy samples for the expression of the kidney injury- and inflammatory markers NGAL, EDN-1, TNFα and IL-1β. Samples were analysed after the preconditioning phase as well as after the reperfusion phase and compared to the t0 biopsy sample (control), which was taken straight after the pig was sacrificed and the kidney was retrieved. The ubiquitously expressed β-Actin served as a housekeeping gene. The relative expressions of the inflammatory markers on mRNA level were calculated with the ΔΔCt method, which is further explained in Chapter 3. For the kidney injury marker NGAL, expression levels after the preconditioning phase were higher for kidneys, which had received HMP+ MSC treatment when compared to the HMP only group, in which the NGAL expression was down regulated when

compared to the control (***Fig. 4.33. a***). The mean expression levels were 0.4 ± 0.35 in former vs. 2.7 ± 2.6 (p=0.13) in latter group. Interestingly, after reperfusion, that effect was reversed, with a down regulation of the NGAL expression in both groups. The mean NGAL expressions were 0.23 ± 0.17 in the HMP group vs. 0.45 ± 0.47 in the HMP+MSC group (p= 0.27, ***Fig. 4.33. b***). Endothelin-1 (EDN-1), which is another kidney injury marker, was higher expressed in the HMP+MSC group than in the HMP only group. This observation was made for both the preconditioning and the reperfusion phase, but was more impressive after reperfusion, with mean expression levels of 1.03 ± 1.02 in the HMP group vs. 2.77 ± 2.1 in the HMP+ MSC group (p=0.1, ***Fig. 4.33. c, d***). A similar observation was made for the expression levels of the inflammatory markers TNFα and IL-1β, which both were expressed in slightly higher amounts in the MSC treated groups than in the HMP only group after the preconditioning phase, and much higher expressed in the former group than in the latter after reperfusion, with mean expression levels of 7.83 ± 9.27 vs. 2.5 ± 1.13 (p= 0.15) for TNFα (***Fig. 4.33. e, f***) and 46.45 ± 66.54 vs. 14.01 ± 13.14 (p= 0.22) for IL-1β (***Fig. 4.33. g, h***).



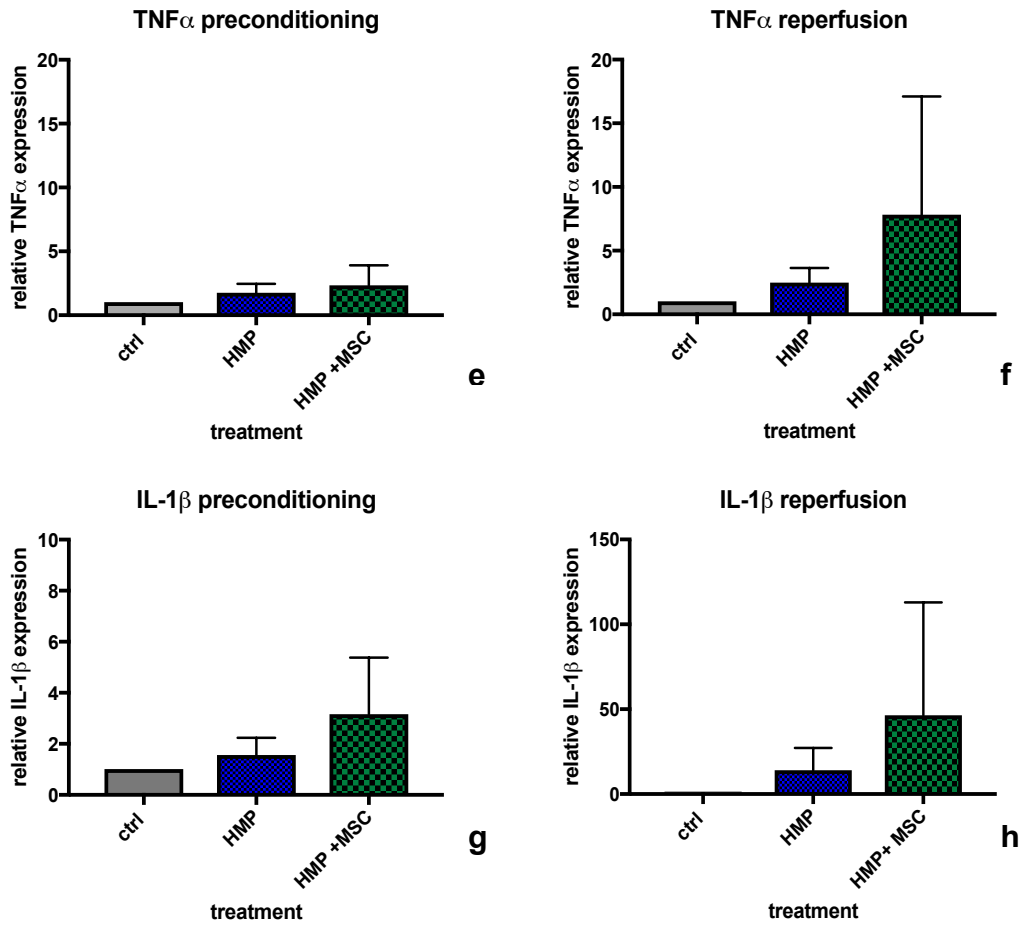


Figure 4.33.: mRNA expression levels of kidney injury markers and inflammatory markers for porcine kidneys after HMP+/- MSCs

Fig. 4.33.: *Expression of the kidney injury markers Neutrophil gelatinase-associated lipocalin (NGAL;* ***a, b****), Endothelin-1 (EDN-1;* ***c, d****), and the inflammatory markers Tumor necrosis factor α (TNFα;* ***e, f****) and Interleukin- 1β (IL-1β;* ***g, h****) on mRNA level after the preconditioning phase and after reperfusion, respectively. Expression levels were determined by RT-PCR and calculated using the ΔΔCt method with β-Actin as a housekeeping gene and the respective t0 biopsy as a control.*

Statistical analyses carried out using Mann-Whitney U test, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).

HMP=Hypothermic MAchine Perfusion, HMP+MSC= Hypothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the perfusate, ctrl= control.

In the HMP+ MSC group, single MSCs could be traced within the glomeruli and tubules of all porcine kidneys, except from the group in which the smallest number of MSCs, 1x10^6 cells, were infused. Using wide field microscopy, single cells could be detected in glomeruli and tubules of frozen sections after the preconditioning phase as well as in frozen sections of the treated kidneys after reperfusion, which indicates that the cells remained at their localization during the reperfusion period. ***Fig. 4.34.*** shows microscopic images taken with a wide field microscope, showing a negative control containing a glomerulus without any cells present (left) and 2 pictures

showing cells present in the glomeruli (middle) and the tubules (right) of the porcine kidneys (cells are highlighted by arrows). As a direct comparison, in between the two images with positive signals, an image of a double- labelled cell was inserted. Unfortunately, it was impossible to quantify the cells within the slides and it was also not possible to proof the viability of the detected cells. However, the fact that dead cells would go into apoptosis and lyse was encouraging for the detected cells carrying the cytosolic and cell membrane dye to be viable.

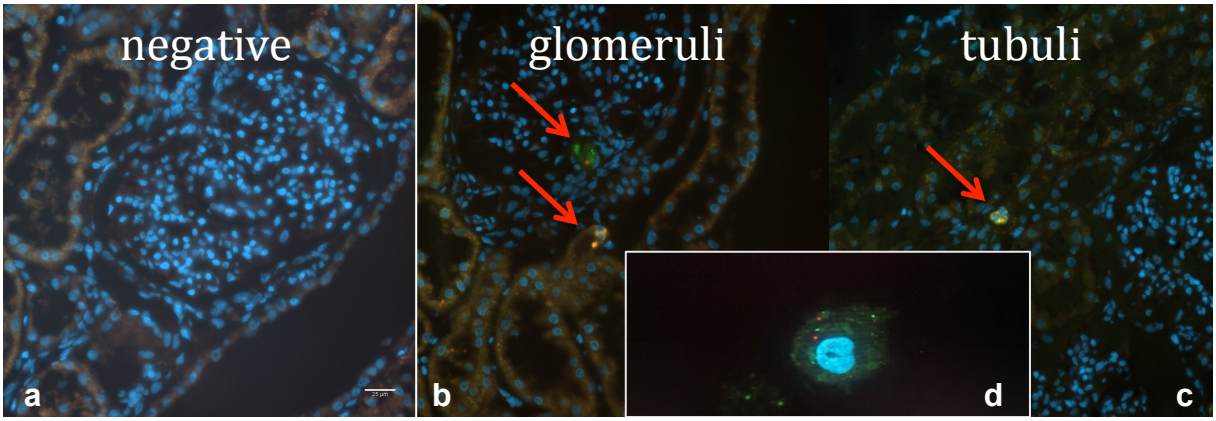


Figure 4.34.: double-labelled human MSCs in porcine kidneys, visualized using wide field microscopy

Fig. 4.34.: *Imaging of frozen porcine kidney sections covered with DAPI (nuclei stained in blue). (**a**) Shows a negative control, which was a kidney undergoing normothermic machine perfusion (NMP) only. In (**b**), 2 fluorescing cells could be visualised within one glomerulum (arrows), but cells could also be detected within the epithelium of some tubuli (**c**). In (**d**) a single double-labelled Mesenchymal Stem Cell is shown as a direct comparison to demonstrate the similarity of the cells found within the kidney.*

This result could be confirmed using immunohistochemichal staining against human MHC class 1 (MHC-1) as shown in ***Figure 4.35.,*** which shows PAS counterstained histology slides at 20x magnification after immunohistochemical staining as described in Materials and Methods**.** A human skin sample served as positive control as it is known to contain high amounts of MHC-1, stained in brown. The negative control was a sample from a kidney that had been perfused hypothermically, without MSC infusion (***Fig. 4.35. A***). As expected, no brown staining was visible unlike in the positive control (***Fig. 4.35. B***). Satisfactorily, the presence of MSCs could be confirmed with this method by detecting brown stained human MSCs within glomeruli (***Fig. 4.35. C***), as well as within tubules (***Fig. 4.35. D***) of porcine kidneys. Immunohistochemical staining revealed that delivered cells seem to not just be stuck within the organs, but, in case of the tubules, to be integrated into the endothelium,

as is visible in ***4.35. D***. The arrows show the brown positive stained MSCs and the cells within the tubules seem to be in between other epithelial cells.

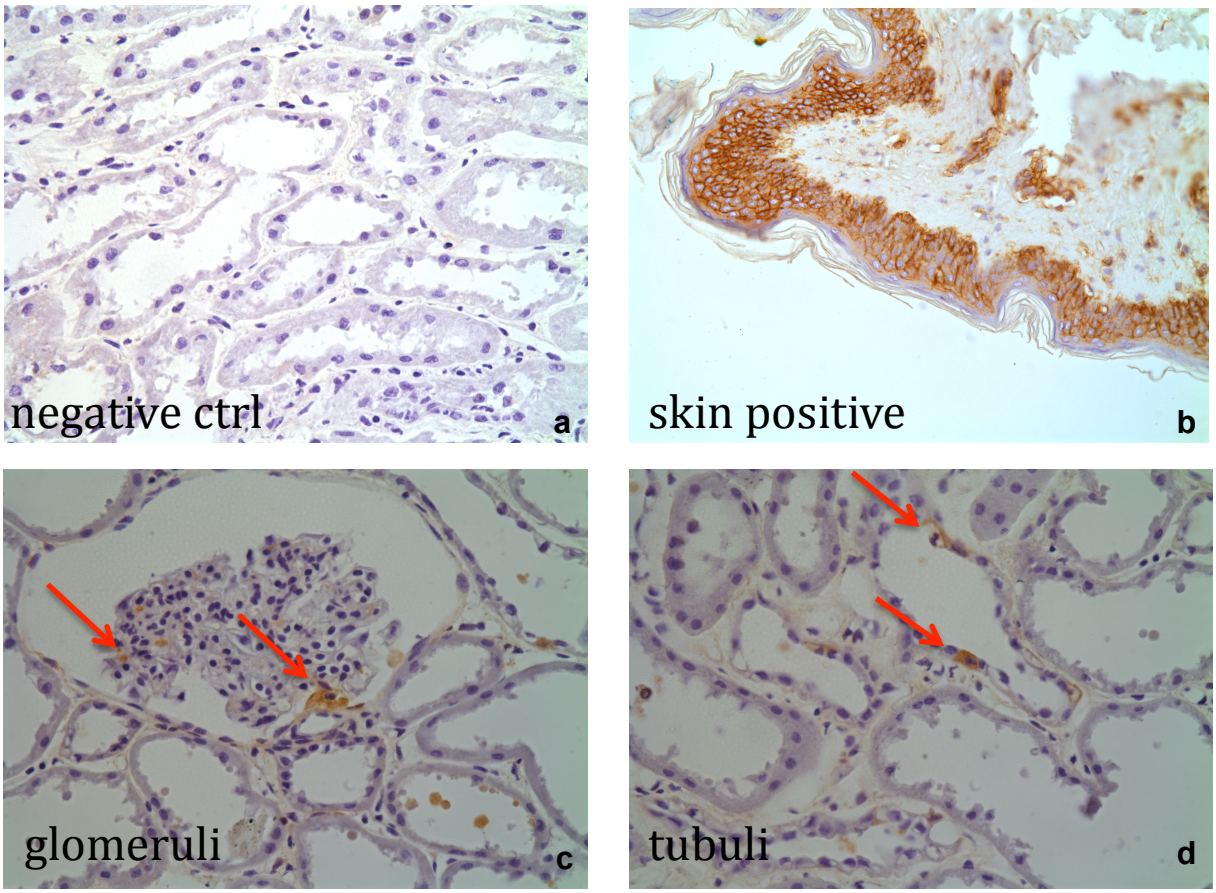


Figure 4.35: Immunohistochemical staining of porcine kidney sections against human MHC class I

Fig. 4.35: *Immunohistochemical staining of porcine slides against human major histocompatibility complex-1 (MHC-1). (**a**) Negative control, (**b**) human skin sample as positive control, (**c**) positive staining for Mesenchymal stem cells (MSCs; arrows) within a glomerulum of a porcine kidney after 4h of hypothermic machine perfusion (HMP) with $5x10^6$ MSCs, (**d**) positive staining of MSCs (arrows) within the tubuli of a porcine kidney after 4h of HMP containing $5x10^6$ cells.*

4.3.1.2. Mesenchymal Stem Cells in a porcine NMP model

For the established NMP model, the same investigations using MSCs were conducted as for the HMP model. The aim was to find out, whether the ex-vivo infusion of MSCs into porcine kidneys would have different effects depending on the temperature of the perfusate with the hypothesis, that the cells would be more active at physiological temperatures.

Kidneys undergoing NMP showed different results to kidneys undergoing HMP as demonstrated in Chapter 4.2., however perfusion parameters and outcomes were

similar again for kidneys undergoing NMP without MSC infusion and kidneys undergoing NMP with MSC infusion.

In ***Fig. 4.36. a-d***, the physiological parameters during the NMP preconditioning phase are illustrated for kidneys undergoing NMP for 4 hours with MSCs (1-5x10^6, n=6) and without MSCs in the perfusate. There was no significant difference between the groups, suggesting that MSCs don't interfere with the physiological function of the kidneys. The RRIs showed the same curves and went down a bit more during NMP in the group without cell infusion. The median resulting flow rates were similar between groups as well, with values of 20.82 ml/min/100g (IQR: 16.88 ml/min/100g; 23.24 ml/min/100g) in the NMP group vs. 21.78 ml/min/100g (IQR: 12.62 ml/min/100g, 25.36 ml/min/100g), p=0.84 (***Fig. 4.36. b***). Interestingly, kidneys receiving MSC treatment during NMP showed lower oxygen consumption rates at the beginning of the perfusion phase, up to 120 minutes, before picking up similar O_2 consumption rates as the NMP controls (***Fig. 4.36. c***). This was reflected in a slightly lower urinary output rate in NMP+MSC kidneys at time points 30 min and 60 min of perfusion, but overall, the urinary output rates were higher in the MSC treatment group, with mean values of 1.81 ml/min (IQR: 1.69 ml/min; 1.94 ml/min) vs. 1.69 ml/min (IQR: 1.52 ml/min; 2.9 ml/min) for the NMP only group (p= 0.74, ***Fig. 4.36. d***).

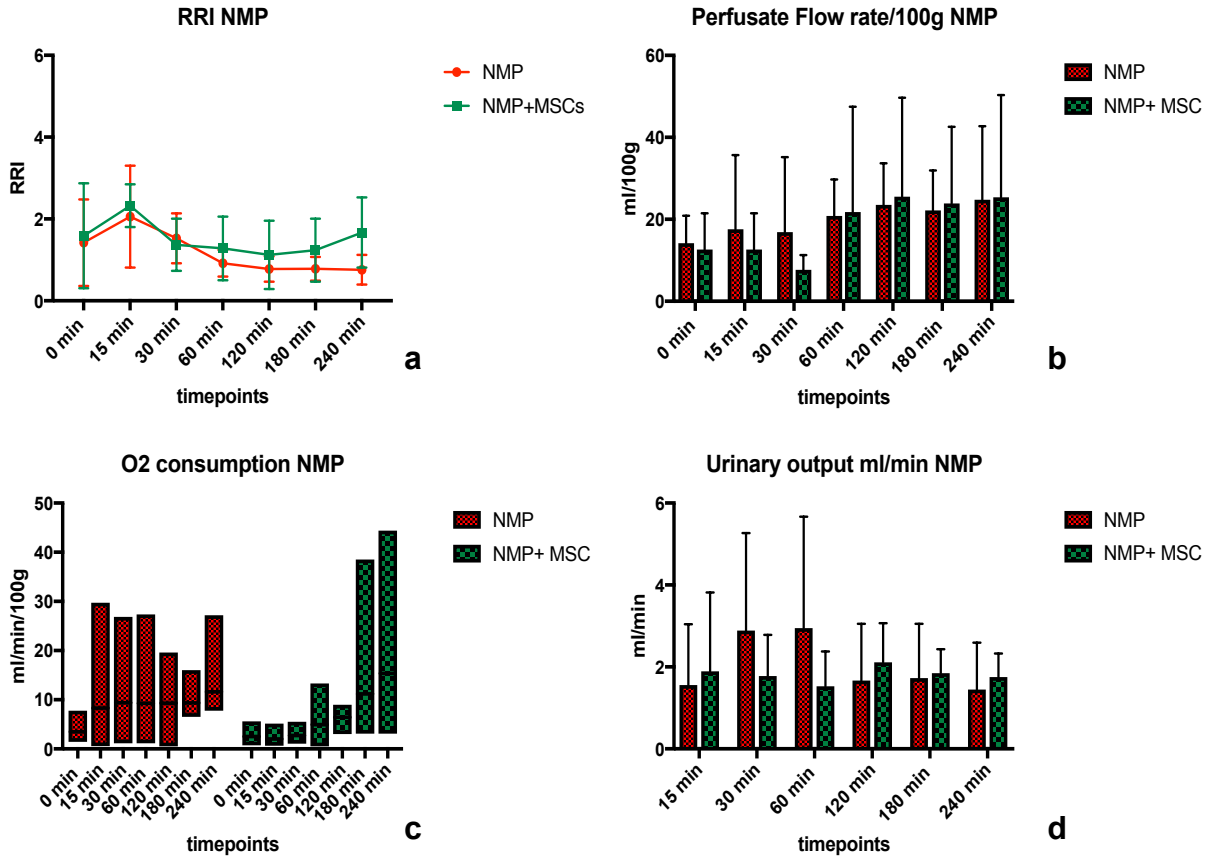


Figure 4.36.: Physiological parameters of porcine kidneys during Normothermic machine perfusion +/- MSC treatment

Fig. 4.36: *Intrarenal resistance indices over time during the normothermic preconditioning period with or without MSC treatment are shown in (**a**). The resulting flow rates during this time at defined timepoints are shown in (**b**). Oxygen consumption rates in ml/min/100g are illustrated in (**c**) and the urinary output rates for both treatment groups in ml/min are shown in graph (**d**).*

Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).

NMP= Normothermic Machine Perfusion, NMP+MSC= Normothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the perfusate.

During the reperfusion phase, interestingly, kidneys with previous MSC treatment showed higher thorough RRIs, compared to the NMP group, with median values of 1.56 (IQR: 1.51; 1.66) vs. 1.07 (IQR: 1.01; 1.12), p=0.12, ***Fig. 4.37. a***. This reflected in higher perfusate flow rates for within the NMP group with median values of 28.18 ml/min/100g (IQR: 24.76; 29.94) vs. 21.03 ml/min/100g (IQR: 20.62; 25.12), with a p-value of 0.48, ***Fig. 4.37. b***. As a consequence, also the oxygen consumption rates in the NMP group were higher than in the NMP+MSC group, with a median of 14.77 ml/min/100g (IQR: 12.46; 15.2) vs. 12.5 ml/min/100g (IQR: 8.02; 13.04), p=0.56 (***Fig. 4.37. c***), which did not influence the urinary output rates too much- they were only slightly higher in the NMP only group than in the NMP+MSC group at the measured

time points during reperfusion, with median values of 3.58 ml/min (IQR: 2.41; 4.51) in the NMP group vs. 2.97 ml/min (IQR: 1.88; 3.75), p= 0.44 (***Fig. 4.37. d***).

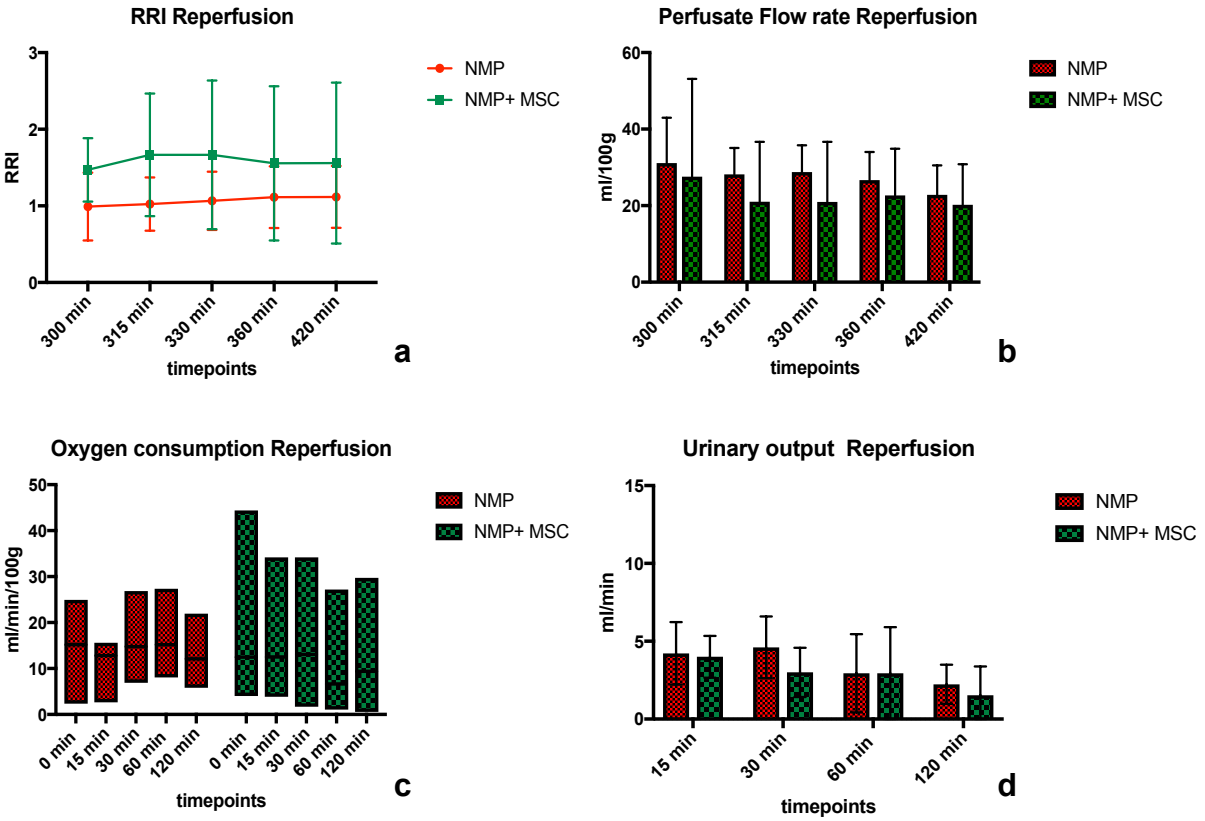


Figure 4.37.: Physiological parameters of porcine kidneys after NMP+/- MSC treatment during reperfusion

Fig. 4.37: *Shows the intrarenal resistance indices (**a**) for both treatment groups during the reperfusion period. (**b**) shows the resulting perfusate flow rates during reperfusion with an insignificant advantage for the normothermic machine perfusion (NMP) group. (**c**) Oxygen consumption rates for NMP vs. NMP+MSC treatment with a higher rate in the NMP+MSC group. (**d**) Urinary output rates for both groups in ml/min were similar in both groups.*

Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).

NMP= Normothermic Machine Perfusion, NMP+MSC= Normothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the perfusate.

Above described results led to higher creatinine-clearance rates in the NMP group compared to the NMP+MSC group, with median rates of 24.91 mmol/min (IQR: 9.91; 33.59) in the former vs. 6.19 mmol/min (IQR: 3.14; 12.75) in the latter group, with a p-value of 0.49 (***Fig. 4.38. a***). The fractional sodium excretion rates were similar in both groups (***Fig. 4.38. b***).

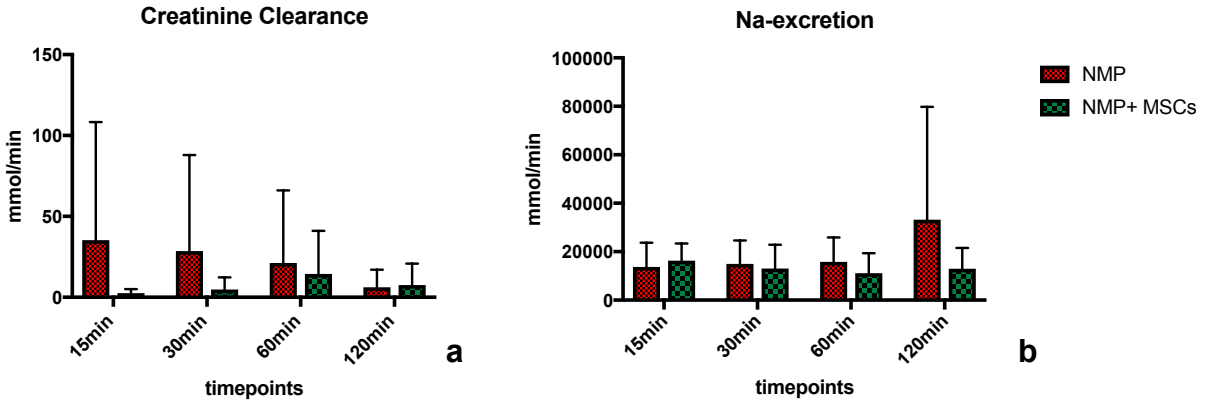


Figure 4.38.: Creatinine clearance and sodium excretion for kidneys after NMP+/-MSC treatment during reperfusion

Fig. 4.38: *Creatinine Clearance rates (**a**) and fractional sodium excretion rates (**b**) during reperfusion revealed an insignificant benefit for the NMP group over the NMP+MSC group.*

Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).

NMP= Normothermic Machine Perfusion, NMP+MSC= Normothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the perfusate.

Perfusate levels of Il-1β were similar in both groups, in perfusate- and urine samples. The median IL-1β levels were 574.7 pg/ml (IQR: 341.3; 1207) in the NMP perfusate group vs. 525.9 pg/ml (IQR: 370.1; 935.4) in the NMP+MSC group with a p-value of 0.8. In urine samples, the mean IL-1β concentrations were 778.2 pg/ml (IQR: 275.3; 1281) in the NMP group vs. 555.2 pg/ml (IQR: 445.8; 1115) in the NMP+MSC group with a p-value of 0.87 (***Fig. 4.39. a, b***).

NGAL concentrations in perfusate- and urine samples were very similar in both groups, with median values of 120.6 pg/ml (IQR: 107.7; 138.2) in the NMP group vs. 122.4 (IQR: 111.7; 148, p= 0.77) in the NMP+MSC group for perfusate samples and 205 pg/ml (IQR: 74.87; 278.9) in the NMP group vs. 256.3 pg/ml (IQR: 150.7; 322.2, p=0.49) in the NMP+MSC group for urine samples (***Fig. 4.39. c, d***).

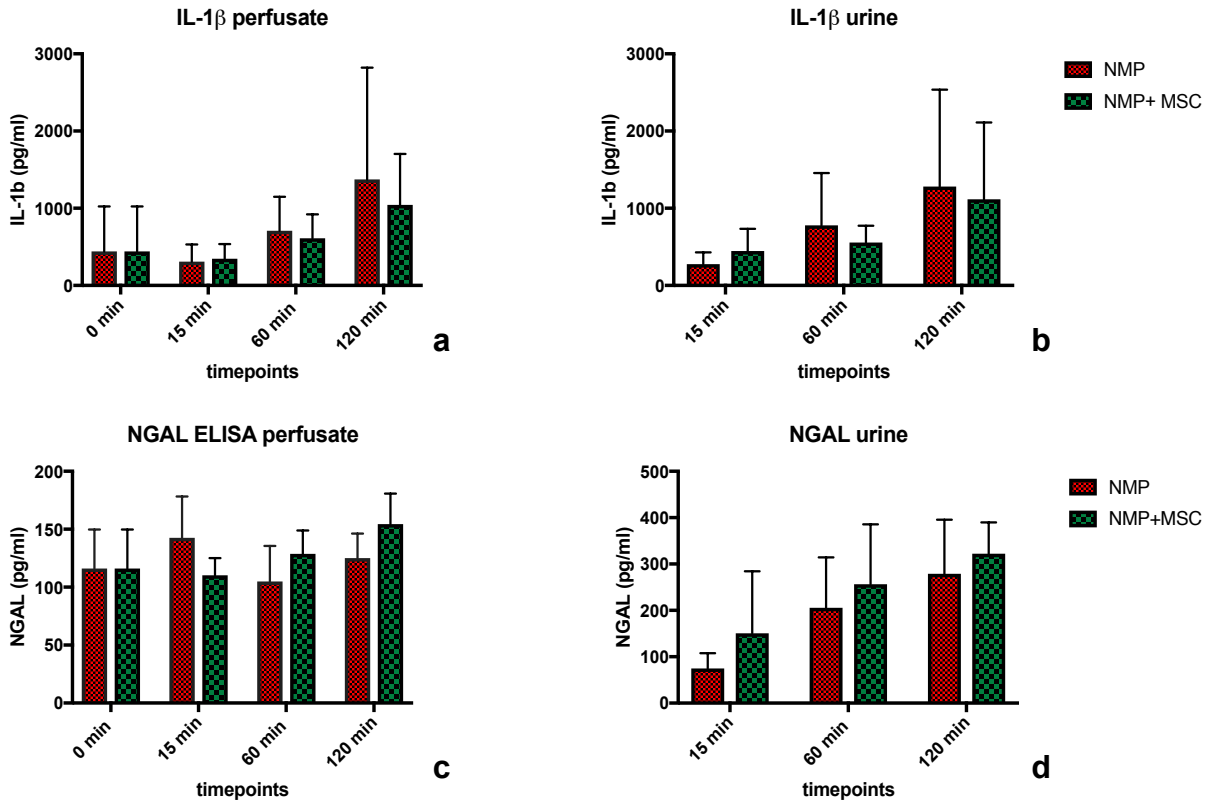


Figure 4.39: IL-1β and NGAL level in perfusate and urine samples of porcine kidneys after NMP+/-MSC treatment

Fig. 4.39: *Interleukin-1β (IL-1β) levels within perfusate- (**a**) and urine samples (**b**), measured by ELISA in pg/ml, were minimally higher in the normothermic machine perfusion (NMP) group. Contrary to this, Neutrophil gelatinase-associated lipocalin (NGAL) levels in perfusate- (**c**) and urine samples (**d**) were slightly higher in the NMP+MSC group (ns).*

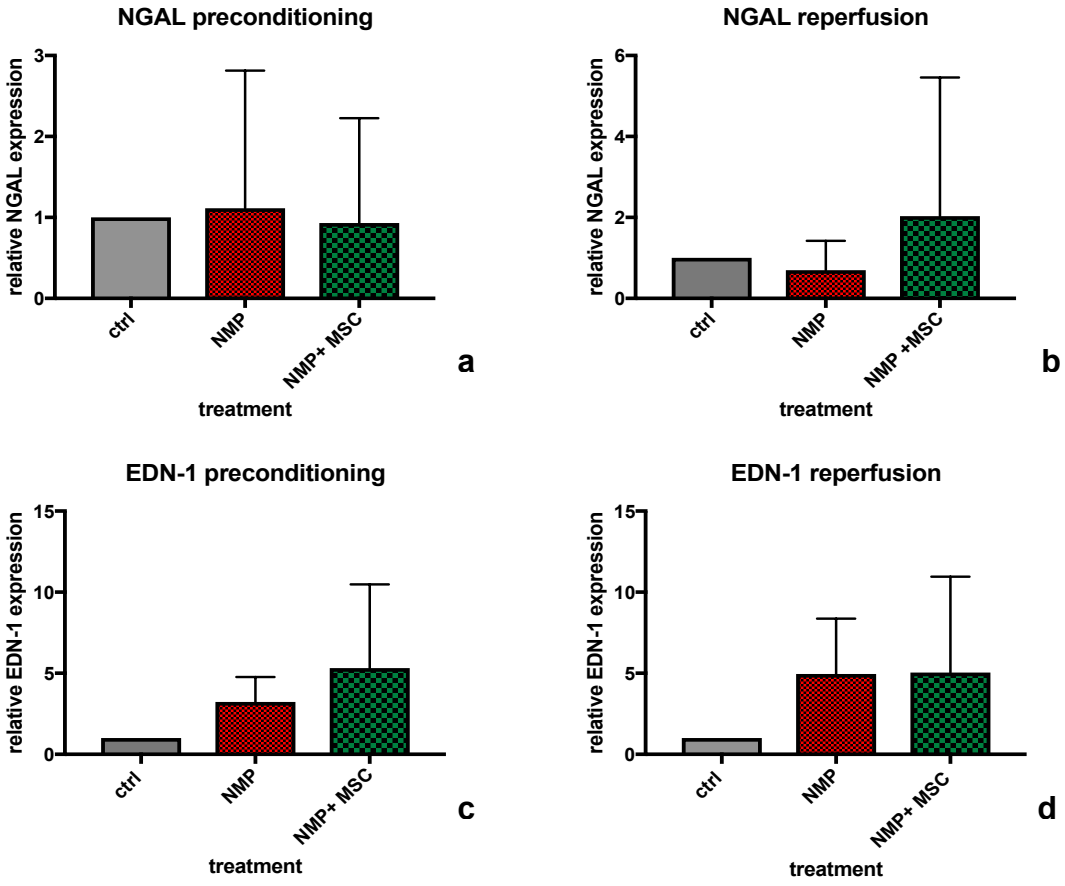
Statistical analyses carried out using ANOVA, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).

NMP= Normothermic Machine Perfusion, NMP+MSC= Normothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the perfusate.

Same as for the study on the introduction of MSCs into the HMP setting, also for the same study on MSCs in an NMP setting, we analysed the expression of the kidney injury markers NGAL and EDN-1 as well as the inflammatory markers TNFα and IL-1β on mRNA level by RT-PCR after the preconditioning phase and the reperfusion phase, compared to the t0 biopsy, respectively.

In the NMP+ MSC studies we observed little differences in expression levels of the inflammatory markers TNFα and IL-1β between the NMP and the NMP+MSC group (***Fig. 4.40. e-h***). NGAL expressions were similar after the preconditioning phase but were higher in the NMP+MSC group after reperfusion, with mean expression levels of 2.03 ± 3.4 vs. 0.7 ± 0.7 in the NMP group (p= 0.48, ***Fig. 4.40. a, b***). For EDN-1, expression levels were higher in the NMP+MSC group after the preconditioning period with mean expression levels of 5.3 ± 5.16 vs. 3.23 ± 1.54 in the NMP group

and returned to similar expression levels with mean values of 4.96 ± 3.4 in the NMP group and 5.05 ± 5.91 in the NMP+MSC group. All expression levels are calculated using the ΔΔCt method and describe the –fold expression level using the housekeeping gene (β-Actin) compared to the control which is set to be 1.



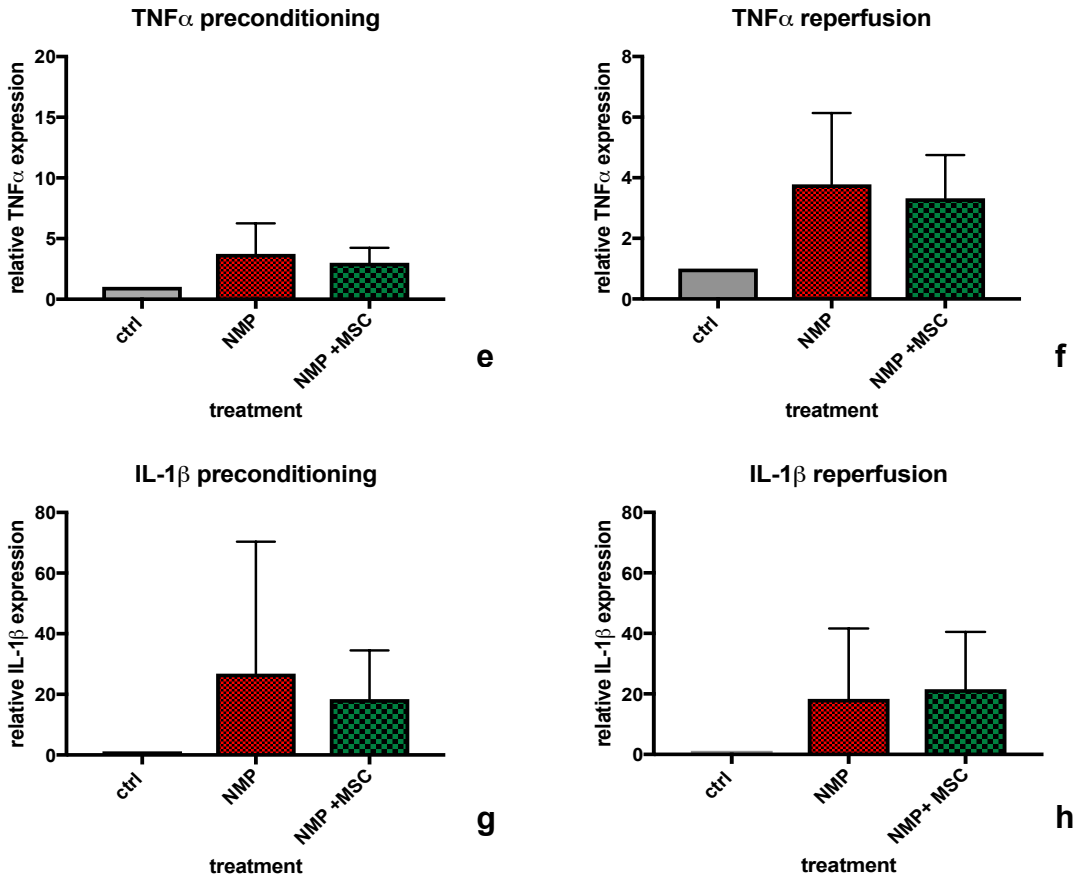


Figure 4.40.: mRNA expression levels of kidney injury markers and inflammatory markers after NMP+/- MSC treatment and after reperfusion

Fig. 4.40. *Expression of the kidney injury markers Neutrophil gelatinase-associated lipocalin (NGAL;* ***a, b****), Endothelin-1 (EDN-1 ;****c, d****) and the inflammatory markers Tumour necrosis factor α (TNFα;* ***e, f****) and Interleukin-1β (IL-1β;* ***g, h****) on mRNA level after the preconditioning phase and after reperfusion, respectively, determined with real time (RT-) PCR and calculated using the ΔΔCt method with β-Actin as a housekeeping gene and the respective t0 biopsy as a control.*

Statistical analyses carried out using Mann-Whitney U test, statistical significance: no statistical significance (ns; no asterisk); p<0.05 (*); p<0.005 (**); p<0.001 (***).

NMP= Normothermic Machine Perfusion, HMP+MSC= Hypothermic Machine Perfusion+ 1-5x10^6 double labelled MSCs in the perfusate.

Also in the study investigating the application of MSCs in normothermic ex-vivo perfusion of porcine kidneys, we were able to trace cells within glomeruli and tubules of treated kidneys, using wide field microscopy (***Fig. 4.41.)*** and immunohistochemical staining against MHC-1 (***Fig. 4.42.***). Also for this group, we could not identify any cells in the group with infusion of 1x10^6 MSCs but in the groups receiving 3- and 5x10^6 cells we were able to trace single cells. However, my subjective observation was that in kidneys after NMP, I could locate less MSC than in the investigated histology slides from kidneys after HMP. Unfortunately, we were not able to exactly quantify the cells within the histology slides, as there were too many factors

influencing a count and also, the facilities in which the perfusion happened, did not allow for a cell count within the perfusate to be performed.

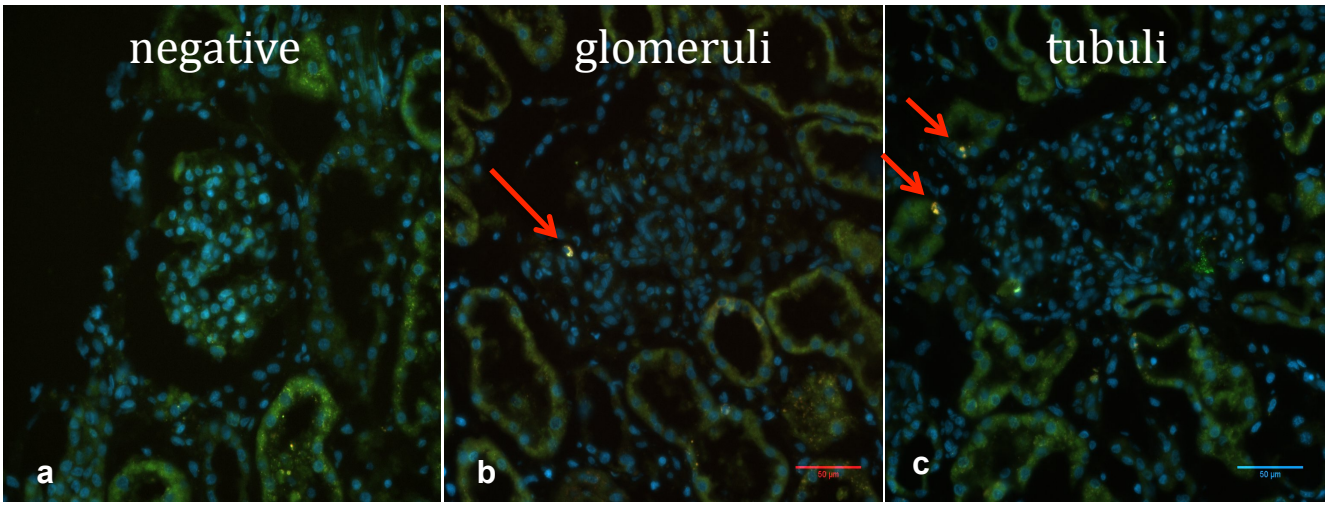


Figure 4.41.: Human double-labelled MSCs in sections of porcine kidneys after Normothermic machine perfusion, visualized using wide field microscopy

Fig. 4.41: *widefield microscopy imaging of frozen porcine kidney sections (4mm) with settings to stimulate the double labelled MSCs (UV and GFP). The slides were covered with DAPI therefore nuclei are shown in blue. (**a**) Picture of a negative control- kidney after normothermic machine perfusion (NMP) without infusion of Mesenchymal Stem Cells (MSCs). (**b**) Kidney after normothermic machine perfusion (NMP) with 5x10^6 MSCs, showing one detected cell within a glomerulum (arrow). (**c**) Showing two detected MSCs within tubuli of a kidney after NMP+ 5x10^6 MSCs (arrows).*

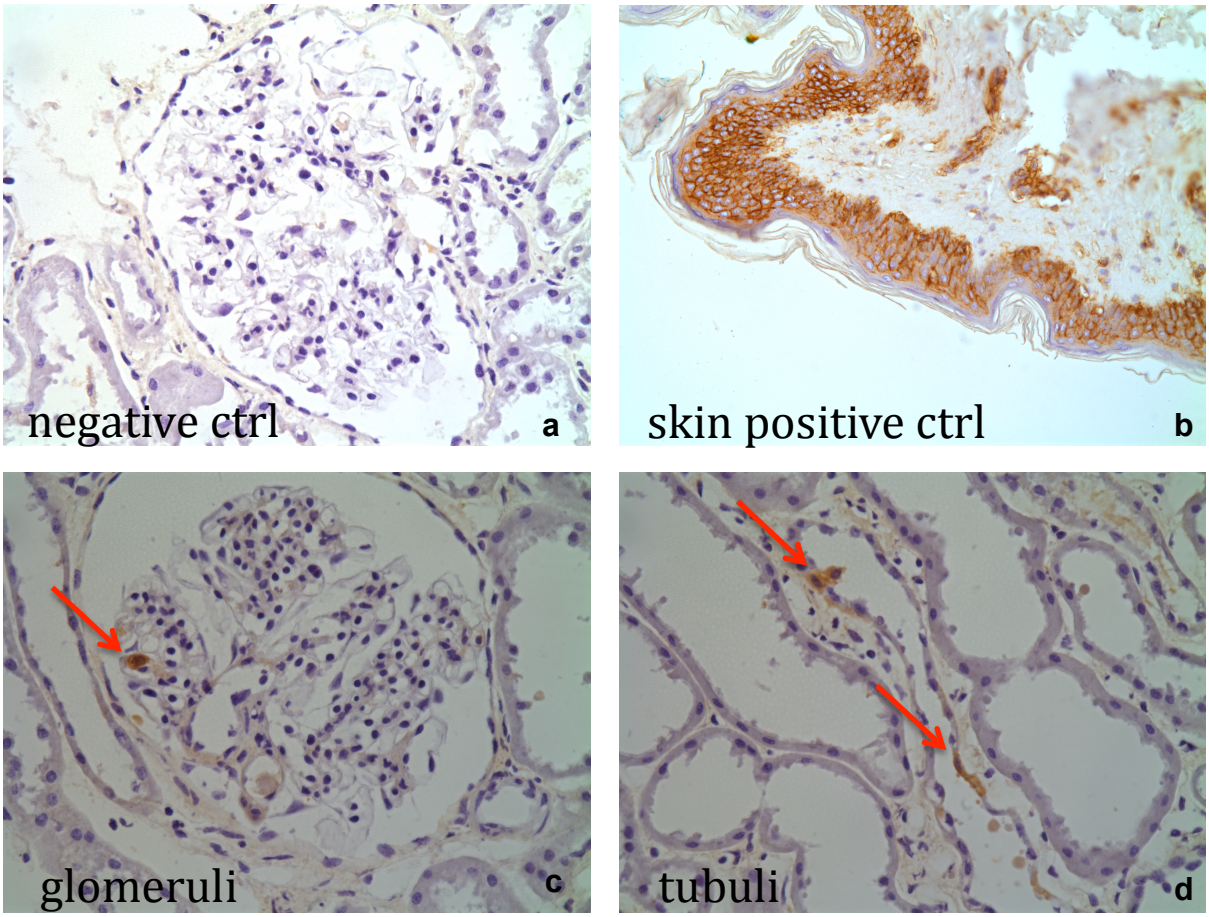


Figure 4.42.: Immunohistochemical staining of porcine kidney sections against human MHC class I

Fig. 4.42.: *Immunohistochemical staining of porcine slides against human Major Histocompatibility Complex-1 (MHC-1). (**a**) Negative control, (**b**) Human skin sample as positive control, (**c**) positive staining for Mesenchymal Stem Cells (MSCs; arrows) within a glomerulum of a porcine kidney after 4h of Normothermic machine perfusion (NMP) with 5x10^6 MSCs, (**d**) positive staining of MSCs (arrows) within the tubuli of a porcine kidney after 4 h of NMP containing 5x10^6 cells.*

4.3.2. Real-time monitoring during porcine ex-vivo kidney perfusion experiments using a micro dialysis device

As mentioned before, our ex-vivo kidney perfusion studies in a porcine model served as a good occasion to perform further tests on the functionality of a portable micro dialysis device to measure glucose- and lactate levels from the extracellular space of kidneys in real time. The measurements were performed by our colleagues from the Department for Bioengineering at Imperial College, under supervision of Prof. Martyn Boutelle. The graphs shown in this Chapter were created by Dr. Isabelle Samper, who is focussing on the development of a portable micro dialysis device as part of her studies for a PhD degree at Imperial College. Dr. Sally Gowers was also part of the team for this project.

In summary, we were able to obtain micro dialysis data on the following experimental groups of porcine kidneys:

- HMP: 8 kidneys, including 3 kidneys with a longer WIT of 45minutes
- NMP: 8 kidneys, including 3 with a longer WIT of 45 minutes
- HMP + MSC : 2 kidneys
- NMP + MSC: 4 kidneys
- cold storage: 2 kidneys, including 1 with longer WIT of 45 minutes

In terms of pairs in direct comparison that was:

- HMP vs. NMP, WIT 20 minutes: 3 kidney pairs
- HMP vs. NMP, WIT 45 minutes: 3 kidney pairs
- HMP vs. HMP+MSC, WIT 20 minutes: 2 kidney pairs
- NMP vs. NMP+ MSC, WIT 20 minutes: 3 kidney pairs
- NMP+MSC vs. CSS, WIT 20 minutes: 1 kidney pair
- HMP vs. CSS, WIT 45 minutes: 1 kidney pair

Our first interest was to find out whether the measurements obtained from the micro dialysis probes were concordant with the glucose- and lactate measurements from the ABG analyser or whether there were any differences that would suggest a discrepancy for the overall results. In ***Fig. 4.43.***, dialysate- and blood gas measurements for glucose- as well as lactate concentrations during ex-vivo NMP followed by reperfusion of one porcine kidney are illustrated. We could show that even though the glucose-levels obtained from the ABG measurements during the preconditioning phase were slightly lower than the levels measured by the micro dialysis device, the pattern of the levels over time for both, lactate- and glucose concentrations during the different treatment periods were adequate and let us assume that the micro dialysis measurements were successfully representing in real time, what was happening inside the perfused kidneys. This was even better highlighted for another comparison conducted on a different kidney after NMP, looking at the reperfusion period only (***Fig. 4.44.***).

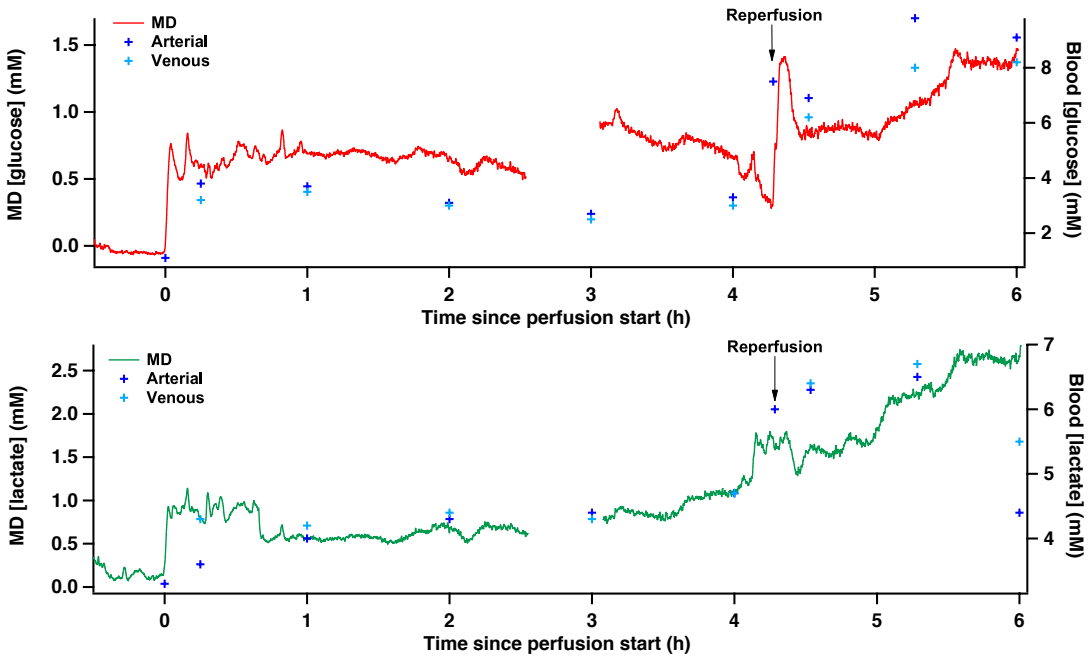


Figure 4.43.: Micro dialysis measurements for a porcine kidney undergoing NMP and reperfusion

Fig. 4.43.: *Glucose- and lactate levels within the microdialysate (green) and the arterial (dark blue) and venous (light blue) blood for a kidney undergoing NMP and reperfusion.*

Each continuous trace represents real-time data recorded at 10 Hz and smoothed with a Savitsky-Golay 201-point filter. The start of the reperfusion phase is indicated by an arrow. Missing data around 2.5 h through perfusion corresponds to time where the system was being recalibrated. Markers indicate venous and arterial levels measured with a standard clinical blood gas analyser. Microdialysate levels read on the left axes, blood levels (arterial and venous) read on the right axes.

Graph created by Dr. Isabelle Samper, Department of Bioengineering, Imperial College London.

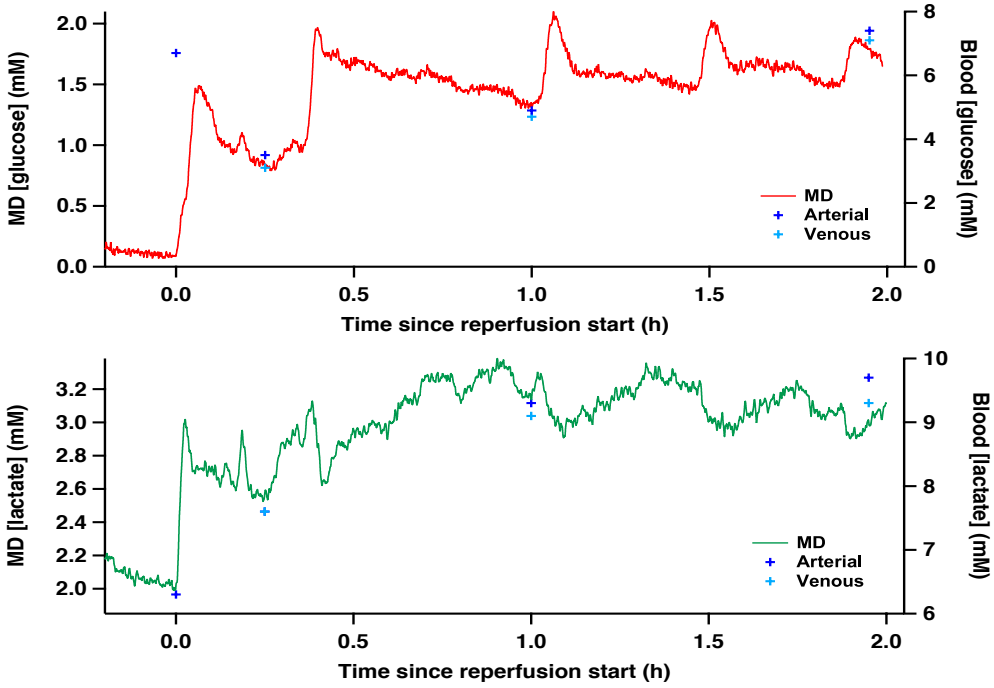


Figure 4.44.: Micro dialysis measurements for a porcine kidney after NMP during reperfusion

Fig. 4.44.: *Dialysate and blood glucose and lactate levels of a NMP kidney undergoing dynamic metabolic changes during reperfusion. Each trace represents real-time data recorded at 10 Hz and smoothed with a Savitsky-Golay 201-point filter. Markers indicate venous and arterial levels measured with standard clinical blood gas analyser. MD levels read on the left axes, blood levels (arterial and venous) read on the right axes.*
Graph created by Dr. Isabelle Samper, Department of Bioengineering, Imperial College London.

Knowing that the results were concordant, first of all, we were interested to see what exactly happened in the extracellular space of our kidneys during the initial hit, namely the warm ischemia time (WIT), which was why we exposed 3 pairs of porcine kidneys to an extended period of WIT (45 minutes). ***Figure 4.45.*** illustrates the median lactate- and glucose concentrations at 5 key time intervals during, and after the period of 45 minutes of WIT. Whilst no significant change of the glucose concentration could be observed during the period of warm ischemia, a significant drop in lactate concentrations could be observed, as well as a further significant drop in lactate concentrations, when the perfusion with cold preservation solution commenced. Both, glucose- and lactate levels dropped to a level during cold perfusion, which remained at a low steady level throughout the observed period of cold storage.

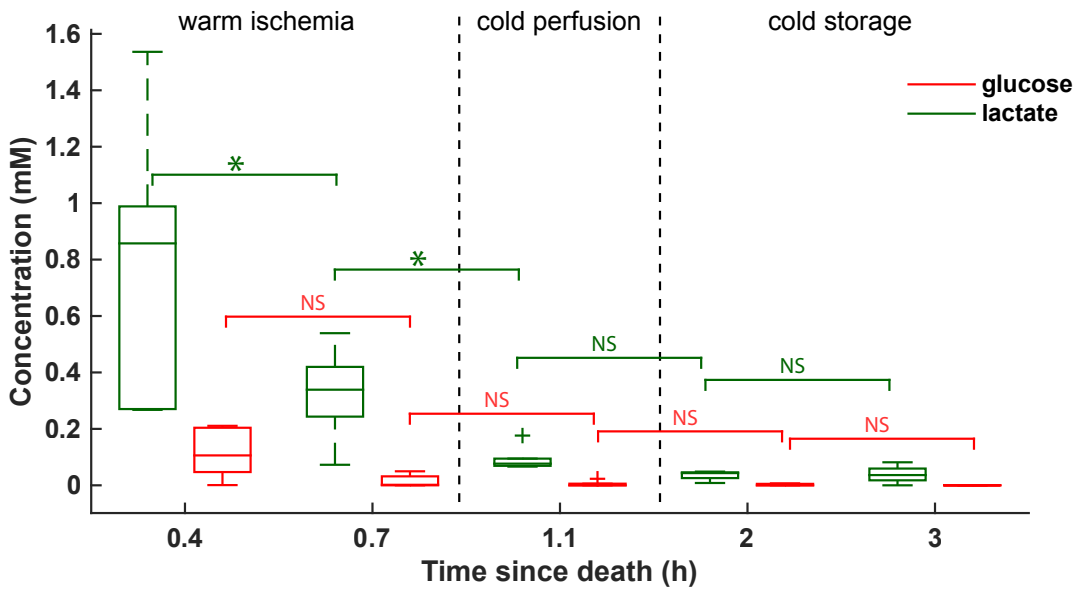


Figure 4.45.: Micro dialysis measurements at 5 key time intervals from 6 porcine kidneys after prolonged cold ischemia time

Fig. 4.45.: *Glucose and lactate median concentrations at 5 key time intervals during passive warm ischemia, cold perfusion and cold storage. Data from 6 kidneys after a long warm ischemic time (WIT), averaged on 2 min intervals. Box plots show median levels and interquartile range. Whiskers indicate 10th and 90th percentiles. Wilcoxon sign rank test (lactate n=6, glucose n=5), significance: * p<0.05, NS = not significant. Graph created by Dr. Isabelle Samper, Department of Bioengineering, Imperial College London*

In a next step, we were of course interested, whether we could detect and confirm differences in findings between our different study groups, starting with the direct comparison between kidneys after a preconditioning period on either NMP or HMP. ***Fig. 4.46.*** Illustrates the median glucose- and lactate concentrations during the preconditioning phase, as well as at two defined time points, namely at 0.5h and 1.5h after start of the reperfusion period for kidneys after NMP (n=10) or HMP (n=10), measured by the micro dialysis device.

Unsurprisingly, both the median glucose- as well as the median lactate levels were significantly lower during HMP than during NMP, which can be explained by a significantly lower metabolism within the kidneys undergoing HMP. Interestingly however, also during the measured time points of reperfusion, there were differences between kidneys after HMP and kidneys after NMP. At 0.5h after start of the reperfusion period, the median glucose level was higher whereas the median lactate level was lower in kidneys after HMP than after NMP. The same observation was made at 1.5h after start of the reperfusion period. The difference was stronger visible

at the latter time point and in that case, almost reached statistical significance (p= 0.052). The result is concordant with what we saw in our comparison between HMP and NMP treated kidneys, indicating a better viability within HMP kidneys at reperfusion, with higher glucose- and lower lactate levels present in the micro dialysate.

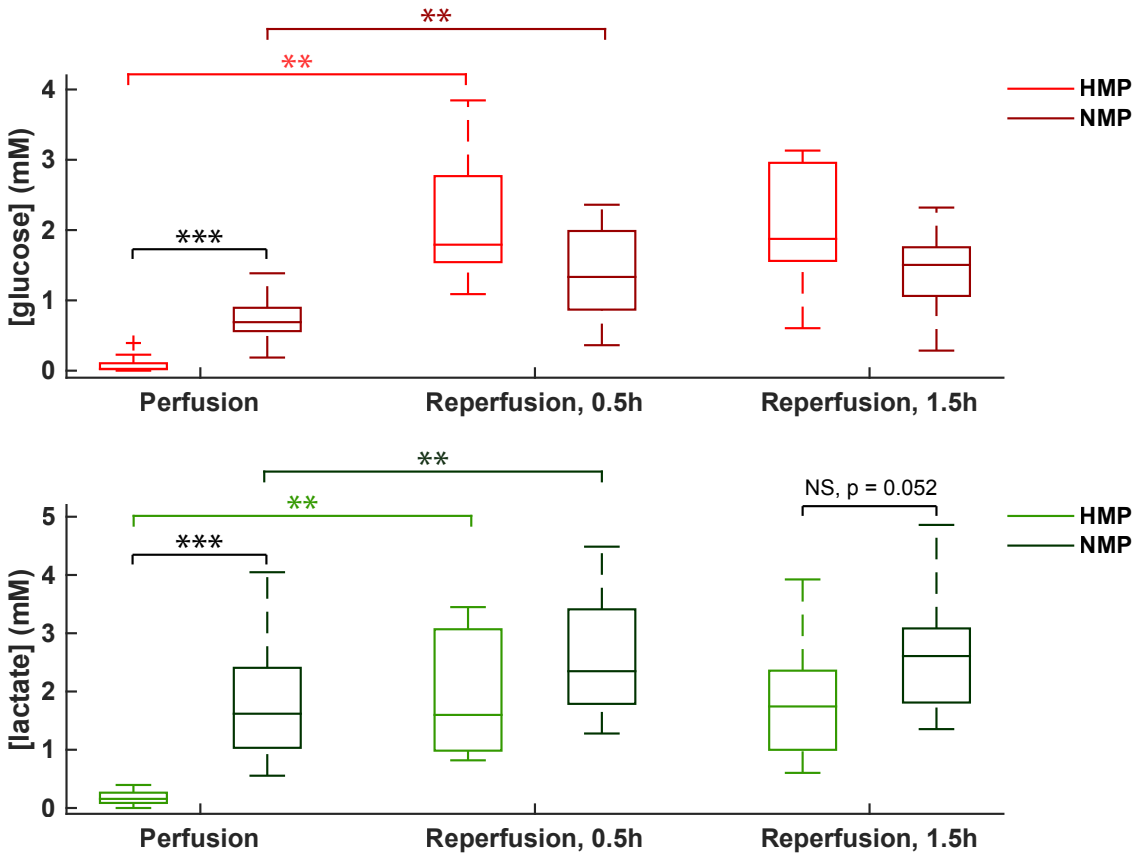


Figure 4.46.: Micro dialysis measurements for porcine kidneys undergoing HMP and NMP

Fig. 4.46.: *Median microdialysate glucose- and lactate levels during perfusion and reperfusion for kidneys undergoing hypothermic- (HMP) and normothermic machine perfusion (NMP) at defined timepoints. Box plots show median levels and interquartile range. Whiskers indicate 10th and 90th percentiles. Mann- Whitney U test between HMP and NMP groups (n=10), Wilcoxon sign rank test within each group (n=10). Significance: *** p<0.001, ** p<0.05, NS = not significant. Graphs created by Dr. Isabelle Samper, Department of Bioengineering, Imperial College London*

In ***Fig. 4.47.,*** the same result is illustrated again for the 10 kidneys at 1 hour after start of reperfusion.

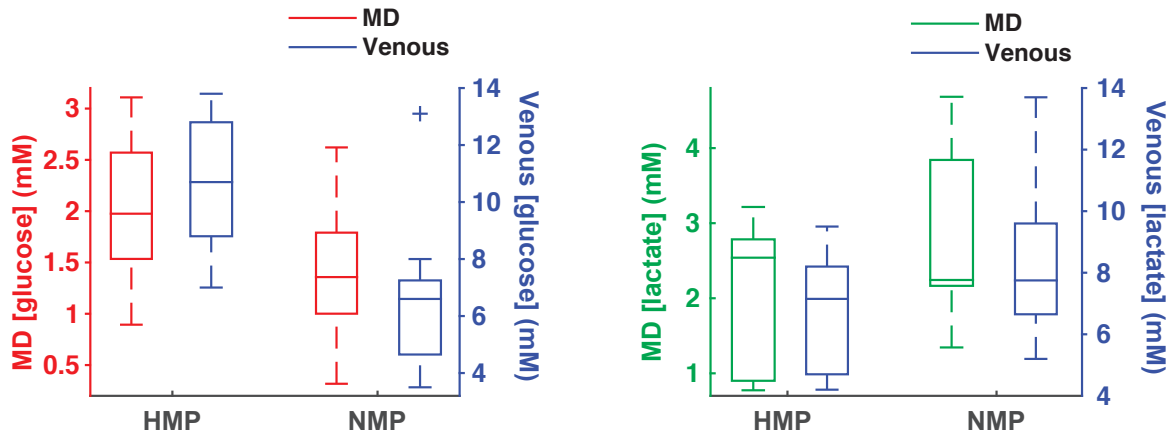


Figure 4.47.: Box plots of micro dialysis results for 10 porcine kidneys after HMP and NMP

Fig. 4.47.: *Median dialysate and venous glucose and lactate levels at 1 h through reperfusion for kidney after hypothermic- (HMP) or normothermic machine perfusion (NMP), n=10. Box plots show median levels and interquartile range of 10 kidneys. Whiskers indicate 10th and 90th percentiles. Microdialysate levels read on the left axes, venous levels read on the right axes. Graphs created by Dr. Isabelle Samper, Department of Bioengineering, Imperial College London*

Fig. 4.48. summarizes the same data but with a subdivision of the kidneys, highlighting the ones which were exposed to a longer WIT as well as the kidneys receiving stem cell treatment during the preconditioning phase. We could not detect any differences in the micro dialysates between kidneys being perfused on HMP or NMP only and those, which were perfused with Mesenchymal Stem Cells. This also confirmed our findings, that Mesenchymal Stem Cells don't alter the immediate physiological function of the kidney, and therefore, are safe to be used as a preconditioning agent within the perfusate.

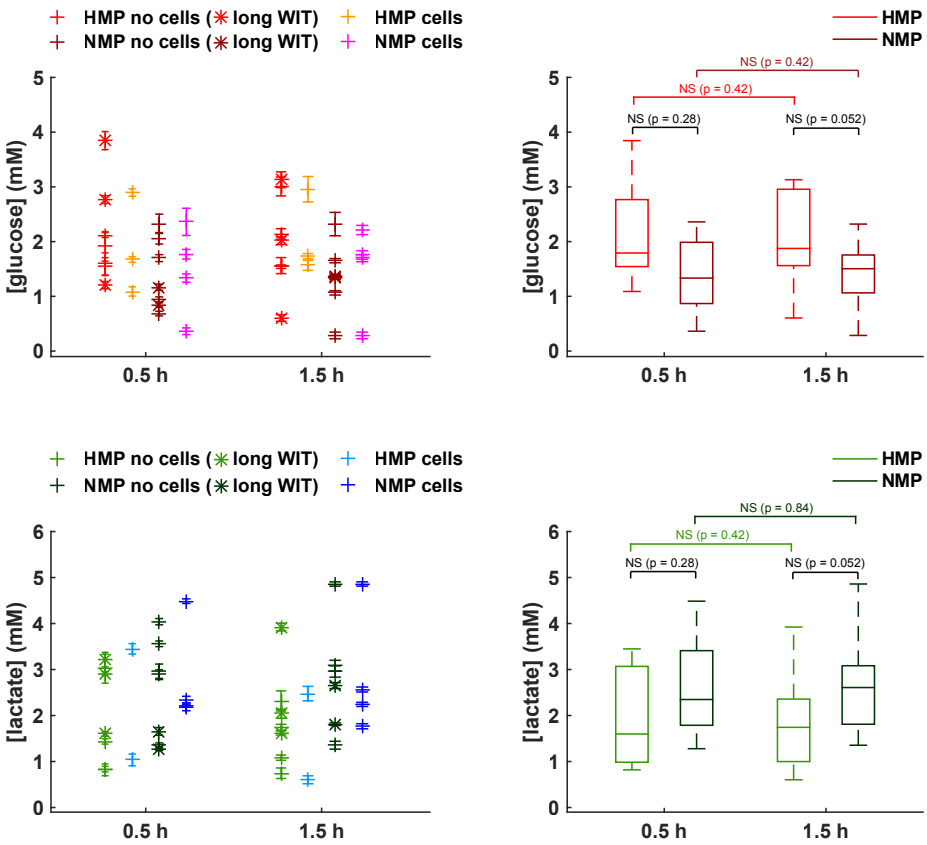


Figure 4.48.: Scatter plot and box plot of micro dialysis measurements for porcine kidneys after HMP and NMP

Fig. 4.48.: *Scatter plot and box plot of dialysate glucose and lactate levels during reperfusion for HMP and NMP kidneys. Data was averaged on 10 min intervals at 0.5 h and 1.5 h through reperfusion for 10 HMP and 10 NMP kidneys. Scatterplot: each marker represents one kidney, error bars show standard deviations for each 10 min interval. Colors indicate whether kidneys were perfused with mesenchymal stem cells. Long WIT kidneys are represented by an asterisk. Box plots show median levels and interquartile range. Whiskers indicate 10th and 90th percentiles. Wilcoxon sign rank test, significance: NS = not significant. Graphs created by Dr. Isabelle Samper, Department of Bioengineering, Imperial College London*

4.3.3. Investigation of the introduction of human Mesenchymal Stem Cells into human models of ex-vivo machine perfusion:

The same protocols for NMP and HMP were applied as for the porcine organs, with the aim to investigate whether it was possible to introduce MSCs into the human ex-vivo machine perfusion models. 4 consecutive single kidneys were used for this purpose, without any comparative experiment. The single kidneys were either perfused hypothermically or normothermically in the same way as mentioned before and numbers of $1x10^6$ and $5x10^6$ double labelled MSCs were added to the circuits, respectively. The purpose of these experiments was simply to see whether we could confirm the presence of double- labelled MSCs within the human organs using wide

field microscopy after ex-vivo perfusion. The numbers of kidneys and their different demographic backgrounds would not have allowed for a reliable comparative study with a treatment- and control groups, which is why this chapter will be held short. The functional parameters of perfused kidneys during HMP and NMP were similar to kidneys in other experimental groups and again, with the single kidneys, no useful comparison could be conducted which is why perfusion characteristics for these kidneys will not be shown here. For every single kidney undergoing perfusion with MSCs, we kept the CIT at exactly 24 hours by either keeping them on ice or declining offers of kidneys for which a longer CIT was expected.

The perfused human kidneys were classified into two groups:

Group 1: 24h of CSS, 4h HMP+ 1x10^6 MSCs (n=1), +5x10^6 MSCs (n=1)
Group 2: 24h of CSS, 4h NMP+ 1x10^6 MSCs (n=1), + 5x10^6 MSCs (n=1)

We could successfully trace MSCs after both, NMP and HMP (+5x10^6 MSCs) within the human kidney grafts using wide field microscopy (***Fig. 4.49. a-c***)

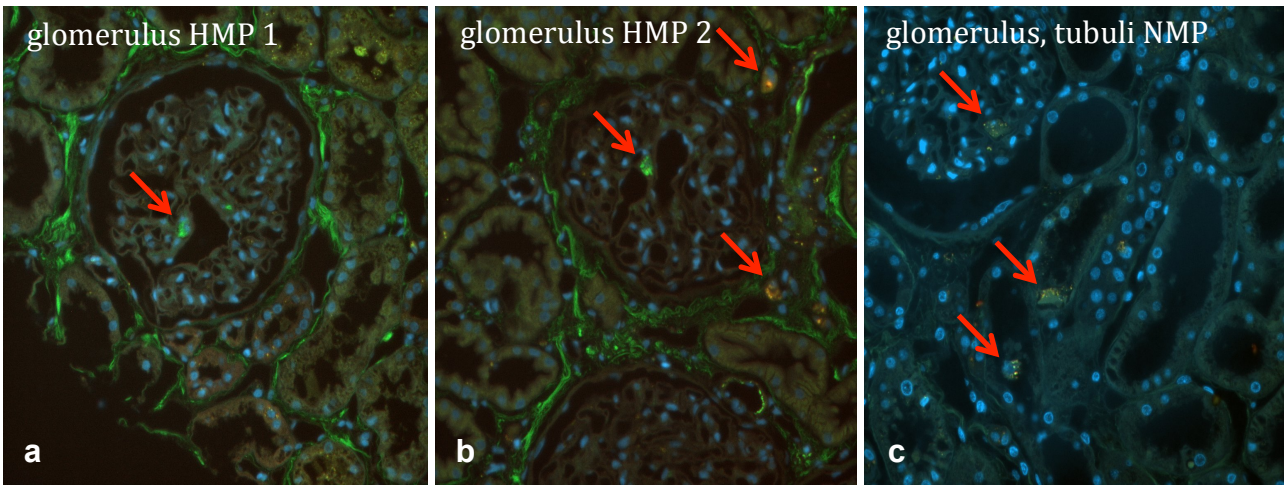


Figure 4.49.: wide field microscopy imaging of frozen human kidney sections after infusion of double labelled Mesenchymal Stem Cells

Fig. 4.49.: *widefield microscopy imaging of frozen human kidney sections (4um) with settings to stimulate the double labelled Mesenchymal Stem Cells (MSCs; UV and GFP). The slides were covered with DAPI, therefore nuclei are shown in blue. (**a**) picture of a fluorescent MSC within the glomerulum of a kidney after 4h of hypothermic machine perfusion (HMP) with 5x10^6 double-labelled MSCs (arrow). (**b**) another frozen section of the same kidney after HMP with 5x10^6 MSCs, showing three detected cells within a glomerulum as well as within the intertubular space (arrows). (**c**) showing two detected MSCs within the tubuli and one cell within the glomerulum of a kidney after NMP+ 5x10^6 MSCs (arrows).*

4.4. Investigation of the effects of ex-vivo delivered Mesenchymal Stem Cells on Ischemia-Reperfusion Injury in a rodent autologous kidney transplantation model.

As described in Chapter 3.4., Material and Methods, rat kidney transplantations were performed according to the protocol and teaching of the method, provided by Dr. Jennifer Smith. 3 rodent kidneys were hypothermically perfused ex-vivo, of which 2 were perfused with $3x10^6$ green fluorescent MSCs in 50mls of Machine Perfusion Solution (modified UW; Bridge to Life). The initial plan for this study was for it to be the starting point of a translational study, starting in a rodent model and translate via a porcine ex-vivo model into a preclinical study in human organs rejected from clinical transplantation. Furthermore, the plan was to investigate the effects of MSCs delivered during ex-vivo kidney perfusion in an *in vivo* model, to see their potential effects on Ischemia- Reperfusion Injury. However, as we did NOT have an animal licence for two years of my time as a PhD student, I was not able to perform research on rodents for that time. Therefore, this was a prototype study to investigate whether MSCs could be detected within rodent kidneys after ex-vivo HMP and where exactly they would be localised. When the animal licence came through, the aim was to learn the method of kidney transplantation in a rat model but the time was not enough to be fully signed off for the procedure. As a consequence to that, the numbers for this study are low as procedures could only take place under supervision of a designated trainer.

Six rat kidney transplantations (n=6) were performed to learn the method and another 4 recipients were transplanted in which the kidney was reperfused after finishing the anastomoses. Unfortunately, also in these cases, we were not able to recover the animal due to the judgement of the dedicated trainer of a fatal outcome. Out of the 4 transplanted kidneys, 1 was left in saline for the interval between retrieval and transplantation. One rodent kidney was treated by machine perfusion alone for the purpose of learning the cannulation and technique of the perfusion, and 2 kidneys were hypothermically perfused ex-vivo, with the perfusate (50ml of MPS) containing $3x10^6$ green fluorescent MSCs (n=2).

The main result for the rodent ex-vivo perfusion model is, that we were able to locate green fluorescent Mesenchymal Stem Cells within the rodent kidney, using wide field microscopy.

Fig. 4.50. shows images of a frozen kidney section. The rodent kidney was hypothermically perfused ex-vivo with a total amount of $3x10^6$ MSCs in a perfusate volume of 50ml (MPS).

The advantage of this model was the positivity of MSCs for green fluorescent protein (GFP), making it much easier than in previous models to distinguish between fluorescence of infused cells and background fluorescence. In ***Fig. 4.50 a,*** the frozen section of a whole rodent kidney is shown at 20x magnification. Covered in DAPI, nuclei are visible in blue. Mesenchymal Stem Cells in bright green are clearly visible, mainly within the glomeruli of the kidney. ***Fig. 4.50. b*** shows one field of that section further magnified, at 40x magnification and green fluorescent MSCs clearly visible within the glomeruli of the rodent kidney (red arrows).

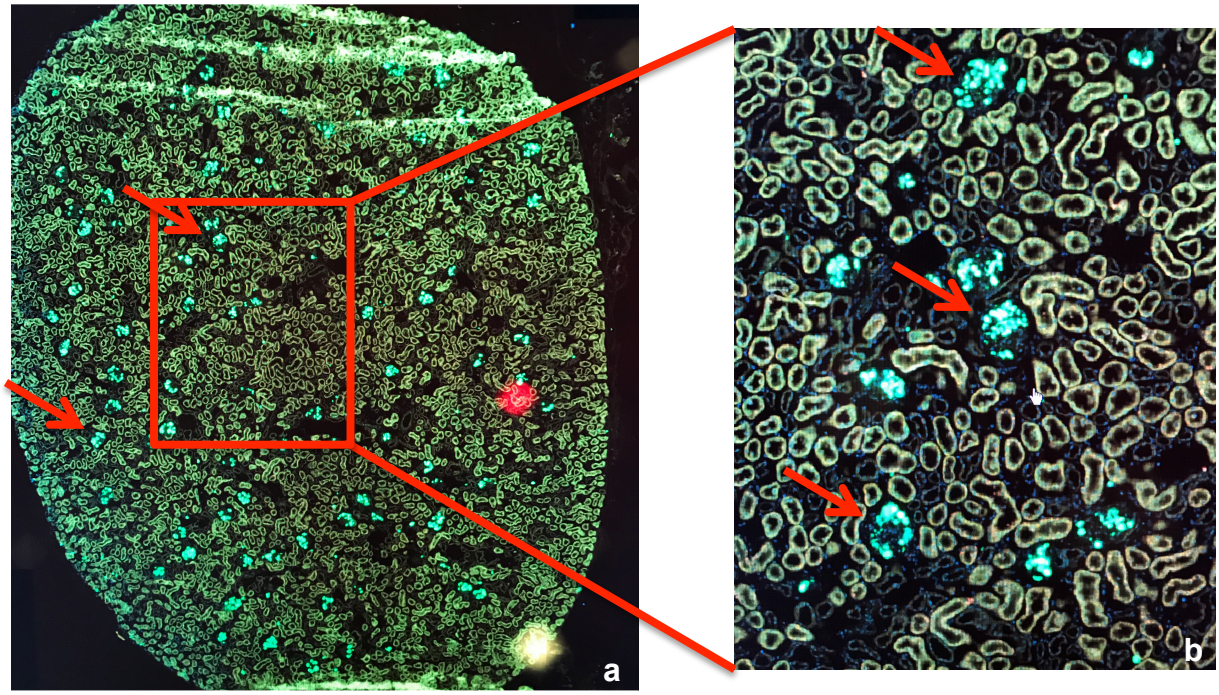


Figure 4.50.: Wide field microscopy image of rat kidney containing GFP positive MSCs

Fig. 4.50.: *widefield microscopy imaging of a frozen rodent kidney section (4um) with settings to stimulate the green fluorescent protein. The slides were covered with DAPI, therefore nuclei are shown in blue. (**a**) Frozen section of a rat kidney cut in half at a 20x magnification. Mesenchymal Stem Cells (MSCs) within the glomeruli were clearly visible in bright green (red arrows). (**b**) 40x magnification of the same section, MSCs within the glomeruli were clearly visible (red arrows).*

Unfortunately, we were not able to perform the next step, namely to see what happens to delivered MSCs when the kidney is transplanted back into a recipient. It would be very interesting to perform an in vivo study and to compare outcomes of recipients undergoing renal transplantation with or without MSCs within the grafts. This will be subject of further investigations.

4.5. Investigation of mechanisms of MSCs and potential differences of GFP+ and WT- MSCs on rodent macrophages in vitro

Our first aim for a series of in vivo experiments in a small animal model was to extract and cultivate rat derived MSCs for autologous application in a machine perfusion model. As the ultimate aim for the in vivo experiments was to trace the cells after administration, we chose to use a Wystar Kyoto (WKY) rat positive for the expression of green fluorescent protein (GFP) as a bone marrow donor. This specific rat has been genetically modified to express GFP on elongation factor 1α by Dr. Anna Garcia-Diaz et al. Prior to the extraction of the MSCs, it was not clear if, for these animals, the expression of the GFP would also be present on bone marrow cells. Therefore, and also to be sure that the genetic modification did not significantly alter the function of the cells, we extracted MSCs from the WKY- wild type rat as a control. We were able to confirm the green fluorescence of the extracted GFP+ MSCs microscopically using a confocal microscope as well as using flow cytometry. According to the definition for MSCs, our cells were furthermore shown to be plastic adherent, to express the cell surface markers CD44, CD29 and CD90 and to lack expression of the hematopoietic markers CD45 and CD34. We were able to show their ability to differentiate into adipocytes and osteocytes upon stimulation with respective differentiation media. All the cells were cultured from passage 0-10 and interestingly, the expression of GFP in the cells from GFP+ rats was strong and the green fluorescence was present up to passage 10 (***Fig. 4.51. a-c***)

In order to investigate whether the age of the bone marrow donor impacts the potential anti-inflammatory or immunomodulatory properties of the MSCs, cells were extracted from donor rats at an age of approx. 6 weeks as well as from donor rats at an age of 6 months, respectively. The supernatants from different cell groups and passages were compared in regards to their immunomodulatory capacities in vitro, with the aim to identify the most promising anti-inflammatory cell type for in vivo experiments.

Mesenchymal stem cells from rats syngeneic to the animals used for the kidney transplant model were used in order to mimic an autologous setting when the cells were perfused into the kidney grafts in later experiments.

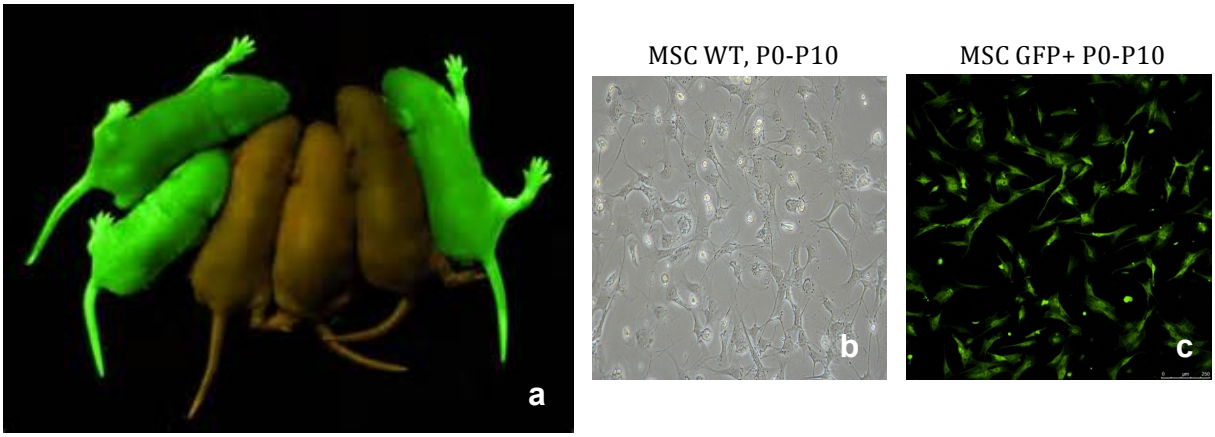


Figure 4.51.: Extraction of GFP+ MSCs from transgenic rats positive for GFP

Fig. 4.51.: *Wystar WKY wildtype and transgenic WKY rats positive for green fluorescent protein (GFP) (**a**) were used as bone marrow donors for the extraction of wildtype MSCs (**b**) and GFP+ MSCs (**c**). Pictures of cells were taken using light microscopy at a 20x magnification (**b**) or using a confocal microscope to detect the fluorescent cells at a 20x magnification (**c**).*

MSCs from WKY wild type and WKY-GFP+ rats at 6 weeks of age (defined as a young donor) and at 6 months (defined as an old donor) were successfully extracted and cry vials containing millions of MSCs from each passage (80 samples) as well as the respective supernatants were stored at -80°C for future experiments. In the passages 0-2, we identified a mixed population of cells with a high percentage of cells positive for the hematopoietic markers CD45 and CD34. This shifts towards a population negative for these markers in later passages. The reason for this is that MSCs are extracted from the bone marrow and that at these early stages of the cell culture it is a mixture of hematopoietic stem cells and MSCs. This is also concordant with most of the literature, in which MSCs are used as a ‚drug' in between passage 3 (P3) and passage 8 (P8). Our cells were shown to be plastic adherent, to express CD90, CD44 and CD29 and to lack expression of CD45 and CD34, which is the minimal criteria to be fulfilled for the identification of the MSC phenotype according to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT)[153]. ***Fig. 4.52. a*** shows the confocal microscopy image of green fluorescent MSCs in passage 2 as well as the flow cytometry plots which show the high percentage of cells positive for haematopoietic markers, indicating that at that early passage, a mixed population of cells was present. ***Fig. 4.52. b*** shows the shift towards a more unique proportion of MSCs, lacking the haematopoietic surface markers CD34 and CD45 in a majority of the cells. Furthermore, the cells could be differentiated into adipocytes and osteocytes in vitro upon stimulation with the

respective differentiation media (***Fig. 4.53. a, b***). Interestingly, the expression of GFP in cells from GFP+ rats was strong, and green fluorescence was present throughout all passages of cells.

In an in vitro study, the effects of supernatants from the MSC culture as well as of the MSCs themselves on rodent bone marrow derived macrophages (BMDM) were investigated. Macrophages were extracted from the bone marrow of a WKY-wild type rat and seeded onto 24-well plates to conduct the experiments as described in Chapter 3.5.. Expressions of the cytokines Mannose receptor-1 (Mrc-1), nitric oxygen synthase (Nos), IL-10 and TNFα were compared between treated and non treated macrophages to identify the most potent MSC phenotype amongst the different groups.

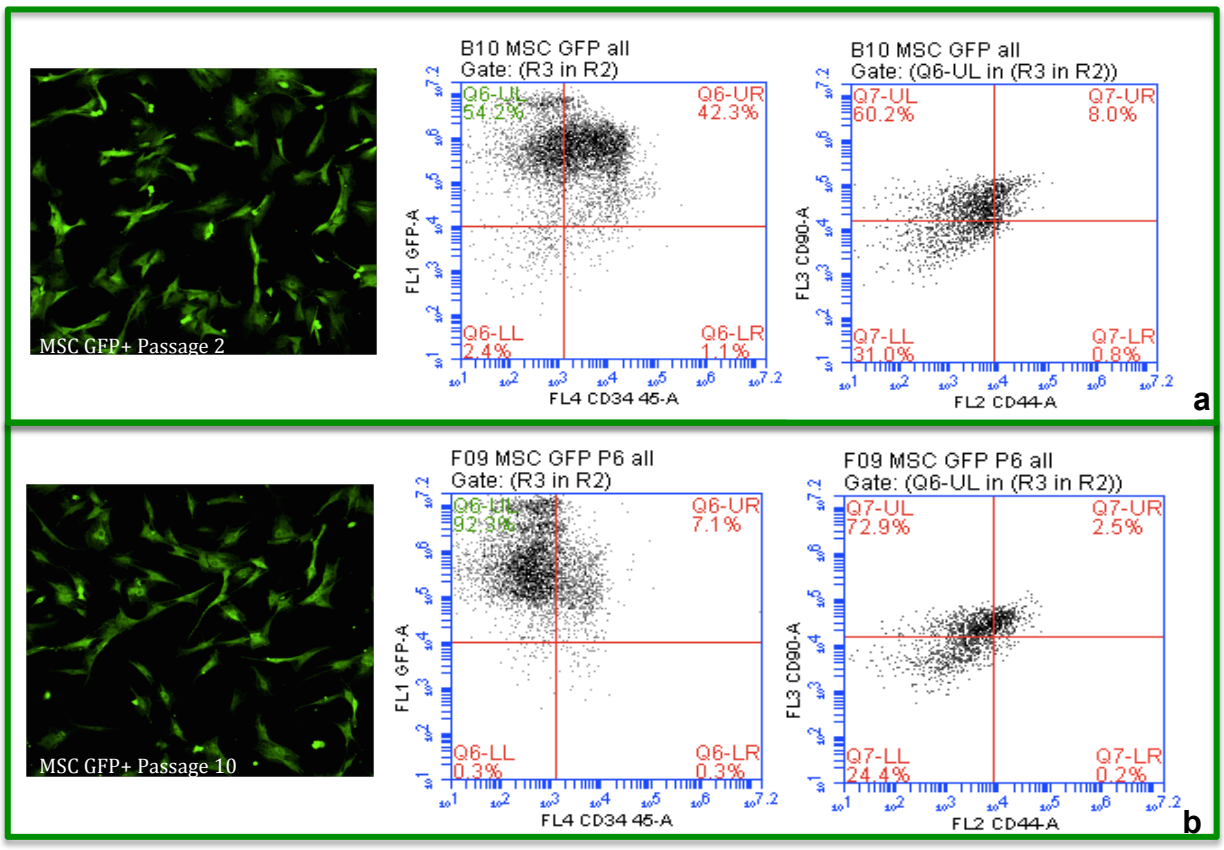


Figure 4.52.: Flow cytometry of extracted MSCs to confirm MSC phenotype

Fig. 4.52.: (***a***) *shows from left to right the confocal microscope image of Mesenchymal Stem Cells expressing green fluorescent protein (MSC-GFP+) in passage 2. Flow cytometry confirmed that the cells were GFP positive but that at that stage, a mixed cell population with 42,3% cells with positive hematopoietic surface markers (CD34, CD45) were present.* (***b***) *Shows from left to right confocal microscope image of MSCs in passage 10. Forward and side scatter of MSCs in passage 6 shows a negative result for the hematopoietic surface markers CD34 and CD45 in the majority of the cells, and a positive result for the MSC specific surface marker CD90.*

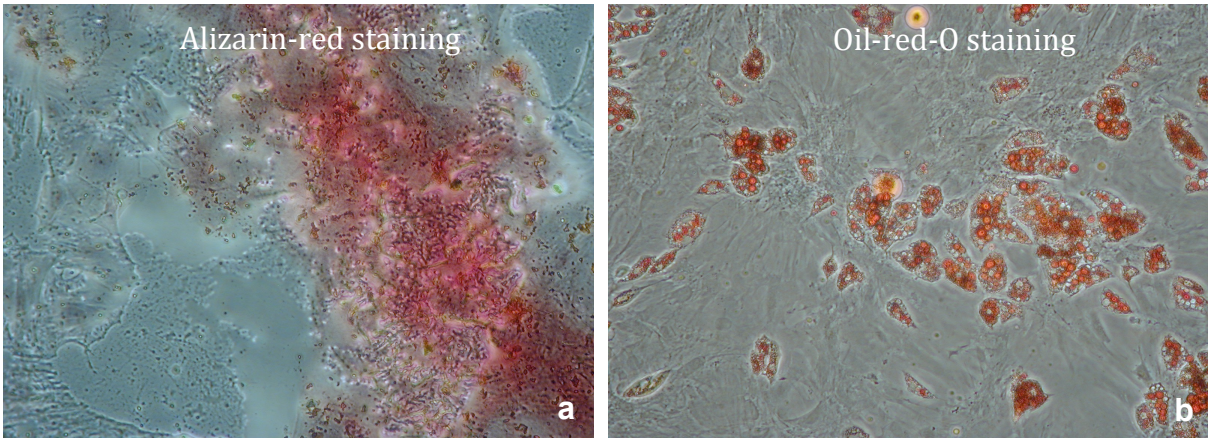


Figure 4.53.: Alizarin-red-staining and Oil-red-O staining to confirm MSC phenotype for rodent MSCs

Fig. 4.53.: *10x magnification of MSCs after stimulation with chondrogenic and adipogenic differentiation media. Alizarin-red-staining for cells after chondrogenic differentiation is shown in (**a**). Oil-red-O staining for adipocytes after differentiation is shown in (**b**).*

We were interested in immunomodulatory effects of different sources and passages of MSCs in vitro. The main interest was, whether MSCs coming from a transgenic donor rat would be altered in their phenotype and have different effects than cells coming from wild type donor rats. Furthermore, the age of the donor rat was a factor we wanted to keep in mind, with the theory that possibly, stem cells coming from a younger donor would have more immunomodulatory potential than the same cells coming from an older donor. Therefore, MSCs were extracted from the bone marrow of wild type as well as GFP positive rats, as explained in detail in Chapter 3.5. 6 week old donor rats served as donors for the ’young’ cell population (y), and 6 month old donor rats served as donors for the ’old’ cell population, respectively. Samples of supernatant from these MSCs were taken for all passages at 24 hours after seeding the same amount of cells per petri dish, to ascertain that a valid comparison can be made, The supernatants were then used as an overnight treatment of bone marrow derived macrophages, which had either been stimulated with lipopolysaccharide (LPS), or not. In our analyses on the different effect of these supernatants on the expression of Mannose receptor 1 (Mrc-1) in macrophages, supernatants coming from WT MSCs were shown to up regulate Mrc-1 expressions more effectively than supernatants coming from GFP+ MSCs. This was significant for native macrophages (no stimulation with LPS), with a median Mrc-1 expression of 1.74 (IQR: 1.18; 2.12) in the WT treated group vs. 0.46 in the GFP treated group (IQR: 0.41; 0.94, p=0.03, ***Fig. 4.54. a***). There was no significant difference in anti-inflammatory potential of

supernatants coming from MSCs from younger donors when compared to older donors, in respect to Mrc-1 expression (***Fig. 4.54. c, d***). Supernatants from WT-MSCs had an up regulatory effect on Mrc-1 expressions of macrophages and could even reverse the inflammatory stimulus of LPS treatment (***Fig. 4.54. a, b***). This experiment indicates, that there are differences between WT and GFP MSCs concerning their paracrine actions.

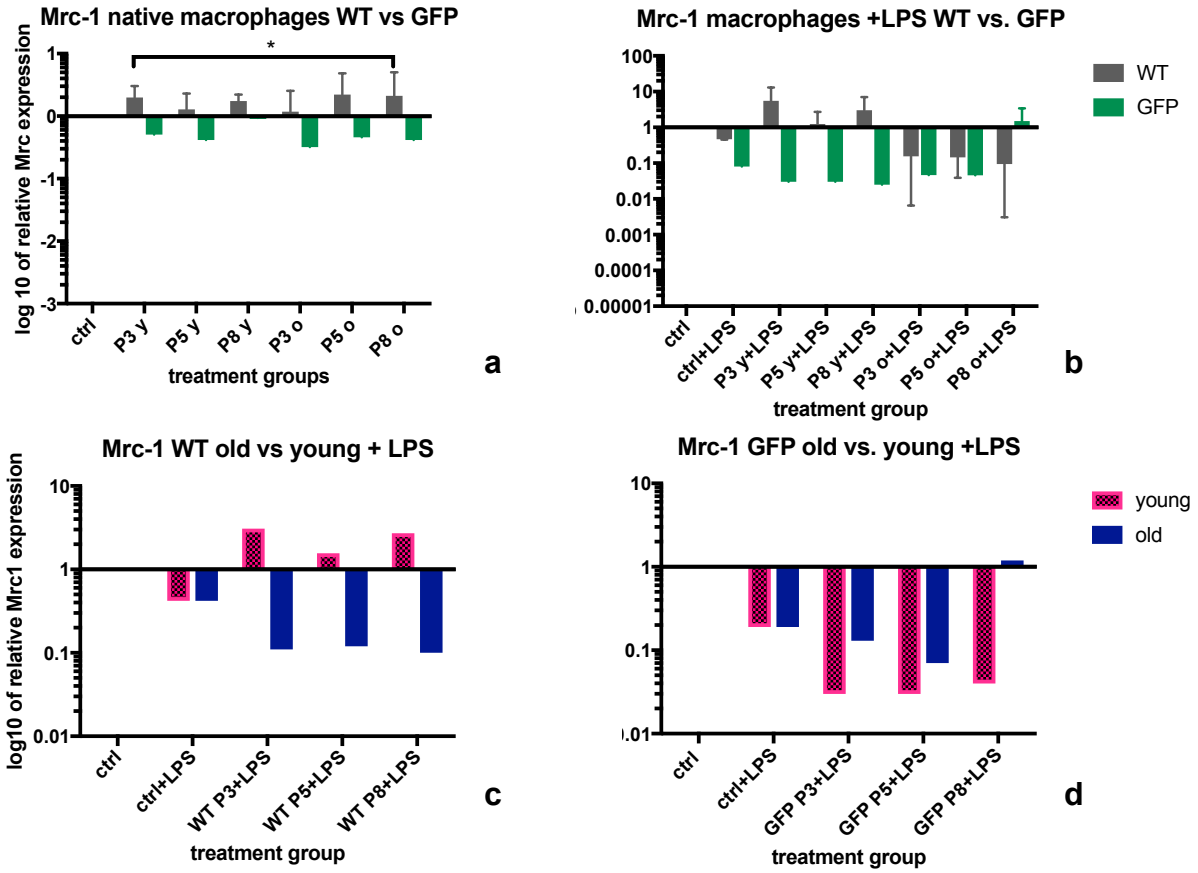


Figure 4.54.: Influence of MSC supernatants on Mrc-1 expression levels in macrophages

Fig. 4.54.: *the graph shows the influence of different supernatants from Mesenchymal Stem Cells on the expression levels of Mannose receptor-1 (Mrc-1) on bone marrow derived macrophages (BMDM) coming from a Wistar Kyoto wildtype (WKY-WT) rat. Supernatants coming from MSCs expressing green fluorescent protein (GFP+MSC) showed a stronger downregulation of Mrc-1 than supernatants coming from WT cells (**a, b**). mRNA levels were calculated using real time (RT) -PCR and the ΔΔCt-method was used for calculations. Untreated macrophages served as control and HPRT-1 was the housekeeping gene. There was no significant difference between the action of supernatants from MSCs coming from older donors when compared to younger donors in this case. However, supernatents from young WT animals caused an upregulation of Mrc-1 and are therefore the most promising in having anti-inflammatory effects (**c, d**). Statistical analysis was performed using Mann Whitney U test.*

Statistical significance: *no statistical significance (ns; no asterisk);* p<0.05 (*); p<0.005 (**); p<0.001 (***).

Mrc-1= Mannose receptor-1, **WT**= supernatants coming from wild-type cells, **GFP**= supernatants from GFP+ cells

old= supernatants from cells from donor rats > 6 months of age, **young**= supernatants from cells from donor rats < 6months of age

To further investigate the paracrine effects of the mentioned MSCs, we looked into the expression levels of further inflammatory markers expressed by macrophages, like inducible nitric oxide synthase (iNOs), TNFα as one of the most classic inflammatory markers, and to look at another anti-inflammatory marker, IL-10. For iNOs, we found, that native macrophages (not stimulated with LPS) treated with supernatants from young donors expressed lower amounts of iNOS than when treated with supernatants from old donors. This observation was made for both, supernatants from WT as well as GFP+ MSCs (***Fig. 4.55. a***). The median expression levels were 0.67 (IQR: 0.49; 0.93) for 'young' supernatant treatment vs. 1.46 (IQR: 0.76; 3.1) for 'old' supernatant treatment in the WT group (p=0.25). The respective median expression levels in the GFP group were 0.39 (IQR: 0.13; 0.89) vs. 34.47 (IQR: 9.17; 63.8, p= 0.06), ***Fig. 4.55. a***). Looking at LPS stimulated macrophages, we saw a significant lower iNOS expression after treatment with supernatants from GFP+ cells than with supernatants coming from WT- cells, within all different age groups and passages, with median expression levels of 482.9 (IQR: 457.7; 723.3) in the WT group vs. 301.1 (IQR: 244.2; 495.6), p= 0.04 (***Fig. 4.55. b***). Considering that LPS stimulated an inflammatory state of the macrophages before the treatment was applied, this means that supernatants from GFP+ cells were more potent to inhibit or reverse the effect from LPS than supernatants from WT cells. Within the experiment with LPS as pretreatment of the macrophages, there was no difference between 'young' and 'old' supernatants anymore.

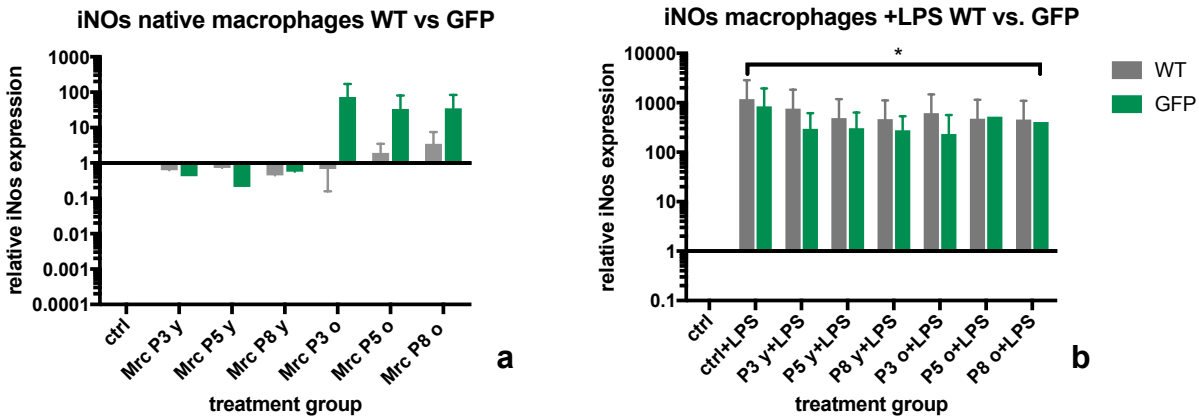


Figure 4.55.: Influence of MSC supernatants on iNOS expression levels in macrophages

Fig. 4.55.: *the graph shows the influence of different MSC supernatants on the expression levels of inducible nitric oxide synthase (iNOS) on bone marrow derived macrophages (BMDM) coming from a WKY-WT rat. Supernatants coming from MSCs from young donors led to lower iNOs expression levels than supernatants coming from MSCs from older donors in native macrophages (**a**). After LPS stimulation of the macrophages, supernatants from GFP+ cells led to a dignificantly stronger downregulation of the inflammatory cytokine iNOs than supernatants from WT cells (p=0.04, calculated using Mann-Whitney U test). mRNA levels were calculated using RT-PCR and the ΔΔCt-method. Untreated macrophages served as control and HPRT-1 was the housekeeping gene.*

Statistical significance: *no statistical significance (ns; no asterisk);* p<0.05 (*); p<0.005 (**); p<0.001 (***).

iNOS = inducible nitric oxide synthase, **WT**= supernatants coming from wild-type cells, **GFP**= supernatants from GFP+ cells

old= supernatants from cells from donor rats > 6 months of age, **young**= supernatants from cells from donor rats < 6months of

Looking at TNFα expression for the same experimental groups, the only and most interesting result was that after LPS stimulation of the macrophages, the expression levels of TNFα were significantly lower in the groups treated with supernatants from GFP positive cells than treated with supernatants from WT-cells (p=0.002). The mean expression levels were 2.98 (IQR: 2.58; 4.1) in the WT treated group vs. 1.04 (IQR: 0.33; 1.23, ***Fig. 4.56. a***). Interestingly, within the GFP treatment group, TNFα expressions could particularly decreased after treatment with supernatants coming from the older donors, however without a significant difference when compared to supernatants from younger donors (***Fig. 4.56. b***).

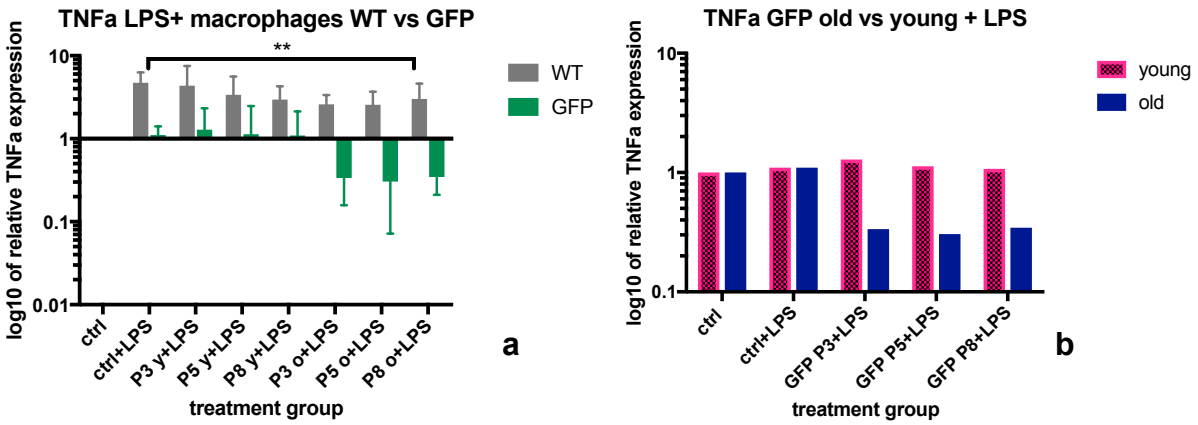


Figure 4.56.: Influence of MSC supernatants on TNFα expression levels in macrophages

Fig. 4.56.: *The graph shows the influence of different supernatants from Mesenchymal Stem Cells on the expression levels of Tumor necrosis factor α (TNFα) on bone marrow derived macrophages (BMDM) coming from a WKY-WT rat. Supernatants coming from MSCs from GFP+ donors led to a significantly better reduction of TNFα expression levels after stimulation with lipopolysacharid (LPS) than supernatants coming from WT-MSCs (p=0.04, calculated using Mann-Whitney U test, (**a**)). Particularly supernatants from older GFP rats led to a decrease of TNFα expression levels (**b**). mRNA levels were calculated using RT-PCR and the ΔΔCt-method. Untreated macrophages served as control and HPRT-1 was the housekeeping gene.*

Statistical significance: *no statistical significance (ns; no asterisk);* p<0.05 (*); p<0.005 (**); p<0.001 (***).

TNFa = Tumor necrosis factor α, **WT**= supernatants coming from wild-type cells, **GFP**= supernatants from GFP+ cells

old= supernatants from cells from donor rats > 6 months of age, **young**= supernatants from cells from donor rats < 6months of age.

Looking at IL-10 to see whether this effect could also be observed in terms of up regulating a more anti-inflammatory acting cytokine, we detected an inverse but similar pattern as for TNFα, namely that treatment of native macrophages with supernatants from GFP positive MSCs were more potent in up regulating IL-10 expression than supernatants from WT cells (***Fig. 4.57. a***). The mean expression levels of IL-10 were 0.43 (IQR: 0.15; 0.8) in the WT treated macrophages vs. 1 (IQR: 0.41; 1.72), p= 0.14. As well as for TNFα, in the case of IL-10, supernatants from MSCs from older donors seem to have more anti-inflammatory effects than supernatants coming from MSCs from younger donors. The mean expression levels were 0.56 (IQR: 0.2; 0.93), which reflects a down regulation of IL-10 for the 'young' group vs. 1.53 (IQR: 1.09; 2.05) for the 'old' group (p=0.057, ***Fig. 4.57. b***). Even though this effect was not so much observed in macrophages which had been stimulated with LPS before, we could see that overall, both WT and GFP+ derived supernatants suppressed IL-10 expression in an inflammatory milieu. This suppression was more effective by supernatants coming from older cells than from younger ones (***Fig. 4.58***).

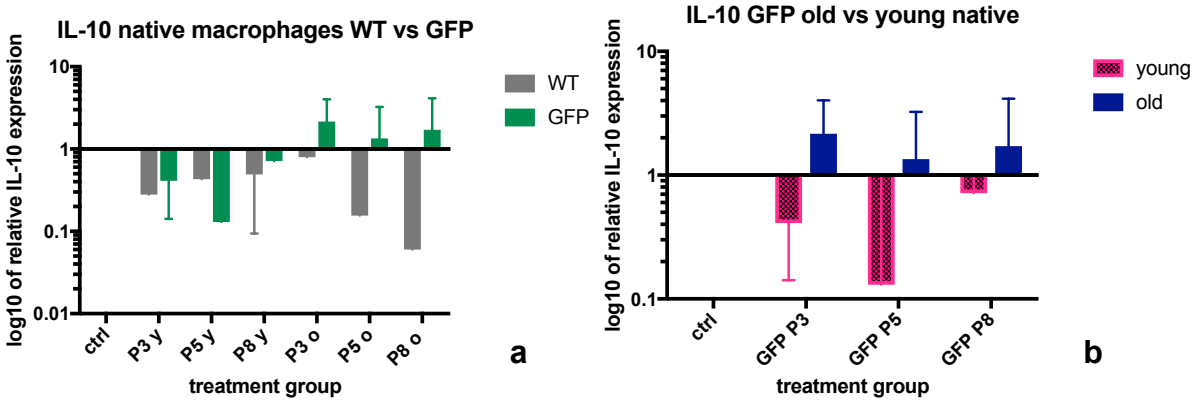


Figure 4.57.: Influence of MSC supernatants on IL-10 expression levels in macrophages

Fig. 4.57.: *the graph shows the influence of different MSC supernatants on the expression levels of Interleukin-10 (IL-10) on bone marrow derived macrophages (BMDM) coming from a WKY-WT rat. Supernatants coming from MSCs from GFP+ donors led to a higher upregulation of IL-10 expression levels in native macrophages than supernatants coming from WT-MSCs (**a**). Particularly supernatants from older GFP rats led to an increase of IL-10 expression levels (**b**). mRNA levels were calculated using RT-PCR and the ΔΔCt-method. Untreated macrophages served as control and HPRT-1 was the housekeeping gene.*

Statistical significance: *no statistical significance (ns; no asterisk);* p<0.05 (*); p<0.005 (**); p<0.001 (***).

IL-10= Interleukin-10, **WT**= supernatants coming from wild-type cells, **GFP**= supernatants from GFP+ cells

old= supernatants from cells from donor rats > 6 months of age, **young**= supernatants from cells from donor rats < 6months of

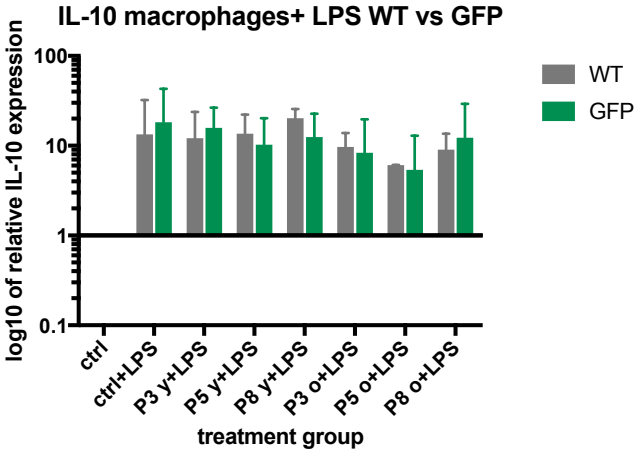


Figure 4.58.: Influence of MSC supernatants on IL-10 expression levels in macrophages

Fig. 4.58.: *the graph shows the influence of different MSC supernatants on the expression levels of IL-10 on bone marrow derived macrophages (BMDM) coming from a WKY-WT rat. After stimulation with LPS, IL-10 levels were upregulated (ctrl+ LPS) and regulated in similar ways by supernatants coming from MSCs from different passages and rats. For both WT and GFP, a downregulation of IL-10, especially by supernatants from 'older' cells was observed. mRNA levels were calculated using RT-PCR and the ΔΔCt-method. Untreated macrophages served as control and HPRT-1 was the housekeeping gene.*

IL-10= Interleukin-10, **WT**= supernatants coming from wild-type cells, **GFP**= supernatants from GFP+ cells

old= supernatants from cells from donor rats > 6 months of age, **young**= supernatants from cells from donor rats < 6months of age

Chapter 5: Discussion

5.1. Project 1: Development of a Normothermic Machine Perfusion circuit for ex-vivo kidney preconditioning on the RM3-perfusion machine and testing of a portable micro dialysis device.

In the past, the research of our group was mainly focussing on ex-vivo organ preconditioning by Hypothermic Machine Perfusion, using the Waters RM3 machine for perfusion experiments. Based on publications about the superiority of HMP above CSS, especially for preservation of kidneys from extended criteria donors[102,316,317], our aim was to further investigate the potential of this preservation method, not only for storage of organs, but also as a method to potentially regenerate damaged organs and thereby expand the donor organ pool in the future. Previously, Hamaoui et al. could demonstrate in a porcine model of ex vivo hypothermic kidney perfusion that graft pretreatment with cytotopic anticoagulants during HMP is feasible, and results in amelioration of perfusion deficits during ex vivo hemoreperfusion[96]. Furthermore, our group was able to demonstrate that improvements of the perfusion quality of organs during HMP could be made by application of a modified preservation solution containing Adenosine and Lidocaine[318]. We could also demonstrate that an application of HMP for ex-vivo perfusion of porcine and human pancreas grafts was successful and feasible for organ viability assessment[98]. These results point out that our group was focussing exclusively on the application of ex-vivo HMP and, for experiments in this field, the facilities were in place and the perfusion circuit using the Waters RM3 machine had been set up in a lab at the Chelsea and Westminster Hospital. Starting my research journey at that point, we wanted to further focus on potential preconditioning methods for organs during HMP. As Mesenchymal Stem Cells had been highlighted for their potential regenerative capacities by numerous publications, but had only been applied in systemic ways, our idea was to try to deliver MSCs to kidney grafts via ex-vivo HMP. The hypothesis behind that idea was to make use of the potential anti- inflammatory and regenerative effects of MSCs directly at the site of injury, in this case a damaged or marginal kidney graft.

During the planning phase for this study, thinking about how and in which condition to deliver MSCs to the perfusate (frozen or fresh from the cell culture), my main concern was the viability of MSCs when added to a circuit of hypothermic machine

perfusion. One concern was the temperature of 4°-6°C and another concern was the composition of the preservation solution used for HMP. Would the cells survive in the cold? Would the cells be viable within the preservation solution over 4 hours? The major concerns were that the cells might either go into apoptosis, or into an inactive state of the cell cycle and therefore might not be able to exhibit potential effects within the organs as well as in a state of normothermia.

Additionally to the concerns about the survival of MSCs in a setting of HMP, more and more data was published on benefits of normothermic machine perfusion of organs, especially shown by Hosgood and Nicholson in numerous studies[319–322] and therefore, the aim was to test the potential use of MSC for ex vivo organ preconditioning in both settings.

In order to do so, an NMP circuit had to be created first. Due to the high cost of perfusion machines and components of perfusion circuits, and also for the conduct of a direct comparison between the treatment groups, the idea was to modify the hypothermic machine perfusion circuit using the Waters RM3 machine in a way to make normothermic machine perfusion of organs possible. All NMP circuits described in the literature were based on components coming from cardiopulmonary bypass technology and so far for the kidney, no commercially available automated normothermic perfusion device exists. For described models of NMP, a circuit consists of a blood reservoir, a centrifugal blood pump, a membrane oxygenator/ heat exchanger and some silicone tubing to connect these elements[323]. In principle, the HMP circuit using the Waters RM3 consisted of similar components, with the difference of having a pulsatile pump instead of a centrifugal pump and lacking a built in membrane oxygenator connected to a heat exchanger.

By introduction of an oxygenator, a heating pump and by using protocols for the composition of the perfusate which had been published by groups focussing mainly on Normothermic Machine Perfusion[322], it was possible to create a circuit for Normothermic Machine Perfusion using the RM3 perfusion device. Unused membrane oxygenators were obtained from the Department of Cardiothoracic Surgery at the Hammersmith Hospital in London. In order to better understand cardiopulmonary bypass technology, I was kindly invited to come to their operating theatres to be taught by perfusionists at the about possible ways to integrate these oxygenators into my NMP circuits. Due to the fact that the oxygenators are usually used to provide an entire adult organism with oxygen, I was aware of the fact that the

rate of delivered oxygen had to be controlled. Therefore, a flow meter was included into the circuit and the oxygen flow rate was kept at the lowest possible (0.2ml/min). Furthermore, a Grant water heating device was interconnected in order to enable to keep the perfusate at a temperature of 35°-37°C.

Fig. 5.1. a) shows the diagram of the Normothermic Machine Perfusion circuit using the Waters RM3 pulsatile perfusion device. In comparison, ***Fig. 5.1. b)*** shows a drawing of the Normothermic kidney perfusion device for long term normothermic kidney perfusion as recently published by Weissenbacher et al., which is based on previously described NMP technology. The latter is the prototype for an automated NMP device for kidneys, which will potentially allow recirculation of urine to enable long term perfusion without fluid loss[323]. It becomes obvious when looking at the two models for NMP, that the circuit established by me using the RM3 machine is a simplified version to the much more sophisticated version used by Weissenbacher et al., consisting only of the main necessary components. A venous cannula outlet was not necessary because the venous outflow is collected passively into the venous reservoir of the RM3 machine, from where it recirculates and flows through the heated part of the cassette followed by the membrane oxygenator. We did not have a urine flow meter in place, the measurements of the urinary output was performed by collecting the urine into a beaker, measuring the volume at the named time points and replacing it by adequate amounts of Ringer's lactate solution. Blood gas analysis was performed by taking samples of venous and arterial blood to a blood gas analyser at named time points of measurement; unlike the device in ***Fig. 5.2. b,*** we did not have an in line blood gas analysis sensor installed. The reason for a simplified version of our NMP circuit was that contrary to research groups focussing purely on NMP, the input of bioengineers who are specialized in that field was not given. Myself as described in this thesis established the NMP model in collaboration with Dr. Bynvant Sandhu, who in line of her research was also following the aim of establishing an NMP circuit in our laboratory.

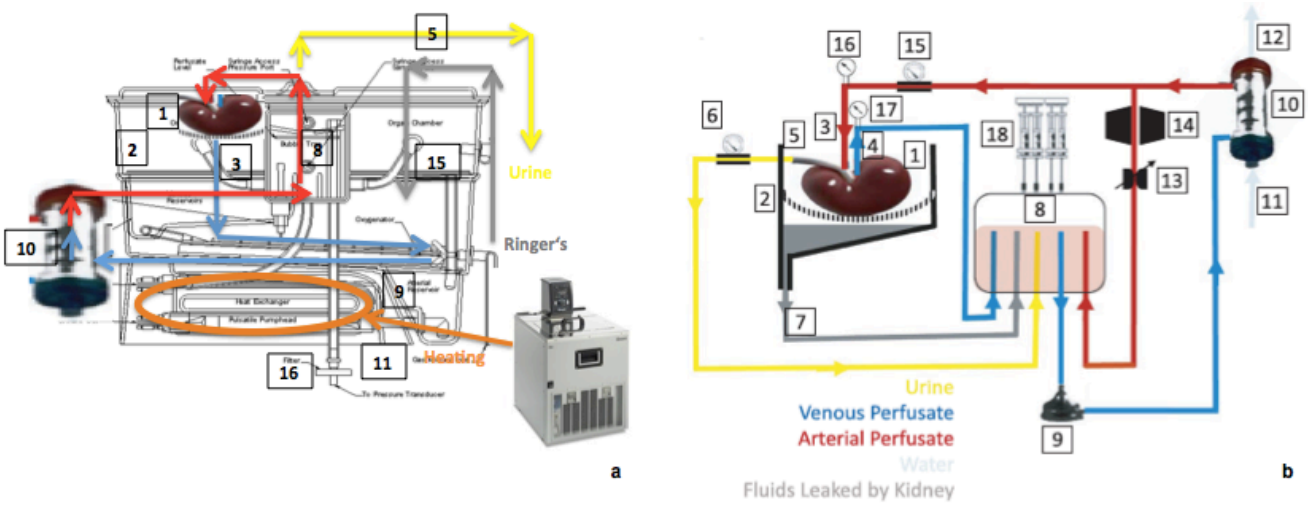


Figure 5.1.: Comparison between the normothermic machine perfusion circuit using the RM3 machine and the prototype for normothermic kidney perfusion by OrganOx Ltd., Oxford, UK

Fig. 5.1. a *shows a diagram of the normothermic machine perfusion (NMP) circuit on the RM3 machine. In direct comparison,* ***Fig. 5.1. b*** *shows the drawing of the prototype device for long- term normothermic machine perfusion of kidneys as published by Weissenbacher et al.*[324] *(OrganOx Ltd, Oxford, UK) earlier this year. The numbers highlight the same components for both circuits, with some components missing on the NMP circuit using the RM3 device (highlighted in red):*
1= Kidney, 2= Organ container, 3= Arterial cannula at kidney inlet, 4= Venous cannula at kidney outlet, 5= Ureter outlet, 6= Urine flow meter, 7= duct for recirculation of fluids leaked by the kidney, 8= Soft- shell reservoir, 9= Perfusion pump (pulsatile for ***a,*** *centrifugal for* ***b****), 10= oxygenator & heat exchanger, 11= Heat exchanger water inlet, 12= Heat exchanger water outlet, 13= Proportional pinch, 14= In line blood gas analysis sensor capable of measuring temperature, Po2, PCo2, pH, 15= Ultrasonic arterial flow meter, 16= Arterial pressuregauge, 17= Venous pressure gauge 18= Syringe pump*

Tests on 4 consecutive porcine kidneys undergoing NMP on our circuit using the RM3 device showed reproducible results, with urinary output rates and oxygen consumption rates suggesting good viability of the kidneys (***Fig. 4.3.***).

Contrary to other Normothermic Machine Perfusion circuits, our circuit was a pulsatile perfusion circuit. For our studies, this was an advantage in terms of a head to head comparison between Hypothermic- and Normothermic Machine Perfusion. Organs within different treatment groups would be treated under very similar circumstances apart from the differences in temperature and the resulting required difference in the perfusate. This could be important, as the effects of pulsatility in ex-vivo machine perfusion have not been fully clarified yet. Gallinat et al. have described beneficial effects of pulsatility during perfusion in 2013. They have described that pulsatility during HMP significantly improved the renal perfusate flow rate as well as the urine production when compared to nonpulsatile HMP. They also observed an increased reduction of perfusate creatinine levels during reperfusion in kidneys which underwent HMP on a pulsatile device. As an underlying molecular mechanism, they found a significant elevation of the expression of the endothelial anti-inflammatory transcription factor Krüppel-like factor 2, as well as endothelial nitric oxide synthase

on mRNA level. Furthermore, significantly higher perfusate levels of the endogenous vasodilator nitric oxide were measured in perfusate samples of their porcine experiments[86].

Another study on machine effects in HMP, conducted by Lindell et al. compared kidneys preserved on HMP using either the LifePort or the RM3 device and found no difference in preservation injury after reperfusion of the organs, as long as the modality of the LifePort perfusion remained on pulsatile perfusion. Interestingly, when the LifePort was changed to nonpulsatile flow, kidneys displayed a significantly greater preservation injury compared to the RM3[324]. However, similar study, comparing the LifePort and the RM3 machine in terms of outcomes found that Machine perfusion using a pressure-driven device (LifePort) generating lower pulse stress is superior to a flow-driven device (RM3) with higher pulse stress for preserving kidney function[325].

For NMP, as mentioned briefly above, there is currently only one commercially available machine on the market (Kidney Assist, Organ Assist, Netherlands) and most research groups are focussing on ex-vivo NMP of the kidney by using circuits based on the principles of cardiopulmonary bypass technology, with continuous flow created by a centrifugal pump. Therefore, no data on a comparison between continuous and pulsatile flow in NMP is available so far. In my opinion, it was a benefit for our studies to eliminate this bias and have pulsatile flow for both perfusion modalities in place. Whether that is beneficial for kidneys undergoing NMP needs to be clarified in further studies. Further studies will also be needed to evaluate the benefits of one modality over the other. One would imagine that an organ like the kidney, which is perfused in a pulsatile way inside the body, would also prefer pulsatile perfusion outside of the body.

Another point worth discussing in this chapter is the size of the oxygenators incorporated into our NMP circuit. The oxygenators were kindly given to us by the Department of Cardiothoracic Surgery at Imperial College after opening them without using them, so these oxygenators were clean, brand new and did not influence our budget. Ordering paediatric oxygenators, which are smaller, would definitely be an option for future studies, however, this would need to be kept in mind for the budget, as these oxygenators are expensive when ordered directly from the manufacturers. As a result of the use of these too big oxygenators, even after implementation of a flow meter to reduce the delivered amount of O_2/CO_2, the resulting arterial O_2 levels

were too high, with levels around 60kPa (physiological range: 10-12.9kPa). However, as all kidneys undergoing NMP were treated under these same circumstances, a valid comparison was possible. Besides this, we speculated that a higher amount of oxygen present in the perfusate than what is needed by the organ was better than a lack of oxygen, and that the oxygen consumption would be a good marker for the condition of the organs.

Fluid lost during NMP was replaced by Ringer's Lactate solution. Although it is a crystalloid solution, isotonic to blood, one of our concerns was, that over the course of time, this would lead to a dilution of the perfusate with a decrease of the Haemoglobin level. However, surprisingly, the haemoglobin levels for all our NMP perfusion experiments remained fairly stable, with a Hb of 57 ± 13.32g/l at the beginning of the NMP period, and a Hb of 56.58 ± 14.58g/l after 4 hours of NMP ($p=0.80$). This result was reassuring in terms of the concerns about haemolysis using a pulsatile perfusion device. We still observed a highly significant increase of K^+ in the perfusate, going from 4.7 ± 1.5mmol/l to 10.42 ± 3.08mmol/l ($p= <0.0001$) but we explained this to be only partly caused by haemolysis and partly by the high K^+ content within Ringer's solution.

The RM3 device was not built to be used for NMP on kidneys and therefore, for future experiments or even clinical trials, it would be better to have a standardised device in place, rather than having circuits which are put together in different ways by different groups. According to my knowledge, Prof. Friend's research group in Oxford is currently working on a fully automated device for long term NMP of kidneys (up to 24 hours) and has successfully shown that urine recirculation is possible in order to avoid fluid replenishment[323]. Fluid balance would certainly be the next step that needs to be considered when constructing a device for NMP of kidneys. For the purpose of our experiments, we accepted the flaws of the created NMP circuit.

The second aim of this first Chapter was to test the worldwide first mobile micro dialysis device which delivers live data on lactate- and glucose concentrations from the extracellular space within kidneys at all time points from the time of insertion of the probes into the kidneys. Previously, our group could demonstrate, in collaboration with the Department of Bioengineering at Imperial College London, that micro dialysis allows for accurate assessment of tissue metabolism and organ viability during the preservation period. Micro dialysis measurements gave an idea about the baseline

ischemic profile for porcine kidneys and helped to characterize metabolic differences between cold static storage and hypothermic machine perfusion[326]. Whereas previous micro dialysis measurements were only possible to be performed in the laboratory, for this study, the progress in technology allowed us to test a mobile micro dialysis device which could be brought along to the abattoir where porcine organs were retrieved. We could demonstrate for the first time that micro dialysis measurements were not only possible in the warm ischemia time during procurement of the organs, but also in the time of transit to the laboratory, followed by measurements during our perfusion experiments. This gave us a good opportunity to obtain great continuous results from one same kidney during different phases of preservation and this could help to elucidate why some preservation methods are superior to other methods. We could demonstrate that the measurements from the micro dialysis device were accurate and in concordance with the measurements from the ABG machine. Flaws of the mobile micro dialysis device are the high sensitivity to external factors like movement, temperature, the resulting long calibration times for the probes, and the delay of 10 minutes for measurements due to the slow flow through tiny tubing to the sensor measuring the glucose- and lactate levels.

Once these problems are solved, data from the micro dialysis device could give important information on what is happening within the graft during warm- and cold ischemia times and results could be of great help in terms of monitoring organs during periods of CSS and-/or preconditioning. It could also be very helpful in estimating the condition of organs for transplantation in the future, which is especially important when it comes to the use and preconditioning of marginal organs.

5.2. Project 2: Investigation and comparison of the effects of current methods of ex-vivo organ preservation on porcine and human kidney grafts after prolonged cold ischemia times.

After setting up the normothermic machine perfusion circuit for experiments on the introduction of Mesenchymal Stem cells into either, the hypothermic or the normothermic machine perfusion circuit, I was not ready to conduct the planned perfusion experiments using Mesenchymal Stem Cells. For the necessary expansion of human MSCs in-vitro I had to wait for Professor Dazzi's group at King's College to kindly provide me with a cry vial of human MSCs, as their group had the approval of the Ethics committee to use human bone marrow samples for research purposes. Whilst waiting for a sample of cells and after having successfully set up the NMP circuit on the RM3 machine, we had the idea to perform a study to directly compare Hypothermic machine perfusion (HMP) and Normothermic machine perfusion (NMP) to each other. The plan was to retrieve porcine kidney pairs or, whenever possible, use human kidney pairs which were rejected from clinical transplantation, and expose them to either method of ex-vivo machine perfusion for the same period of time. Outcomes would then be evaluated by directly comparing the function of the organs during a reperfusion period of 2 hours using whole blood. This idea seemed a logical consequence to having set up two ex-vivo machine perfusion settings using the very same machine and having pairs of kidneys coming from either one donor pig, or even from the same human donor.

In recent years, machine perfusion of kidneys has been in the focus of interest with a variety of strategies driven by the aim to not only optimize organ preservation, but also to enable preassessment of graft viability and repair of damaged organs prior to transplantation. Hypothermic machine perfusion and Normothermic machine perfusion both have been studied extensively and are being introduced into clinical practice [90].

HMP has been demonstrated to be superior to CSS, especially in organs coming from extended criteria donors, with a reduced risk of delayed graft function and improved graft survival in the first year after transplantation. This effect could be demonstrated for kidneys from brain dead donors (DBD), as well as for kidneys from deceased donors (DCD) [102,104,105,107]. As mentioned in Chapter 5.1., our group had previously demonstrated that in HMP, graft pretreatment with cytotopic anticoagulants or modified perfusion solutions is feasible and that perfusion deficits in

ex-vivo haemo-reperfusion models could be improved when compared to non treated controls[327]. For HMP, our group was furthermore able to demonstrate, that ex-vivo perfusion and assessment of human and pancreas grafts was possible[98]. The only logical flaw of the hypothermic perfusion models is however that an assessment of the function of a graft would only be possible once reperfusion was mimicked- or in clinical application, reperfusion takes place within the recipient. Thereafter, it is difficult, if not impossible, to directly influence the function and outcomes for an organ, because many external factors, e.g. the medical- and sensitization history of a recipient come into action to influence the end result.

In recent history, normothermic machine perfusion (NMP) has been investigated as an alternative method to HMP, with the theory that in an active state of an organ its function can be better assessed and, if necessary, be improved by adding treatments to the system. Most of the protocols for NMP in the literature describe packed red blood cells as the main component for the perfusate. Red cells have to be blood type (AB0) compatible with the organ undergoing NMP, otherwise a cross reaction could lead to thrombosis and malperfusion of the organ. This is also relevant for porcine perfusion experiments, which is why it was important to use autologous blood for these experiments. With this in mind, one potential flaw of NMP in clinical transplantation could be the use of third party derived red cells. This could lead to additional sensitization of recipients. Therefore, research groups with a focus on NMP have also performed some studies using alternative perfusion solutions with artificial, acellular oxygen carriers instead of packed red cells[328–331].

NMP aims to keep an organ in a functional state, thereby keeping up an aerobic metabolism within the organ. Using NMP, we might be able to effectively revive damaged organs ex vivo in the future. Several studies have found that NMP has a potential to lower the rates of DGF, potentially by mitigating the severity of IRI. This has led to the establishment of a multicentre randomized controlled trial in the UK, comparing NMP and CSS in kidneys from DCD donors[320,332,333]. So far, NMP has been designed to ‚resuscitate' kidney grafts after SCS, hence, in on-going clinical trials, it is mostly carried out for up to one hour before implanting an organ, whilst the patient is prepared for transplantation. After performance of NMP, kidneys are flushed with cold preservation solution, packed and placed back onto SCS[122,321] before implantation. In my opinion, the impact of flushing an organ twice with cold preservation solution must not be overlooked, and should be investigated properly

before drawing conclusions about the outcomes after NMP. It could be that cooling down, rewarming, cooling down and rewarming of an organ has an impact which we might not see in the immediate phase post transplantation, but which influences the outcomes on the long term.

Browsing through the literature at the start of my research on different methods of ex-vivo organ preconditioning, it appeared to me that the field is currently split into two groups: research groups focussing purely on hypothermic machine perfusion on one side, and groups who are focussing on normothermic machine perfusion on the other side. There are some groups working on variations of the two, e.g. subnormothermic perfusion of organs, but there is actually not much data existing on a direct comparison between the two methods. It seemed to me, that the newer technology behind NMP attracted a lot of attention and and whilst it seems logical to agree with Professor Friend's quotation: 'An organ must never learn that it left the body', I had the feeling that conclusions about the superiority of NMP towards other preservation methods had been drawn too early. The well-established and investigated method of HMP for preservation of organs ex-vivo seemed to be 'out of fashion', without conduction of thorough comparative studies between HMP and NMP. In most of the literature, the different methods of machine perfusion are being compared to CSS.

Only three studies so far aimed to directly compare the two methods, one of them reaching back into the year 2002[315]. In these studies, slightly better outcomes were observed for kidneys in the NMP group, but the differences were not significant. In two of the studies, the times of the different preconditioning methods were different between the groups, and in none of the studies was the reperfusion phase performed with both kidneys on the same device allowing for a direct comparison of the outcomes under the same circumstances[315,322,334].

In our study comparing HMP to NMP, both kidneys were reperfused on the same machine after the preconditioning period, receiving the same rates and pressures of arterial inflow. This made it possible to perform a first head to head comparison, which had not been performed before in this way. In the porcine arm of this study, we also included some kidneys after CSS only. The number of these kidneys was lower than the number for the actual study groups. This was due to the fact that each experiment was very time and money consuming. Furthermore, we made the observation that the kidneys after CSS showed very stable perfusion patterns, with similar results between the samples at reperfusion and so considered the sample

size of n=4 for CSS to be a good reference for comparison. We did not include these organs into the statistical analysis. In an ideal case scenario, we would have had an equal amount of porcine kidneys in all three groups.

Studying pairs of porcine kidneys, which were exposed to a WIT of 20 minutes followed by a CIT of 24 hours and either 4 hours of NMP or HMP followed by 2 hours of reperfusion with whole blood, we found that kidneys after HMP showed better physiological results than kidneys after NMP. Urinary output rates as well as oxygen consumption rates were significantly higher in kidneys after HMP than in kidneys after NMP. This might be a consequence of the higher intrarenal resistance indices during NMP than during HMP.

During NMP, Eprostenol was applied in hourly doses, as recommended in the protocol published by Nicholson et al., to support the microcirculation within the kidney. Furthermore, the red cell based perfusate was fully heparinized to prevent the formation of blood clots or micro embolism. However, the ideal dose for both drugs in a red cell based NMP perfusate might still have to be determined. Some disturbance to the microcirculation of NMP kidneys might have occurred, resulting in higher values for the RRI than in kidneys undergoing HMP, even though macroscopically there was nothing visible to suggest that. The perfusate for HMP, in contrast to the perfusate for NMP, does not contain any third party derived red blood cells. Indicated by a rosy colour of the perfusate after some time of HMP, a washout of donor-derived red blood cells takes place over time, maybe contributing to the effect of the observed decrease in RRI values over time. In our study, the increased RRI values observed in kidneys undergoing NMP furthermore led to a significantly lower perfusate flow rate in these kidneys during reperfusion (***Fig. 4.8.***). Another interesting observation was that at the end of the reperfusion period, lactate levels in the perfusate were highest in kidneys after cold static storage, followed by kidneys after NMP and lowest for kidneys after HMP, suggesting the lowest degree of hypoxia for the latter group (***Fig. 4.11.***).

Looking at tissue samples and analysing them for expression of kidney injury markers as well as markers of inflammation at mRNA level was another interest of ours, to potentially have better explanations for observed results. After performance of a literature review at the time of starting our experiments, we chose to look at the expression of Neutrophil gelatinase-associated lipocalin, Endothelin-1, Tumour necrosis factor-α and Interleukin-1β on mRNA level using quantitative real time PCR

(RT-PCR) as a method. NGAL has been considered to be one of the most promising biomarkers of acute kidney injury[335,336], and given my own involvement in some research concerning that biomarker for monitoring and prediction of delayed graft function after renal transplantation[337], its inclusion as a marker into my experiments was the first logical idea. Endothelin-1 (EDN-1) has been described as another marker for ischemia-reperfusion injury in kidneys[338] and the fact that it was found to be mostly secreted by endothelial cells during stress and hypoxic damage[339], led us to include EDN-1 into our analysis of mRNA expression levels within tissue samples of experimental kidneys. To complete the panel for mRNA analyses, I wanted to include Tumour necrosis factor-α and Interleukin-1β as general markers of inflammation. For human organs only, the expression levels of Kidney Injury Marker-1 were included because of simultaneous analysis of perfusate- and urinary samples for this well known marker of kidney injury using human KIM-1 ELISA kits. Such a kit for KIM-1 was not available for porcine samples. Therefore, this marker was not part of the porcine mRNA analyses either.

For the porcine perfusion experiments, the results of these analyses showed a significantly higher expression of EDN-1 in kidneys after HMP than in kidneys after NMP. Also, the inflammatory markers IL-1β and TNFα showed a trend to be higher expressed in kidneys after HMP than in kidneys after NMP. Only NGAL was lower expressed in kidneys after HMP than in kidneys after NMP. In fact, NGAL was down regulated in both groups after reperfusion when compared to the internal control, but stronger in kidneys after HMP than after NMP. The underlying reasons for these findings are unclear but it was interesting that despite better physiological function of kidneys after HMP compared to NMP, these kidneys had a more 'pro-inflammatory' expression pattern on mRNA level overall. In contrast to EDN-1, TNFα and IL-1β, which are markers of inflammation and hypoxia, NGAL has been described to be of prognostic value in concern to acute kidney injury. After both HMP and NMP the NGAL expression was down regulated in comparison to the control, namely the untreated sample after retrieval. This could mean that the perfusion phase was of benefit for the organs, more so after HMP than NMP when we consider NGAL to be a predictive marker for acute kidney injury. Unfortunately, NGAL expressions in samples after CSS was below the detection limit using RT-PCR so no comparison to kidneys after CSS could be made.

Some of my described results seem to contradict each other. Possible explanations for the higher degree of mRNA expression of inflammatory markers in kidneys after HMP, despite better physiological outcomes, could be that a certain degree of inflammation is necessary to protect the organs from damage. Another explanation for this observation could be that kidneys after HMP experience the impact of reperfusion at a time point, when kidneys after NMP have already recovered from the phase of warming up. The resulting IRI becomes evident at that point on a molecular level in kidneys after HMP. However, the same would be the case for kidneys after CSS, in which we did not see this radical up regulation of inflammatory parameters at reperfusion. To complete our studies, also perfusate- and urine samples were analysed for the inflammatory markers IL-1β and NGAL by ELISA, but there was no difference found between the groups. The creatinine clearance, as well as the sodium excretion rates, as markers of functionality of the kidneys, were not different between the groups, even though there was a trend towards a higher creatinine clearance in HMP kidneys. In order to further clarify and validate our results, a longer period of reperfusion would be necessary. In an ex-vivo model with limited amounts of whole blood for reperfusion, this is however not possible. In vivo experiments would be the next logical step to validate our results.

After performing our experiments on MSCs in ex-vivo organ perfusion settings, we decided to expand the study group and to include control kidneys, which either underwent NMP or HMP only (n=6) into our calculations and to add them to the initial groups of 9 kidneys, respectively. We wanted to see, whether we could confirm the result of better physiological function of kidneys after HMP than after NMP with a higher number of samples, including kidney pairs from different donor pigs but undergoing the same methods of machine perfusion. Indeed, we could confirm these findings as well as the results for the expression of the kidney injury markers as well as the markers of inflammation, some of them with higher statistical significance, which was reassuring. Also for the same study using porcine kidneys after a prolonged CIT of 45 minutes, our initial results could be confirmed, lacking statistical significance due to small sample size (n=3 per group). However, this was another important and reassuring result and it suggested, that a prolonged warm ischemia time for the porcine kidneys did not alter the results.

Kidneys after HMP showed the best histological results, with good preservation of tubular structures, followed by NMP and CSS. This again points towards the idea that

a certain degree of inflammatory response might be of protective value for the organs. In our porcine study, we included 4 kidneys after CSS. These kidneys were not included into statistical analyses, as we were purely focussing on a direct comparison between NMP and HMP. However, 4 kidneys after CSS served as a control group to estimate how kidneys after machine perfusion perform in comparison to kidneys, which remained on ice in the meantime.

To summarize our results for porcine experiments on NMP vs. HMP, ***Table 5.1.*** shows an overview of the major findings during reperfusion in a simplified version. Arrows pointing up or down do not indicate an increase or a decrease of the 'total' amount of the listed subjects, but point out the relative outcomes between the groups. This purely serves as a simplified overview to better understand our results.

PORCINE PERFUSION STUDY	NMP (n=9)	HMP (n=9)	CSS (n=4)
Urinary output	↑	↑↑*	↑
Oxygen consumption	↑	↑↑*	↑
Perfusate flow rate	↑	↑↑*	↑↑
Lactate levels perfusate	↑	↑↑	↑↑↑*
EDN-1 expression	↑↑	↑↑↑*	↑
NGAL expression	↓	↓↓	–
IL-1β expression	↑↑↑	↑↑↑	↑
TNFα expression	↑↑	↑↑	↑

Table 5.1.: Overview of outcomes during reperfusion for porcine kidneys after NMP or HMP as a preconditioning method

Table 5.1. *shows, in a simplified version, an overview of the main findings during the reperfusion phase for porcine kidneys after either normothermic machine perfusion (NMP) or hypothermic machine perfusion (HMP) with n=9 kidneys in each group. For the purpose of comparison the findings for kidneys after cold static storage, with a lower number of n=4 are also included.*

The arrows serve as a relative quantification in comparison between the groups. Stars next to the arrows indicate that a statistically significant result was observed.

A similar pattern of results was found, when the groups were expanded and kidneys from other experiments but after the same treatment were included.

As we were also getting human kidneys referred to us, which had been declined for clinical transplantation due to various reasons, e.g. arteriosclerosis or malignancy within the donor, we decided to repeat the experiment on a direct comparison between HMP and NMP using these kidneys in a similar setup to the porcine model. The only difference was that for these human kidneys it was very hard to obtain

blood group compatible packed red cells or whole blood at the time of receiving the organs. We purchased some expired red cells as well as whole blood of certain blood types at the times from NHSBT, but by the time we got organs for an experiment referred to us, these blood products were out of range in terms of the electrolyte content as well as the pH (measured by the ABG machine). We were not able to use that blood for our perfusion experiments. Another consideration was that usually the expired products were of a certain blood type and it was a matter of luck to get kidneys from the same blood type with matching blood referred to us at the same time. Therefore, we decided to conduct the experiments with Krebs-Henselleit Buffer for the NMP preconditioning phase, as well as for the reperfusion phase, and to purely focus on the influence of the difference in temperature and mechanistics for these experiments. The limitation was the same as for the oxygenation of the perfusate for HMP, that we were not able to measure the oxygen content of the perfusate, nor the oxygen consumption, because the ABG machine was set to analyse human blood and did therefore not read KHB samples.

The next limitation to the study using human organs was an advantage and a step forward at the same time. Kidneys were coming from different donors with different age, clinical history and, of course, different cold ischemia times. The demographics are summarized in ***Table 4.1.*** At first sight, after performing the perfusion experiments, we noticed similar results for intrarenal resistance indices as in the porcine experiments, with a downward trend in RRIs during HMP, whereas RRIs increased towards the end of NMP. Again, the question had to be raised, whether a more generous application of vasodilators should have been given. However, to start with, we wanted to stick to the published protocol and to the protocol used for porcine organs. The observation for the RRIs led to the consequence of significantly lower RRIs within the HMP group than within the NMP group during reperfusion (***Fig. 4.22.***). Interestingly, however, looking at the perfusate flow rates during the preconditioning period, we observed higher perfusate flow rates in kidneys during NMP than during HMP. The physiological temperature with capillaries being wide might be the reason for this, additionally to the fact that in contrast to the porcine organs, the perfusate was an acellular fluid. During the reperfusion period, this effect was reversed and kidneys after HMP featured higher perfusate flow rates per 100g of tissue.

So far, results from the human studies seemed to be concordant with the results for the porcine experiments. However, looking at the urinary output rates, human kidneys after NMP produced higher rates at all measured time points than human kidneys after HMP. This was surprising as it was the opposite result to what we had observed in the porcine model (***Fig. 4.23***). These results were not statistically significant and with the aim to find an explanation for the contrast to the porcine study, we looked into the backgrounds of the kidneys. The first apparent observation was, that exactly 3 pairs out of the 6 had cold ischemic times of up to 24 hours, whereas the other three pairs had cold ischemic times of 24 up to a maximum of 38 hours. So we decided to analyse with respect to the clinical cut-off time of 24 hours and divided the groups in two subgroups. After doing so, we found interesting results, namely again, the exact opposite, depending on which group kidneys were in.

First of all, after HMP as a preconditioning method, kidneys with shorter CITs (up to 24 hours) showed higher urinary output rates than kidneys with longer CITs (24-38 hours). For kidneys after NMP, the opposite was the case, with kidneys after shorter CITs producing less urine than kidneys after longer CITs (***Fig 4.24.***) upon reperfusion. This led us to analyse for a direct comparison between HMP and NMP dependant on the CIT. Indeed, kidneys with CITs up to 24 hours, showed similar results as observed in the porcine model, in which CIT were kept at 24 hours for all kidneys. Kidneys after HMP had higher urinary output rates than kidneys after NMP within this group. Contrarily, kidneys after CIT of 24 hours and more had much better urinary output rates after NMP at reperfusion when compared to the HMP group (***Fig. 4.25***).

Interestingly, this also reflected in mRNA expressions of kidney injury markers and inflammatory cytokines. KIM-1 was included into this study as a well-known kidney injury marker. Again, we discovered inverse results depending on the CITs of the kidneys. For KIM-1, the overall result of mRNA expression was higher after HMP than after NMP. When split into the subgroups, KIM-1 was down regulated after NMP and up regulated after HMP in the short CIT group, and vice versa in the long CIT group, when compared to the internal control (tissue sample after retrieval).

As illustrated in ***Figure 4.26.***, results for EDN-1 could be confirmed with significantly higher expression levels after HMP than after NMP in the CIT<24 hours group and the same observation was made for the inflammatory parameters IL-1β and TNFα. Again, the fact that at the same time in these same groups, the physiological

parameters of the respective kidneys were better, led to the conclusion, that a certain degree of inflammation might have protective effects on the organ. The only contrary observation to the porcine experiments was made when we looked at the expression profile of NGAL: it was significantly higher expressed in HMP treated kidneys after CIT<24 hours than after NMP, whereas it was down regulated in the respective porcine cohort. ***Table 5.2.*** shows a simplified summary of our most important findings for the study using human organs. Subdivided into groups with different cold ischemic times, the arrows indicate the relative trend for the results between samples, not the absolute trend. This should demonstrate the overall pattern of results as a summary.

To complete the human experiments, we also looked at periodic acid Schiff (PAS) stained histology sections, with the help of Professor Terry Cook. There were no histological differences detectable after the reperfusion period between the two groups. He suggested that the time of reperfusion was too short to see microscopically visible effects. For that reason we did not blindly rank these slides for the performance of a ranking, as especially for a non-histopathologist, an evaluation of the histology performed in the same way as for the porcine samples would have been a nearly impossible undertaking. It would however be very interesting to perform a study like this in a clinical setting to find out which modality for preconditioning is better.

HUMAN PERFUSION STUDY	NMP CIT 12-24h	NMP CIT 24-48h	HMP CIT 12-24h	HMP CIT 24-48h
Urinary output	↓	↑	↑	↓
KIM- expression	↓	↑	↑	↓
EDN-1 expression	↓	↑	↑*	↓
NGAL expression	↑	↓	↑*	↑
IL-1β expression	↓	↑	↑	↓
TNFα expression	↓	↑	↑*	↓

Table 5.2.: Overview of the outcomes during reperfusion of human kidneys after HMP or NMP as a preconditioning method.

Table 5.2. *shows an overview of the main findings during the reperfusion phase for human kidneys after either normothermic machine perfusion (NMP) or hypothermic machine perfusion (HMP) and subdivided for the duration of cold ischemia times (CIT) of the pairs, with n=3 kidneys in each group.*
The arrows serve as a relative quantification in comparison between the groups. Stars next to the arrows indicate that a statistically significant result was observed.

From a transplant immunological point of view, despite positive reports on NMP in various clinical pilot studies, we have to think about the fact, that blood products are necessary to perform NMP and that, even though flushed out, residual erythrocytes can remain within the organs and lead to immunisation of the recipients.

Our overall conclusion to this chapter is that in a direct comparison of HMP vs. NMP using porcine kidney pairs, organs after HMP performed better in terms of physiological function during the reperfusion phase. The reasons for this are unclear and one limitation of this study is definitely the short period of reperfusion, during which a final conclusion might not be possible. Contrary to the function of the porcine kidneys on a molecular level it seemed like overall, kidneys after HMP showed a more pro-inflammatory pattern than kidneys after NMP. Again, the impact of the gene expression patterns might only have manifested after the two-hour reperfusion time but due to the limitations of an ex-vivo reperfusion model, a longer period of observation was not possible. With the results of our work, we can only speculate, that an inflammatory response on mRNA level within organs after HMP might have a protective effect and therefore, lead to a better function of these organs. A longer period of reperfusion is necessary to further investigate this, ideally in an in vivo porcine transplant model.

Having performed the same study on human organs for which a consistent background in terms of the cold ischemia times and the medical history of donors was not possible, our results in this translational model furthermore demonstrated that the outcomes were similar to the outcomes in the porcine model for human kidneys with cold ischemic times equivalent to the porcine model. For organs with longer cold ischemia times, the results were contrary, suggesting that in terms of finding the perfect method for preconditioning organs in transplantation, there might not be just one way forward suitable for all organs. Much rather, there might be a need for tailored preconditioning methods, depending on the origin of a graft, as well as on the individual recipient's needs. One major limitation for both, the porcine as

well as the human experimental model was the limited number of organs, with 9 (+6 unpaired) kidney pairs in the porcine study group and 6 kidney pairs in the human study group. The results might have been different with larger numbers of organs.

One other thing to consider will be the establishment of either method of organ preservation in clinical practice. In times of shortage of staff within the NHS, we also have to think about the economical benefits and the possibilities to provide a service in terms of the required staff. Whilst studies on NMP as well as on HMP currently provide good results in clinical trials, especially NMP is a method which requires a lot of expertise. At least until there is a commercially available automated machine, a separate team of professionals is needed to look after an organ whilst it is being perfused normothermically. In contrast to this, HMP of kidneys is already in clinical use for a while and it is possible to be performed in a fully automated way. The clinicians only need to connect the organ to the machine and have it in a safe place. Therefore, HMP is simpler and more cost effective at the moment.

Despite of this, a ‚resuscitation' of organs deemed untransplantable might not only be possible using NMP. Nicholson et al. described the successful transplantation of organs which were about to be discarded from clinical transplantation, mainly due to poor perfusion at retrieval and were successfully transplanted after an episode of normothermic machine perfusion[123]. During my time focussing on perfusion of human organs, it sometimes occurred that only a single human kidney was referred to me by NHSBT. In cases in which the organs had been rejected due to poor perfusion, I was interested whether an improvement could be achieved by performing a period of HMP. Indeed, for most of these kidneys, during HMP, the patchy areas cleared up and also a decrease in RRIs could be observed. Perfusion data of kidneys randomly perfused are not shown in this thesis but as an example, which is shown in ***Fig. 5.2. a*** *and* ***b****,* in in one particular case, a kidney from a 45 year old DBD was rejected from clinical transplantation due to poor perfusion and when I received the kidney in my lab, I found that there was a lower pole artery leading to an unperfused area. The RRI for the kidney was around 1 when I started HMP and dropped down to 0.5 at the end of 4 hours. The darker area cleared up nicely and at the end of perfusion. I am suggesting, that after HMP, this kidney could have been used for clinical transplantation.

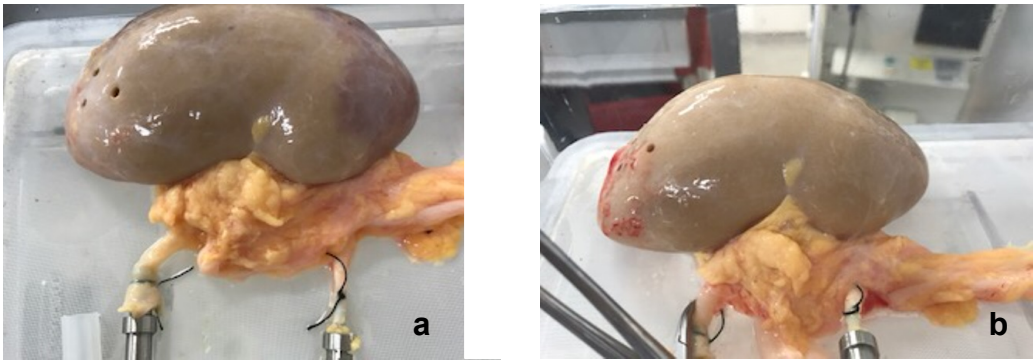


Figure 5.2.: A human kidney before and after 4 hours of Hypothermic machine perfusion

Fig. 5.2. a *shows a kidney from a 45 year old DBD donor which had been discarded from clinical transplantation due to bad perfusion. Upon retrieval in the lab, the organ was benched and a demarcation was visible on the lower pole. A lower pole artery was discovered and also connected to the machine before performance of 4 hours of hypothermic machine perfusion (HMP). After the 4 hours of perfusion, the demarcation was not visible anymore (**b**) and the perfusion parameters read by the Waters RM3 device suggested the kidney to be in good condition (RRI around 0.5).*

This is just one example, which indicates, that testing of organs, not also on NMP but also on HMP is feasible and could lead to rescuing some organs from being put into the bin.

As a next step, I think we should perform clinical trials in which organ pairs undergo either, an episode of NMP or HMP, instead of comparing each method to CSS. We might find differences in outcomes depending on the medical history of individual organs as well as recipients and this could give answers to some more unanswered questions. Even with the progress being made in organ preservation and perfusion, there are still many unanswered questions. More studies will need to be done to find the optimal conditions for duration of perfusion in the cold phase, optimal perfusion solution, degree of oxygenation, and the addition of pharmacologic agents, to improve organ function and expand the organ pool.

5.3. Project 3: Investigation of the introduction of MSCs into ex-vivo organ preservation systems as a preconditioning method for porcine and human kidney grafts (translational model).

As described in detail in Chapter 1.3., a lot of studies are focussing on the administration of Mesenchymal Stem Cells as a potential agent to alleviate IRI at this moment. MSCs have been shown to have anti-inflammatory and regenerative capacities and furthermore, to home to sites of injury[213,264,265,299]. In most of the studies found in the literature, MSCs have been delivered systemically. One of the major problems with this was that MSCs were trapped within capillaries of the lungs before reaching the target organ[294]. Furthermore, the efficacy of delivered MSCs has been found to be dependent on the timing of administration peri-transplantation. Liu et al. described that MSCs administered immediately after ischemia or 1 hour post ischemia yielded the highest renoprotective and anti-inflammatory effects. They furthermore postulated that MSC treatment for acute kidney injury is most effective when applied prior to the development of a potent inflammatory microenvironment. In their study, urinary NGAL levels were described as a marker for the estimation of when to infuse MSCs because NGAL was found to correlate well with the inflammatory state of kidneys[340].

As a start to our studies, we first had to find a way to obtain Mesenchymal Stem Cells. Commercially available Mesenchymal Stem Cells are available and can be ordered in batches from companies like Lonza or StemCell. However, to order stem cells is expensive. Therefore, we asked Professor Dazzi from King's College, who is a specialist in the field of Stem Cell research, whether he and his group would be interested in collaborating with us for this project. His group has the necessary Ethics approval for the use of human bone marrow for research purposes in place. Furthermore, they have the facilities to extract MSCs from human bone marrow samples and also the necessary protocols to prove MSC phenotype by flow cytometry. After receiving the sample with the frozen cells, I had to expand them in cell culture using the protocol provided by Prof. Dazzi's group. The next question was how to label the cells to be able to trace them after the perfusion of kidneys ex vivo. The recommendation of Prof. Dazzi's group was to transfect the cells with green fluorescent protein. However, cell transfection is a very delicate method, which requires a lot of experience and we did not know how transfection would affect the

phenotype of MSCs altogether. Hence, we decided to choose a less invasive method for labelling of the cells. We came across a method of double labelling the MSCs with green cell membrane dye and red cytosolic nano particles. This method was described by another group focussing on research in the same field at a conference. The PKH67 fluorescent cell linker kit (Sigma-Aldrich) stably incorporates a green fluorescent cell dye with long aliphatic tails (PKH67) into lipid regions of cell membranes[341,342]. Qdot® Nano crystals (molecular probes®, life technologies™) Qdot® Nano crystals are fluorescent substances that absorb photons of light and then re-emit them at a different wavelength, depending on their size. The Nano crystals are available in different sizes to result in different fluorescent colours. In our case, choosing the Qdot® far red Nano crystal kit, the double-labelling of the cells was supposed to result in MSCs featuring a green cell membrane and red particles within the cytosol. This was supposed to enable us to definitely distinguish delivered MSCs from cells of kidney donor origin. Unfortunately, the Qdot® labelling did not result in a strong red signal, even after prolongation of the recommended incubation time. Only single and small Nano crystals could be visualized within our cells. Thanks to Stephen Rothery from the Imperial College FILM facility, I was able to learn about wide field microscopy and about how to use the best possible filters and software settings to visualize the dyed cells. I found that the PKH67 staining worked very well and the result was MSCs with a bright green cell membrane and some small red crystals within the cytosol.

Not knowing whether the cell dye would fade during machine perfusion experiments, the first series of experiments was focussing on introducing MSCs in numbers between 1 and $5x10^6$ cells into the HMP setting. There are no clear guidelines or recommendations on an ideal cell number when introducing MSCs as a treatment for IRI into an isolated organ. Our chosen number, $1\text{-}5x10^6$ was a result of findings in the literature, where similar numbers of cells had been used for systemic infusions to target damaged organs. Hence, $1\text{-}5x10^6$ cells seemed more than enough when aiming to treat an isolated organ. Aiming to perform our experiments in a translational model, starting out with the perfusion of porcine kidneys, the question was, whether perfusion of porcine organs with human MSCs was feasible. Trying to extract porcine Mesenchymal Stem Cells from a porcine femur obtained in the abattoir, in a non-sterile environment, resulted in growth of fungus in culture and was not possible for further experiments. Noort et al. had demonstrated that porcine MSCs have

comparable characteristics and functionality with human MSCs[343] and therefore we decided to use human MSCs for both, porcine as well as human kidney perfusion experiments. For the purpose of investigations, whether MSCs could be traced within perfused organs, using human MSCs for porcine organs gave us the advantage of not only tracing cells via the fluorescent cell dye, but to confirm the findings with immunohistochemical staining against human proteins. At first, we tried to distinguish the human cells by staining against human Vimentin, which is a cytoskeletal protein found in MSCs. Unfortunately, the antibody cross reacted with porcine Vimentin. In the next step, we tried to stain against human MHC class-1 and this time, the staining was specific for the human cells only. As described in Chapter 3.3., we were able to trace human MSCs using wide field microscopy and to confirm the findings by immunohistochemical staining. It was interesting to find, that MSCs were detectable within glomeruli, but also on some of the pictures seemed to be incorporated into the endothelial cell layer of the tubules. Indeed, several mechanisms of the acceleration of regenerative capacities of MSCs have been identified, ranging from wound healing and angiogenesis by secretion of pro-angiogenic factors to the differentiation into endothelial cells and/or pericytes[344].

A combination of ex vivo machine perfusion and delivery of MSCs has not been described in the literature yet. We are the first group to demonstrate that it is possible to deliver MSCs into an organ by ex vivo hypothermic- as well as normothermic machine perfusion and to trace the cells within the organs after doing so. The next question was, whether the infusion of MSCs had an impact on the function of the organs. If yes, would the duration of the perfusion and the number of applied cells have an influence on the effect? Another question was whether potential effects would be triggered by direct cell-cell interactions in an indirect way, for example by secretion of chemokines or microvesicles[345].

With an aim to answer the above questions, we performed studies on the use of MSCs in ex-vivo machine perfusion of porcine and human kidneys, using the same protocols for HMP and NMP as described in Chapter 2.2., and introducing 1-5x10^6 MSCs into the respective perfusates. This time however, both kidneys from one donor pig were perfused hypothermically or normothermically, one receiving a bolus of MSCs 15 minutes after start of ex-vivo perfusion and the other kidney serving as control.

6 consecutive porcine kidney pairs were hypothermically perfused, one kidney receiving MSC treatment on the machine and the other kidney serving as an internal control. After analysing our results, we found that during 4 hours of HMP, there were no differences between treated and non treated kidneys in terms of functional parameters, e.g. IRR and flow rate. The graphs for each group looked almost identical, which is demonstrated in ***Fig. 4.29***. Also during the reperfusion phase, no differences of physiological parameters between the two groups were found. The same findings were made when we looked at the results of MSC treatment during normothermic machine perfusion. There were no differences between kidneys undergoing NMP only and kidneys undergoing NMP with MSCs infusion in terms of functional parameters, e.g. urinary output, RRIs and perfusate flow rates. The finding, that kidney pairs which had been treated with the same perfusion modality showed almost identical results during reperfusion, was at the same time reassuring for described differences in outcomes after either perfusion method, to be real. During ex vivo HMP, it seemed like an infusion of MSCs, no matter in which number, did not immediately influence the function of the kidneys during mimicked reperfusion. Out of the 6 kidneys undergoing HMP with MSC treatment, 2 kidneys were perfused with $1x10^6$cells, 2 kidneys received $3x10^6$ MSCs and 2 kidneys were infused with $5x10^6$ cells. We found, that infusion of $1x10^6$ MSCs resulted in difficulties to find any cells within the kidney histologically. Comparing HMP to HMP+MSCs in 6 porcine kidney pairs, we did not find any differences between the groups in terms of functional outcomes at reperfusion. In my opinion, this was not a very surprising result considering that cells under hypothermic conditions are supposed to be metabolically inactive.

To further investigate whether MSCs had an effect on the kidneys when introduced into the HMP model, we performed the same analyses for tissue-, perfusate- and urine samples as for previous perfusion studies. The same markers for kidney injury and inflammation were chosen in order to have a good idea about how to interpret the results. Perfusate samples after 2 hours of reperfusion analysed by ELISA revealed a trend towards higher concentrations of IL-1β and NGAL in kidneys after MSC treatment. This could indicate some on-going inflammatory processes within these organs. For the analyses of kidney injury markers NGAL and EDN-1, as well as the inflammatory markers IL-1β and TNFα on mRNA level, we subdivided the results into expression levels after the preconditioning phase, and results after reperfusion.

We were interested, whether patterns of expression of these genes would change depending on the time of the organ's exposure to MSCs. ***Table 5.3.*** summarizes the major findings of experiments on the introduction of MSCs into the HMP setting.

PORCINE HMP PERFUSION + MSCs	HMP	HMP REP	HMP+MSC	HMP+MSC REP
Urinary output	–	↑	–	↑
Oxygen consumption	–	↑	–	↑
RRI	↓	↓	↓	↓
Perfusate flow rate	↑	↑	↑	↑
IL-1β perfusate	–	↑	–	↑↑
IL-1β urine	–	↑	–	↑↑
NGAL perfusate	–	↑	–	↑
NGAL urine	–	↑	–	↑↑
EDN-1 expression	↑	↑	↑	↑↑
NGAL expression	↓	↓	↑↑	↓
IL-1β expression	↑	↑	↑↑	↑↑
TNFα expression	↑	↑	↑	↑↑

Table 5.3.: Overview of main findings after HMP +/- MSCs of porcine kidneys.

Table 5.3. *shows an overview of the main findings after the preconditioning phase using ex-vivo hypothermic machine perfusion (HMP) with or without addition of 1-5x10^6 Mesenchymal Stem Cells/500mls of perfustate for porcine kidneys. Findings are shown for post 4h of preconditioning using HMP as well as for after a period of reperfusion with whole blood and at physiological temperatures. The sample size for each group was n=6 kidneys. Arrows serve as a relative quantification in comparison between the groups. Stars next to the arrows indicate that a statistically significant result was*

On mRNA level NGAL was higher expressed after the preconditioning period in MSC treated kidneys but returned to equal levels as in the HMP only group after reperfusion. All other inflammatory markers were higher expressed in the MSC treatment group. These results were not statistically significant but as discussed in the previous chapter, the results might point towards some inflammatory processes, potentially triggered by MSCs within the graft.

Similar functional findings were made for MSCs in the NMP setting. The intrarenal

resistance indices (RRIs) during the preconditioning phase as well as during reperfusion were slightly higher in the MSC treated group than in the NMP group, which resulted in insignificantly lower oxygen consumption- and perfusate flow rates in that group. This could be the result of MSCs being stuck within the micro capillaries of the kidneys. Mentioned differences in this case did not lead to any differences in the urinary production rates of the organs within different treatment groups. This indicates that the function of the kidneys was not influenced much by the presence of MSCs (***Fig. 4.37***). ***Table 5.4.*** shows an overview of observed functional findings as well as for expression patterns of investigated markers for experiments on MSC infusion into kidneys undergoing NMP.

PORCINE NMP PERFUSION + MSCs	NMP	NMP REP	NMP+MSC	NMP+MSC REP
Urinary output	↑	↑	↑	↑
Oxygen consumption	↑	↑	↑	↑
RRI	↓	↓	↓	↑
Perfusate flow rate	↑	↑	↑	↑
IL-1β perfusate	–	↑	–	↑
IL-1β urine	–	↑	–	↑
NGAL perfusate	–	↑	–	↑↑
NGAL urine	–	↑	–	↑↑
EDN-1 expression	↑	↑	↑↑	↑
NGAL expression	↑	↑	↑	↑↑
IL-1β expression	↑↑	↑	↑	↑
TNFα expression	↑	↑	↑↑	↑

Table 5.4. .: Overview of main findings after NMP +/- MSCs of porcine kidneys.

Table 5.4. *shows an overview of the main findings after the preconditioning phase using ex-vivo normoothermic machine perfusion (HMP) with or without addition of 1-5x10^6 Mesenchymal Stem Cells/ 500mls of perfustate for porcine kidneys. Findings are shown for post 4h of preconditioning using NMP as well as for after an additional period of reperfusion with whole blood and at physiological temperatures. The sample size for each group was n=6 kidneys. Arrows serve as a relative quantification in comparison between the groups. Stars next to the arrows indicate that a statistically significant*

Not much is known about the exact mechanisms of action of MSCs. Newest studies postulate that the regenerative effects of MSCs do not rely on their differentiation and ability to replace damaged tissues, but are primarily mediated by the paracrine release of factors, for example so called extracellular vesicles (EVs). EVs are composed of micro vesicles and exosomes, and MSC-derived EVs can contain genetic and protein material that, upon transferring to recipient cells, can activate repair mechanisms to ameliorate inflammatory processes, for example renal injury[346]. There are studies suggesting that not the actual cells, but conditioned media containing anti-inflammatory and proangiogenetic factors like hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF-A) and insulin-like growth factor-1 (IGF-1) would be sufficient to ameliorate IRI[347]. Other studies suggest the opposite, namely the necessity for MSCs to be in place in order to excite their anti-inflammatory actions via paracrine rather than endocrine mechanisms[292]. If and how MSCs or secreted chemokines, growth factors or extracellular vesicles are attracted to sites of injury is still unclear.

We were the first to show that delivery of MSCs into an isolated organ via both ex-vivo HMP as well as NMP is possible and that MSCs seem to be located within glomeruli and tubules of the treated organs. Even after the reperfusion period with whole blood for two hours, cells could still be detected within the organs, which is a promising result, suggesting that MSCs 'home' to sites of injury within these organs. In case of the tubules, the microscopic appearance even suggests an integration of MSCs into the tubular epithelial layer (as shown in ***Fig. 4.34.***, ***Fig. 4.35***, ***Fig. 4.41.*** and ***Fig. 4.42.*** for porcine experiments and ***Fig. 4.49.*** for human kidneys). The fact that our observations could be confirmed in a translational model, ranging from bright green cells positive for GFP within rodent kidneys after HMP (see Chapter 3.4.), via human MSCs traced within porcine kidneys after HMP as well as NMP, and ending with human cells in human kidneys, again after HMP and NMP, is very encouraging for the result to be valid. MSCs infused during HMP looked viable within the kidneys Another encouraging fact was, that microscopic imaging on the wide field microscope was performed with the help of Dr. Steven Rothery from the FILM facility at Imperial College, who, without knowing any backgrounds about the project, pointed out the positive signals of double-labelled MSCs to me.

MSCs could only be detected successfully in both HMP and NMP, when a number of either $3x10^6$ or $5x10^6$ was added to the perfusate, not when a number of $1x10^6$ cells were used. This does not necessarily mean that there are no cells present at the lower number, but that it is harder to detect them. Having histology slides at a thickness of 4um, from a kidney with a weight of approximately 300g and a volume of 10x5x2cm, finding cells back within these slides is comparable to looking for needles in a haystack. It was therefore not surprising, that when applied into a much smaller organ, like a rodent kidney, the cells seem to be omnipresent and easy to find. It would certainly be good for future studies, to have flow cytometry facilities in place to also analyse the perfusate for cells before start of perfusion and afterwards, in order to estimate the total number delivered to an organ. This method could then also be used to confirm the number of MSCs within an organ.

In our ex-vivo perfusion models, MSCs could successfully be delivered into porcine and human kidney grafts and be traced even after the reperfusion period of two hours by wide field microscopy as well as by immunohistochemical staining. The cells looked viable. After double- labelling, it requires an intact cell membrane and cytosol for the cells to be visible, which was the case. Furthermore, the microscopic appearance of traced cells was very similar to their microscopic appearance before administration (adequate in size and shape). Further studies, especially in-vivo studies will be necessary as a next step to further evaluate the potential effects of MSCs on the outcomes of recipients. Preclinical studies within the pig could be helpful, but also clinical trials would be an option as the application of MSCs has been shown to be safe and feasible without any significant side effects in many studies. Furthermore, our results support these findings. To my imagination, ideally in the future, patients who go onto the waiting list for an organ could donate their own bone marrow for the extraction of MSCs so that these cells could be used as to precondition and improve the quality of the organ for transplantation. Besides repair of damage due to IRI, this would potentially even lead to better immunological long-term outcomes.

5.4. Project 4: Investigation of the effects ex-vivo delivered Mesenchymal Stem Cells on Ischemia-Reperfusion Injury in a rodent autologous kidney transplantation model.

The discussion of this project will be relatively short, as the project could not be completed to the extent I aimed for. Due to an unexpected expiry of our project licence just after my completion of the necessary animal training, we were not able to perform any procedures on rodents for two years. This meant that once our licence was reinstalled, I could only then start to be trained to perform renal transplants in the rat. Also, for the training, we were dependent on Dr. Jenny Smith, who is the only designated trainer according to our animal facility. Due to the fact that Dr. Smith has retired, training was performed in 3 blocks of 1 week each. During the phase of being trained in doing the procedure, one pair of a donor rat and a recipient animal takes up the whole day. Hence, per week of training I was able to perform 4 renal transplants (Monday-Thursday).

The challenges of rat kidney transplantation are the vascular anastomoses, including an end-to-end anastomosis of the renal vein, as well as the anaesthetic management of the animals. Occasionally, a rat would arrest on the operating table due to a too high dose of anaesthetic or due to a lack of fluid when bleeding occurred. Sometimes, for no apparent reason, the kidney did not perfuse properly after releasing the clamps. Unfortunately I didn't manage to get to the point where I could successfully recover an animal from surgery. Therefore, our only result for this study, which was still very interesting was, that we could deliver green fluorescent MSCs to rodent kidneys and find them afterwards, mainly within the glomeruli using wide field microscopy (***Fig. 4.50.***). This was a promising result and can be accounted as the pilot project for the next step, which will be to transplant these kidneys back into recipient rats to look at outcomes in vivo. The question will be whether delivered cells will remain within the transplanted kidney or whether they will relocate somewhere else within the recipient rat and whether outcomes in terms of creatinine clearance and graft survival will be superior in MSC treated kidneys compared to kidneys without MSC perfusion. Another question to be answered will be whether MSCs locate into glomeruli due to chemotactic factors, which attract them or whether they simply can't pass the basal membrane of the glomeruli and get trapped there. Also, a higher resolution of the microscopic imaging would be necessary in order to identify,

where exactly within the glomeruli the MSCs are located. All these remaining questions will be subject to subsequently planned future studies.

5.5. Project 5: To investigate mechanisms of MSCs and potential differences of GFP+ and WT- MSCs on rodent macrophages in vitro

The immunosuppressive properties of MSCs have been extensively studied both in vitro and in animal models of immune-mediated disorders. Clinical trials are currently underway in which MSCs are employed to treat various human immunological diseases as well as inflammatory processes, e.g. ischemia- reperfusion injury[348]. The molecular mechanisms leading to immunomodulatory and anti-inflammatory effects of MSCs on different immune cells are still unclear and there have been several reports aiming to elucidate the MSC-macrophage interaction in pathology. In that context, 'MSC- educated' macrophage activation has been proposed by some groups in the past[303,349,350]. Although there is a large body of evidence on the immunomodulatory effects of MSCs, the genetic determinants of the immunosuppressive effects of these cells is largely ignored.

A study conducted by Behmoaras et al. in 2014 found, that soluble factors present in supernatants of mesangial cells from WKY rats could differentiate macrophages into M1 or M2 depending on the genetic background of these cells. Indeed, MC supernatants from different genetic backgrounds polarised WKY macrophages in different ways[351]. As mesangial cells as well as Mesenchymal Stem Cells are of a similar origin, we were interested in the question, whether MSCs extracted from a transgenic WKY rat (positive for the expression of green fluorescent protein), would have different immunomodulatory effects in vitro than MSCs coming from a wild- type animal. The reason to investigate that was also, to evaluate possible in-vitro studies using these cells adequately.

As a first step, we aimed to perform a similar study as had been performed by our colleagues, and to investigate the effect of different MSC supernatants on bone marrow derived macrophages from WT animals. Furthermore, we were also interested on the differences between the passages of MSCs as well as the influence of the age of the bone marrow donor. The aim was to find the most potent anti-inflammatory cell phenotype, as this could be essential information in case treatment with autologous MSCs becomes a reality in the future. Cells could be extracted at a younger age as insurance and stored for a later stage of life, and different passages could be cultured specifically.

To start with, in this supernatant- transfer experiment, we looked at the expression pattern of Mannose receptor 1 (Mrc-1) of bone marrow derived macrophages after

over- night treatment with the different supernatants. An up-regulation of Mrc-1 indicates a shift from a more pro- inflammatory macrophage phenotype, the so-called M1 phenotype, into a more anti-inflammatory 'M2' phenotype[351]. In our experiments, we could confirm different influences of supernatants coming from MSCs of different genetic backgrounds on native macrophages, as well as on macrophages, which had been given an inflammatory stimulus with LPS. In the case of Mrc-1, we demonstrated, that supernatants coming from wild type MSCs, but not supernatants from GFP+ MSCs stimulated an up regulation of Mrc-1 on native macrophages. For LPS stimulated macrophages, which showed a down regulation of Mrc-1 after stimulation, treatment with supernatants from the 'young' passages over night was even able to reverse the effect of LPS and lead to an up regulation of the Mrc-1. This was not the case for supernatants from GFP+ cells, suggesting less anti-inflammatory potency of these cells (***Fig. 4.54***).

We then looked at the expression levels of iNOs, also known as Nos2 as a marker for the more pro-inflammatory macrophage phenotype and observed a down regulation in native macrophages after treatment with both, supernatants from WT- as well as GFP+ cells from young donors, and an up regulation in the native macrophages treated with supernatants coming from older donors for supernatants from both phenotypes, however less so for the WT treated group. Interestingly for LPS stimulated macrophages, the opposite was the case, after the up regulation of iNOs due to LPS stimulation, supernatants from GFP cells of all groups induced a higher down regulation of iNOs expression levels on the macrophages than supernatants from WT cells. This is an interesting observation, suggesting many complex cascades going on that lead to different outcomes, depending on the inflammatory milieu the macrophages are in. Similar observations were made for the expression of TNFα on the macrophages. After stimulation with LPS, supernatants from GFP+ cells, in particular from MSCs from older donors led to significantly lower TNFα levels than supernatants from WT cells, with a down regulation of TNFα by the supernatants of GFP+ MSCs from older donors (***Fig. 4.56.***).

For the expression levels of IL-10, which is more a marker for the anti-inflammatory M2 macrophage phenotype, again supernatants from GFP+ MSCs and here again, from older cell passages, seemed to have the best effect on an up regulation of IL-10 in native macrophages. No essential differences in IL-10 expression levels were detected after LPS stimulation (results not shown).

Overall, in our supernatant transfer study, we can confirm the results described by Behmoaras et al., namely that different paracrine effects are observed, depending on the genetic background of the cells and that most likely, different cytokine patterns are excreted by the cells, depending on their genetic background, age of the donors etc. The fact that triplicates of either the 'younger' supernatants seem to have similar effects as well as the triplicates of 'older' supernatants furthermore confirms this theory.

In summary, our experiment suggests, that with exception to Mrc-1, supernatants from GFP+ cells had a better anti-inflammatory effect on the macrophages than supernatants from WT cells, and in particular supernatants from 6 month old GFP+ donors showed stronger results in that respect than supernatants coming from 6 week old donors.

It would be interesting for future experiments to measure the exact concentrations of cytokines present in these supernatants and as a next step, to perform a similar experiment investigating the effects of the same different groups of MSCs on macrophages on a direct cell-cell interaction level. This might help to further understand the mechanisms of action of MSCs.

Nevertheless, the different phenotypes of MSCs will have to be considered in clinical studies, not in the matter of transgenic cells, but more in the matter of different genetic backgrounds and ages of potential bone marrow donors.

In summary, these results show the importance of genetic determinants of Mesenchymal Stem Cell activation and their potential anti-inflammatory effects. This must be taken into consideration in understanding the unique regulatory effects of these cells on activated macrophages in the pathophysiology of ischemia-reperfusion injury.

Chapter 6: Conclusions

6.1. Project 1: Development of a Normothermic Machine Perfusion circuit for ex-vivo kidney preconditioning on the RM3-perfusion machine and testing of a portable micro dialysis device.

The conclusion to this Chapter is that we were able to successfully set up a circuit for ex-vivo Normothermic Kidney Perfusion using the Waters RM3 perfusion machine. Furthermore, we were able to show that the first portable micro dialysis device, developed by the Department of Bioengineering at Imperial College London reliably measures glucose- and lactate concentrations from the extracellular space of the kidney and that this could be an important tool in the future of kidney transplantation.

6.2. Project 2: Investigation and comparison of the effects of current methods of ex-vivo organ preservation on porcine and human kidney grafts after prolonged cold ischemia times.

We were the first to perform a head to head comparison between Hypothermic (HMP) and Normothermic ex-vivo kidney perfusion (NMP) in a translational model. HMP of porcine kidneys resulted in improved physiologic parameters including significantly increased urinary output rates, despite showing a higher up regulation of inflammatory markers on mRNA level. Translation into a human model showed controversial results, dependent on the donor demographics of perfused kidneys. This leads to the conclusion, that HMP as a well established method for ex-vivo organ preservation and preconditioning must not be overlooked too quickly and that the way forward in the field of ex-vivo organ preservation might have to be individually tailored to different types of organs and their respective background. Clinical studies directly comparing the two investigated preservation methods as well as long term clinical data are urgently needed for further clarification.

6.3. Project 3: Investigation of the introduction of MSCs into ex-vivo organ preservation systems as a preconditioning method for porcine and human kidney grafts (translational model).

We are the first to show that Mesenchymal Stem Cells (MSC) can be delivered to kidneys in during ex-vivo Hypothermic machine perfusion as well as during

Normothermic machine perfusion. Delivered MSCs are located within glomeruli and tubules of treated kidneys. Whilst the cells don't influence the organ's immediate physiological function, an effect on the expression of inflammatory markers was observed. Changes in levels of IL-1β as well as RNA expression patterns of cytokines suggest that MSCs do have an effect on the kidney grafts and whether this leads to a positive or a negative outcome on IRI in transplantation needs to be determined in further (in vivo) experiments. Further investigations in vivo will be necessary to investigate the effects of ex-vivo delivered MSCs on the outcomes of transplanted organs. The application of Mesenchymal Stem Cells in an ex vivo hypothermic machine perfusion setting is possible and does not immediately influence functional parameters of the kidney grafts.

6.4. Project 4: Investigation of the effects ex-vivo delivered Mesenchymal Stem Cells on Ischemia-Reperfusion Injury in a rodent autologous kidney transplantation model.

Ex-vivo delivered MSCs could be localized within glomeruli of treated rodent kidneys. Their effects in vivo could not be investigated in this project due to the absence of a project licence. However, I was able to learn the technique for the performance of kidney transplantation in the rat and this pilot study helped setting up an experimental protocol for further in vivo studies in a rodent model.

6.5. Project 5: To investigate mechanisms of MSCs and potential differences of GFP+ and WT- MSCs on rodent macrophages in vitro

MSCs from transgenic WKY rats positive for the expression of GFP show strong green fluorescence throughout passages 0-10 and therefore, application in a rodent model of kidney transplantation will be promising to reveal possible effects of these cells on ischemia-reperfusion injury (IRI). Supernatants from MSCs do influence the immunogenic profile of macrophages suggesting chemotactic immunomodulatory effects of the cells. The immunomodulatory effect of MSC supernatants is furthermore dependent on the genetic background of the MSC donor. In order to find the most promising anti-inflammatory MSC phenotype, further studies are needed.

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APPENDICES

1. Animal Licence Natalie Vallant

SOCIETY OF Biology

This is to certify that

Natalie Vallant

has been assessed as having satisfactorily completed accredited training for personnel working under the Animals (Scientific Procedures) Act 1986

Modules: **One, Two, Three & Four**

Species: **Rat, Mouse, Guinea Pig, Hamster, Rabbit & Gerbil & General Principles**

Training organised by: **CBS Imperial College**

Certificate Number: **IMP/14/108**

Date: **18.07.14**

Course Organiser

For the Society of Biology

This is not a licence to perform procedures under the Animals (Scientific Procedures) Act 1986

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18 December 2012

Mr Vassilios Papalois
Chief Renal and Transplant Services
Imperial College Healthcare NHS Trust
Imperial College Renal and Transplant Centre
Hammersmith Hospital
Du Cane Road
W12 0HS

Dear Mr Papalois

Study title:	**Use of Machine Perfusion for Improving Allograft Viability**
REC reference:	**12/SW/0302**
IRAS project ID:	**84202**

Thank you for your e-mail of 2 December 2012, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Kirsten Peck, nrescommittee.southwest-exeter@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Covering Letter		
Evidence of insurance or indemnity		28 July 2012
Investigator CV		
Investigator CV - Karim Hamaoui		
Other: Donor Consent - Solid Organ and Tissue Donation - UK transplant Registry		
Protocol	4	18 September 2012
REC application	3.4	18 September 2012
Response to Request for Further Information		02 December 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/SW/0302	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Kirsten Peck

pp **Dr Denise Sheehan**
Chair
NRES Committee South West - Exeter

Enclosures: *"After ethical review – guidance for researchers" [SL-AR2]*

Copy to: Ms Becky Ward, Imperial College Healthcare NHS Trust (Hammersmith Hospital, Charing Queen Charlotte & Chelsea